Studies on the Development of Antimicrobial Tolerance in Monospecies and Binary Biofilms

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Director of Studies

Abstract

Biofilms are formed by a spectrum of microorganisms, including *Pseudomonas aeruginosa* PAO1 and *Escherichia coli* ATCC 10000, and provide a means for these organisms to protect themselves against antimicrobial agents. Several mechanisms have been proposed to explain the phenomenon of tolerance within biofilms, including delayed penetration of the antimicrobial into the biofilm extracellular matrix, slow growth rate of organisms within the biofilm, other physiologic changes brought about by interactions of the organisms with a surface and the biofilm phenotype. However, none of these mechanisms on its own can explain the development of resistance in biofilm cells. The practical implications of biofilm formation are that alternative control strategies must be devised both for testing the susceptibility of the organisms within the biofilm and treating the established biofilm to alter its structure.

The primary objective of this study was to induce tolerance in monospecies biofilms towards selected antimicrobial agents (including Zinc Pyrithione (ZnPT), Sodium Pyrithione (NaPT), Cetrimide, Benzisothiazolone (BIT) and Thiomersal) and investigating the possibility of cross-resistance between these biocides. This was followed by observation of the action of BIT in binary biofilms and subsequent susceptibility of component species this biocide. Monoculture of *Ps. aeruginosa* PAO1 biofilms grown on the Sorbarod filters were passaged in the presence of sub-minimum inhibitory concentrations (MIC/4) of the biocides in a chemically defined medium (CDM). During 10 passages in increasing MIC/4, a gradual increase was observed in the MIC value of up to 3-fold and 4-fold from the original value (for ZnPT and Cetrimide). In the case of BIT, there was an overall increase of 17-fold for the adherent cells and 10-fold for the eluate cells. The difference in MIC values between biofilm and eluate cells was partially explained by the presence of thiol groups in the EPS that surrounds the biofilm, which quenched the effect of BIT. Once the resistant cells were passaged in biocide-free media, the MIC started to decrease. The results from cross-resistance studies in the case of ZnPT and BIT exhibited a marked increase in the MIC when compared to the other three biocides for both types of cells. On exposure of binary biofilms (two species biofilms, composed of *E. coli* and *Ps. aeruginosa*) to BIT, the original, pre-exposure value for *Ps. aeruginosa* was 5 $\mu g\ mL^{-1}$ for both biofilm and eluate cells. After 5 passages in increasing the concentration of the biocide, there was an overall increase in MIC of 13.2-fold and 12.4-fold for biofilm and eluate cells, respectively. For *E. coli* cells growing as binary biofilms, the original MIC was 5 $\mu g\ mL^{-1}$ for biofilm and eluate cells. The MIC increased in a step-wise fashion until Passage 5, at which point there were increases of 12.4-fold for biofilm cells and 10.5-fold for eluate cells. Once, the cells were cultured in the absence of biocide, a significant decrease in the MIC was observed, indicating that the mechanism of resistance was dependent upon the exposure of the biocide. Analysing the outer membrane profiles of both adherent and eluate (mono- and binary species) cells illustrated marked differences between sensitive and resistant biofilm and eluate cells.

This project yielded novel information and techniques regarding the use of passage approaches to develop antimicrobials tolerance and resistance in both monospecies and binary species biofilms of medically important bacteria. The results from these experiments suggest that it is possible to use these models to investigate the results of environmental exposure of bacteria to sub-MICs of biocides and develop an understanding of their subsequent tolerance and resistance characteristics.

This piece of work is dedicated to the memory of my father

Daifallah Al-Hmoud

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Contents

List of Figures

List of Tables

Chapter One:

Introduction

Our perception of bacteria as unicellular life forms is deeply rooted in the pure culture paradigm. Since bacteria can, in strict sense, be diluted to a single cell and studied in liquid culture, this mode of operation has been exploited and used to study many bacterial activities. Although this traditional way of culturing bacteria in liquid medium has been instrumental in the study of microbial pathogenesis and enlightening as to some of the facets of microbial physiology, pure culture growth is rarely how bacteria exist in nature (Davey & O'Toole, 2000). Direct observation of a wide variety of natural habitats has established that the majority of microbes persist attached to surfaces within a structured biofilm ecosystem and not as free-floating organisms (Costerton *et al.*, 1995). Moreover, it is becoming clear that these natural assemblages of bacteria, within the biofilm matrix, function as a cooperative consortium, in a relatively complex and coordinated manner (Costerton *et al.*, 1995). Hence, although microorganisms can have an independent planktonic existence, an interdependent lifestyle in which they function as an integral part of a population or community is also possible and is, in fact more typical.

What is a biofilm?

Biofilms are assemblages of cells growing on the surface of (usually) solid substrata, which are frequently (though not always) submerged in an overlying aqueous phase, such as that of stream or pond (Palmer & White, 1997). Therefore the cells within a biofilm are confined close to a substratum surface and are able to interact with molecules or ions that tend to accumulate there (Langley & Beveridge, 1999). Biofilms can comprise a single microbial species or multiple microbial species and can form on a range of biotic and abiotic surfaces. Although mixed-species biofilms predominate in most environments, single-species biofilms exist in a variety of infections and on the surfaces of medical implants (Dickinson & Bisno, 1993; Adal & Farr, 1996; Archibald & Gaynes, 1997).

These single-species biofilms are the focus of most current research. *Pseudomonas aeruginosa* has emerged as the most studied single-species, biofilm-forming, Gram-negative bacterium.

Studies indicate that biofilms are a stable point in a biological cycle that includes initiation, maturation, maintenance and dissemination. Bacteria seem to initiate biofilm development in response to specific environmental cues, such as nutrient availability, resistance to antimicrobial agents, predation, and other factors (Lawrence *et al.*, 1991). Although these conditions vary widely, the Gram-negative organisms undergo a transition from free-living, planktonic cells to sessile, surface-attached cells in response to a nutrient-rich medium. These biofilms continue to develop as long as fresh nutrients are provided, but when they are nutrient deprived, they detach from the surface and return to a planktonic mode of growth (Kolter *et al.*, 1993; O'Toole *et al.*, 2000). Presumably, this starvation response allows the cells to search for a fresh source of nutrients and is driven by well-studied adaptations that bacteria undergo when nutrients become scarce. Therefore, the starvation response pathway can be subsumed as a part of the overall biofilm developmental cycle (Kolter *et al.*, 1993; Sawyer & Hermanowicz, 1998).

Formation of Biofilms

A biofilm is the culmination of a series of processes (**Figure 1**)

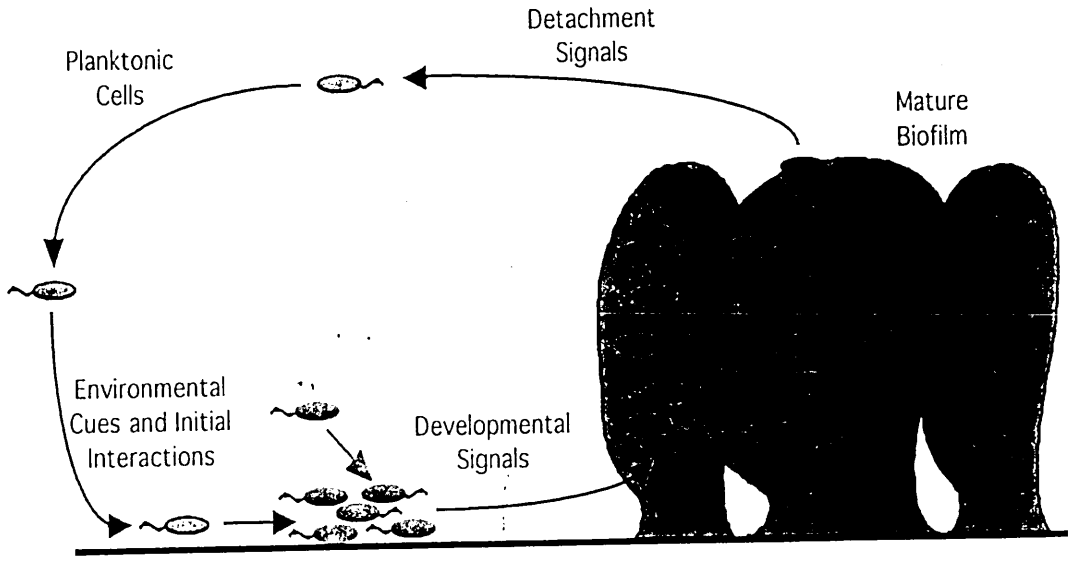


Figure 1: Model of biofilm development. Individual planktonic cells can form cell-to-surface and cell-to-cell contacts resulting in the formation of microcolonies. The hallmark architecture of the biofilms form in an acylhomoserine lactone-dependent process. Cells in the biofilm can return to a planktonic lifestyle to complete the cycle of biofilm development (O'Toole *et al.*, 2000).

Initiation of Biofilm Formation

Biofilm formation is thought to begin when bacteria sense environmental conditions that trigger the transition to a life on a surface (Palmer & White, 1997; O'Toole & Kolter 1998; Pratt & Kolter, 1998; Watnick & Kolter, 1999; O'Toole *et al.*, 2000). These environmental signals vary among microorganisms. For example, *Ps. aeruginosa* will form biofilms under almost any conditions that allow growth. On the other hand, some strains of *Escherichia coli* will not form biofilms in minimal medium unless supplemented with amino acids (Pratt & Kolter, 1998; Watnick *et al.*, 1999). In addition to the nutritional

content of the medium, other environmental cues that can influence biofilm formation include temperature, osmolarity, pH, iron, and oxygen (Stanley, 1983; Wang *et al.*, 1996). This process starts when floating (planktonic) bacteria encounter a surface, which can occur actively involving motility or chemotaxis by the bacteria, or because of the passive diffusion of the bacteria, or because of convective flow (Hoyle & Costerton, 1991). Bacteria are of colloidal dimension and possess a net negative charge and a variable degree of cell surface hydrophobicity (Allison & Gilbert, 1994). Since both surfaces possess an overall negative charge then, for the attachment to take place, the resulting electrostatic repulsion barrier must be overcome by attractive forces. The eventual outcome of this interplay between competing forces is governed by thermodynamic principles (Absolom *et al.*, 1983) and is described by the DLVO theory, which derives its name from the authors who proposed it (Derjaguin & Landau, 1941; Verwey & Overbeek, 1948). When microorganisms arrive in the vicinity of a surface, they are subject to short-range attraction forces (Hydrophobic and Van der Waals forces), which are capable of holding the microorganisms at the solid-liquid interface, balancing the strong electrostatic repulsive forces (Busscher & Weerkamp, 1987; Busscher & Van der Mei, 1997). This first stage of adhesion is termed reversible adhesion and the cells are said to be at their "secondary energy minimum". Cells at this stage have been shown to exhibit Brownian motion and can be easily removed from the surface by washing (Meinders *et al.*, 1995).

Cells may subsequently become firmly attached to the surface through the action of surface structures such as fibrils and pili. Flagella or flagellar driven motility was found to have a role in the initial cell-to-surface interactions for both *E. coli* and *Ps. aeruginosa* (Pratt & Kolter, 1998; O'Toole & Kolter, 1998; Tolker-Nielsen & Molin, 2000). Evidence has been elucidated indicating that for *E. coli* type I pili, flagella and curli are required for the initial surface attachment (Pratt & Kolter, 1998; Vidal *et al.*, 1998). For *Ps. aeruginosa*

the polar localized type IV pili evidently have a role in microcolony formation, as they enable this organism to move on the substratum. Strains unable to make functional type IV pili attach to the surface and form a monolayer, much as the wild type does, yet they are unable to form the microcolonies that are a hallmark of early biofilm development in the bacterium (Fletcher *et al.*, 1993; O'Toole & Kolter, 1998). The surface structures are able to overcome the repulsive forces that exist between the two negatively charged surfaces, by virtue of their shape and rigidity (Beachey *et al.*, 1988). Having overcome the secondary energy minimum, the cells bind irreversibly at the primary energy minimum where Van der Waals forces overcome the electrostatic repulsion (Gilbert *et al.*, 1993). In addition, LPS, an important component of the Gram-negative bacterial outer membrane, also plays a role in initial surface attachment in *Ps. aeruginosa* (Davies *et al.*, 1993; Davies & Geesey, 1995). Of the two major species of LPS produced, the loss of the B-band LPS (but not A-band LPS) reduced the cells ability to interact with hydrophilic surfaces and increases attachment to the hydrophobic surfaces. A-band mutations had only mild effects on attachment (Makin & Beveridge, 1996). For *E. coli*, loss of LPS results in a decreased ability to attach. However, because these LPS mutants are also defective in flagellum-mediated motility and type I pilus production, it is difficult to determine if the loss of LPS has a direct or indirect role in biofilm development.

Observing *Ps. aeruginosa* before attachment reveals that the organism swims along the surface almost as if it is scanning for an appropriate location for initial contact. It appears that, once bacteria initiate surface contact, they come to rest on the surface. However, time-lapse microscopy reveals that, once *Ps. aeruginosa* forms a monolayer on an abiotic surface, the bacteria continue to move. This movement occurs only on the surface and uses twitching motility instead of swimming. Twitching motility is dependent on type-IV pili, and it has been proposed that, by extending and retracting their pili,

bacteria can push or pull themselves across a surface (Bradley, 1980; Semmler *et al.*, 1999). Furthermore, there are indications that cells move by twitching motility only when they are in contact with other cells, suggesting that this is a form of community behaviour (Semmler *et al.*, 1999).

Maturation of the biofilm

Once cells have attached to a substratum, growth and division occur in three dimensions. This process of growth and division along with continuous introduction of microorganisms can be seen as a type of differentiation during which the biofilm changes from a monolayer of cells to a thicker structure with different cell types in locations determined by an interplay of architecture and environmental conditions (Palmer & White, 1997). Two properties are often associated with surface-attached bacteria: increased synthesis of extracellular polymeric substances (exopolysaccharide; EPS) and the development of antibiotic resistance (Anwar *et al.*, 1989; Gacesa, 1998). Prigent-Combaret *et al.* (1999) showed that flagellin synthesis is decreased in biofilm-associated cells, while production of colanic acid, an EPS made by *E. coli*, is increased. The situation appears to be similar in *Ps. aeruginosa.* Alginate is an EPS that is found in *Ps. aeruginosa* biofilms (Gacesa, 1998; Watnick & Kolter, 2000). Transcription of *algC*, a gene involved in the production of alginate, is increased approximately four-fold in biofilm-associated cells as compared with planktonic cells (Garrett *et al.*, 1999). Furthermore, for many years, researchers have noted that pulmonary isolates of *Ps. aeruginosa* are mucoid due to the production of copious amounts of alginate (Govan & Fyfe, 1978; Govan & Deretic, 1996; Gacesa, 1998). Recently, Garrett *et al.* (1999) noted that flagellae are absent from these mucoid isolates. In addition, they showed by mutational analysis, that while alginate synthesis is positively regulated by the alternative sigma factors σ^{22}, this sigma factor

negatively regulates the synthesis of flagellum. This suggests that when synthesis of EPS or alginate increased in biofilm associated cells, flagellar synthesis decreased.

Another important step in biofilm development is the formation of the characteristic biofilm architecture (Davey & O'Toole, 2000). Although numerous techniques have been utilized to document the biofilm architecture of bacteria, until recently it was not clear if this structural complexity was regulated or the consequence of stochastic processes. Production of the quorum-sensing molecules known as acyl-homoserine lactones (acyl-HSLs) has been demonstrated in both natural and cultured biofilms (McLean *et al.*, 1997; Davies *et al.*, 1998; De Kievit & Iglewski, 1999). The importance of acyl-HSLs in single-species biofilms has been clearly demonstrated (Allison *et al.*, 1998; Davies *et al.*, 1998; De Kievit & Iglewski, 1999). In *Ps. aeruginosa*, acyl-HSLs are responsible for defining the separations between bacterial "*pillars*" in the three-dimensional structure of the biofilm. Davies *et al.* (1998) showed that a *lasI* mutant, although still capable of early cell-surface interactions, does not develop the hallmark architecture of *Ps. aeruginosa* biofilms. These mutants that do not produce acyl-HSLs form biofilms in which the cells are closely packed together and are easily disrupted by sodium dodecyl sulphate (SDS). This suggests that formation of biofilm architecture is not a stochastic process, but is controlled as part of a complex regulatory system (Davies *et al.*, 1998; O'Toole *et al.*, 2000).

Detachment and Return to the Planktonic Mode of Growth

If the bacteria were unable to escape the biofilm, the biofilm would become a death trap when the nutrient supply was exhausted, environmental conditions became unfavorable, or a predator entered the community. In multispecies biofilms, the loss of key members of the community may result in a shift to a dramatically different population

(Palmer & White, 1997). However, once the bacterium is encased in EPS, abandoning the biofilm becomes a significant task. At such times, a polysaccharide lyase may provide the bacterium with an escape method. Boyd and Chakrabarty (1994 & 1995) reported that the enzyme alginate lyase may play a role in the detachment phase in *Ps. aeruginosa.* They showed that over-expression of alginate lyase could increase the rate of detachment and cell sloughing from biofilms. Analysis of newly detached cells showed that compared to cells in the biofilm they had a low hydrophobicity, which steadily increased with time following detachment (Gilbert *et al.*, 1993). Under certain circumstances in *Ps. aeruginosa*, the alginate lyase activity may be preferentially enhanced resulting in increased detachment of bacteria into the surrounding environment and dispersal of the microbes to new locations. This enzyme is capable of cleaving the bond between two uronic acids by an eliminase mechanism, to yield two shorter alginate polymers (Boyd *et al.*, 1993; Schiller *et al.*, 1993). Further action of the lyase results in continued depolymerisation of the alginate to oligosaccharides. Detachment of single bacteria and sloughing of pieces of biofilm, limit any further increase in biofilm thickness. Consequently, the rate of biofilm development will achieve a steady state condition with biofilm-accumulation being approximately equal to biofilm loss by detachment and sloughing (Marshall, 1992; Gilbert *et al.*, 1993). Sometimes parts of the substratum may also be released in this process (e.g. intestinal mucosa and biodegradable materials; Gilbert *et al.*, 1993; Macfarlane *et al.*, 1997). Complete loss of all cells is probably rare as the remains of the biofilm can act as a new substratum for other microorganisms. Thus, the cycle of attachment, shown in **Figure 1**, is completed.

The Role of Alginate Exopolysaccharide

Alginate and *Pseudomonas aeruginosa*

Ps. aeruginosa is a widespread microorganism that can be found in a variety of habitats including soil and freshwater streams. In these environments the microbes are found predominantly attached to solid surfaces. With *Ps. aeruginosa*, the study of EPS has focused on alginate, a polymer of uronic acid and guluronate, because of its presumed importance in the infection of cystic fibrosis lung (Govan & Fyfe, 1978; Govan & Deretic, 1996). Not all *Ps. aeruginosa* strains produce alginate and some may be capable of synthesizing alternate exopolysaccharides. Alginate is a linear (1→ 4) linked EPS of β-D-mannuronic acid and the C-5 epimer α-L-guluronic acid (**Figure 2**; Evans & Linker, 1973; Boyd and Chakrabarty, 1995).

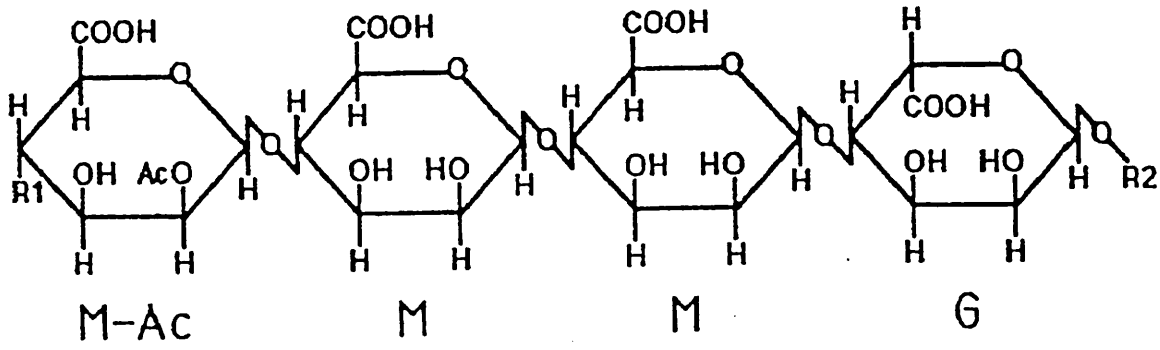


Figure 2: Structure of the alginate exopolysaccharide produced by *Ps. aeruginosa*. M, mannuronic acid; G, guluronic acid; M-Ac, acetylated mannuronic acid (Boyd & Chakrabarty, 1995).

In bacteria, the mannuronic acid residues can be acetylated at the 2' and/or 3' positions (Davidson *et al.*, 1977; Sutherland, 2001). The activation of a critical alginate promotor, *algD*, by *Ps. aeruginosa* has been shown to occur during nitrogen limitation, during membrane perturbation induced by ethanol, and when cells were exposed to media of high osmolarity (Davies *et al.*, 1993). The pathway of alginate biosynthesis by *Ps. aeruginosa* is summarized in **Figure 3**.

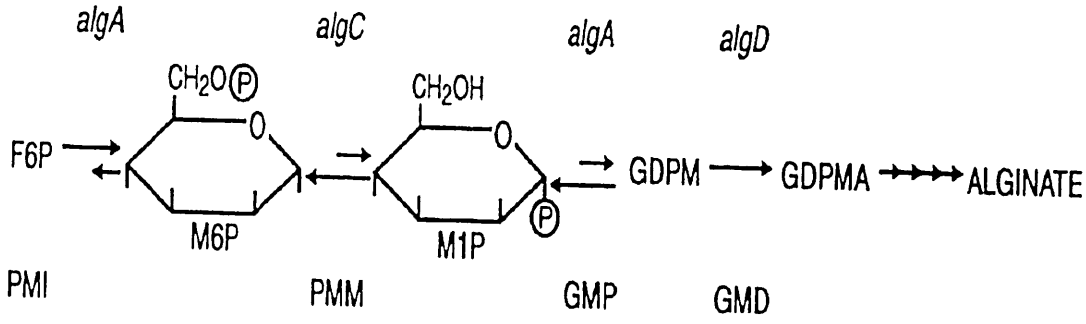


Figure 3: Alginate biosynthetic pathway in *Ps. aeruginosa*. Enzymes catalyzing the known reactions in the pathway are as follows: PMI, phosphomannose isomerase; PMM, phosphomonnomutase; GMP, GDP-mannose pyrophosphoylase; and GMD, GDP-mannose dehydrogenase. The genes encoding these enzymes are indicated above the respective name. The known reaction intermediates in the pathway are F6P, fructose 6-phosphate; M6P, mannose 6-phosphate; M1P, mannose 1-phosphate. GDPM, GDP-mannose; and GDPMA, GDP-mannuronic acid. The steps between the formation of GDP-mannuronic acid and that of alginate include polymerization, acetylation, export and epimerization (shown by arrows) (Davies *et al.*, 1993).

Biofilms and Alginate

A knowledge of biofilm structure is important to our understanding and interpretation of biofilm processes and to our ability to predict the influence a biofilm may have on its environment. The first direct observations concerning microbial biofilms were generated from direct light and electron microscopic observations and revealed that biofilm microbes were enveloped in copious amounts of a fibrous hydrated exopolysaccharide matrix (Geesey *et al.*, 1977). Bacteria experience a certain degree of shelter and homeostasis when residing within a biofilm, and one of the key components of

this microniche is the surrounding extrapolymeric substance matrix (Davey & O'Toole, 2000). This matrix is composed of a mixture of components, such as EPS, protein, nucleic acids, and other substances. The best studied of these components is EPS. Most bacteria are able to produce polysaccharides, either as wall polysaccharides (capsules) or as extracellular excretions into the surrounding environment (EPS). The EPS composition determines many important properties of biofilm such as density, porosity, diffusivity, and metabolic activity (Zhang *et al.*, 1999). More information about the composition of EPS will contribute to a better understanding of the physical and physiological behaviour of biofilms in environmental systems. The chemical compositions of the EPS are usually reported in the literature to be heterogeneous. Carbohydrate predominates and represents up to 65% of extracellular materials (Horan & Eccles, 1986), although other substances are also present, such as proteins, nucleic acids and lipids (Goodwin & Forster, 1985). Their exopolymer component ratios vary, depending on the sample source and extraction technique (Morgan *et al.*, 1990; Zhang *et al.*, 1999).

As described before, the EPS is clearly an integral part of the structural organization of biofilms. EPS has been proposed to play an important role in initial adhesion, as well as in securing and anchoring of bacteria to solid surfaces (Costerton *et al.*, 1985; Mai *et al.*, 1993; Boyd & Chakrabarty, 1995). However, Allison and Sutherland (1987) queried the extent to which EPS is involved in the initial adhesion. Non-EPS producing bacterial strains, including those of *Ps. aeruginosa*, can attach to solid surfaces but are unable to form mature biofilms (Nivens *et al.*, 1994). Hentzer *et al.* (2001) showed that a mucoid (i.e. alginate or EPS overproducing) *Ps. aeruginosa* strain PAO1 developed a highly differentiated biofilm, resulting in a more structurally heterogeneous biofilm than that produced by a comparable non-mucoid strain. A qualitative and quantitative analysis of biofilm architecture shows that the non-mucoid strain (PDO300) forms microcolonies very

early in biofilm development, although fewer cells are attached to the substratum at the initial attachment stage.

EPS has also been shown to adsorb dissolved organic compounds, such as diclofop methyl (a herbicide) and other xenobiotics, from the bulk fluid, thereby providing a mechanism by which the community can concentrate essential nutrients and growth components (Wolfaardt *et al.*, 1998). The EPS matrix also has the potential to physically prevent access of certain antimicrobial agents into the biofilms. Alginate enhances resistance to antimicrobial agents by acting as an ion exchanger, thereby restricting diffusion of compounds from the surrounding milieu into the biofilms, by binding the compounds and/or by inactivating them (Bayer *et al.*, 1991 & 1992; Gilbert *et al.*, 1997). This characteristic largely depends on the nature of both the agent and the EPS matrix. The effect appears to be most pronounced with the antibiotics that are hydrophilic and positively charged, such as aminoglycosides (Nichols *et al.*, 1988 & 1989). Hentzer *et al.* (2001) also showed that the mucoid biofilms are more resistant to the antibiotic tobramycin than biofilms formed by the non-mucoid strain. This difference in antibiotic susceptibility to tobramycin is not seen in planktonic cells of the mucoid strain (PAO1) and the non-mucoid strain (PDO300). This suggests that the alginate overproduction by PAO1 in the cystic fibrosis lung may play a role in forming biofilms that are more resistant to antimicrobial stresses at that site. In addition, the extrapolymeric matrix produced by biofilm bacteria has been shown to inhibit phagocytosis by cells of the immune system (Meluleni *et al.*, 1995). The host defense response to *Ps. aeruginosa* grown in biofilms is greatly reduced compared to free cells. EPS has also been reported to sequester metals, cations, and toxins (Flemming, 1993). An additional study by Ophir and Gutnick (1994) examined the role of EPS in protection from desiccation. They demonstrated that EPS can protect bacteria from dehydration, as it can bind several times

its own volume of water and only slowly becomes desiccated (Roberson & Firestone, 1992; Ophir & Gutnick, 1994). The presence of acetylated uronic acids in bacterial alginate increases its hydration capacity. In addition, it has been reported that EPS provides protection from a variety of environmental stresses, such as UV radiation, pH shifts and osmotic shock (Flemming, 1993). Elasir and Miller (1999) found that the EPS matrix protected the cells from DNA damage. Thus, alginate is a general protection mechanism for both individual bacteria and those associated with biofilm.

Collective Behaviour of Bacteria

Shapiro (1998) proposed the view of bacteria as interactive organisms capable of significant collective activity. Complex differentiation and collective behaviour have been demonstrated for a number of different microorganisms under a variety of different situations. Biofilms represent biological systems with a high level of organization, where bacteria form structured, coordinated, functional communities. Indeed, it has been suggested recently that such communities could be viewed holistically as a "*multicellular organism*" in which there is a cellular division of labour that may be facilitated by both specific spatial organization and sophisticated signaling networks among the member populations (Caldwell *et al.*, 1997a; Shapiro, 1998). In a number of articles, Caldwell *et al.* (1997a & b) have discussed the complex interactions that form the basis of coexistence in these sessile communities. Viewing bacteria from the biofilm community perspective is providing us with novel insights into microbial biology and ecology.

Nutrient Availability and Metabolic Cooperation

Confocal scanning laser microscopy (CSLM) showed that biofilms are highly hydrated open structures containing a high fraction of EPS and large void spaces between microcolonies (Lawrence *et al.*, 1991). *Ps. aeruginosa* biofilms formed on solid surfaces exposed to a continuous flow of liquid, develop into complex mushroom and stalk-like structures (Costerton *et al.*, 1995). The highly permeable water channels penetrating throughout the biofilm in the areas surrounding the microcolonies have been compared to a primitive circulatory system (Costerton *et al.*, 1994 & 1995; Davey & O'Toole, 2000). They provide an effective means of exchanging nutrients and metabolites with the bulk aqueous phase, enhancing nutrient availability as well as removal of potentially toxic metabolites (Costerton *et al.*, 1995). The metabolic characteristics of bacteria within a biofilm community are distinct from those of their planktonic counterparts. The elaborate architecture provides the opportunity for metabolic cooperation, and niches are formed within these spatially well-organized systems. Microenvironments can form, and due to the differing conditions within the biofilm, the cells become physiologically diverse (van Loosdrecht *et al.*, 1990). Consequently, the bacteria are exposed to an array of distinct environmental signals within a biofilm. For instance, cells situated near the centre of a microcolony are more likely to experience low oxygen tensions. Microelectrode measurements have shown that the oxygen concentration and pH fall in a biofilm as the substratum is approached (Xu *et al.*, 1998; Okabe *et al.*, 1999). In mixed biofilms, which are more representative of biofilms occurring in nature, bacteria distribute themselves according to who can survive best in the particular microenvironment and also, based on the symbiotic relationships that develop between the groups of bacteria present (Moller *et al.*, 1996 & 1998). Thus, the bacteria in multispecies biofilms are not randomly distributed, but organized to best meet the needs of each species within structure. For

example, the degradation of complex organic matter into methane and carbon dioxide during anaerobic digestion requires the interaction of at least three guilds of bacteria. Fermentative bacteria initiate the catabolism, producing acids and alcohols that are often readily utilized as substrates by acetogenic bacteria. Finally, the methanogens obtain energy from converting acetate, carbon dioxide, and hydrogen into methane (Schinck, 1997). In another model system, a much closer coupling between two metabolically interesting species was observed (Nielsen *et al.*, 2000). In this model system *Burkholderia sp.* LB4000 was capable of degrading 3-chlorobiphenyl to 3-chlorobenzoate, whereas *Pseudomonas* sp. B13 (FR1) could mineralize 3-chlorobenzoate, but was unable to degrade 3-chlorobiphenyl. When the consortium was grown on 3-chlorobiphenyl medium, it consisted predominantly of mixed colonies, but when it was grown on citrate medium (metabolisable by both organisms), it consisted predominantly of separate microcolonies of the two species. When a citrate-grown consortium was fed 3-chlorobiphenyl medium, the structure changed towards mixed microcolonies within two days after the substrate shift. Hence, very efficient cooperations and mutual dependence can evolve within a biofilm. In fact, biofilms provide an ideal environment for the establishment of syntrophic relationships (Schink, 1997; Okabe *et al.*, 1999). At the same time, biofilm formation may require coordination with, interactions of, and communication between multiple bacterial species (Kaiser & Losich, 1993; Davey & O'Toole, 2000). From the previous examples, one can demonstrate that the spatial organization of the different organisms in multispecies biofilms reflects syntrophic and microhabitat localized processes. However, it is not fully understood what controls the spatial organization of the microorganisms in biofilms, but at least two hypotheses, that are very different, but not mutually exclusive, have been offered.

The first hypothesis is mechanistic, which explains various structural forms in biofilms as a result of differences in local substrate availability (Wimpenny & Colasanti, 1997). According to this model, heterogeneous biofilm structures develop when biofilm growth occurs under substrate-transfer-limited conditions. Local consumption of substrate creates substrate gradients, whereby organisms situated on "*mounds*" have more substrate available than organisms situated in "*valleys*".

The second hypothesis is a morphogenetic one, which suggests that bacteria in biofilms differentiate and express genes that directly control the spatial organization of the organisms (Costerton *et al.*, 1995). It has been suggested that cell-to-cell communication plays a role in determining the spatial organization of the bacteria in sessile communities (Shapiro, 1998). Intercellular communications mediated by the quorum-sensing molecules (known as acyl-homoserine lactones (acyl-HSLs)) has been shown to be involved in regulation of specific gene expression as a function of population density, and could be an example of a community regulator (Costerton *et al.*, 1995; Shapiro, 1998). Quorum sensing is a mechanism used by a large number of Gram-negative bacteria to monitor their cell density and, in response, to regulate the expression of specific genes. As an example, *Ps. aeruginosa* uses quorum sensing to regulate virulence factor production (Latifi *et al.*, 1995 & 1996; De Kievit & Iglewski, 1999). Comparison of biofilms produced by a wild-type strain of *Ps. aeruginosa* with those of quorum-sensing mutants revealed that cell-to-cell signaling is required for the formation of a normal *Ps. aeruginosa* biofilm. A *Ps. aeruginosa* mutant deficient for synthesis of an acyl-HSLs produced flat and undifferentiated biofilms, but addition of the acyl-HSLs to the medium resulted in production of a structured biofilm typical of the wild-type strain (Davies *et al.*, 1998). Thus, *Ps. aeruginosa* uses quorum sensing to elaborate the complex structure.

Quorum sensing systems make use of two components: a small diffusible signaling molecule known as the autoinducer (AI) and a positive transcriptional activator, or R-protein. At low cell density the AI is produced at a basal level; however, as the cell density increases, so does the concentration of AI. Once a threshold level of AI is reached, sufficient AI/R-protein complexes accumulate, enabling the activation of target genes (Fuqua *et al.*, 1994; Bassler, 1999; De Kievit & Iglewski, 1999). Fuqua *et al.* (1994) described the minimum bacterial unit necessary to develop one of these cell-density responses as a quorum, and applied the term Quorum Sensing Transcriptional Activation to the process. Two distinct quorum-sensing systems have been identified in *Ps. aeruginosa*: the *las* system and the *rhl* system. The *las* system consists of the transcriptional activator *LasR* and its cognate AI, 3-oxo-C_{12}-HSL[N-(3-oxododecanoyl)-$_L$-homoserine lactone). The *rhl* system is composed of the transcriptional activator *Rh1R* and its cognate AI, C_4-HSL (N-butyryl-$_L$-homoserine lactone) (Latifi *et al.*, 1996). Data indicate that in the development of a normal *Ps. aeruginosa* biofilm, only the *las* quorum-sensing system is essential (Davies *et al.*, 1998). The *lasI* gene codes for an enzyme that directs the synthesis of N-(3-oxododecanoyl) L-homoserine lactone (the las system) (O'Toole *et al.*, 2000). Recently, acyl-homoserine lactones have been detected in naturally occurring biofilms (McLean *et al.*, 1997).

Although little is known of the role of intercellular signaling in multispecies biofilms, it may differ significantly from that observed in single-species biofilms. These signals may be especially important in favourable environments where surfaces are heavily colonized and competition for attachment to the surface is fierce. These signals may be defined broadly as any actively or passively transported bacterial products that alter the state of neighboring microbes (Gray, 1997). These might include bacterial metabolites, acyl-HSLs, secreted proteins, genetic material such as DNA or RNA, or as yet

undiscovered bacterial products (Watnick & Kolter, 2000). These signals may alter the distribution of specific bacterial species in the biofilm, alter protein expression in neighbouring cells, introduce new genetic traits into neighbouring cells, or lure and incorporate bacteria into the biofilm for subsequent consumption. The last function of intercellular communication in multi-species biofilms is both fascinating and as yet un-elucidated. However, there are laboratory models of lethal interspecies bacterial communication (Shi & Zusman, 1993; Riley, 1998). *Myxococcus xanthus*, for instance, is known to prey on *E. coli*. On soft agar plates, *E. coli* moves towards *M. xanthus*; its chemotactic machinery is required for this directed movement. The hypothesis is that *M. xanthus* secretes a signal that lures *E. coli* to its death (Shi & Zusman, 1993). The bacteriocins are another example of cell-cell signals that result in lethal interspecies interactions. These are bacterially derived antibacterial proteins that act against closely related species (Riley, 1998). In fact, mathematical models predict that bacteriocin production would be most advantageous in a spatially structured environment such as a biofilm (Durrett & Levin, 1997), suggesting that these secreted proteins may have evolved specifically for the biofilm environment. The impact of intercellular communication on multi-species biofilms is potentially far reaching, and whether it is beneficial or detrimental to the recipient, it is a critical factor in the diversity and distribution of bacteria in a biofilm.

The complexity of microbial community organization cannot be achieved by a single hypothesis or explanation. Although the mechanistic hypothesis does not take into account the cell mobility toward substrate (i.e. chemotaxis), the organization found (especially in syntrophic multispecies biofilms) suggests an active involvement of chemotactic motility during the development of community structures (Tolker-Nielson & Molin, 2000). However, the bacteria as individuals must react to nutrient gradients. At the

same time, bacterial signaling does occur and intercellular interactions of this type often affect the regulatory activities in the cells resulting in coordinated performances (Caldwell *et al.*, 1997b; Shapiro, 1998).

Biofilms and Antimicrobial Resistance

Many studies have focused on adhesion and biofilm formation by planktonic monocultures, e.g. *Ps. aeruginosa* (Costerton *et al.*, 1995). Although prominent in nature, much less information is known about mixed population biofilms. Bacteria do not have uniform colonization and physiological properties (Fletcher, 1991), a feature that enables them to utilize different ecological niches. Therefore, one would predict that increasing species diversity of planktonic bacterial communities would lead to increased species diversity and overall cell density within biofilms.

Growth as a biofilm serves to protect the bacteria (Davey & O'Toole, 2000). Resistance to antimicrobial agents is a general feature of all biofilms that are the major cause of recalcitrant infections (Gander, 1996; Costerton *et al.*, 1999). The host defense response to *Ps. aeruginosa* grown in biofilms is greatly reduced compared to that of free cells. The biofilm mode of growth protects the sessile bacteria from concentrations of antibiotics and biocides, which would kill planktonic cells (Stewart *et al.*, 1998). Biofilm bacteria can be up to 1000-fold more resistant to antibiotic treatment than the same microorganism grown planktonically (Gilbert *et al.*, 1997), but the mechanisms by which the biofilm-grown bacteria attain this resistance are still a matter of speculation.

Do biofilm cells express mechanisms that contribute to biofilm resistance?

Costerton *et al.* (1987) published a landmark review article that was the first to articulate the general phenomenon of biofilm resistance to antimicrobial agents. Fifteen

years later, the question of how bacteria in biofilms manage to evade killing by antiseptics, antibiotics and antimicrobial components of the host defences remains largely unanswered. Mah and O'Toole (2001) suggest the possibility that multiple resistance mechanisms operate in concert within a single biofilm community.

Mechanisms by which Biofilms Confer Resistance towards Antimicrobial Agents

Delayed penetration of the Antimicrobial

One obvious difference between planktonic cells and biofilm cells is the presence of polysaccharide matrix enveloping the community that retards diffusion of antimicrobials into the biofilm (Brooun *et al.*, 2000). This diffusion limitation may be the result of either transport limitation (i.e. the inability of the antimicrobial molecules to diffuse through the polymer matrix) or inactivation of the antimicrobial molecule by the matrix material. However, direct measurements of diffusion rates show that at least some antibiotics equilibrate throughout the biofilm within minutes or hours of initial exposure (Nichols *et al.*, 1989; Darouichie *et al.*, 1994; Stewart, 1996). *Ps. aeruginosa* expresses a β-lactamase, and a combination of retarded diffusion and an enzyme that destroys the antibiotic at the rate at which it arrives at the cell surface could explain resistance to ampicillin (Stewart, 1996). However, this would not explain resistance to quinolones, for example. Retarded diffusion alone can postpone, but not prevent the death of biofilm cells from quinolones. Suci *et al.* (1994) investigated the penetration of ciprofloxacin into *Ps. aeruginosa* biofilms. They demonstrated that penetration of the antibiotic was significantly impeded by the biofilm. Hoyle *et al.* (1992a) examined the ability of *Ps. aeruginosa* mucoid exopolysaccharide (MEP) to bind tobramycin. They found that tobramycin diffusion across the biofilm-fluid interface and into the biofilms was the primary reason that bacterial cells were dispersed from biofilms and that these dispersed cells were 15 times

more susceptible to tobramycin than were cells in the intact biofilms. However, other studies (Nichols *et al.*, 1989; Evans *et al.*, 1990a) found that tobramycin efficacy against biofilm cells could not be explained solely by the decreased diffusion into the biofilms. They proposed that the effect could also be due to the extremely slow growth rates of bacteria within the depths of the biofilm due to the depletion of organic nutrients, inorganic ions, and oxygen.

Alteration of the Cellular Growth Rate

A second mechanism of biofilm-reduced susceptibility requires that at least some of the cells within a biofilm experience a nutrient limitation that causes them to enter a slow-growing or starved state (Brown *et al.*, 1988 & 1990; Gilbert *et al.*, 1990). Bactericidal action generally decreases with lower growth rate, and slow growth is considered to be one of the components of biofilm resistance (Ashby *et al.*, 1994; Costerton *et al.*, 1999). Evans *et al.* (1990b) examined the effect of a quaternary ammonium compound (Cetrimide) on *E. coli* biofilms. The organisms were most resistant at the slowest growth rates. At growth rates above $0.3h^{-1}$, sessile and planktonic cultures were equally susceptible. Moreover, newly divided daughter populations were generally more sensitive than their heterogeneous donor cultures. This suggests some sensitisation of the cells at or shortly after division. Evans *et al.* (1991) also examined the effect of ciprofloxacin on biofilms of *Ps. aeruginosa* and *E. coli* using a similar test method. They found, for *Ps. aeruginosa*, that intact biofilm cells were more resistant than biofilm cells that had been removed from the surface and tested in suspension. However, biofilm growth rate *per se* had a negligible effect on susceptibility. Newly formed daughter cells, those that had recently detached from the biofilms, were more susceptible than other populations, indicating that ciprofloxacin activity was influenced by the bacterial cell cycle. This might be expected

due to the mode of action of this antibiotic on the DNA gyrases of the target cell. Other studies (Anwar *et al.*, 1992a) examined the interaction of *Ps. aeruginosa* with tobramycin and piperacillin and found that cells in older biofilms were significantly more resistant than those in younger biofilms of the same culture, suggesting that it may take some time for the particular properties of the biofilm to develop (Anwar *et al.*, 1992b). The cells of new biofilms are still metabolically active, few layers have formed and polysaccharide synthesis may not yet be maximal. All of these studies provide evidence that antimicrobial susceptibility is affected by cell growth rate and in some cases by phase of growth.

Biofilm Phenotype

The third mechanism of reduced biofilm susceptibility, which is more speculative than the preceding hypotheses, is that at least some of the cells in a biofilm adopt a distinct, and relatively protected, biofilm phenotype. This phenotype is not the result of a nutrient limitation. Whereas, the first two mechanisms both involve transport, in the first case for the antimicrobial agent itself and in the second case for a nutrient, there is no requirement for any transport limitation in the "*resistant biofilm phenotype*" hypothesis. The practical importance of this mechanism, should it prove true, is profound because it implies that reduced susceptibility of biofilm is genetically programmed (Cochran *et al.*, 2000a).

Clonal Selection of Resistant Phenotypes

With respect to treatment with many antibiotics, then for many agents (i.e. staphylococci and fusidic acids (Chopra, 1976); pneumococci and sulphonamides (Wolf & Hotchkiss, 1963); Enterobacteriaceae and triclosan (McMurray *et al.*, 1998a)), resistance can develop as a result of single point mutation. These often occur spontaneously with a frequency of approximately $1:10^8$ divisions. However, within a biofilm, such cells will not

only be protected against phagocytosis, but they will also be capable of rapid division when bathed in the degradation products of their dead neighbours. In this latter respect, the glycocalyx will act as a nutrient reservoir for the stressed community and circumvent the normal iron restriction of growth that would be imposed within a mammalian host. The appearance of sub-lethally treated biofilms with small clusters of surviving cells is supportive of the validity of clonal selection in biofilm resistance or tolerance development. However, clinical data do not support this view, since a number of studies have demonstrated that the susceptibility of recovered treated biofilms is not too dissimilar from that of the "wild-type" (Nickel *et al.*, 1985; Gristina *et al.*, 1987).

Multidrug Efflux Pumps: Their Contribution to Antibiotic Resistance in *Pseudomonas aeruginosa* Biofilms

All known organisms have multidrug resistance efflux pumps (MDRs) that can extrude chemically unrelated antimicrobials from the cell (Nikaido, 1996; Paulsen *et al.*, 1996). In *Ps. aeruginosa*, four efflux pumps have been described, MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY (Li *et* al., 1995; Kohler *et al.*, 1997). Among these four pumps, MexAB-OprM is the only one believed to be constitutively expressed in wild-type *Ps. aeruginosa*. As such, the MexAB-OprM MDR is largely responsible for high levels of "*intrinsic resistance*" of this microorganism to antibiotics, including tetracycline, chloramphenicol, quinolones, novobiocin, macrolides, trimethoprim, β-lactams and β-lactamase inhibitors (Poole, 1994; Li *et al.*, 1995; Nikaido, 1996). The pump extrudes antimicrobials across the outer membrane, which explains its ability to confer resistance to β-lactams that target the cell wall synthesis. The pump is composed of three different peptides: a MexB translocase belonging to the resistance-nodulation-division (RND) family of solute/proton antiporters, an outer membrane porin, OprM; and a membrane

fusion protein, MexA, that docks MexB to OprM (Gotoh *et al.*, 1995). The resistance of *Ps. aeruginosa* biofilms to numerous antimicrobial agents that are substrates subject to active efflux from planktonic cell suggests that efflux pumps may substantially contribute to the innate resistance of biofilms. However, biofilms can show very high levels of resistance (for example, a minimal bactericidal concentration of 1 mg mL^{-1} in the case of *Pseudomonas* and tobramycin), and it is unclear whether mechanisms operating in planktonic cells that confer significantly lower levels of resistance play a role in biofilms. De Kievit *et al.* (2001) examined the expression of two *Ps. aeruginosa* efflux pumps, MexAB-OprM and MexCD-OprJ, throughout the course of biofilm development. Furthermore, they assessed the contribution of four *Ps. aeruginosa* MDR efflux pumps to biofilm resistance to a number of antibiotics. A comparison of biofilms formed by cells in which MexAB-OprM was expressed, overexpressed, or completely absent demonstrated that the pump plays a minor role in resistance to aztreonam, gentamycin, tetracycline, and tobramycin. However, although the biofilm formed by the MexAB-OprM-overexpressing strain (OCR1) exhibited increased resistance to ciprofloxacin compared to the deletion strain, it was still more sensitive than the wild type. For the other three characterized efflux pumps of *Ps. aeruginosa*, in almost every instance, deletion/or hyperexpression of the pump did not markedly alter biofilm antibiotic resistance, with the one exception being the MexEF-OprN hyperexpression strain (K1240), which exhibited increased resistance to ciprofloxacin. Together, these findings indicated that elevated expression of the four MDR pumps play little role in the innate antibiotic resistance of *Ps. aeruginosa* biofilms. Maira-Litrán *et al.* (2000a) investigated the expression of the multiple antibiotic resistance (*mar*) efflux operon in *E. coli* biofilms. Their results suggested that while *mar* expression was greatest within the depths of a biofilm (where growth rates are suppressed), its probable induction within biofilms cannot explain the elevated levels of antibiotic resistance

observed.

Both De Kievit *et al.* (2001) and Maira-Litrán *et al.* (2000a) demonstrated that the expression of MDR pumps (for *Ps. aeruginosa*) and the *mar* efflux operon (for *E. coli*) were heterogeneous throughout the biofilm population, with maximal expression occurring in cells located at the substratum. In another study, Poole (1994) proposed that secondary metabolites are the natural substrates for the pumps. Therefore, increased expression of these pumps at this location (substratum) may be required to ensure sufficient efflux of secondary metabolites, thereby preventing toxic accumulation. These findings are similar to those of Brooun *et al.* (2000), who found no difference in *Ps. aeruginosa* biofilm resistance to ciprofloxacin between cells over expressing the MexAB-OprM pump and those lacking it.

From this discussion, one can confirm (to a certain extent) that the four characterized efflux pumps do not play a marked role in the antibiotic-resistance phenotype of *Ps. aeruginosa* biofilms, and thus the mechanisms underlying this phenomenon remain an enigma.

Biocides - The Other Antimicrobials

Biocides are inorganic or synthetic organic molecules used to protect materials or processes from microbiological degradation or to disinfect, sanitize, or sterilize objects or surfaces. They are used in a wide variety of processes and matrices: industrial process waters, cosmetics, shampoo, food, marine antifouling paints, plastics, wood, and swimming pools, to name but a few (Chapman, personal communication).

Biocides exert their antimicrobial action against targets, which are less specific than those commonly associated with antibiotics. Several factors are known to influence the activity of the active ingredients in antiseptics, disinfectants, and preserved products

(Collective term used here: biocides) (Russell & McDonnell, 2000). These include the period of contact with microorganisms, presence of interfering materials (e.g. organic or inorganic soils), formulation effects, temperature of contact and the nature and type of target microorganisms (Denyer & Stewart, 1998). In addition, the efficacy of antimicrobial products depends on, and varies significantly with, formulation effects, which can often enhance activity despite the presence of lower levels of the biocide (McDonnell & Russell, 1999). All these factors are important in evaluating the modes of action of, and mechanisms of resistance to, biocides and are often regrettably overlooked. Their mechanisms of action can be divided into two broad categories:

I. **Electrophiles**, which are divided into:

- Oxidants (e.g. Halogen and Peroxy Compounds).
- Electrophiles (e.g. Formaldehyde, Formaldehyde-releasers, Isothiazolones, Bronopol and Cu, Hg, Ag compounds).

II. **Membrane Active**, which is divided into:

- Lytic (e.g. Quaternary Ammonium Compounds, Biguanides, Phenols and Alcohols).
- Protonophores (e.g. Parabens, Weak Acids and Pyrithiones).

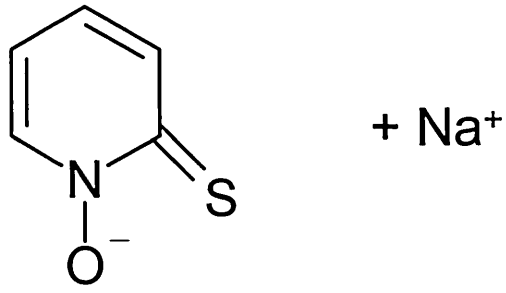
Throughout this project, two different types of biocides have been used; the Pyrithiones and the Isothiazolones.

The Pyrithione Biocides

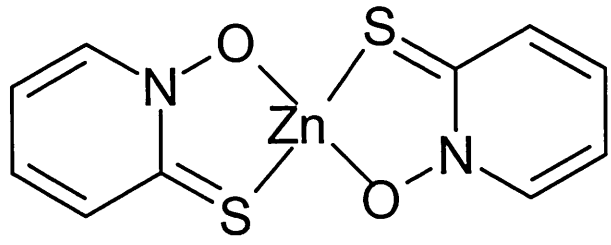
Sodium Pyrithione and Zinc Pyrithione (NaPT & ZnPT, respectively, also known as sodium and zinc omadine) are the sodium salt and zinc chelate of 1-hydroxypyridine-2-thione (hydroxypyrithione) (Shaw *et al.*, 1950). Sodium Pyrithione exists as a pyrithione anion and a sodium cation when solubilized (Nelson & Hyde, 1981; Dinning *et al.*, 1998a;

Figure 4). Zinc Pyrithione exists in the monomeric form as two pyridine rings bound to a central zinc atom by bonds between the zinc atom and the sulphur and oxygen molecules of the pyridine ring structures (Nelson & Hyde, 1981; Dinning *et al.*, 1998a; **Figure 4**). Zinc Pyrithione may also exist as a dimer (Barnett *et al.*, 1977; Dinning *et al.*, 1998b). In the dimeric form, the zinc atoms of the two monomers have valencies of five, allowing two monomers to become adjoined *via* zinc-oxygen bridges (Zn_2PT_4). ZnPT and NaPT are the most abundantly used of the pyrithione group of antimicrobial compounds. Both of them have been shown to possess a wide spectrum of antimicrobial activity against fungal and bacterial species (Khattar *et al.*, 1988). They are widely applied in the cosmetics and fuel industries as preservatives (Nelson & Hyde, 1981; Khattar & Salt, 1993) and are incorporated as anti-dandruff agents in shampoos (Nakajima *et al.*, 1993). Both NaPT and ZnPT exhibit excellent metal chelating properties and ZnPT is the most utilized metal chelate of hydroxypyrithione (Marks *et al.*, 1985). The behaviour of NaPT and ZnPT in solution enables transchelation to occur when these compounds are in the presence of transition metal ions (i.e. Cu^{2+} and Fe^{3+}) (Edrissi *et al.*, 1971). As a result of their metal chelating properties, they are also employed in the mining industry for the extraction of metals from ore samples.

Membrane activity by NaPT and ZnPT has been exhibited in both eukaryotic and prokaryotic microorganisms. Dinning *et al.* (1998c) developed a spectrophotometric assay for the quantification of the pyrithiones, which was based on the chelation of copper II ions by the two biocides (ZnPT & NaPT). From this assay, Dinning and colleagues succeeded in determining the distribution of these biocides in the Gram-negative bacteria *Escherichia coli* NCIMB 10000 and *Pseudomonas aeruginosa* NCIMB 10548. They founded that sodium pyrithione was exhibited only in the cytosol of these two microorganisms. However, ZnPT was assayed in the cytosol of both bacteria and was



Sodium Pyrithione (NaPT)



Zinc Pyrithione (ZnPT)

Figure 4: Schematic chemical structures of Sodium Pyrithione (NaPT) and Zinc Pyrithione (ZnPT).

found in the cell envelope of *Ps. aeruginosa*. These findings suggest that the pyrithione biocides are active within bacterial cells as well as at the cell membrane. Khattar *et al.* (1988 & 1989) reported the inhibition of thymidine and uracil transport processes by the pyrithiones. Other studies (Dinning *et al.*, 1998a) showed that NaPT and ZnPT are poor inhibitors of substrate catabolism. On the other hand, the sub-minimum inhibitory concentrations (sub-MICs) of biocide greatly reduce intracellular ATP levels in both *E. coli* and *Ps. aeruginosa*. This is thought to be due to the action of NaPT and ZnPT on the Gram-negative bacterial membrane. In another study, Dinning *et al.* (1998b) reported the interactions between the pyrithiones and the bacterial phospholipid head group structures, at both a practical and a theoretical level. From the molecular modeling, Dinning observed stable interactions for both NaPT and ZnPT (monomer and dimer forms) with the bacterial membrane phospholipid phosphatidylethanolamine of *Ps. aeruginosa* and *E. coli*. Hydrogen bonding between the phospholipid head group and NaPT was observed. ZnPT interacts more strongly with the phospholipid head group than NaPT, suggesting interactions with orthophosphoric acids group and ammonium groups of the phospholipid head group.

Finally, an article by Al-Adham *et al.* (1998) suggested that both pyrithiones induce the leakage of intracellular material (potassium ions and OD_{260nm} absorbing material) from exposed cultures of *E. coli* and *Ps. aeruginosa*, as well as affecting nutrient uptake in bacteria by the inhibition of membrane-bound metabolic processes.

Cetrimide, which is employed extensively in urology and gynaecology as an antiseptic in the form of aqueous and alcoholic solution is also one of the membrane active biocides. It was used throughout this project as a positive, membrane active control.

BIT and its Mode of Action

Benzisothiazolone (BIT) is one of the non-chlorinated isothiazolones, which are widely used as industrial and environmental biocides (Collier *et al.*, 1991). BIT (the basis of the Proxel range of biocides) is a skin sensitising agent, a property that prevents its use in pharmaceutical, cosmetic and toiletry preparations. The commercial product can be used as a preservative in a variety of products, for example paints, adhesives and detergents, and it rapidly degrades in the environment (Anon, 1996). Benzisothiazolone can effectively control microbial growth in metal-working fluids, where bacterial counts can be reduced to zero after a four-day contact period (Singer, 1976). The antimicrobial activity of BIT is strongly antagonised by exogenous thiol-containing agents. The biocide is thought to interact oxidatively with accessible thiols within the cell (Fuller *et al.*, 1985; Collier *et al.*, 1990a & c). For thiol-interactive agents, the major interactive sites are within the cytosol (e.g. glutathione, cysteine etc), with relatively little thiol being situated at the cell surface/membrane. In prokaryotic cells many important cellular functions are located at the cell envelope, which is enriched in thiol group, relative to eukaryotic cells. Glutathione (GSH) is the most abundant thiol-containing target in microbial cells at >10 mM (Owens & Hartman, 1986), and will probably be the major target for BIT, disrupting intracellular redox balance (Collier *et al.*, 1991; Fuller *et al.*, 1985). BIT does not possess significant bactericidal property and it is primarily bacteriostatic in action (Collier *et al.*, 1990a).

Fuller *et al.* (1985) identified S-(2-carbamoylphenylthio)-y-L-glutamyl-L-cysteinyl-glycine (**Figure 5**; Collier *et al.*, 1990b) as an initial by-product of the interaction between glutathione (GSH) and BIT and suggested that bacteria reacted with most thiol containing compounds to form disulphide adjuncts. Further interaction with thiols caused the release of oxidized thiol dimers and a reduced, ring-opened form of BIT (mercaptobenzamide). In

an analogous fashion to the initial reaction with thiol the mercaptobenzamide was able to provide further sources of thiol for the unreacted biocide to give BIT dimers. For BIT, the reversibility of thiol-adjunct formation would favour partitioning of the agent to the cytosol (Collier *et al.*, 1990b & c).

Thiomersal (merthiolate; Sodium-o-(ethylmercurithio)-benzoate) is also a thiol-interactive agent. It 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 and Gilbert, 1991). Thiomersal was used as a thiol interactive positive control agent in this study.

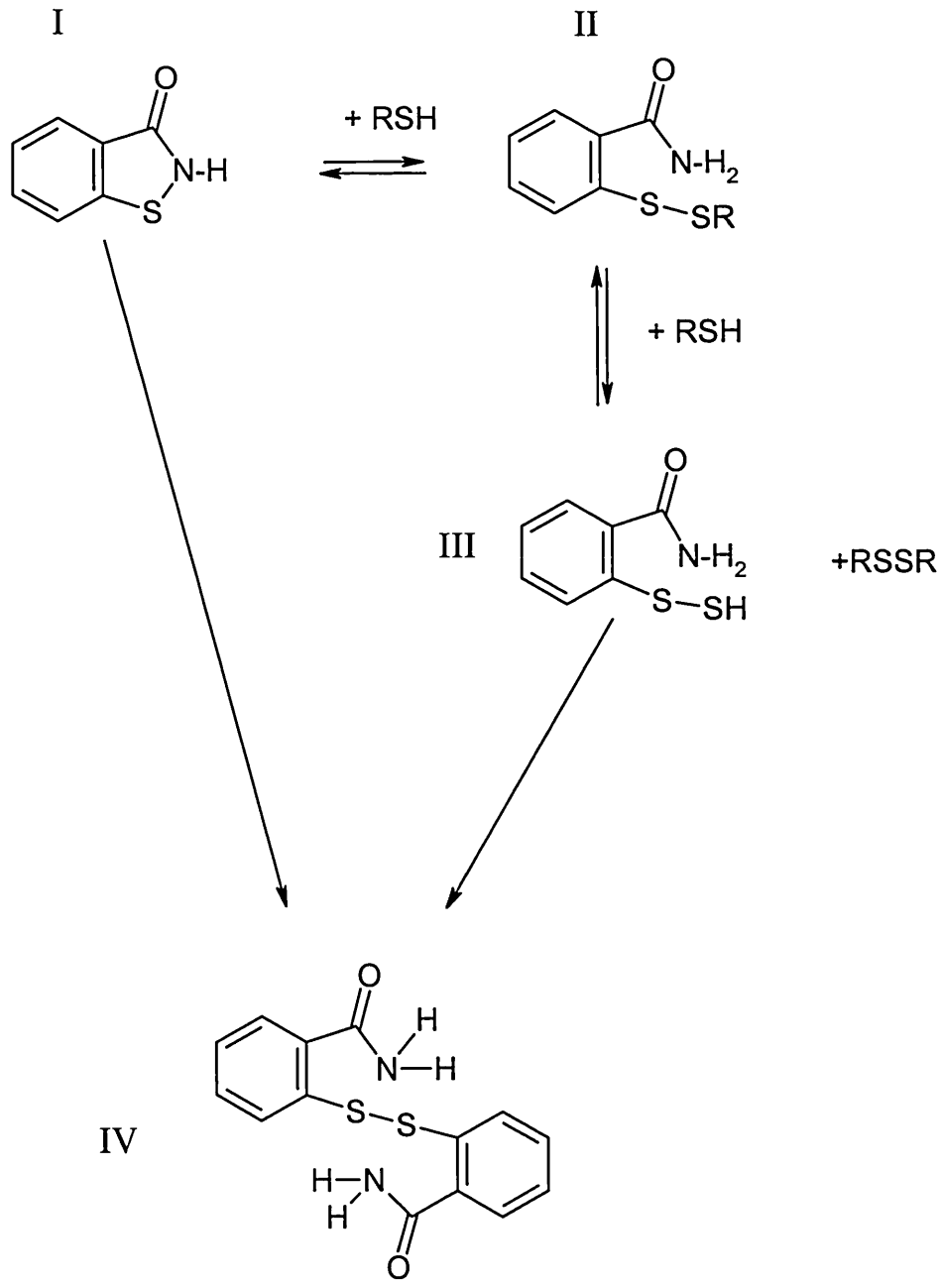


Figure 5: Schematic reaction pathway proposed by Fuller (1986) for the interaction of a thiol group (RSH) with benzisothiazolone; I, BIT. II, reacted BIT (S-(2-carbamoylphenylthio)-y-L-glutamyl-L-cysteinyl-glycine(uBIT)). III, mercaptobenzamide (uBIT). IV, BIT dimer (Collier *et al.*, 1990b).

Resistance towards Biocides

Biocide resistance is a little studied field, especially when compared to antibiotics, due mainly to economics. The speciality chemical industry cannot afford to invest resources to the same extent as the pharmaceutical industry. On the other side, microbial biofilms are notorious for their high level of resistance towards biocidal treatments (Brown & Gilbert, 1993; Gilbert *et al.*, 2001). The mechanisms associated with such resistance are complex. These mechanisms involve not only the reaction-diffusion limitation of biocide-access to the underlying cells (Stewart, 1994; Stewart *et al.*, 1994; Huang *et al.*, 1995), but also the expression of spatially heterogeneous, less susceptible phenotypes, caused either by growth as a biofilm *per se* (Brown *et al.*, 1988; Gilbert *et al.*, 1990) or through the expression of high cell density (Davies *et al.*, 1998), or starvation, phenotypes (Foley *et al.*, 1999). Unfortunately, there are not enough studies, which generate kinetic data that are capable of systematic evaluation of the relative extent of biofilm recalcitrance across a range of chemical biocides. Das *et al.* (1998) used a microtitre plate methodology, which showed that the susceptibility of *Staphylococcus epidermidis* and *E. coli* changed markedly within minutes of cellular attachment to a surface and that the extent of change was dependent upon the nature of the biocidal agent.

The Sorbarod Model: A Simple *in vitro* Model for Growth Control of Bacterial Biofilms (Hodgson *et al.*, 1995)

Researchers have developed many techniques to control the growth of biofilms. These models include the Robbins Device (McCoy *et al.*, 1981), inoculated discs (Prosser *et al.*, 1987), the Constant Thickness Biofilm Fermenter (Wimpenny *et al.*, 1989) and the Perfused Biofilm Fermenter (Gilbert *et al.*, 1989). However, many of these protocols suffer from a lack of appropriate planktonic controls (Brown *et al.*, 1988), which are, in general, broth cultures growing significantly faster than the biofilms. Therefore, these techniques cannot differentiate between the effects of growth rate and adherent growth. Controlling the growth rate in adherent populations is only possible with Perfused Biofilm Fermenter (Gilbert *et al.*, 1989). In this approach the growth rates may be standardized for both the attached and eluate populations. Although, it was shown by Hodgson *et al.* (1995), that this technique is unsuitable for the long-term culture and growth rate control of *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms. Based on the Perfused Biofilm Fermenter, Hodgson and colleagues developed a simplified method, the Sorbarod model. Relatively high cell numbers can be harvested from this model, which facilitates biochemical investigations of biofilm populations and the related eluate cells, which are released spontaneously from them.

Biofilm culture on Sorbarod (**Figure 6**)

This model was first developed as supports for micropropagation of plant tissue (Conkie, 1988; Donkin & Price, 1989), but it has not previously been used in bacteriology. The Sorbarod biofilm model is composed of silicone "PVC" tubing containing a single Sorbarod. A Sorbarod filter consists of a cylindrical paper sleeve, encasing compacted concertina of cellulose fibres. The packed cellulose filling provides a large surface area for

bacterial adhesion whilst the extensive inter-fibre spaces avert system blockage. Biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa* were grown within Sorbarod filter plugs, which were perfused with culture medium. Over several days pseudo-steady state was established; the growth rates obtained for the biofilms were reproducible, measurable and similar to likely *in vivo* growth rates (Brown & Williams, 1985b). For each experiment cultured Sorbarods were sacrificed periodically and viable counts performed to determine the numbers of adherent cells. The high yield of cells from the Sorbarod method generates sufficient material for biochemical analysis. Using SDS-PAGE technique, significant differences have been seen. These include, in *Staphylococcus aureus,* the repression of 48 kD cell envelope protein in Sorbarod grown attached populations and cells eluted from the Sorbarod, relative to the planktonic populations. Also, an increased expression of 21 kD protein was observed in biofilm and eluted populations. The simplicity of this device makes it easy to establish and operate in large numbers in any standard microbiology laboratory. This gives the approach great potential for use in screens for biofilm activity, and the design and testing of biocides and antibiotics.

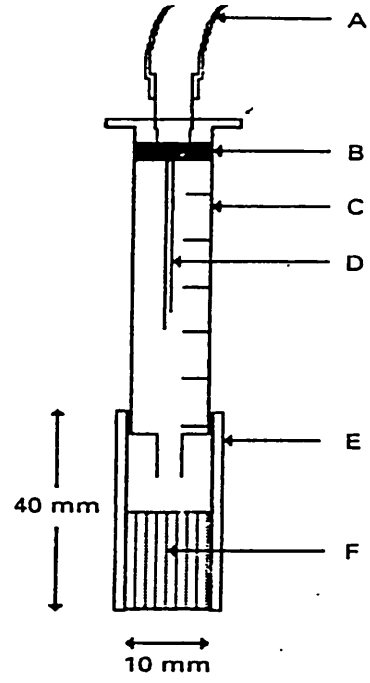


Figure 6: Schematic diagram of the Sorbarod biofilm model showing silicone tubing to peristaltic pump and media vessel (A), rubber plunger seal (B), 2 mL disposable sterile syringe (C), syringe needle (D), PVC tubing (E) and Sorbarod (F) (Hodgson *et al.*, 1995).

Resistance of *Pseudomonas aeruginosa* to Isothiazolones as Observed by Brözel and Cloete (1994)

Previous work by Brözel and Cloete (1994) suggested that exposure of *Pseudomonas aeruginosa* PAO1 to Kathon^{TM} (a mixture of 1.15% 5-chloro-N-methylisothiazolone and 0.35% N-methylisothiazolone, CMIT & MIT respectively), results in a gradual increase in minimum inhibitory concentrations (MIC) to increasing

concentrations of these biocides. They proposed that *Ps. aeruginosa* acquires resistance to isothiazolone by a process of adaptation, such that the outer membrane protein T (T-OMP) is suppressed. This protein was not detected in resistant or induced cultures. Production of T-OMP was suppressed upon 24-hour exposure to isothiazolone. It was also undetected in resistant cells cultured for three consecutive times (72 hours) in the absence of isothiazolone. This could be due to a phenotypic switch to the suppressive state. A phenotypic adaptation is an alteration, which demonstrates a change in response to environmental stimuli. This phenotypic adaptation has a genetic base, but does not necessarily require a genetic alteration.

Brözel and Cloete (1994) gave two possible explanations for the increased resistance due to the absence of 35 kD outer membrane protein (T-OMP). The first was that T-OMP itself is a more susceptible target to reaction with isothiazolone than are the other outer membrane proteins, resulting in the subsequent loss of protein function. In their second explanation, Brözel and Cloete assume that the isothiazolone might be a structural analogue to a T-OMP substrate. Therefore, it could enter through the outer membrane *via* T-OMP. Nikaido (1992) demonstrated that most outer membrane proteins of *Ps. aeruginosa* have limited rates of substrate uptake. If we accept the assumption that isothiazolone is a T-OMP substrate analogue, then its rate of uptake would be limited. This explains why isothiazolone is bacteriostatic at lower concentrations. Therefore, cells deficient in T-OMP (Opr T) would be more resistant to isothiazolone as they lacked the port of entry for the bactericide. Winder *et al.* (2000) observed a similar increase in the minimum inhibitory concentrations between sensitive and resistant cultures against the isothiazolone biocides. However, there was a reduction in the MIC within 72 hours of passaging in the absence of biocide. Analysis of the outer membrane profiles (OMP) from sensitive and resistant cells illustrated the loss of T-OMP in the tolerant cells. The protein

was suppressed within the early passages in the presence of the isothiazolone biocide and reappeared within the 72 hours of passaging in the absence of biocides. Therefore, Winder *et al.* (2000) suggested that the loss of T-OMP is associated with the observed resistance towards the isothiazolone biocide, but it was not the sole cause of that resistance. In another study, Abdel Malek *et al.* (2002) attempted to repeat this observed increase in resistance with the biocides, NaPT and ZnPT, and the positive control, Cetrimide. The observed pattern of resistance was similar to that for the isothiazolone biocides, wherein there was a step-wise increase in resistance corresponding to the sub-MIC of biocide applied. However, upon removal of biocide the observed MIC did not return to the pre-exposure value, suggesting that the developed resistance is irreversible. The onset and development of resistance was accompanied by the loss of T-OMP from outer membrane profiles, which suggested that this is a non-specific membrane channel, whose production within the cell is sensitive to biocide. The T-OMP was observed to reappear when the cells were passaged in the absence of pyrithione. These results indicate that the pyrithione biocides have similar resistance profiles in *Ps. aeruginosa* to those exhibited by the isothiazolones, but the acquired resistance to the pyrithiones is irreversible.

Aims

The aims of this study were to investigate interactions in monoculture and mixed species biofilms and subsequent susceptibility of component species to selected antimicrobial agents (including Pyrithiones and Isothiazolones). The examination of the growth characteristics of *Pseudomonas aeruginosa* PAO1 and *Escherichia coli* 10000 (planktonic and biofilm cultures), and the establishment of the most suitable method of minimum inhibitory concentration (MIC) of biocide determination must be achieved in order to estimate the development of resistance in monoculture biofilms. However, as naturally occurring bacterial biofilms rarely occur as monocultures, it is important to determine a suitable method for induction of resistance towards these biocides and to investigate the relative abundance of species within binary biofilms (*Ps. aeruginosa* and *E. coli*). Cell components of both eluate and biofilm cells (resistant and control cells) will then be observed *via* sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and two-dimensional (2D-PAGE) gel electrophoresis for changes in protein expression, which may explain the development of resistance to antibiotics in the medically important bacterium, *Ps. aeruginosa*.

Chapter Two:

General Experimental Methods

Protocols for Bacterial Growth and Maintenance of Cultures

Maintenance and Growth of Cultures

Pseudomonas aeruginosa ATCC 15692 (PAO1) and *Escherichia coli* ATCC 10000 were used throughout the project. The original cultures of these two bacteria were obtained from the National Collections of Industrial and Marine Bacteria (NCIMB), Aberdeen, UK. Cultures of these organisms were maintained on nutrient agar (Oxoid CM3) slopes in quadruplicate. The four-labelled slopes were numbered from 1 to 4. Slope number 1 was used to inoculate overnight cultures for experimental purposes and slope number 2 was the backup slope in case of contamination. Slope number 3 was used for further subculturing and slope number 4 was kept as part of a stock culture collection. The maintenance of culture in quadruplicate ensured that the stock bacterial cultures were not ruined by a single occurrence of contamination. Inoculated agar slopes were maintained at room temperature, in a darkened cupboard, following incubation at 37°C for 24 hours. These were replaced at monthly intervals. Cultures were grown in R2A media supplemented with 1% glycerol (Reasoner and Geldrich, 1985) and chemically defined media (CDM, replacing 0.5% succinic acid with glycerol; Dinning, 1995) for 24h at 37°C in an orbital incubator (Gallenkamp, INA-305) at 190 rpm. Culture identification was determined using Gram-stain reaction and the API 20 NE test (Biomerieux, France).

Reagents

Sigma, Poole, Dorset: Ammonium persulphate; casein; coomassie brilliant blue R-250; dithiothreitol; 5,5'-dithio-bis (2-nitrobenzoic acid); DNase; ethylenediaminetetra-acetic acid; glutathione; glycerol; lysostaphin; N-laurylsarcosine; 2-mercaptoethanol; phenyl-methyl-sulphonyl-fluoride (PMSF); protease peptone number 3; Pyruvic acid; RNase; sodium carbonate; sodium thiosulphate; soluble starch; Thiomersal; TEMED; TRIZMA base; TRIZMA hydrochloride; N-P- $_{P}$-tosyl-$_{L}$-lysine chloromethyl ketone (TLCK); urea; yeast extract.

BDH Chemicals, Poole, Dorset: ammonium chloride; calcium chloride ($CaCl_2$); D-glucose; dipotassium orthophosphate; ethanol; ferric ammonium citrate; glycerol; hydrochloric acid (HCl); Igepal CA-630; magnesium sulphate heptahydrate ($MgSO_4.7H_2O$); potassium dihydrogen phosphate (KH_2PO_4); Resolyte (pH 3.5 to 10.0); Resolyte (pH 4.0 to 8.0); silver nitrate; sodium chloride (NaCl); sodium hydroxide (NaOH).

Fisher, Scientific UK Ltd (Loughborough, Leics): glacial acetic acid; methanol (HPLC).

Zinc Pyrithione was a kind gift from Zeneca Specialities plc (Biocides Research, Blackley, Manchester) and Nipa Ltd (BIT). Cetrimide was supplied by Rhone-Poulenc (Cheshire) and Sodium Pyrithione from Fluka.

Sterilisation and Preparation of Media

R2A medium

This medium gives excellent plate counts, but appears inadequate for laboratory subculture of bacterial isolates. It was prepared according to the Reasoner and Geldrich (1985) protocol, supplemented with 1% glycerol. It is similar to modified Henria medium and casein-peptone starch medium, but does not contain Fe as $FeCl_3$ or $FeSO_4$. R2A medium was prepared by adding 0.5g yeast extract, 0.5g protease peptone, 0.5g casein acid hydrolysate, 0.5g glucose, 0.5g soluble starch, 0.3g sodium pyruvate, 0.3g K_2HPO_4 and 0.05g $MgSO_4.7H_2O$ to 900 mL deionised water. The volume was made up to 1L by distilled water. The pH was adjusted to 7.2 with either crystalline K_2HPO_4 (increase the pH) or KH_2PO_4 (decrease the pH). 1.5% technical agar (Oxoid number 3) was added as a gelling agent when required (pouring plate). This medium was sterilized by autoclaving at 121°C, 15 psi for 15 minutes.

Chemically defined medium (CDM)

CDM was prepared according to Dinning (1995), replacing 0.5% succinic acid with glycerol. This medium is made up from 4 solutions; solution A, solution B, solution C and solution D. Solution A was prepared by adding K_2HPO_4 (2.56g), KH_2PO_4 (2.08g) and NH_4Cl (1.00g) to 900 mL of distilled water. The volume was made up to 1L with distilled water. The pH was adjusted to 6.8 with either 1M HCl or 1M NaOH. The solution was sterilized by autoclaving at 121°C, 15 psi for 15 minutes. Solution B was prepared by adding ferric ammonium citrate (1.00g) and $CaCl_2$ (0.1g) to 100 mL of distilled water. This was sterilized by filtration through a 0.22 μm pore size cellulose acetate filter (Merck Eurolab Ltd,

Lutterworth) under vacuum. Solution C is a 1M glycerol solution and was prepared by adding 46.45g of glycerol to 400 mL of distilled water. The volume was made up to 500 mL with deionised water. The pH was adjusted to 6.0 with the addition of either 0.1M HCl or 0.1M NaOH. This solution was sterilized by autoclaving at 121°C, 15 psi for 15 minutes. Finally, solution D was prepared by adding $MgSO_4.7H_2O$ (0.5g) to 900 mL distilled water. This was made up to 1L by distilled water. The solution was sterilized by autoclaving at 121°C, 15 psi for 15 minutes. The 4 solutions were prepared, and CDM was completed by the aseptic addition of 5 mL solution B, 15 mL solution C and 10 mL solution D to 1L solution A. As a gelling agent, 15.45g technical agar (Oxoid number 3) was added when required.

Chromogenic *E. coli*/Coliform Medium

Chromogenic *E. coli*/Coliform Medium CM956 (Oxoid LTD., Basingstoke, Hampshire, England) is a differential agar, which provides presumptive identification of *E. coli* and coliforms in food and environmental samples. The agar base uses two enzyme substrates to improve differentiation between *E. coli* and other coliforms. One chromogen allows specific detection of *E. coli* through the formation of purple colonies. This substrate is cleaved by the enzyme glucuronidase, which is produced by approximately 97% of *E. coli* strains. The other chromogen is cleaved by the enzyme galactosidase, which is produced by the majority of coliforms, resulting in rose/pink colonies (Sartory & Howard, 1992).

This medium has been used in this project to differentiate between two types of bacteria: *Ps. aeruginosa*, which gives straw colonies and *E. coli*, which gives purple colonies (**Figure 7**). Aliquots (55.8g) of this medium were suspended in 1L of distilled water. This medium was sterilized by autoclaving at 121°C, 15 psi for 15 minutes.

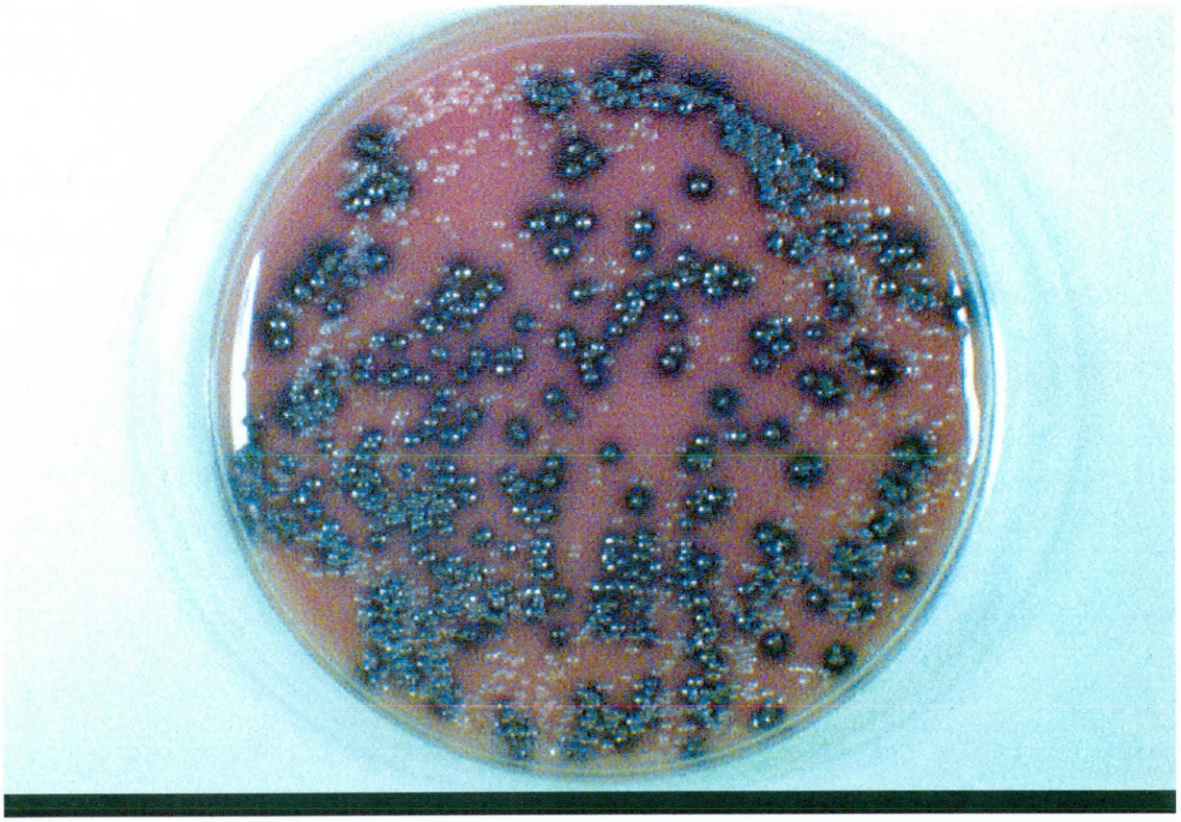


Figure 7: Chromogenic *E. coli*/Coliform plate showing two different coloured colonies; the purple ones represent *E. coli* ATCC 10000 and the straw ones represent *Ps. aeruginosa* PAO1.

Methods for Isolating Pure Cultures

Streak Plate

In the streak plate technique, which is a commonly used method for isolating pure cultures of bacteria, a loopful of bacterial cells is streaked across the surface of a sterile solidified agar plate that contains a nutrient medium. The plates are then incubated under favourable conditions to permit the growth of the bacteria. The key principle of this method is that, by streaking, a dilution gradient is established across the face of the plate as bacterial cells are deposited on the agar surface. Because of this dilution gradient, confluent growth occurs on part of the plate where the bacterial cells are not sufficiently separated and individual isolated colonies develop in other regions of the plate where few enough bacteria are deposited to form separate microscopic colonies that can easily be seen with the naked eye. Each well-isolated colony is assumed to arise from a single bacterium and therefore to represent a clone of a pure culture.

Spread Plate

In the spread plate method a drop of a liquid containing a suspension of microorganisms is placed on the centre of an agar plate and spread over the surface of the agar, using a sterile glass rod. The glass rod is normally sterilized by being dipped in alcohol and flamed to burn off the alcohol. When the suspension is spread over the plate, individual microorganisms are separated from others in the suspension and are deposited at discrete locations. To accomplish this separation, it is often necessary to dilute the suspension before application to the agar plate to prevent overcrowding and the formation of confluent growth rather than the desired

development of isolated colonies. After incubation, isolated colonies are picked up and streaked onto a fresh medium to ensure purity.

Enumeration of Bacteria

In order to assess the rate of microbial reproduction, it is necessary to determine the numbers of microorganisms present. There are a variety of methods that can be employed for enumerating bacteria including viable counting and turbidometric procedures.

Viable Count Procedures

The viable count plate method is one of the most common procedures for the enumeration of bacteria. In this procedure, serial dilutions of a suspension of bacteria are plated onto a suitable solid growth medium. The dilution procedure influences the overall counting process. The plates are incubated to allow the bacteria to grow and form colonies. Multiplication of a bacterium on a solid medium results in the formation of a small colony visible to the naked eye. The formation of visible colonies generally takes 16 to 24 hours. It is assumed that each colony arises from an individual bacterial cell. Therefore, by counting the number of colonies that develop **colony-forming units (CFUs)** and by taking into account the dilution factors, the concentration of the bacteria in the original sample can be determined. Countable plates are those having between 30 and 300 colonies. Fewer than 30 colonies are not acceptable for statistical reasons, and more than 300 colonies on a plate are likely to produce colonies too close to each other to be distinguished as individual CFUs (Roszak & Colwell, 1987a, Dupray *et al.*, 1993; Salvesen & Vadstain, 2000).

Turbidometric Procedures

Because particles within certain size limits scatter light in proportion to their concentration, when a beam of light passes through a suspension of bacteria, the amount of light transmitted is reduced due to the turbidity of the solution. Measuring the amount of light that passes through a suspension of microorganisms with a spectrophotometer can be used for estimating cell mass, since the amount of light absorbed or scattered by the microorganisms is proportional to the cell density. Spectrophotometers provide an accurate and rapid way to estimate the dry weight (mass) of bacteria per unit volume of culture. An increase in cell mass, which can be equated with increases in the number of bacterial cells, is useful for establishing a growth curve for a bacterial population (Atlas, 1988; Fuchs & Kroger, 1999).

Agar Diffusion Technique

A useful modification of the agar diffusion assay, which allows direct determination of MICs in the gradient plate method. For this assay, square petri dishes, y cm across (BDH; 20 cm x 20 cm), were required. The plates were labelled and CDM agar (25 mL) containing a known concentration of antimicrobial agent was added (1.5 x MIC conc. of biocide). The agar was allowed to set in the form of a wedge and the plates are then laid flat. A second 25 mL volume of the CDM agar (without the addition of biocide) was poured at the surface of the first layer and allowed to set in an upright position. Plates were then stored to allow diffusion of biocide to occur, giving a concentration gradient across the plates. The surface of the plate was then inoculated with biocide resistant *Pseudomonas aeruginosa* or *Escherichia coli.* Following incubation for 24 hours, the zone of inhibition (**x**) was measured and the MIC calculated from **Equation 1:**

$$\text{MIC } (\mu g\ mL^{-1}) = ((y-x) / y) \,.\, [B] \qquad \textbf{(Equation 1)}$$

Where [B] = concentration of biocide ($\mu g\ mL^{-1}$)

The point, at which growth was ceased, indicated the MIC.

Validation of the Flow Rate in a Peristaltic Pump

The peristaltic pump (Autoclude model VL) is essential equipment in this project in order to accurately deliver known volumes and flow rates of media to Sorbarod biofilms. Therefore, it was necessary to undertake an accurate calibration procedure. Four types of silicone peristaltic pump tubes were used, each with different diameter. The arbitrary speed range was set between 0 and 99 and the volume of water throughput was measured into a volumetric cylinder. This step was repeated for 4 tubes with 21 different speed settings. Five replicate measurements for each speed were made with one-minute intervals between each measurement. The volume of the water was plotted against the speed to produce a standard curve (**Figure 8**). This data was used to determine which combination of tube and peristaltic pump speed were used in subsequent biofilm experiments.

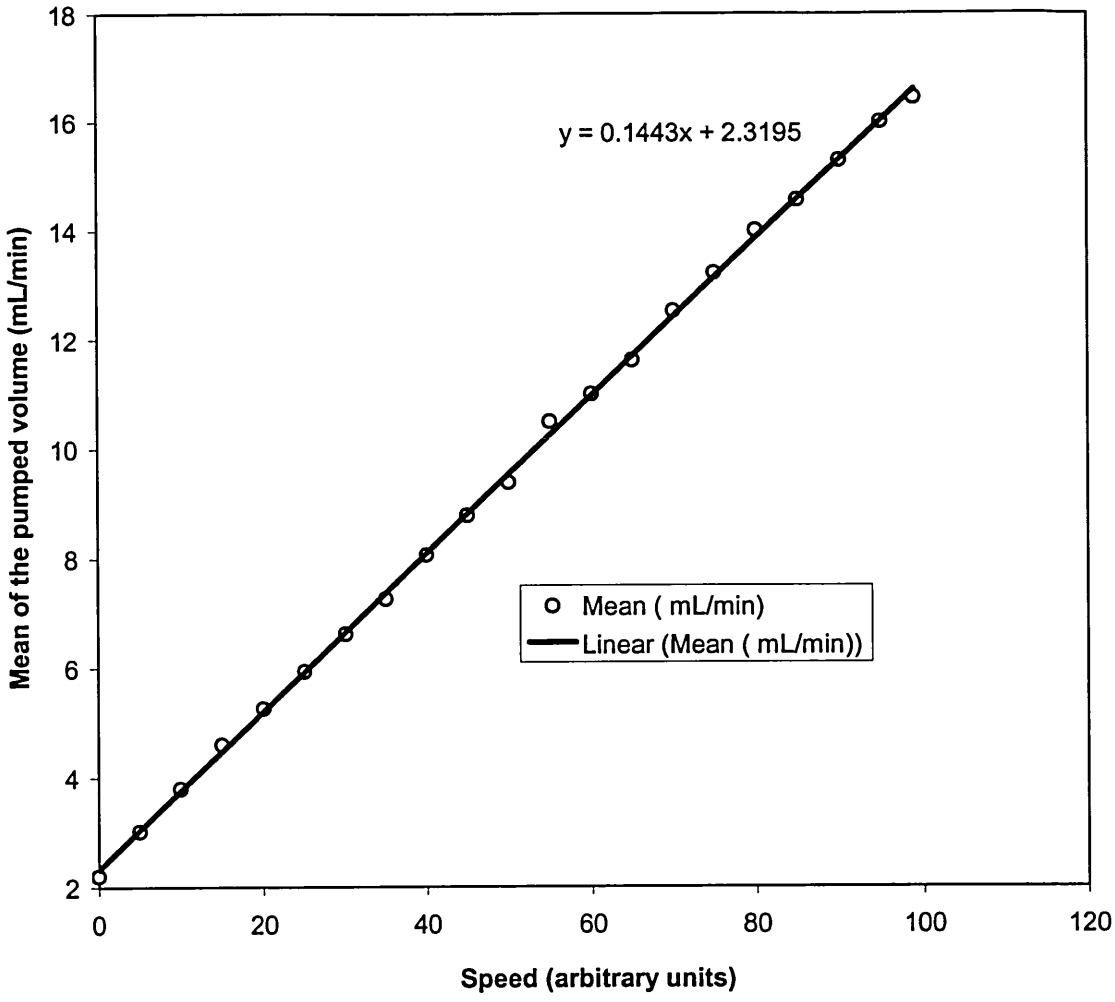


Figure 8: The standard curve for the peristaltic pump calibration. Error bars are calculated as the standard deviation of each data point. n = 5 replicates. (o) Mean of the pumped volume (mL min^{-1}).

Preparation of Cell-Free Extract

The leakage of the cytosolic constituents (e.g. free bases, inorganic phosphates) from the cell into the bacterial bathing solution can be measured by measuring the absorbance (E260nm). This is used as an indicator of cellular disruption. The process of homogenisation has to be validated in order that a sufficient time period is applied to disrupt the Gram-negative envelope of the cells.

Preparation of Cells

Sterile R2A medium (25 mL) was inoculated with a pure culture of *Pseudomonas aeruginosa* PAO1. The flask was incubated overnight at 37°C, 190 osc/min in an orbital incubator (Gallenkamp INA-305). Aliquots (5 mL) of the overnight culture were centrifuged at 5000 rpm (IEC Centra-4B) for 20 minutes. The pellet was washed with phosphate buffer (pH 7.2), this was repeated three times, and then the pellet was resuspended in 2 mL phosphate buffer. The prepared cells were homogenised on ice for 10 seconds, using a hand-held homogeniser (Ultra Turrax T8, S8N-5G, IKA labortechnik, Staufer, Germany). In order to remove any cell debris from the supernatant (cytosol), the cell suspension was centrifuged at 13000 rpm (MSE Microcentaur) for 1 minute. Using a Jenway 6105 UV/vis spectrophotometer, the optical density of the supernatant was measured at E260nm. A fresh phosphate buffer was used as a blank. The homogenisation and subsequent OD reading were repeated at 10-second intervals up to one minute, and every minute up to 5 minutes, until the optical density reading ceased increasing. By plotting homogenising time (H_t) against E260nm, the minimum required time of homogenisation in order to yield the maximum absorbency was determined as 2.5 minutes for *Pseudomonas aeruginosa.*

Bicinchoninic Acid (BCA) Assay for Protein Estimation

Bicinchoninic acid, sodium salt (BCA-Na), is a stable, water-soluble compound capable of forming an intense purple complex with cuprous ions (Cu^{1+}) in an alkaline environment. This reagent forms the basis of an analytical method capable of monitoring cuprous ion produced in the reaction of protein with alkaline Cu^{2+}. The bicinchoninic acid assay was originally described by Smith *et al.* (1985). It is dependent on the conversion of Cu^{2+} to Cu^{1+} under alkaline conditions and is therefore, similar to the traditionally used Lowry method (Lowry *et al.*, 1951). However, the stability of the reagent (BCA) and resulting chromophore also allows for a simplified, one-step analysis. 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 (Smith *et al.*, 1985).

The end-point colour stability of the BCA assay was assessed by performing the BCA method at the incubated temperature (37°C for 30 minutes), and then observing absorbance at 562 nm. 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).

Preparations of Solutions for the Standard Assay

A stock of reagent A was prepared, (BCA-Na_2, 1% (w/v); sodium carbonate, 2% (w/v); sodium tartarate, 0.16% (w/v); sodium hydroxide, 0.4% (w/v) and sodium bicarbonate, 0.95% (w/v) dissolved in 50 mL of distilled water). If needed, appropriate addition of NaOH (50%) or solid $NaHCO_3$ was made to reagent A to adjust the pH to 11.25. Reagent B comprises of copper sulphate (4% w/v). Reagent A and B are stable indefinitely at room temperature.

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 colouration developed.

Preparation of Standard Curve

A stock of a 1 mg mL^{-1} Bovine Serum Albumin (BSA) was prepared in deionised water. Aliquots were withdrawn from BSA stock solution 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 absorbency was measured at 562 nm (Novaspec II, LKB) and plotted against known protein estimations to produce a standard curve (**Figure 9**).

Analysis of Samples

Aliquots of the test samples (10 µL) were diluted to a total volume of 50 µL with dH_2O. An aliquot of reagent C (1 mL) was added to those samples, which were incubated at 37°C for 30 minutes. Their absorbencies were measured at 562 nm (versus a reagent blank) and compared against the standard curve (**Figure 9**) in order to estimate protein concentration.

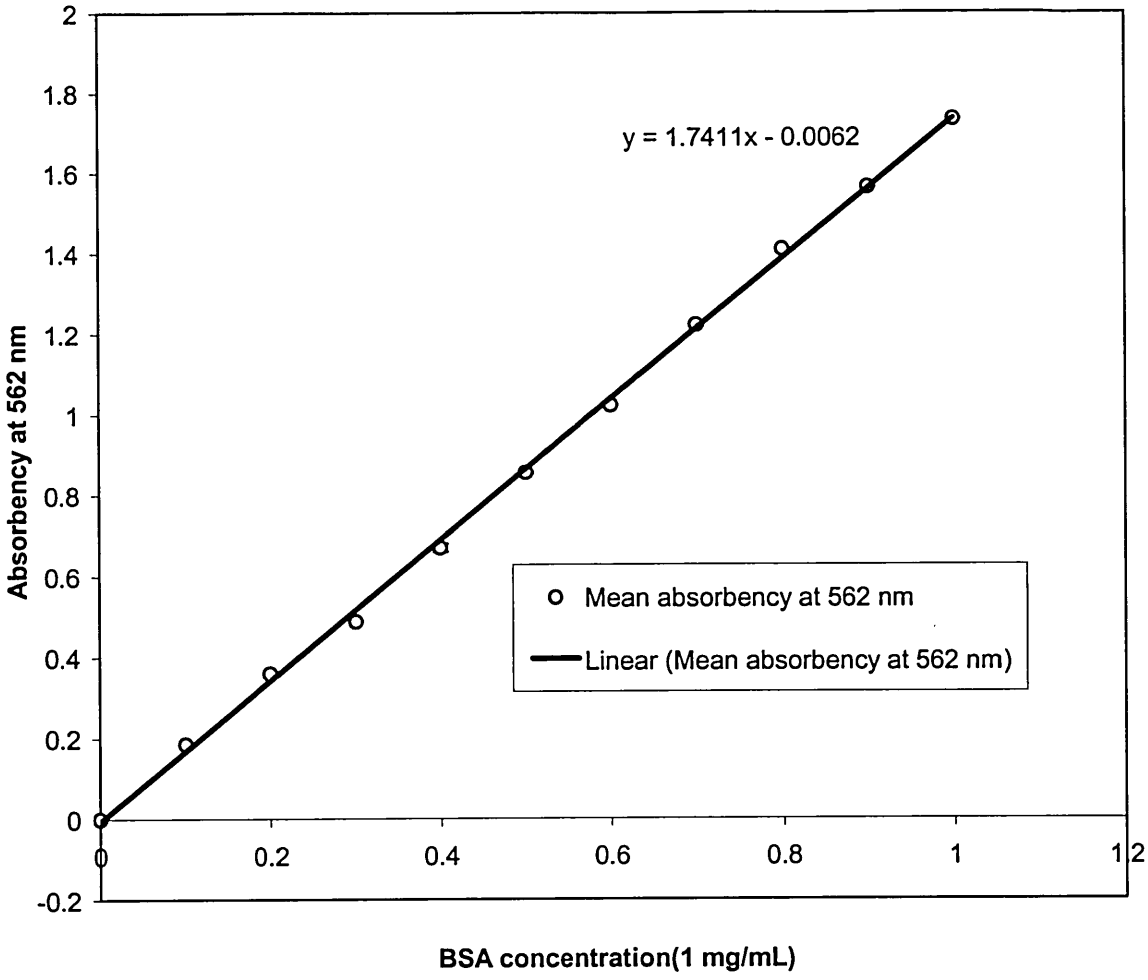


Figure 9: The standard curve for protein estimation. Error bars are calculated as the standard deviation of each data point. n = 3 replicates. (o) Mean absorbency at 562 nm.

Preparations of Outer Membrane Proteins and SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) for Biofilm and Eluate Cells

Outer Membrane Protein (OMPs) Preparations

Two methods for the preparation of OMPs were used in this project. The first method was that described by Hodgson *et al.* (1995) for biofilm cells and the second was that of Pugsley *et al.* (1986) for eluate cells. However, experimentation showed that the Hodgson *et al.* (1995) method could be appllied for both types of cells.

Biofilm Cells

OMPs were prepared for SDS-PAGE analysis according to Hodgson *et al.* (1995). Cultured Sorbarods were sacrificed. Cells were washed twice in 0.9% (w/v) sterile normal saline and harvested by centrifugation (MSE Microcentaur) at 10000 rpm, for 10 minutes. Cells were resuspended in 1 mL of 0.9% (w/v) saline containing 100 µg of lysostaphin and incubated at 37°C for 2h in an orbital incubator (Gallenkamp, INA-305) at 24 rpm. In order to inhibit proteolytic activity of bacterial enzymes released during lysostaphin treatment, phenylmethylsuphonyfluoride (PMSF) and N-P-p-tosyl-L-lysine chloromethyl ketone (TLCK) were added to give final concentrations of 2 mmol L^{-1} for both. DNase and RNase were added to give final concentrations of 0.1mg mL^{-1} each. After centrifugation at 10000 rpm for 20 minutes, the supernatant fluid was decanted and stored at -20°C.

Eluate Cells

OMPs were prepared for SDS-PAGE analysis according to Pugsley *et al.* (1986). The eluate suspension was centrifuged (IEC Centra-4B) at 5000 rpm for 20 minutes. The cells

were washed three times in 25 mmol L^{-1} Tris buffer (pH 7.4) containing 1mmol $MgCl_2$. After homogenisation for 2.5 minutes on ice, sarcosine (Sigma) was added to give a final concentration of 2% (w/v), and samples were kept in ice for 20 minutes. The insoluble outer membranes were sedimented out at 13000 rpm (MSE Microcentaur) for 1h at 4°C. The supernatants were removed and the pellets were washed with 1 mL distilled water. The pellets were resedimented out at 13000 rpm for 1h at 4°C. Samples were stored in the freezer (-20°C) until required. Prior to SDS-PAGE analysis the pellet was resuspended in 100 μL of Tris-HCl buffer (pH 6.8). Protein samples were diluted 1:1 with sample (cracking) buffer (sodium dodecyl sulphate, 2% (w/v); mercaptoethanol, 5% (v/v); glycerol, 10% (v/v); bromophenol blue, 0.125% (w/v); Tris-HCl, 0.5M; pH 6.8; made up to 10 mL with distilled water) and heated at 100°C in a water bath (Gallenkamp, England) for 5 minutes immediately prior to loading of the gels.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Preparation of Separating Gel Mixture

Three stock solutions were required to prepare this mixture:

(i) Stock acrylamide solution: 73g, acrylamide and 2g, bis-acrylamide dissolved in 250 mL distilled water.

(ii) Stock separating buffer: 1g, SDS and 45.5g, Tris buffer (2-amino-hydroxymethyl-propane-1,3-diol) dissolved in less than 250 mL of distilled water, the pH was adjusted to 8.0 with HCl and then made up to 250 mL with distilled water.

(iii) Stock ammonium persulphate solution 10% (w/v); 1.00g, ammonium persulphate was dissolved in 10 mL of distilled water.

10 mL of stock acrylamide, 12 mL of distilled water and 7.5 mL of stock separating buffer were added together and degassed. Then, 45 µL of stock ammonium sulphate and 15 µL of N,N,N',N'-tetramethyl-ethylenediamine (TEMED) were added to begin the polymerisation process. The mixture was mixed gently to avoid re-gassing, and poured into a prepared gel cast (0.5 mm thick: mini-PROTEAN II, Biorad). Then, the gel was overlaid with water-saturated butan-2-ol, and left to set for approximately 1h.

Preparation of Stacking Gel Mixture

Stock stacking gel buffer: 1g, SDS and 15.1g Tris buffer dissolved in less than 250 mL distilled water, the pH was adjusted to 6.8 and the stock was made up to 250 mL with distilled water. 750 µL, stock acrylamide; 3 mL, distilled water and 1.25 mL stacking gel buffer were mixed together and degassed. Then, 15 µL of ammonium persulphate stock solution and 5 µL of TEMED were added and mixed gently to avoid re-gassing. All the above stock solutions are stable for several weeks in brown glasses, at 4°C.

After nearly an hour, the separating gel was set and the butan-2-ol was removed from the polymerised gel. To remove any traces of the alcohol, the surface was rinsed with distilled water. This was followed by pouring the stacking gel over the separating gel and allowing it to set for approximately 45 minutes, with a comb (0.5 mm, 10 wells, Biorad) in place. Once, the stacking gel had set, the comb was removed gently, and the wells were washed with distilled water. The polymerised gel was transferred to a mini-PROTEAN II system, (Biorad), which was filled with reservoir buffer (0.192 M, glycine (28.8g); 0.025M, Tris (6.0g); and 0.1% (w/w), SDS (2.0g), were made up to 2L with deionised water, the pH should be at about 8.3 without adjustment, and the solution is freshly prepared each time), ensuring the wells were completely filled with the buffer.

After loading the molecular weight markers (5 μL) and the test samples (10 to 20 μL) into the wells using a pipette (Sealpipette Jencons), a constant current (35 mA) was applied to the gel by a Powerpack 300 (Biorad) until the bromophenol blue front reached the bottom of the gel (between 1h-1.5h). Once the bromophenol blue front reached the bottom, the Powerpack 300 was switched off and the gel was gently removed from its cast and immersed in Coomassie brilliant blue R250 stain or in the fixing solution (Silver stain).

Protocols for the Preparation of Two-Dimensional Gel Electrophoresis for Protein Separation on the Basis of Isoelectric Points and Molecular Weights

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 distilled water and placed in fresh potassium hydroxide solution (KOH, 0.4g; ethanol, 20 mL). The tubes were rinsed in distilled 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 was prepared to contain;

1. Urea (ultrapure, Sigma, Poole); 5.5g.
2. Acrylamide stock (acrylamide, 14.19g; bis-acrylamide, 0.8g in 50 mL dH_2O); 1.33 mL.
3. Igepal CA-630 stock (10% (v/v)); 2 mL.
4. Resolyte (BDH, Poole, pH 3.5 to 10.0); 0.3 mL.

5. Resolyte (BDH, Poole, pH 4.0 to 8.0); 0.2 mL.
6. Distilled water; 1.97 mL.

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 non-equilibrium pH gradient electrophoresis (NEPHGE) method, ammonium persulphate (10% w/v; 20 μL) 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 (urea, 4.81g; distilled water, 10 mL; 20 μL) 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 distilled water.

Isoelectric Focusing of Protein Samples

The tube gels were placed in the 2D gel rig and the bottom reservoir chamber was filled with freshly prepared 0.02M NaOH. An aliquot (20 μL) of lysis buffer was added to the surface of the tube gels. This buffer was prepared as follows:

1. Urea; 9.5 M.
2. Igepal CA-630; 10% (v/v).
3. Dithiothreitol (DTT; Sigma, Poole); 1 M.
4. Resolyte (BDH, Poole, pH 3.5 to 10.0); 0.3 mL.
5. Resolute (BDH, Poole, pH 4.0 to 8.0); 0.2 mL.
6. Dissolved in 3 mL distilled water.

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) as below:

1. Urea; 8M.
2. Resolyte (BDH, Poole, pH 3.5 to 10.0); 250 μL.
3. Dissolved in 5 mL distilled water.

The top reservoir chamber was filled with freshly prepared 0.01M 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. The tube gels were then removed from glass tubes, rolled in parafilm and placed in 5 mL SDS sample buffer, for 30 minutes. This buffer was prepared to contain:

1. Glycerol; 10% (w/v).
2. DTT; 15% (w/v).
3. SDS (Sodium Dodecyl Sulphate, Sigma, Poole); 2.3% (w/v).
4. Tris-HCl (pH adjusted to pH 6.8); 0.0625M.

The tubes were then stored in the freezer for later analysis.

Preparation of Slab Gel (Second Dimension Protein Separation)

Stock acrylamide solution, 10 mL (73g, acrylamide and 2g, bis-acrylamide dissolved in 250 mL distilled water), stock separating buffer, 7.5 mL (1g, SDS and 45.5g, Tris buffer (2-amino-hydroxymethyl-propane-1,3-diol) were dissolved in less than 250 mL of distilled water, the pH was adjusted to 8.0 with 1M HCl and then made up to 250 mL with distilled water) and 12 mL distilled water were mixed together and the whole solution was 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 (0.5 mm thick: mini-PROTEAN II, Biorad). The gel was overlaid 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 distilled 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 few drops of 0.1% bromophenol blue were added along the surface of the tube gel. The gels are then placed into a vertical slab gel apparatus (a mini-PROTEAN II system, Biorad). The upper and lower buffer chambers were filled with reservoir buffer (glycine, 0.192 M; Tris-Base, 0.025 M; SDS, 0.1% (w/v)). A constant current (35 mA) was applied to the gels by a Powerpack (LKB) for approximately 3h, or until the marker dye (the bromophenol blue) front reached the bottom of the gel. The gel was removed from the mini-PROTEAN II system and silver stained.

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

Coomassie Brilliant Blue R250 Staining

This is a two-step staining technique and is capable of detecting approximately 2.33 μg of protein per band. The gel was transferred to the stain solution (0.2g, coomassie brilliant blue; 125 mL, methanol; 25 mL, glacial acetic acid; and 100 mL, distilled water). The coomassie dye was dissolved in the ethanol component first, the acid and water were added later (if dissolved in a different order, the dye's staining behaviour may alter). The gels were allowed to take up the stain for 3 to 4 h, with gentle agitation using an orbital shaker (Stuart

Scientific, UK). De-staining of the gels was achieved by immersion them in the de-stain (450 mL, methanol; 100 mL, glacial acetic acid; 450 mL, deionised water), and mixing thoroughly. The stain and de-stain are best used when freshly made. After about 24 h, with gentle agitation using an orbital shaker (Stuart Scientific, UK) and several changes of de-staining agent, the gel background became colourless and left the protein bands stained blue (Smith, 1984).

Silver Staining

This stain is useful when the protein concentration is low, as it can detect between 0.1 and 1 ng of protein per band (Walker, 1984b). The gel was immersed in the fixing solution (see below) for a minimum of 60 minutes; it could also be left overnight if desired. Then, it was transferred to wash A for 20 minutes; this step was repeated 3 times. The gel was placed in pretreat solution for one minute exactly, and then rinsed with distilled water for 20 seconds. This step was repeated 3 times. The gel was immersed in the impregnate solution for 20 minutes, and then washed twice with distilled water for 20 seconds each. Then, the gel was placed in the develop for as long as required (between 2-6 minutes), until the bands were clearly visible. The gel was rinsed with distilled water twice for approximately 20 seconds, and was transferred to stop solution for 10 minutes. Finally, the gel was placed in wash B for 20 minutes. The gel could be stored in this solution for 3 to 4 weeks at 4°C (container wrapped with foil).

The Preparation of Silver Stain's Solutions

Fixing solution (50% methanol (HPLC), 100 mL; 12% glacial acetic acid, 24 mL; 37% formaldehyde, 100 µL. This was made up to 200 ml with distilled water).

Wash A (50% ethanol, 250 mL; made up to 500 mL with distilled water).

Pretreat solution (0.04g sodium thiosulphate.$5H_2O$; made up to 200 mL with distilled water).

Impregnate solution (0.4g of silver nitrate (anhydrous) and 37% formaldehyde, 150 µL. This made up to 200 ml with distilled water).

Develop solution (12g of sodium carbonate (anhydrous); 37% formaldehyde, 100 µL and 0.0008g of sodium thiosulphate.$5H_2O$. This was made up to 200 mL with distilled water).

The above solutions only last for two weeks, maximum.

Stop solution (50% methanol, 100 mL; 12% glacial acetic acid, 24 mL and it was made up to 200 mL with distilled water).

Wash B/Store (50% methanol, 250 mL; made up to 500 mL with distilled water).

Reconstitution of Pharmacia Low Molecular Weight (LMWt) Markers

Low MWt Calibration Kit (for SDS Electrophoresis)

This is a lyophilised mixture of 6 highly purified well-characterized proteins, for use in molecular weight determination in the presence of the detergent SDS. The size range of these proteins is between 14,400 and 94,000 Daltons.

Phosphorylase B : 94,000 Daltons

Albumin : 67,000 Daltons

Ovalbumin : 43,000 Daltons

Carbonic anhydrase : 30,000 Daltons

Trypsin inhibitor : 20,100 Daltons

β-lactalbumin : 14,400 Daltons

Reconstitution

(I) For Coomassie Brilliant Blue Detection, 200 µL of 1x sample buffer (0.0625 M, "Tris"-HCl; 2% SDS; 10% (v/v) glycerol; 0.1 M, DTT and 0.01% bromophenol blue) was added to the kit.

(II) For Silver Staining, the aliquots were diluted by at least 50-fold in sample buffer and reconstituted as above.

For best reproducibility, any unused reconstituted protein solution was discarded. If necessary it could be stored for up to 3 months at –80°C.

Denaturing of proteins

Proteins were heated in a boiling water bath (Gallenkamp) for 5 minutes.

Loading

Proteins markers were loaded in a range of 1to 8 µL.

Two Dimensional SDS-PAGE Standards

Bio-Rad's 2-D SDS-PAGE Standards are formulated to provide a two-dimensional protein pattern with detection either by silver or Coomassie staining. The standard proteins are defined by isoelectric point (pI) and molecular weight. Added to the sample, they are used to determine pIs and molecular weights of sample proteins or to serve as a marker for 2D gel matching. The characteristic pattern of main and minor spots of each standard protein makes

them easy to identify among sample spots. The molecular weight range is from 17,500 to 76,000 Daltons.

Conalbumin	: 76,000 Daltons
Albumin	: 66,200 Daltons
Actin	: 43,000 Daltons
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	: 36,000 Daltons
Carbonic anhydrase	: 31,000 Daltons
Trypsin inhibitor	: 21,500 Daltons
Myoglobin	: 17,500 Daltons

Loading

Using silver staining: 1-5 μL per mini gel.

Extraction of Exopolysaccharides in Biofilms

Microbial extracellular polymeric substances (EPS) are high molecular-weight mucous secretions of bacteria and microalgae. They ranged from tight capsules, which closely bind cells, to the loosely attached slime material. Biofilm EPS possesses many important functions in water and wastewater treatment, including anchoring the microorganisms near food sources, protecting them from dehydration and toxic substances, and providing ion exchange properties due to negatively-charged surface functional groups which allow them to bind cationic species such as heavy metals (Sutherland, 2001).

Many methods have been used to extract EPS from different bacterial cultures and activated sludges for analysis. These extraction methods have included use of ammonium

hydroxide, sodium hydroxide, ethylene-diaminetetraacetic acid (EDTA), sulphuric acid, trichloroacetic acid, boiling benzene, ultrasonication, blending, high-speed centrifugation, and extraction by boiling or autoclaving (Brown & Lester, 1980). However, most of these extraction studies were performed on activated sludge (Brown & Lester, 1980; Gehr & Henry, 1983; Morgan *et al.*, 1990; Urbain *et al.*, 1993; Jia *et al.*, 1996; Frølund *et al.*, 1996); very little research has been conducted on biofilm samples. The method, which had been applied in this study, was extracting EPS by using EDTA. Four steps were involved in extracting and collecting EPS from the biofilm samples (Zhang *et al.*, 1999):

(Step 1) Washing (recover slime material). Biofilm samples (about 1g) were put into centrifuge tubes along with 25 mL deionised water. The tubes were shaken gently, and then centrifuged at 3500 rpm (6000g, IEC Centra-4B) for 10 minutes. The liquid was decanted from the centrifuge tubes, and collected as the slime material.

(Step 2) Stripping (recover capsule-bound material). After the washing step, the tubes containing the biofilm pellets were filled with another 25 mL deionised water. The contents were blended in a vortex (WhiriliMixer^{TM}, Fisons scientific equipment, Instruments Ltd. Dyce, Aberdeen) at high speed for 1 minute to recover the capsule-bound material. Liquid from the washing step and the stripping step were combined and brought to a volume of 50 mL.

(Step 3) Extraction (separate the stripped EPS material from the cells) using EDTA (Brown and Lester, 1980). An aliquot of 2% EDTA (10 mL) was added to 10 mL of the combined sample and left quiescently for 3 hours at 4°C. The centrifuge tubes were then centrifuged at 13000 rpm (MSE Microcentaur) for 30 minutes at 4°C.

(Step 4) Filtering and collecting the EPS. The supernatant obtained on centrifugation after the extraction step was filtered through 0.22 μm cellulose acetate filters to ensure that samples were free of cells. The pellets were discarded.

Statistical Methods

All experiments in this study 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 in the data set.

Chapter Three:

Studies on the Growth of *Pseudomonas aeruginosa* PAO1

Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium that can be found in a variety of habitats including soil and fresh water streams (Boyd & Chakrabarty, 1995). This bacterium can grow well in water because of its simple nutritional requirements: it will even grow in distilled water (Favero *et al.*, 1971) and recreational waters will provide ample nutrients. *Ps. aeruginosa* is of particular interest as a human pathogen, which rarely causes infection in the healthy host. However, it infects patients with predisposing conditions, such as burns, open wounds (Anwar *et al.*, 1985), immunosuppression or indwelling medical devices (Costerton *et al.*, 1987). This bacterium can also infect the lungs of cystic fibrosis (CF) patients (Govan & Deretic, 1996). In all these environments, the microbes are found predominantly attached to solid surfaces forming biofilms. Biofilms are surface-attached communities of bacteria embedded in an extracellular matrix of biopolymer substances and are involved in many types of chronic infections (Costerton *et al.*, 1995 & 1999; Davey and O'Toole, 2000; O'Toole *et al.*, 2000). Initial microscopic observations of postmortem lung tissue and the sputa of patients suggested that *Ps. aeruginosa* forms biofilms in the CF lung (Lam *et al.*, 1980; Speert *et al.*, 1987; Govan & Deretic, 1996). Biofilm bacteria are physiologically distinct from free-swimming bacteria of the same species. The differences between sessile and planktonic bacteria seem to result from a combination of factors including the inaccessibility of underlying cells to the external environment, the surrounding glycocalyx and the intrinsic physiological properties of biofilm cells compared to planktonic cells (Costerton *et al.*, 1987; van Loosdrect *et al.*, 1990; Brown & Gilbert, 1993). Many biofilms may essentially be considered to be layered, with an aerobic layer overlying an anoxic or anaerobic layer. Biofilm properties change with biofilm depth because of transfer limitations

of dissolved oxygen (DO) and nutrients and because of varying biological processes (Bishop & Yu, 1998; Zhang & Bishop, 2001). Growth rate, growth temperature and nutrient limitation all influence the stability, production of virulence factors and resistance to host defences of mucoid and non-mucoid *Ps. aeruginosa* (Ombaka *et al.*, 1983). Growth rate and iron availability appear to be of major importance in bacterial pathogenesis. Nearly all bacteria require iron in order to multiply and bacterial doubling times *in vivo* appear to be slow (Eudy & Burrough, 1973). Therefore, the ability to acquire iron will alter doubling times which in turn influence the attainment of critical bacterial populations, which may be crucial in determining the outcome of an infection (Brown & Williams, 1985a & b). Nutrient limitation can also have profound effects on heterotrophic bacteria. The growth of these bacteria in natural environments is inhibited by periods of insufficient levels of energy and nutrients (Gilbert *et al.*, 1990). Such inhibition may reduce the growth rate of the bacteria to such an extent that they may be considered to have growth rates that approximate zero. The survival strategies of bacteria in their natural environment under starvation conditions have been identified (Roszak & Colwell, 1987b) and suggest that bacterial cultures undergo a series of physiological or phenotypic changes, which enable the survival of some of the cells. Rapid multiple divisions of starved cells, which lead to the formation of ultramicrobacteria (<0.3 μm in diameter) have been observed (MacDonnell & Hood, 1982). It is assumed that such a reduction-division in response to starvation improves the chances of individual genomes surviving by the rapid formation of multiple copies (Novitsky & Morita, 1976; Morita, 1982; Gilbert *et al.*, 1990). Also, a series of rapid metabolic adjustment have been reported during nutrient-limited conditions (Turner *et al.*, 2000). The accumulation of a novel nucleoside, guanosine-3^{1}-diphosphate-5^{1}-diphosphate (guanosine tetra phosphate; ppGpp) occurs in response to amino acid deprivation (Sarubbi *et al.*, 1988; Leitch, 1998) and triggers the

stringent response. There is a reduction in the rate of rRNA and phospholipid accumulation and a general stimulation of amino-acid synthesis and binding of uncharged tRNA (instead of aminoacyl-tRNA) to ribosomes. The response is mediated by ppGpp accumulation, a product of the *relA* (relaxed control) gene. In cells (*rel*$^{-}$) that lack the stringent response, as a consequence of a mutation in the *relA* gene, rRNA accumulates. Starvation-induced ribosomal breakdown is considerably slower in *relA*$^{-}$ mutants that are unable to produce ppGpp (Sarubbi *et al.*, 1988; Matin *et al.*, 1989; Gilbert *et al.*, 1990; Leitch, 1998; Turner *et al.*, 2000).

It was suggested that the stringent response may be at least partially responsible for the increased resistance to antibacterial agents at slow growth rates (Gilbert *et al.*, 1990; Leitch, 1998; Whitehead *et al.*, 1998; Greenaway and England, 1999a). Also implicated are alternative sigma factors, one of which (σ^{S}, encoded by the *rpoS* gene) directs gene expression during stress conditions and slow growth (Greenaway and England, 1999b). However, it has recently been proposed that the sigma factors RpoS and AlgT may play a transient role in protecting thin, but not thick, biofilms of *Ps. aeruginosa* against hydrogen peroxide (Cochran *et al.*, 2000b).

The Growth Characteristics of *Pseudomonas aeruginosa* PAO1

In order to assess the rate of microbial reproduction, it is necessary to determine the numbers of microorganisms present. The growth of *Pseudomonas aeruginosa* can be assessed by several different methods, including the total count, the viable count and turbidometric procedures. The viable plate count method is one of the most common procedures for the enumeration of bacteria. It is used to determine the viable population in a bacterial culture. In this procedure, serial dilutions of a bacterial suspension are plated onto a suitable solid growth

medium in order to determine the number of colonies. It is assumed that each colony arises from an individual bacterial cell. Therefore, by counting the number of colonies that develop and by taking into account the dilution factors, the concentration of bacteria in the original sample can be determined as colony-forming units (CFU) (Roszak & Colwell, 1987a; Dupray *et al.*, 1993; Salvesen & Vadstein, 2000). A major limitation of the viable plate count procedure is its selectivity. It measures only those cells that are capable of growth on the given plating medium under the set of incubation conditions that are used. Sometimes cells are viable, but nonculturable (VNC) unless rigorous steps are taken to acclimate the microorganisms to laboratory culture conditions (Cooper *et al.*, 1968; Colwell *et al.*, 1985; Duncan *et al.*, 1994; Bloomfield *et al.*, 1998). Bacteria can also be enumerated by direct counting procedures (total count or microscopy), that is, counting without the need to first grow the cells in culture. However, the difficulty in establishing the metabolic status of the observed bacteria, that is, whether the cells are living or dead, is a major limitation of this procedure. Measuring the amount of light that passes through a bacterial suspension with a spectrophotometer can be used for estimating cell mass, since the amount of light observed or scattered by the bacteria is proportional to the cell density (Fuchs and Kroger, 1999). An increase in cell mass, which can be equated with increases in the number of bacterial cells, is useful for establishing a growth curve for a bacterium. At low densities, the absorbency is roughly proportional to the cell number, but at higher densities, there is a significant deviation from linearity. Therefore, this procedure is only accurate when the absorbency at 470 nm is less than 0.5, above this point the sample must be diluted (Lawrence and Maier, 1977).

The aim of this chapter was to undertake a series of experiments designed to elucidate the growth characteristics and parameters of *Pseudomonas aeruginosa* PAO1 grown under varying conditions of nutrient supplement and nutrient limitation. The outcome of these

experiments was designed to enable more accurate growth control of this microorganism in subsequent experimentation.

Experimental Approach

The Overnight Culture of *Pseudomonas aeruginosa* PAO1

25 mL of a sterile medium (R2A or CDM), in a 100 mL Erlenmeyer flask, was inoculated with a pure culture of *Pseudomonas aeruginosa* PAO1 (single colony, from a streak plate). The flask was incubated overnight in an orbital incubator (Gallenkamp INA-305) at 37°C, 180 osc/min.

The Test Culture of *Pseudomonas aeruginosa* PAO1

A fresh aliquot (25 mL) of medium (R2A or CDM) was inoculated with 250 µL of *Pseudomonas aeruginosa* overnight culture; this was performed in duplicate (flasks A & B). The flasks were incubated at 37°C, 180 osc/min, in an orbital incubator (Gallenkamp INA-305).

Assay for Growth

1 mL of test culture was aseptically removed (from flask A) and the optical density (turbidity) was measured at 470 nm using a spectrophotometer (Novaspec II LKB) against a sterile media blank. The sample was aseptically replaced back into the flask, so that the volume of the flask did not significantly alter. This was performed at hourly intervals for 24 hours. If the absorbency was greater than 0.5, a dilution of the culture was made (1:10), (Lawrence and Maier, 1977). This diluted sample was discarded after reading the optical

density. To calculate the optical density for this sample, E470 was observed and multiplied by 10; thus giving the actual optical density of the sample.

Viable Count

1 mL of the identical test culture (flask B) was aseptically removed and serially diluted in the range of 10^{-1} to 10^{-10}. Aliquots (100 µL) of the dilutions were spread plated onto appropriate agar plates (CDM or R2A agar). These plates were prepared in triplicate (three plates for each dilution). The plates were incubated overnight at 37°C. After 24 hours, colonies were counted in each plate and viable counts were calculated according to **Equation 2:**

$$\textbf{CFU mL}^{-1} = \textbf{N (1/DF)} \qquad \textbf{(Equation 2)}$$

Where; **N**= Mean of counts on the plates (must be in the range of 30-300 colonies per plate to be counted. If it was above 300, colonies cannot be distinguished as individual colony-forming unit, "TNTC". Fewer than 30 colonies are not acceptable for statistical reasons). **DF**= Dilution factor.

Results & Discussion

Figure 10 presents the growth curve of *Pseudomonas aeruginosa* in R2A medium. The lag phase in this medium was short, lasting 1 hour. The log or exponential phase lasted between 3 and 4 hours. Therefore, mid exponential phase was at 3.5 hours after inoculation. The doubling time of *Pseudomonas aeruginosa* in R2A medium was 24 minutes. This type of growth was expected in this kind of medium (R2A is a nutrient-enriched medium).

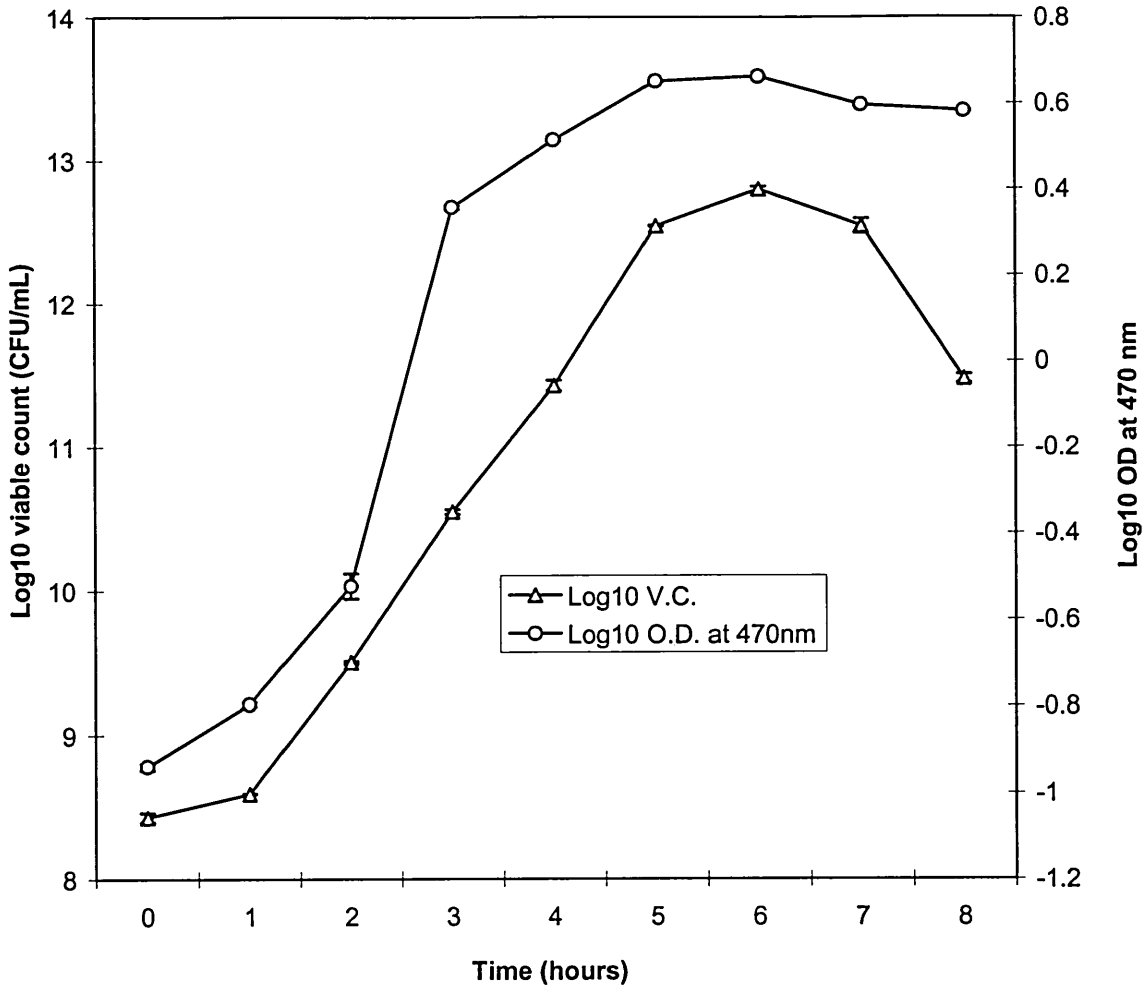


Figure 10: The Observation of Growth of *Pseudomonas aeruginosa* PAO1 in R2A medium. Error bars are calculated as the standard deviation of the individual data points. n = 3 replicates. (o) $\log_{10}$ OD at 470 nm; (Δ) $\log_{10}$ viable count (CFU mL^{-1}).

Figure 11 presents the growth curve of *Pseudomonas aeruginosa* in CDM. CDM is a chemically defined medium, where the nutrient supply is generally lower than that in R2A medium. Therefore, it was expected that the growth would be considerably slower. The cells in this medium were in lag phase for 2 hours before they entered the exponential phase. The exponential phase was very long, lasting between 2 and 21 hours after inoculation. Mid-exponential phase was at 11.5 hours after inoculation. The generation time in CDM was calculated to be 480 minutes (8 hours). In comparing the generation times for *Ps. aeruginosa* PAO1 in both media, one can observe the great gap between the doubling time in R2A medium (24 minutes) and that in CDM (480 minutes). The doubling time is the unit of measure of the microbial growth rate. R2A medium is a nutrient-enriched medium, which has a very good supply of nutrients. Therefore, the lag phase in this medium was short; it only took the bacteria one hour to prepare for reproduction under optimal conditions. In the log phase, bacterial reproduction occurred at a maximal rate and the microbial population was continuously doubling. On the other hand, the growth rate in CDM was much slower than that in R2A medium. The nutrients sources in this medium were very limited and it took the bacteria two hours to prepare for their proliferation. During the exponential phase, the bacterial growth was not rapid, and the increase in the bacterial biomass against time was slow. This result suggests that CDM was sufficiently stringent to be used in further experimentation. CDM is a defined medium; that is a medium in which the concentrations of all components are known. This medium includes an organic carbon growth substrate, glycerol; a source of nitrogen (NH_4Cl and ferric ammonium citrate) and water. It also includes potassium, magnesium, calcium, iron and chloride as trace elements (Chapter 2). Therefore, it is relatively easy to use this medium as a nutrient limited medium by controlling variation of

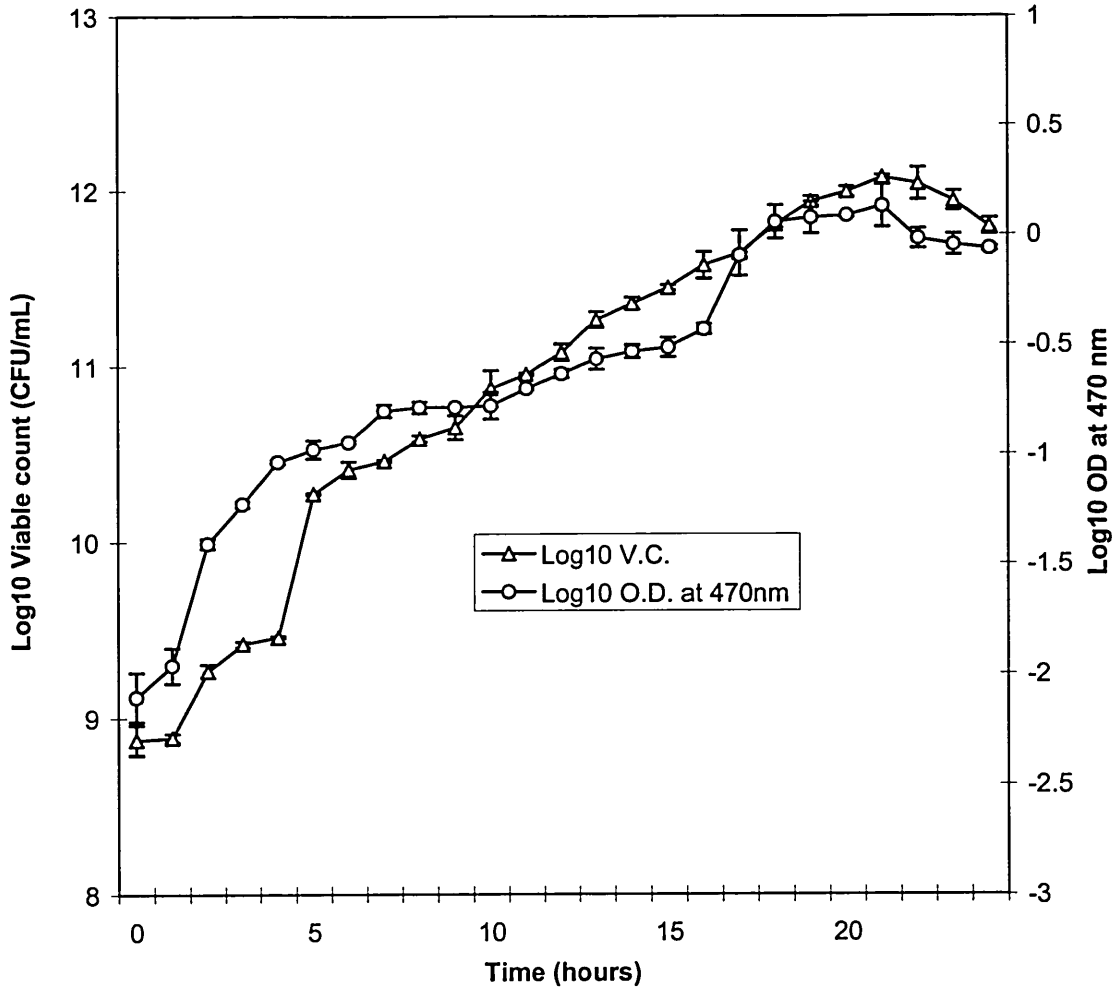


Figure 11: The Observation of Growth of *Pseudomonas aeruginosa* PAO1 in CDM. Error bars are calculated as the standard deviation of the individual data points. n = 3 replicates. (o) $\log_{10}$ OD at 470 nm; (Δ) $\log_{10}$ viable count (CFU mL^{-1}).

each component such that one is restrictive and the remainder are present to a controlled excess.

The Control Experiment for *Pseudomonas aeruginosa* PAO1 Biofilm Culture

An overnight culture (OD 470nm = 1.00) of *Ps. aeruginosa* PAO1 grown in CDM was used to inoculate (10mL) a pre-wetted (with the addition of 5mL 0.9% (w/v) sterile normal saline) sterile Sorbarod filter, held within sterile PVC tubing (**Figure 6**). This was perfused with CDM at 37°C, using a peristaltic pump and tubing calibrated to give a flow rate of 5 mL min^{-1}. Numbers of cells eluted (into a sterile receiving reservoir) from the Sorbarod filters were estimated with time by performing viable counts until the rate of loss of cells from the filter had decreased to a constant steady-state value, which was maintained for several days. This was performed in triplicate. Pseudo-steady states were established at which the growth rate of the biofilm was reproducible, measurable and significantly slower than in broth culture. At 103 hours after achieving steady-state, Sorbarod filters were removed and stored at -18°C for later analysis. Since steady-state had been achieved in both the biofilm and eluate populations, growth rate could be calculated from a knowledge of the elution-rate. The mean of the logarithm of viable counts for the three biofilms was plotted against time (h) to establish the growth curve of *Ps. aeruginosa* biofilm (**Figure 12**).

Viable cell number

An aliquot (100 μL) of eluate culture was aseptically removed and used for preparation of serial dilutions in the range of 10^{-2} to 10^{-10}. Aliquots (100 μL) were spread plated onto sterile CDM agar plates, in triplicate. The plates were incubated at 37°C for 2 days and the subsequent colonies were counted.

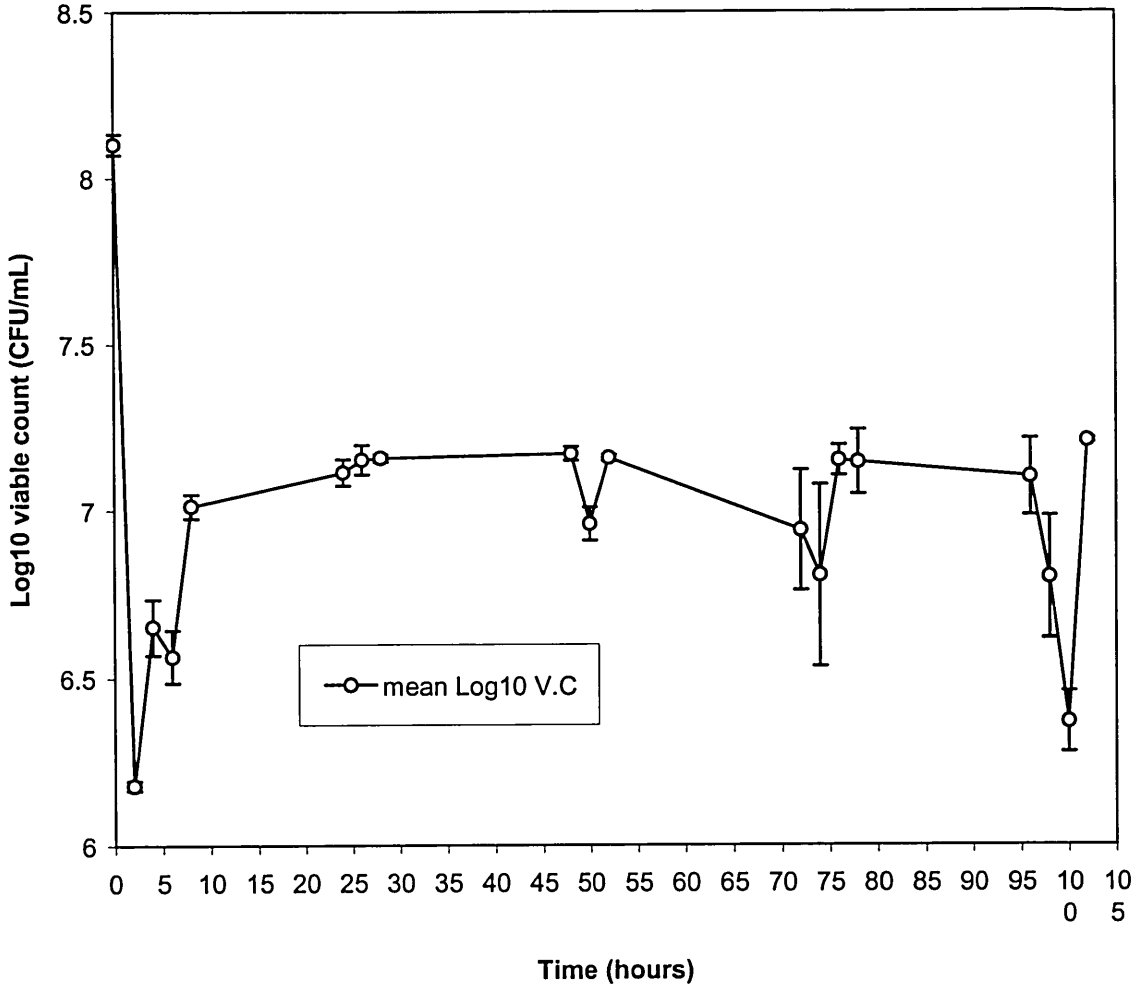


Figure 12: Graph of Mean Biofilm Eluate Counts (*Ps. aeruginosa* PAO1 perfused with CDM) over Time. Error bars are calculated as the standard deviation of the individual data points. n = 3 replicates. (o) $\log_{10}$ viable count (CFU mL^{-1}).

Defining Culture Media to Support Microbial Growth

In order to grow, microorganisms require a suitable environment, including a growth medium that can support their nutritional needs. Additionally, the culturing of microorganisms requires careful control of various environmental factors, including temperature, which is normally maintained within narrow limits by using a temperature-controlled incubator. By understanding the growth requirements of a given microbial species, it is possible to establish the necessary conditions *in vitro* to support the optimal growth of that microorganism (Gilbert *et al.*, 1987).

Many bacterial species can be grown in the laboratory on a defined medium, that is, on a medium in which all the components are known. Such a medium usually includes an organic carbon growth substrate, such as glucose, protein, or mineral nutrient-including a source of nitrogen and phosphorus, and water (Lee *et al.*, 1982). Some microorganisms require a complex medium for growth, that is, a medium made with constituents whose composition is not totally known and may in fact vary from one batch to another (e.g. R2A medium). There are many different types of media used for growing bacteria and fungi in pure culture.

The aim of this study was to grow *Ps. aeruginosa* PAO1 under conditions of specific nutrient-limitation in batch and biofilm cultures. This was primarily performed to gain fuller and more complete understanding of the requirements for carbon and nitrogen exhibited by this bacterium (as planktonic and sessile populations). Hence, the results from this experiment will eliminate or reinforce the possibility that biofilm-reduced susceptibility towards antimicrobial agents was a result of nutrient limitation (second mechanism, Chapter 1) and not biocide exposure. *Ps. aeruginosa* was cultured in chemically defined medium (Dinning, 1995), which was limited for sources of carbon and nitrogen at separate time intervals. The

growth of *Ps. aeruginosa* planktonic cultures was observed (until entering the stationary phase) at E_{470nm} using a spectrophotometer (Novaspec II LKB) and that for biofilm cultures was estimated (using the Sorbarod model; Hodgson *et al.*, 1995) from a knowledge of the elution rate (after achieving steady-state).

Planktonic Cultures

Preparation of overnight culture

Sterile CDM, 25 mL in Erlenmeyer flasks (100mL), was inoculated with two loopfuls of pure culture of *Pseudomonas aeruginosa* PAO1, from a nutrient agar streak plate. This was incubated at 37°C in an orbital incubator (180 osc min^{-1}) overnight. This culture was used to inoculate the nutrient limited flasks.

Preparation of nutrient limited experimental cultures

Sterile CDM was prepared by the addition of various controlled concentrations of nutrients to be limited. Aliquots of these media, 25 mL in Erlenmeyer flasks (100 mL), were inoculated with 250 µL of an overnight culture of *Pseudomonas aeruginosa* PAO1 in CDM. Cultures were then incubated at 37°C in an orbital incubator (180 osc min^{-1}).

Limitation of carbon

The limitation of carbon was achieved by limiting the concentration of glycerol (solution C). Seven flasks were prepared to contain glycerol in a concentration range of 0M to 1M.

Limitation of Nitrogen

The limitation of nitrogen was achieved by limiting the concentrations of NH_4Cl (solution A) and ferric ammonium citrate (solution B) to the chemically defined medium (CDM). Eleven flasks were prepared with concentrations ranging from 0M to $7.39x10^{-4}M$.

Assay for Growth

Aliquots (2 mL) of the nutrient limited cultures of *Pseudomonas aeruginosa* PAO1 were aseptically removed at one-hour intervals from zero time. These samples were observed at E_{470nm} using a spectrophotometer (Novaspec II LKB) blanked against a 2 mL sterile medium in a 1cm path length cuvette. The samples were aseptically returned to their respective flasks (in order to ensure no alteration in the surface : volume ratio and hence, in overall aeration of the cultures). This technique was repeated until stationary phase was observed.

Experimental samples with an E470nm greater than 0.5 were diluted ten-fold with sterile media and the true E470 values were calculated (this prevented deviations from linearity in optical densities above this value; Lawrence and Maier, 1977). These samples were not returned to their flasks but discarded. The absorbance at E_{470nm} was plotted against time and the K_s (substrate constant) was determined from the Lineweaver-Burk plot.

Biofilm Cultures

Biofilm cultures of *Pseudomonas aeruginosa* PAO1 were grown within Sorbarod filter plugs, which were perfused with CDM (Hodgson *et al.*, 1995).

Preparation of overnight culture

The overnight culture was prepared in the same way as for planktonic cells.

Preparation of nutrient limited experimental cultures

Sterile CDM (4L) in a Pyrex flask (5L) was prepared by the addition of various controlled concentrations of nutrients to be limited; this was performed in duplicate. The Sorbarod filter was pre-wetted with 5 mL of 0.9% (w/v) sterile normal saline and then inoculated with 10 mL of an overnight culture of *Ps. aeruginosa* PAO1 from a syringe in a dropwise fashion. The rubber plunger seal was withdrawn from a sterile, disposable 2 mL syringe. The syringe was introduced into the opposite end of the PVC tubing containing the Sorbarod and a sterile disposable needle (0.8 x 40 mm) inserted through the rubber seal. Media inlet tubing was attached *via* the needle and the sterile nutrient limited medium was delivered to the unit. Duplicates of these units were established and placed within a 37°C incubator.

Limitation of carbon

The limitation of carbon was achieved by limiting the concentration of glycerol (solution C). Glycerol concentrations were prepared in a concentration range of between 0.2M to 1M.

Limitation of nitrogen

The limitation of nitrogen was achieved by limiting the concentrations of NH_4Cl (solution A) and ferric ammonium citrate (solution B). NH_4Cl and ferric ammonium citrate were prepared as a separate solution (solution E). The concentrations of solution E were prepared in a concentration range of between 0.2M to 1M.

Assay for Growth

Aliquots (100 mL) of the eluate were collected in (125 mL) Erlenmeyer flasks at 2-hour intervals from zero time until 8 hours and from 23 hours to 31hours. These were serially diluted in the range of 10^{-1} to 10^{-10}. Aliquots (100 µL) of the dilutions were spread plated onto sterile CDM agar plates in triplicate. The plates were incubated overnight at 37°C and after 48 hours, colonies were counted and viable counts were calculated (Chapter 2). The data (viable counts) were plotted against time (h). The eluate cultures were then centrifuged at 5000g (IEC Centra-4B) for 2 hours in order to remove the cells (pellets). The cells were washed twice in Tris buffer (0.5M, pH 6.8). The treated cells were then stored for later analysis. The Sorbarod biofilms were also stored in a freezer (-80°C) for later biochemical analysis (SDS-PAGE and 2D-PAGE).

Results & Discussion

For planktonic culture

Growth rates of *Ps. aeruginosa* PAO1, under nutrient limitation were affected dramatically (**Figure 13 & 14**). Cultures were more responsive to carbon limitation than to nitrogen limitation. The minimum concentration at which growth rate changes were observed

was 0.6M for carbon and $1.478x10^{-4}$M for nitrogen. For carbon, the lag phase duration varies from one concentration into the another. The duration was 19 hours for 1 M, 20 hours for 0.8M and 21 hours for 0.6M, but the lag phase duration was the same (7 hours) for the lowest concentrations of carbon (0.4M, 0.2M, 0.1M and 0M). For most carbon concentrations, there was no clear onset of stationary phase after 26 hours of inoculation. The critical carbon concentration was between 0.6M and 0.4M. For nitrogen, the duration of lag phase was the same for all concentrations and lasted for 8 hours. The 9^{th} hour after inoculation was the beginning of the exponential phase. This phase was very long, lasting between 9 hour and 27 hour. Mid-exponential phase was at 18 hours after inoculation. The onset of the stationary phase was at 28 hours. The critical nitrogen concentration was not that obvious (in comparison to that for carbon), but it could be between $1.478x10^{-4}$M and $7.39\ x10^{-5}$M. The results obtained from the limitation studies enabled the determination of the bacteria's requirement for each nutrient. The formula was adapted from the Michaelis-Menten equation, where:

$$V = V_m \cdot [s]/ (K_m + [s])$$ (Michaelis and Menten, 1913, **Equation 3**)

Equation 3 is substituted by the **Equation 4**:

$$\mu = \mu_{max} \cdot c/ (K_s + c)$$ (Monod,1949, **Equation 4**)

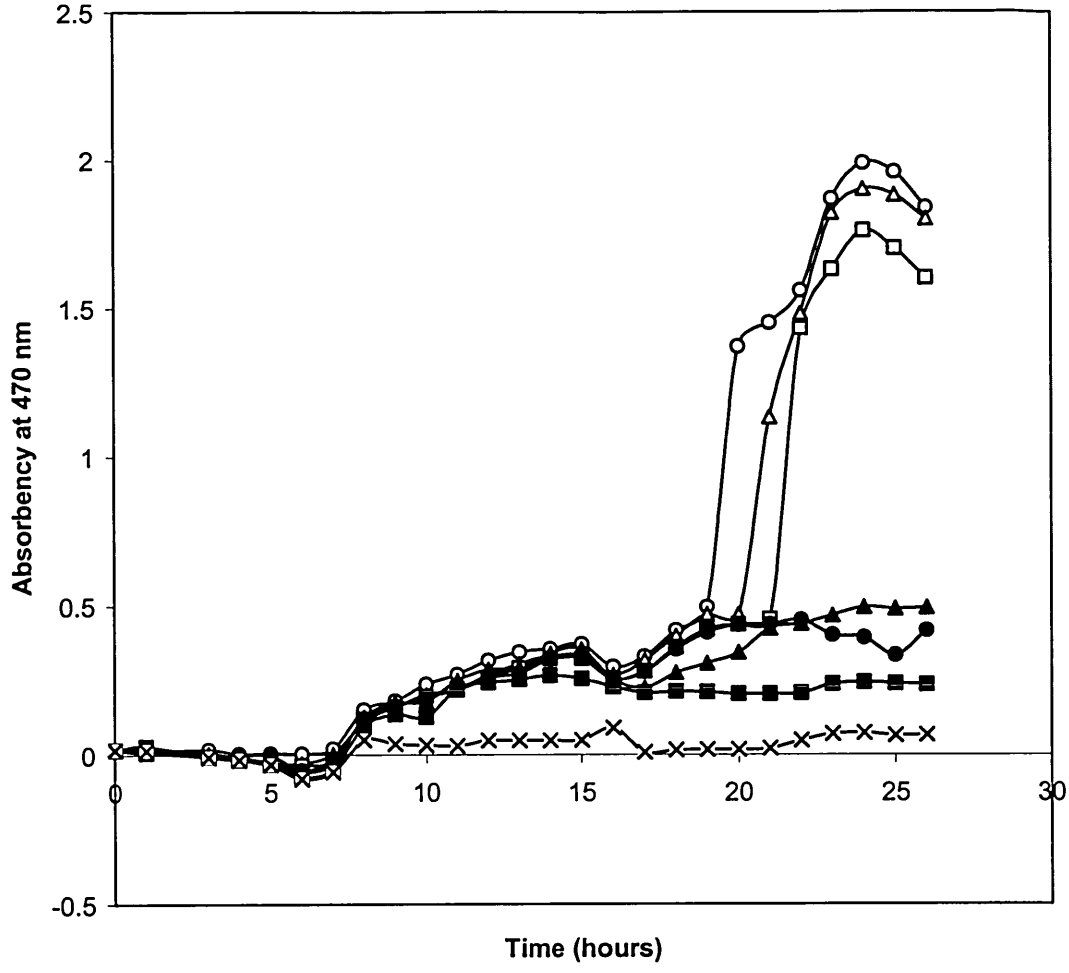


Figure 13: Growth of *Pseudomonas aeruginosa* PAO1 under varying conditions of carbon limitation. Carbon concentration (o) 1M; (Δ) 0.8M; (□) 0.6M; (●) 0.4M; (▲) 0.2M; (■) 0.1M; (×) 0M. Values are the mean of 3 replicates.

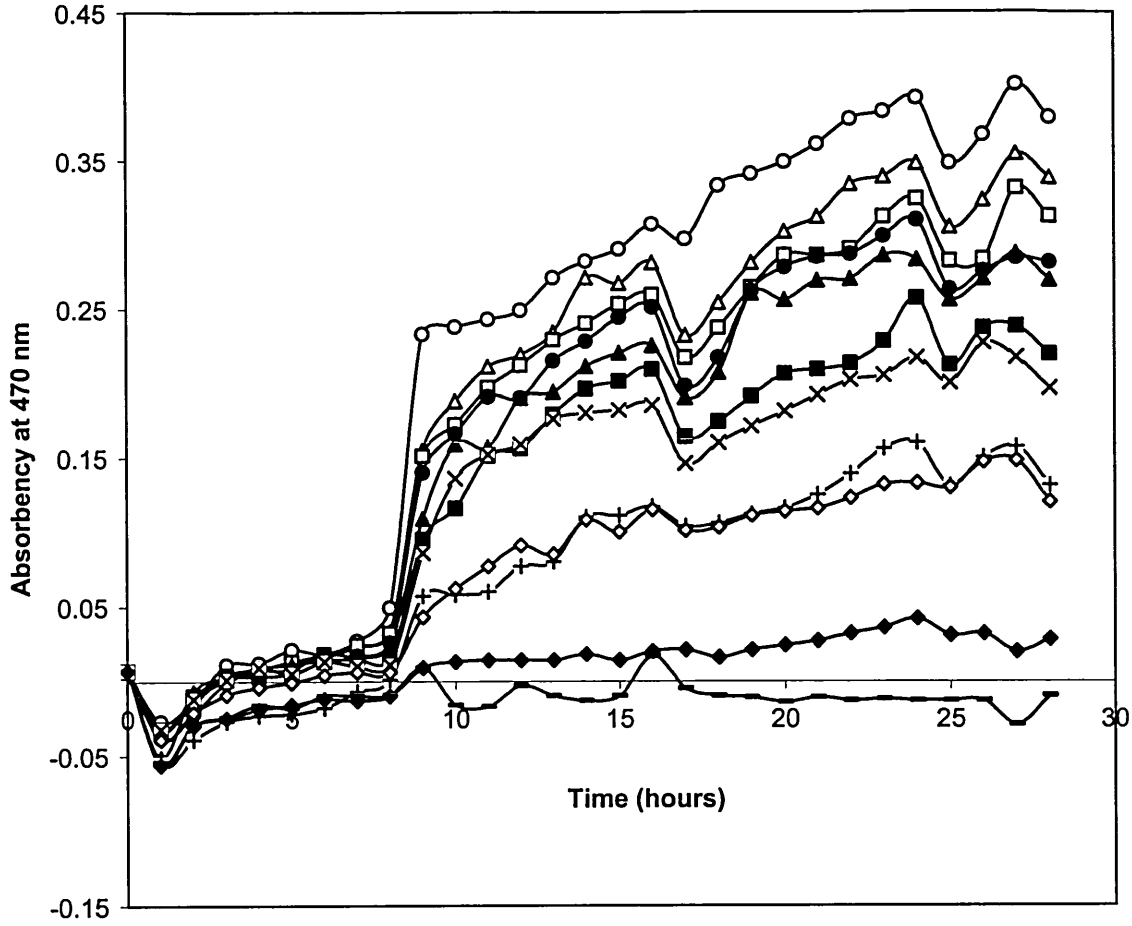


Figure 14: Growth rate of *Pseudomonas aeruginosa* PAO1 under varying conditions of nitrogen limitation. Nitrogen concentration (o) 7.39 x 10^{-4}M; (Δ) 6.651 x 10^{-4}M; (□) 5.591 x 10^{-4}M; (●) 5.173 x 10^{-4}M; (▲) 4.434 x 10^{-4}M; (■) 3.695 x 10^{-4}M; (×) 2.956 x 10^{-4}M; (+) 2.217 x 10^{-4}M; (✧) 1.478 x 10^{-4}M; (◆) 7.39 x 10^{-5}M; (-) 0M. Values are the mean of 3 replicates.

This manipulation treats the microorganism as a "bag" of enzymes, which responds to nutrient limitation, as would a single enzyme. A Lineweaver-Burk plot (Lineweaver and Burk, 1934), (**Figure 15 & 16**) of the reciprocal of rate of growth ($1/\mu$) against the reciprocal of the substrate concentration (1/[s]) was created. The point of intersection where the slope intercepts on the x-axis is the reciprocal of the K_s (Monod constant) of the culture. Thus, K_s is indicative of the cultures requirement for that specific nutrient. In general, the lower the K_s value the greater the requirement that organism has for that specific nutrient. The K_s value was 0.935M for carbon and 1.43M for nitrogen. Therefore, *Ps. aeruginosa* PAO1 has a greater requirement for a carbon source than for a nitrogen source (**Table 1**).

Table 1: Table of K_s and μ_{max} values for *Ps. aeruginosa* PAO1 in CDM as determined *via* the growth rate effect method.

Substrate	**K_s (M)**	**μ_{max}**
Carbon	0.935	0.0875
Nitrogen	1.43	0.160

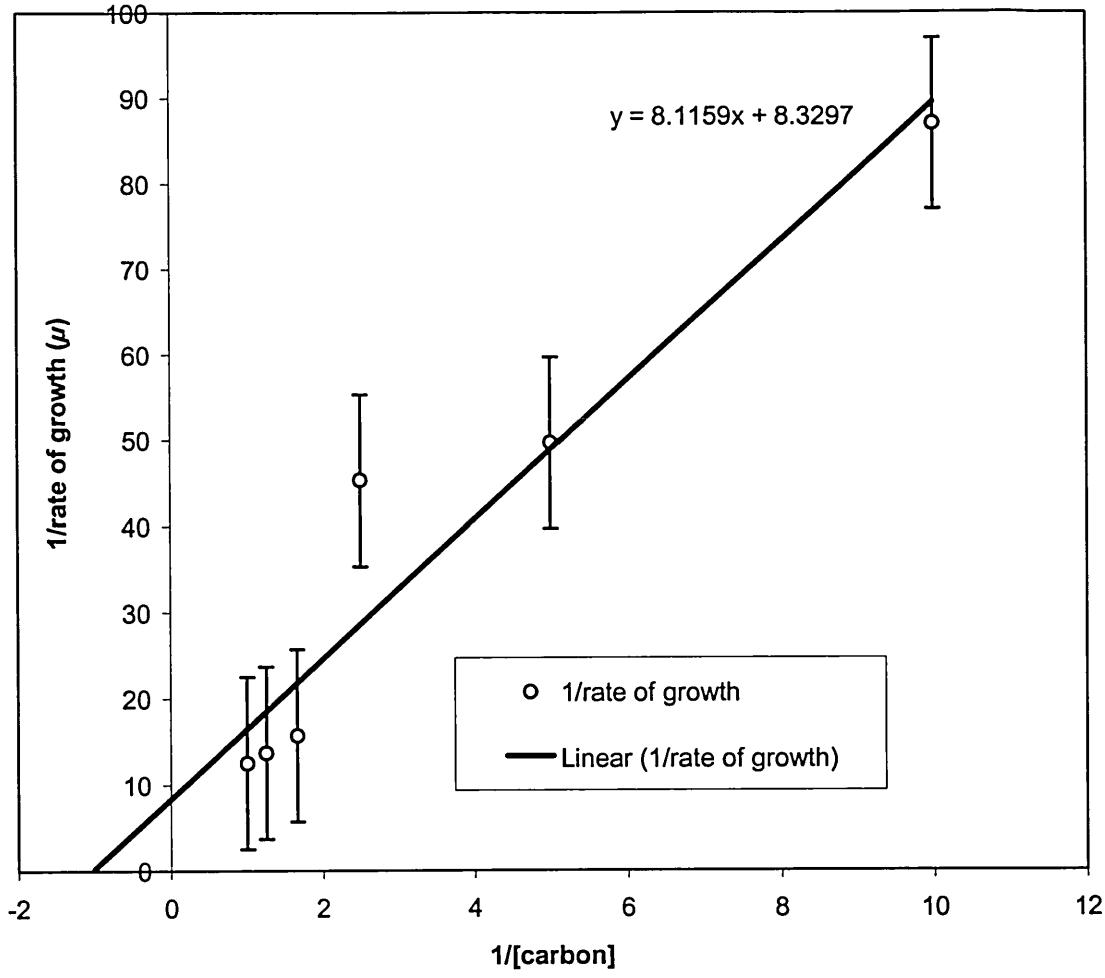


Figure 15: Lineweaver-Burk plot of carbon limitation of *Ps. aeruginosa* PAO1in CDM. The error bars are calculated as the standard error of the data set. (o) 1/rate of growth.

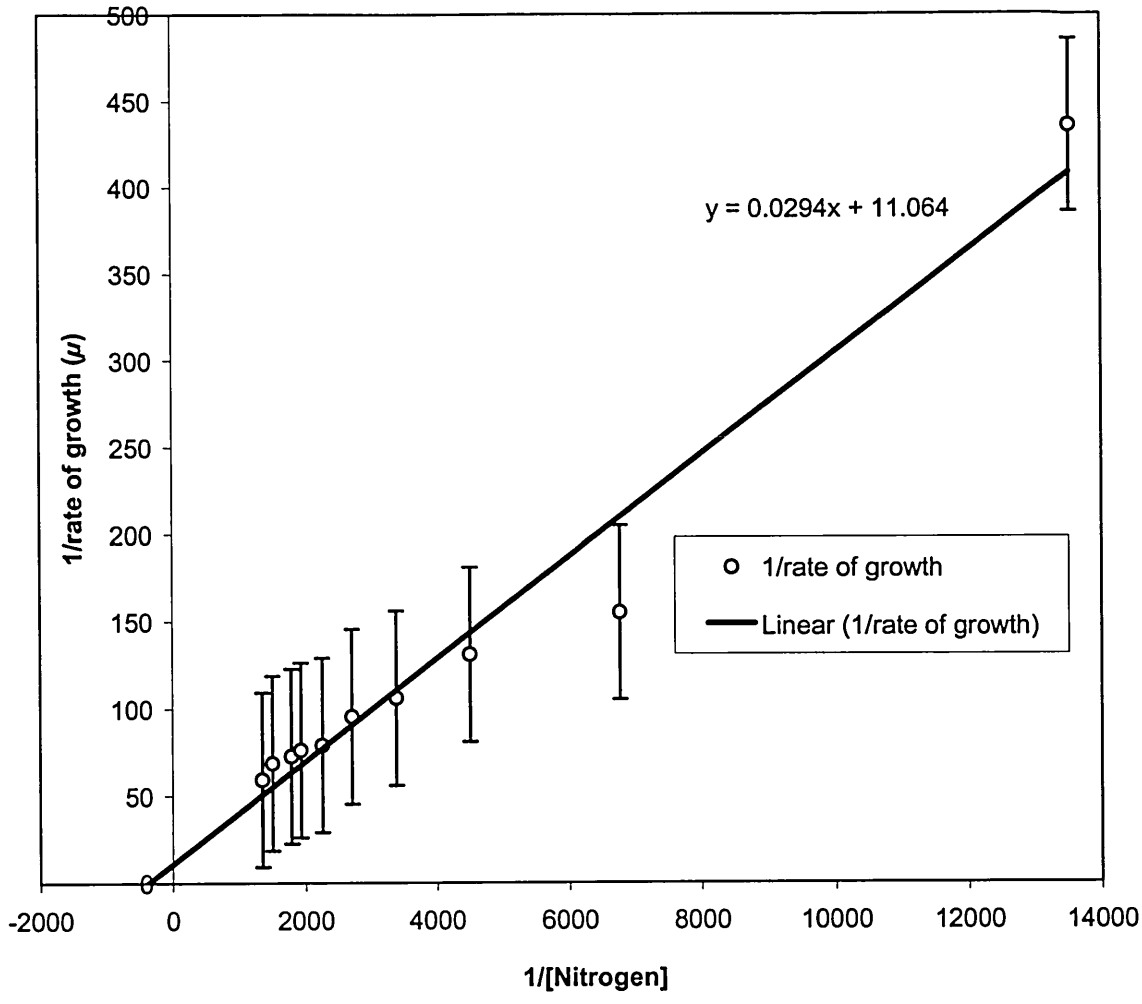


Figure 16: Lineweaver-Burk plot of nitrogen limitation of *Ps. aeruginosa* PAO1 in CDM. The error bars are calculated as the standard error of the data set. (o) 1/rate of growth.

For biofilm cultures (as determined by observation of eluate cell numbers)

Due to the time-consuming nature of the establishment and attainment of steady-state of test biofilms, it is difficult to assess biofilm growth by direct observation of cell numbers on the biofilm itself. However, the rate of production of eluate cells from a biofilm allows an estimate of the actual biofilm growth rate, due to their being a product of binary fission occurring within the biofilm. This assumption allows for the direct estimation of biofilm growth by the determination of eluate viable counts against time. **Figures 17** and **18** represent the growth curves of *Ps. aeruginosa* biofilms in CDM under nutrient limited conditions. The eluate cell numbers decreased in a step-wise fashion with decreasing concentrations of the nutrient. However, the growth rates of biofilm cells under various nutrient limitations (carbon and nitrogen) were not affected to the same extent as those for planktonic cells (**Figures 13** & **14**). The apparent inability to induce classical nutrient limitation in *Ps. aeruginosa* biofilms may be due to the presence of a polysaccharide matrix enveloping and protecting the biofilm community. Biofilm EPS can retain and capture nutrients, ions and other compounds and may be responsible for concentrating extracelluar factors at the surface of biofilm-bound bacterial cells (Costerton *et al.*, 1987; Boyd and Chakrabarty, 1995). This phenomenon suggests that nutrient gradients exist within bacterial biofilms, wherein, cells at the exterior surfaces of the biofilm are in relatively nutrient rich conditions and those in the interior are in relatively nutrient poor conditions (Huang *et al.*, 1998). Similar gradients (or their inverse) can be envisaged for oxygen and other gasses (Xu *et al.*, 1998; Okabe *et al.*, 1999) as well as bacterial metabolic bye-products. These results counter the hypothesis, which suggests nutrient limitation as a mechanism by which biofilms might reduce their susceptibility towards antimicrobial agents (Chapter 1).

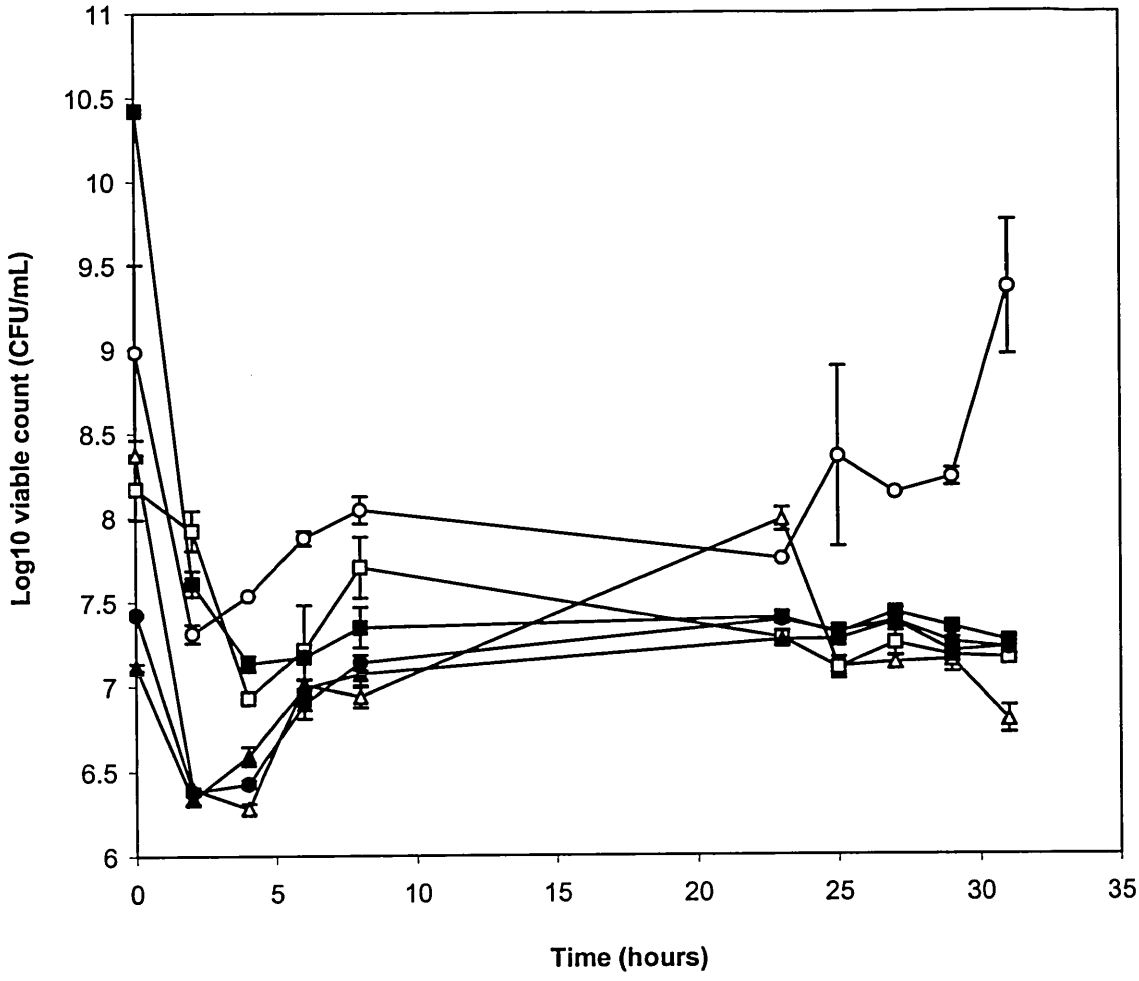


Figure 17: Eluate production (log_{10} of viable count (CFU mL^{-1})) by *Pseudomonas aeruginosa* PAO1 biofilms under varying carbon limitation. Error bars are calculated as the standard deviation of individual data points. n = 3 replicates. Carbon concentration (o) 1M; (Δ) 0.8M; (□) 0.6M; (●) 0.4M; (▲) 0.3M; (■) 0.2M.

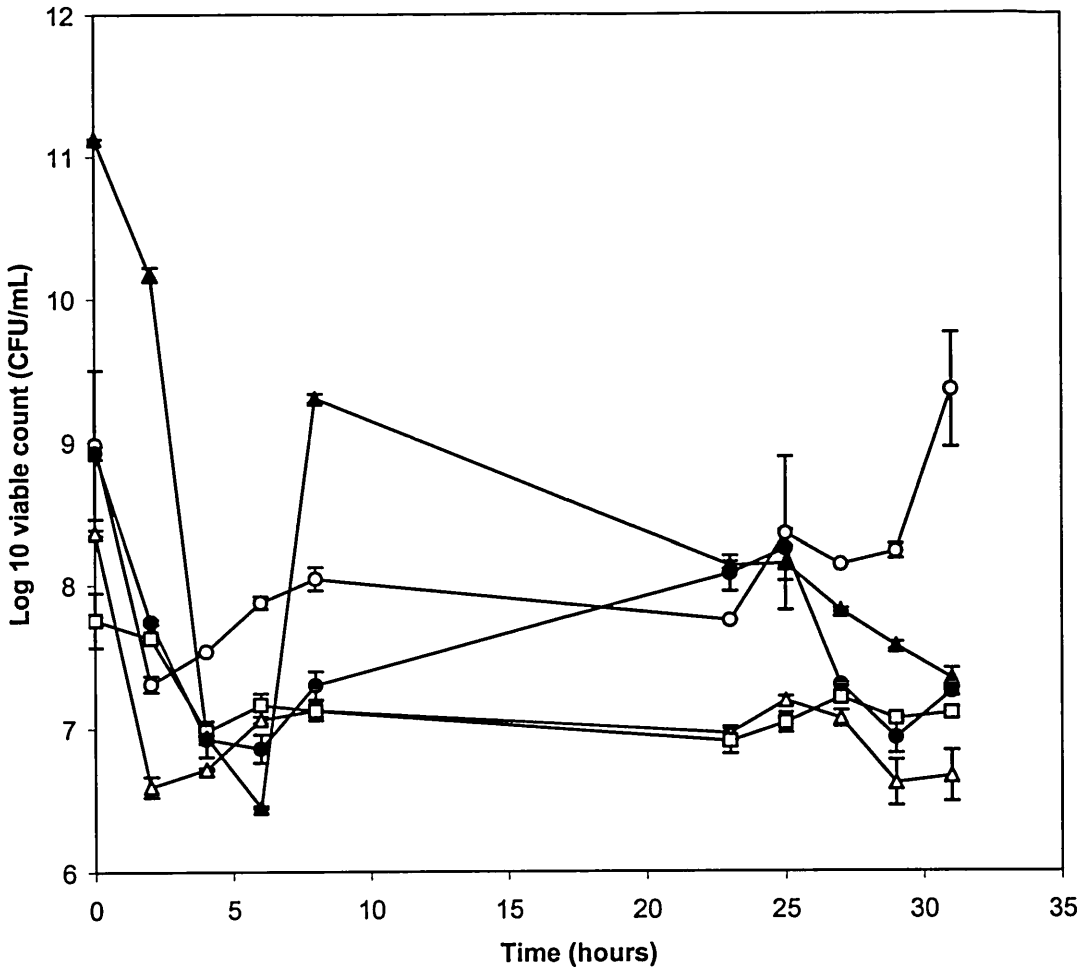


Figure 18: Eluate production (log_{10} of viable count (CFU mL^{-1})) by *Pseudomonas aeruginosa* PAO1 biofilms under varying nitrogen limitation. Error bars are calculated as the standard deviation of the individual data points. n = 3 replicates. Nitrogen concentration (o) 1M; (Δ) 0.8M; (□) 0.6M; (●) 0.4M; (▲) 0.2M.

Conclusions

The growth rates of *Ps. aeruginosa* (as planktonic cultures) were affected by the reduction of the levels of carbon and nitrogen. However, the requirement for carbon was more than that for nitrogen. Carbon limitation causes an increase of cross-linking in the peptidoglycan layer of Gram-negative bacteria, amongst other phenomena. This is important as many antimicrobial agents are cell wall active and these changes may be important in the development of bacterial resistance to the antibiotics (Mason & Egli, 1994). Previous work by Leitch (1998) showed that the limitation of nitrogen sources (K_s = 0.125M) affects the growth rate of *E. coli* more than that for carbon sources (K_s = 0.5M). Leitch (1998) performed this study in a different type of CDM (Leitch/Collier CDM, Leitch & Collier, 1996), where the major nitrogen source for *E. coli* was the amino acid L-tryptophan. Therefore, it is possible that this amino acid may be used for protein synthesis and does not contribute to the overall growth of the microorganism. Although both *Ps. aeruginosa* and *E. coli* are Gram-negative bacteria, however, their response to nutrient limitations appears to be markedly different.

For *Ps. aeruginosa* biofilm cultures (growing on Sorbarod filters), the story was different. Upon exposure of those cultures to conditions of nutrient limitation, there was a small reduction in the growth rate. These results will underpin further work using the Sorbarod model (Hodgson *et al.*, 1995) to induce resistance in *Ps. aeruginosa* biofilm cultures towards selective biocides, eliminating the possibility that any observed tolerance will be due to a nutrient-limitation phenomenon in biofilm cultures.

Chapter Four:

Assessment of Antimicrobial Activity of Selected Biocides and Induction of Tolerance in *Pseudomonas aeruginosa* PAO1 Biofilms to these Biocides

Introduction

A number of inhibitory chemicals are employed for the control of microbial growth. Chemicals that kill microorganisms or prevent their growth are called antimicrobial agents. Concentration and contact time are critical factors that determine the effectiveness of an antimicrobial agent against a particular microorganism (McDonnell & Russell, 1999; Russell & McDonnell, 2000). Microorganisms vary in their sensitivity to particular antimicrobial agents (Russell, 1999). Generally, growing microorganisms are more sensitive than organisms in dormant stages, such as spores (Brown *et al.*, 1990; Gilbert *et al.*, 1990; Knott *et al.*, 1995; Turner *et al.*, 2000). Many antimicrobial agents are aimed at blocking active metabolism and preventing the organism from generating the macromolecular constituents needed for reproduction (Neu, 1992; McManus, 1997). Because resting stages are metabolically dormant and are not reproducing, they are unaffected by such antimicrobial agents (Russell & Chopra, 1996; Turner *et al.*, 2000). Similarly, viruses are more resistant than other microorganisms to antimicrobial agents because they are metabolically dormant outside host cells (Brown, 1984).

Antimicrobial agents are used in a wide variety of applications. They are classified according to their application and spectrum of action (Chapman, personal communication; Russell & McDonnell, 2000). Germicides are chemical agents that kill microorganisms, but not necessarily bacterial endospores (Sagripanti & Bonifacino, 2000). Such chemicals may exhibit selective toxicity. Whereas germicides kill growing microorganisms, microbiostatic agents merely inhibit the multiplication of bacteria, but do not lead to actual killing or lysis of the cells (Goessens, 1993). When the microbiostatic agent is removed, microorganisms resume their growth. Disinfectants can be either germicides or microbiostatic agents that kill or prevent the growth of pathogenic microorganisms.

Antiseptics are similar to disinfectants, but may be applied safely to living tissue (Russell & McDonnell, 2000). Biocides are a collective term for antiseptics, disinfectants and preservatives (Russell *et al.*, 1998).

The antimicrobial agents used in medical practice are aimed at eliminating infecting microorgainsms or preventing the establishment of an infection (Brown & Gilbert, 1993; Chen & Stewart, 2000). In medicine, biofilms are responsible for numerous difficult-to-manage infections (Costerton *et al.*, 1999). Antimicrobial agents are widely used to control biofilm formation, but they are found to be less effective against biofilm cells than they are against cells of the same microbial strain grown in conventional suspension cultures (Costerton *et al.*, 1987; Gilbert & Brown, 1993; Dodds *et al.*, 2000). Antibiotics, which are defined as antimicrobial substances produced by microorganisms, were discovered by Sir Alexander Fleming. They have been used in medicine only since the mid-1940s (Barbosa & Levy, 2000). Although many of the antimicrobial compounds used today are in fact produced by microorganisms, and therefore are actually antibiotics, some are produced partly or entirely by chemical synthesis. Even though not of microbial origin, similar compounds synthesised by organic chemists are usually also called antibiotics (Lancini & Parenti, 1982). In order to avoid problems in terminology the all-inclusive term antimicrobial is often used. The biochemical differences in the cell structures of bacterial (prokaryotic) cells and eukaryotic cells form the basis for the effective use of antibiotics against bacterial infections (Lynn & Solotorovsky, 1981). The bacterial cell wall, with its unique peptidoglycan layer structure, and the 70s ribosome represent two major sites against which antimicrobial agents may be directed (Bryan, 1988; Russell, 1997). Most of the common antibiotics used in medicine for treating bacterial infections are inhibitors of cell wall, protein synthesis or DNA replication (Bryan, 1988; Neu, 1992; Goessens, 1993). For example, cephalosporins, aminoglycosides, tetracyclines and quinolones are used to

treat endocarditis, meningitis, tuberculosis and pneumonia (Foley and Gilbert, 1996). Some antibiotics are more selective than others with respect to the bacterial species that they inhibit. A narrow-spectrum antibiotic may be used against a particular pathogen or a particular bacterial species. In contrast, the broad-spectrum antibiotic inhibits a relatively wide range of bacterial species (Lancini & Parenti, 1982). However, concern is mounting in the medical field about the overuse of antibiotics because the undesired side effect is the selection for disease-causing antibiotic-resistant strains (Brown, 1999; Barbosa & Levy, 2000). The reason for concern about how we use antibiotics is that numerous bacterial strains have acquired the ability to resist the effects of some antibiotics, with some bacterial strains, generally those containing R plasmids, having multiple antibiotic resistance mechanisms (Russell, 1997; Levy, 1998; Mulamattahil *et al.*, 2000). Plasmid-encoded bacterial resistance has emerged to various antibiotics such as β-lactams, aminoglycosides, aminocyclitols, tetracyclines, macrolides and chloramphenicol (Russell, 1997).

Antibiotic resistance and biocide resistance may be linked, but this is not always observed clinically (Royal Pharmaceutical Society of Great Britain, 1997). The role of plasmids in encoding resistance to biocides was considered by Russell (1985). The overall conclusion was, that apart from certain specific examples such as silver and other metals, plasmids were not normally responsible for high levels of biocide resistance associated with certain species or strains. It has been proposed that intrinsic resistance in Gram-negative bacteria has the greatest significance (Russell, 1997). Resistance to both antibiotics and biocides in Gram-negative bacteria is more likely, where less specific mechanisms are involved (e.g. the outer membrane may act as a non-specific exclusion blanket, thereby preventing the uptake of chemically unrelated molecules; Russell *et al.*, 1997; Russell *et al.*, 1998). Russell *et al.* (1998) developed stable chlorhexidine (CHX)

resistance in some strains of *Ps. stutzeri*. The CHX-resistance strains showed a variable increase in resistance to quaternary ammonium compounds (QACs) and to triclosan. Additionally, these CHX-resistant strains also demonstrated a variable increase in resistance to polymyxin B, gentamicin, nalidixic acid, erythromycin, and ampicillin. Cell envelope changes were observed microscopically, implicating the outer membrane as being involved in this reduced susceptibility. However, in bacteria grown planktonically, it is now known to be the combined action of multidrug resistance (MDR) efflux pumps and decreased OM permeability that confers this resistance (Ma *et al.*, 1994; Masuda *et al.*, 1995).

Efflux is increasingly implicated as a resistance mechanism. There is evidence that *Ps. aeruginosa* can efflux triclosan and that this represents an important intrinsic tolerance mechanism to the bisphenol (Chuanchuen *et al.*, 2001; Russell, 2001). The intrinsic resistance of *Ps. aeruginosa* to numerous antimicrobial agents is even more pronounced when this microorganism is found growing as a biofilm. Antimicrobial resistance is a trait typical of most biofilm microorganisms and it has been speculated that biofilms are the causative agent of upto 65% of bacterial infections (Potera, 1999). The mechanisms by which microorganisms in a biofilm evade killing by biocides and antibiotics are of obvious practical interest and are just beginning to be discovered. It is now clear that there must be multiple resistance mechanisms (Mah & O'Toole, 2001; Chapter 1).

There are three types of hypothesised mechanisms of reduced biofilm susceptibility to antimicrobial agents. The first of these is failure of the antimicrobial agent to penetrate the full depth of the biofilm; this is due to the presence of a polysaccharide matrix enveloping the biofilm community. However, the inherent mobility of antimicrobial agents within the biofilm is unlikely to be restricted enough to account for the profoundly reduced susceptibility of biofilm microorganisms. It is only when the antimicrobial agent is

reactively neutralized in the surface layers of the biofilm, faster than it diffuses into the biofilm interior that penetration limitation occurs (Dodds *et al.*, 2000). Studies have shown that many antibiotics diffuse completely through the biofilm, but with a reduced rate of transfer (Darouichie *et al.*, 1994; Nichols *et al.*, 1989; Stewart, 1996). The ability of these agents to penetrate depends on their not being prone to deactivation in the biofilm. Therefore, penetration is most viable as a resistance mechanism when dealing with thick biofilms and highly reactive antimicrobials. Even when the antimicrobial agent is not particularly reactive or when the biofilm is very thin, biofilm microorganisms can display remarkable resistance to killing. In these cases, some other resistance mechanism must be at work because penetration-limitation is not a tenable theory under these conditions (Dodds *et al.*, 2000).

A second mechanism of biofilm reduced susceptibility requires that at least some of the cells within a biofilm experience a nutrient limitation that causes them to enter a slow-growing or starved state (Brown *et al*, 1988; Gilbert *et* al., 1990; Desai *et al.*, 1998). It is well known that the physiology of biofilm cells is remarkably heterogeneous and varies according to the location of individual cells within the biofilm (Sternberg *et al.*, 1999). Cells located at the biofilm surface presumably have adequate supplies of nutrients and are metabolically active, while deeply embedded cells are likely to be metabolizing more slowly due to potential nutrient and oxygen limitations (Huang *et al.*, 1998; Xu *et al.*, 1998). Because many antimicrobial agents require actively metabolizing cells to be effective, the presence of slow growing or dormant cells is thought to represent a resistant population (Brown *et al.*, 1988). Tresse *et al.* (1995) examined the susceptibility of agar entrapped *E. coli* cells to a beta-lactam and aminoglycoside antibiotics under different oxygen tensions. They found that oxygen deficiency in the gel layer of the agar contributed to the enhanced resistance of the bacteria to the aminoglycoside, but not to the

beta-lactam, and proposed that the effect was due to the lowered uptake of the antibiotic by the oxygen deprived cells. This would be particularly relevant for thicker biofilms, where cells within the biofilm could become oxygen limited.

The third mechanism of reduced biofilm susceptibility suggested that bacteria growing in a biofilm undergo distinct phenotypic changes associated with surface-attached growth that render them more resistant (Cochran *et al.*, 2000a). At present, very little is known about the genotypic and/or phenotypic changes that occur as cells transition from planktonic to the biofilm mode of growth (De Kievit *et al.*, 2001). However, the practical importance of this mechanism, should it prove true, is profound because it implies that reduced susceptibility of biofilm cells is genetically programmed. Dagostino *et al.* (1991) speculated that association of bacteria with a surface during biofilm formation causes a number of physiological responses as a result of the repression or induction of genes. They were able to demonstrate that a specific gene was induced by surface associated bacterial cells, but that this induction did not occur in liquid media. Also, the work of Cochran *et al.* (2000a) showed that reduced susceptibility of attached *Ps. aeruginosa* cells to two oxidative biocides was recognized as an inherent phenotypic change and not just a transport artefact. Brooun *et al.* (2000) showed that cells liberated from a *Ps. aeruginosa* PAO1 biofilm into growth medium were considerably more resistant to tobramycin than planktonic cells. This experiment suggested that cells become intrinsically more resistant when growing in the biofilm and retain part of this resistance even outside the biofilm.

Therefore, one can suggest that biofilms are probably protected by multiple resistance mechanisms (Stewart, 2001). Mah & O'Toole (2001) suggest the possibility that multiple resistance mechanisms operate in concert within a single biofilm community. In practical terms this means that the problem of biofilm control by antimicrobial agents is indeed complex. In addition to overcoming antimicrobial penetration failure and

surmounting regions of starved or nutrient-limited cells, one must also cope with an inherently resistant biofilm phenotype.

Antimicrobial Susceptibility Testing

As soon as antibiotics became commercially available to medical practitioners, the need for antimicrobial susceptibility testing immediately became apparent. Determination of the antimicrobial susceptibility of a pathogen is important in aiding the clinician to select the most appropriate agent for treating this disease. The most common approach to antimicrobial susceptibility testing is to determine the minimum inhibitory concentrations (MIC) using tube dilution procedures (Ceri *et al.*, 1999). The MIC measures the actions of antibiotics against planktonic microorganisms and serves as an important reference in the treatment of many acute infections. Application of MICs in the treatment of chronic or device-related infections involving bacterial biofilms is often ineffective (Costerton *et al.*, 1995). MIC is, in effect, a range of concentrations depending on the dilution series used. Measurement of the MIC should be a first line technique of discovery for the biocide and preservative scientist, but in many laboratories, it is used as an indicator of activity rather than something of real substantive value (Lambert & Pearson, 2000).

A current definition of the MIC is the "*lowest concentration, which resulted in maintenance or reduction of inoculum viability*" (Carson *et al.*, 1995). The determination of the MIC involves a semi-quantitative test procedure which gives an approximation to the least concentration of an antimicrobial needed to prevent microbial growth. In the recent past, the method used tubes of growth broth containing a test level of preservative, into which an inoculum of microbes was added. The end result of the test was the minimum concentration of antimicrobial, which gave a clear solution (i.e. no visible

growth) (Davidson & Parish, 1989).

The aims of this chapter were to determine the characteristics of inhibition of the selected biocides against *Ps. aeruginosa* PAO1. In particular, the determination of the planktonic MIC was undertaken in order to allow for an estimate of in-use MIC for subsequent biofilm experiments to be determined. The use of the passage technique upon biofilm cultures of *Ps. aeruginosa* PAO1 in order to observe potential development of tolerance or resistance towards the selected biocides was another aim of this chapter.

Experimental Approach

Tube Dilution Method

The tube dilution or serial dilution method, according to Bloomfield (1991), provides a quantitative assessment of active concentrations of antimicrobials. This test determines the concentration of an antibiotic that is effective in preventing the growth of the pathogen and gives an indication of the dosage that should be effective in controlling the infection. A standardized microbial inoculum is added to tubes containing serial dilutions of an antimicrobial agent and the growth of the microorganism is monitored as a change in turbidity. In this way, the breakpoint or MIC of the antimicrobial agent, that is the lowest concentration that prevents growth of the microorganism *in vitro*, can be determined. The MIC is dependent on the conditions under which the experiment is run. These conditions should be specified when reporting the results. The most important conditions that have been observed are the incubation temperature, the organism and the inoculum size (Lambert & Pearson, 2000). 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). The MIC can be used for determining the

antimicrobial agent sensitivity of both aerobic and anaerobic microorganisms. This test is used to establish the concentration of an antimicrobial agent that will inhibit growth, but not to determine whether the antibiotic is microbiocidal.

Determination of MIC

Initially, a screening for the MIC point is performed. Aliquots (9 mL) of media (R2A or CDM) were prepared and doubling concentrations (or 10 fold increase) of biocide were added in 1 mL aliquots (**Table 2**). Tubes were inoculated with 100 µL of a 16h (overnight) culture of *Ps. aeruginosa* PAO1 grown at 37°C, 180 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 approximate value is determined an arithmetical series of not less than 8 dilutions is employed in order to establish the exact MIC. In determining the exact MIC value, the choice of dilutions ranged between the two concentrations where growth was visible and absent (**Table 3**).

Table 2: Determination of screening MIC for Sodium Pyrithione against *Ps. aeruginosa* PAO1 in R2A medium. n = 3 replicates.

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

+ = Presence of Growth.

- = Absence of Growth.

Table 3: Determination of exact MIC for Sodium Pyrithione against *Ps. aeruginosa* PAO1 in R2A medium. n = 3 replicates.

Biocide concentration (µg mL^{-1})	**Growth**	
	19h	**48h**
0	+	+
30	+	+
35	+	+
40	+	+
45	+	+
50	+	+
55	-	-
60	-	-
65	-	-
70	-	-

+ = Presence of Growth.

- = Absence of Growth.

Table 4: Summary of MIC results obtained against *Ps. aeruginosa* PAO1 in R2A medium. n = 3 replicates.

Biocide	Screening MIC (μg mL^{-1})		Exact MIC (μg mL^{-1})	
	19h	48h	19h	48h
BIT	64	64	55	55
Thiomersal	16	16	13	13
Zn Pyrithione	16	16	3	3
Na Pyrithione	32	64	55	55
Cetrimide	64	*	*	*

* = Observed Resistance at all Test Concentrations.

Table 5: Summary of MIC results obtained against *Ps. aeruginosa* PAO1 in CDM. n = 3 replicates.

Biocides	Screening MIC (µg mL^{-1})		Exact MIC (µg mL^{-1})	
	19h	**48h**	**19h**	**48h**
BIT	16	16	8	8
Thiomersal	1	1	0.1	0.1
Zn Pyrithione	8	8	2.5	2.5
Na Pyrithione	64	64	55	55
Cetrimide	32	32	20	20

Results & Discussion

R2A medium is a nutrient-enriched medium. This medium has accessible thiol groups with which BIT and thiomersal interact oxidatively. The antimicrobial activity of BIT is strongly antagonised by thiol-containing materials (Fuller *et al.*, 1985; Collier *et al.*, 1990a). This will lead to a greater observed MIC for this biocide in order to inhibit the growth of the bacteria. Hence, the true MIC is hidden by an interaction between BIT and thiomersal biocides and exogenous thiol.

CDM is a chemically defined medium. This medium has a source of transition metal ion (Fe^{+3} in solution B), which causes the chelation of the pyrithione biocides. The behaviour of NaPT and ZnPT in solution enables transchelation to occur when these compounds are in the presence of transition metal ions (i.e. Cu^{+2} and Fe^{+3}) (Edrissi *et al.*, 1971). This will cause a reduction in the MIC..

Although the same method was performed for both media (R2A and CDM), MIC values vary considerably between the two media, which may be the result of the differing constituents of each medium (**Tables 4 & 5**). For example, the MIC value for BIT was 55 $\mu g\ mL^{-1}$ in R2A medium and 8 $\mu g\ mL^{-1}$ in CDM. For thiomersal, this value was 13 $\mu g\ mL^{-1}$ and 0.1 $\mu g\ mL^{-1}$ in R2A and CDM, respectively. It was difficult to observe any growth in CDM against thiomersal. This is thought to be a result of the combined effect of nutrient limitation (by growing the cells in a chemically defined medium) and biocide exposure. In comparing the MIC values between ZnPT and NaPT, the exact MIC value for NaPT was 55 $\mu g\ mL^{-1}$ (in both types of media), which is significantly higher than that for ZnPT (3 $\mu g\ mL^{-1}$ in R2A and 2.5 $\mu g\ mL^{-1}$ in CDM). This indicates that ZnPT demonstrates a greater activity towards *Ps. aeruginosa* PAO1 than NaPT. Cetrimide, which is also a membrane active biocide, was used throughout this experiment as a

positive control.

Winder *et al.* (2000) performed the same experiment for *Ps. aeruginosa* against the isothiazolone biocides. This group also undertook the same test against the pyrithione biocides (Abdel Malek *et al.*, 2002). For BIT, the exact MIC value was 60 μg mL^{-1} in R2A medium and 5.8 μg mL^{-1} in CDM. For thiomersal, this value was 9.5 μg mL^{-1} in R2A medium. It was impossible to observe any growth in CDM. These results are similar to the results, which were obtained from this test. For the pyrithione biocides, the exact MIC for NaPT was 40 μg mL^{-1} and 30 μg mL^{-1} in R2A and CDM, respectively. For ZnPT, the MIC was 4 μg mL^{-1} in R2A medium and 3.5 μg mL^{-1} in CDM. Growing *Ps. aeruginosa* in R2A medium against cetrimide did not yield any result; but in CDM, the MIC was 10 μg mL^{-1} (**Table 6**).

Al-Adham *et al.* (1998) determined the MIC values for selected biocides. Among those biocides were the pyrithiones and BIT. *Ps. aeruginosa* cultures were grown in nutrient broth (Oxoid CM1). The MIC was 100 μg mL^{-1} for NaPT, 13 μg mL^{-1} for ZnPT, 80 μg mL^{-1} for BIT and 128 μg mL^{-1} for cetrimide. These values are much higher than the values obtained from this study. This is due to growing *Ps. aeruginosa* in richer media.

Table 6: Summary of MIC results obtained against *Ps. aeruginosa* PAO1 in CDM and R2A media from three different studies. n = 3 replicates.

Biocide	Medium	MIC (μg mL^{-1}) for study number		
		1	2	3
BIT	R2A	55	60	-
Thiomersal	R2A	13	9.5	-
ZnPT	R2A	3	-	4
NaPT	R2A	55	-	40
Cetrimide	R2A	*	-	*
BIT	CDM	8	5.8	-
Thiomersal	CDM	0.1	**	-
ZnPT	CDM	2.5	-	3.5
NaPT	CDM	55	-	30
Cetrimide	CDM	20	-	10

1 = the results obtained from this study; 2 = the results obtained from Winder *et al.* (2000); 3 = the results obtained from Abdel Malek *et al.* (2002); * = observed resistance at all test concentrations; ** = no growth in all test concentrations; - = not performed in that study.

MIC Determination for Biofilm Cultures

A biofilm of *Ps. aeruginosa* PAO1 was established on a Sorbarod filter (Chapters 1 & 3; Hodgson *et al.*, 1995). This biofilm was perfused with CDM at 37°C. This was only run for 48 hours (to reach steady-state). At 48 hours, the Sorbarod filters were sacrificed (the adherent cells were removed and resuspended in 0.9% (w/v) sterile normal saline) and the MICs for five selected biocides (ZnPT, NaPT, Cetrimide, BIT & Thiomersal) were determined according to the tube dilution method (Bloomfied, 1991) using a standard inoculum (100 μL). This was performed for the eluate and attached cells (**Table 7**).

Table 7: Summary of MIC results obtained in CDM for *Ps. aeruginosa* PAO1 Biofilm and Eluate cells. n = 3 replicates.

Biocide	Eluate MICs (μg mL^{-1})		Biofilm MICs (μg mL^{-1})	
	19hr	48hr	19hr	48hr
BIT	1	1	4	4
Thiomersal	1	1	1	1
ZnPT	8	8	8	8
NaPT	64	64	64	64
Cetrimide	16	16	32	32

Induction of resistance

The idea of induction of resistance in *Ps. aeruginosa* PAO1 to selected biocides was taken from a method previously described by Brözel and Cloete (1994) (Chapter 1). The Sorbarod Model was established according to the model described previously by Hodgson *et al.* (1995) (Chapters 1 & 3). However, the induction of tolerance in biofilm cultures (growing on Sorbarod filters) has not been performed before. Therefore, this combination could be considered an original and useful idea to enable our better understanding of the development of resistance in biofilms. The Sorbarod filter was pre-wetted with 5 mL of 0.9% (w/v) sterile normal saline then inoculated with 10 mL from an overnight culture of *Ps. aeruginosa* PAO1. The rubber plunger seal was withdrawn from a sterile disposable 2 mL syringe. The syringe was introduced into PVC tubing containing the Sorbarod filter and a sterile, disposable needle (0.8 x 40 mm) inserted through the rubber seal. This unit was placed within a 37°C incubator. Media inlet tubing was attached *via* the needle and sterile CDM was delivered to the unit. This medium was prepared to contain a quarter strength concentration of biocide of the previously established MIC (MIC/4). After 48 hours, the eluate culture for the last hour was collected and the biofilm filter was sacrificed (the adherent cells were removed and resuspended in 0.9% (w/v) sterile normal saline, 10 mL) and the MIC for both eluate and biofilm cells were redetermined (Passage 1). An aliquot (1 mL) of the cultures was spread plated onto sterile CDM gradient plates. This was performed for both eluate and biofilm cells. The plates were incubated at 37°C for 48 to 72 hours (Chapter 2). The point at which growth ceased on the surface of the agar was used to indicate the approximate MIC. The colonies growing in this region (the region with the greatest concentration of the biocide) are the most resistant and therefore, were inoculated into the next passage. These colonies were resuspended in 0.9% (w/v) sterile

normal saline (25 mL) and used to inoculate a new Sorbarod filter. A new unit was set up in 37°C incubator and a fresh CDM media (4L in 5L Pyrex flask) containing MIC/4 of the newly established MIC was delivered to this unit. This was repeated until 9 successive passages had been performed in the presence of increasing MIC/4 of the biocide. Although the MICs may continue to increase with further exposure to the biocide, the cultures were deemed sufficiently tolerant for our experimental purposes, at this point. The resistant biofilm colonies on the gradient plate (Passage 10) were removed and resuspended in 10 mL, 0.9% (w/v) sterile normal saline, as noted above. This was used as an inoculum for the next passage (Passage 11). In this passage, the Sorbarod filter was perfused with a fresh CDM containing no biocide. The MIC was redetermined by the tube dilution method (Bloomfield, 1991). This was repeated for two successive passages (Passages 12 & 13), without the addition of biocide. This passaging process was repeated for four selected biocides (ZnPT, NaPT, BIT & Cetrimide) in CDM.

The experimental aim of this study was to evaluate the efficacy of selected biocides on biofilms grown within Sorbarod filter plugs, which were perfused with medium. The antimicrobial effects of the se biocides were assessed in this simplified biofilm system (Hodgson *et al.*, 1995) by comparing sub-minimum inhibitory concentrations (sub-MIC) of eluate and biofilm (attached) cells.

Results & Discussion

Figures 19 to **22** illustrate the MIC values for the passaging process for the four selected biocides. In all cases both the biofilm and eluate cells exhibited increased MICs after each passage. During 10 passages in increasing MIC/4, the biofilm and eluate cells exhibited gradual increases in MIC of up to 3- or 4-fold from the initial value (ZaPT & Cetrimide). For BIT the increase in MIC over 10 passages was17-fold for adherent cells and 10-fold for the eluate cells. The same passaging process was performed in CDM against thiomersal, but no results were established from that process. This may be due to the combined stress effect of growing the cells in a chemically defined medium and biocide exposure.

Exposure to Zinc Pyrithione (ZnPT)

There was no difference in MIC values for both biofilm and eluate cells (**Figure 19**). The initial MIC against ZnPT in CDM was 4 $\mu g\ mL^{-1}$ (Passage 1). At Passage 3 the MIC value started to increase (6 $\mu g\ mL^{-1}$), after which, the MIC value gradually increased with each progressive exposure to the biocide, until Passage 10 at which point the MIC was 17 $\mu g\ mL^{-1}$. This is an increase of 4.25-fold from the initial MIC. The following passage (Passage 11) was the first passage performed in biocide-free medium and the MIC value immediately decreased to 13 $\mu g\ mL^{-1}$, which remained constant for the two successive passages. These results are summarized in **Table 8**.

Table 8: Summary of the average MICs for *Ps. aeruginosa* PAO1 biofilm and eluate cells in CDM when exposed to the four Biocides. n = 3 replicates.

Biocide	Type of Cells	MIC (μg mL^{-1}) of passage number		
		1	**10**	**13**
BIT	Biofilm	2	34	16
	Eluate	2	20	16
ZnPT	Biofilm	4	17	13
	Eluate	4	17	13
NaPT	Biofilm	48	51	51
	Eluate	40	51	51
Cetrimide	Biofilm	18	56	51
	Eluate	14	56	51

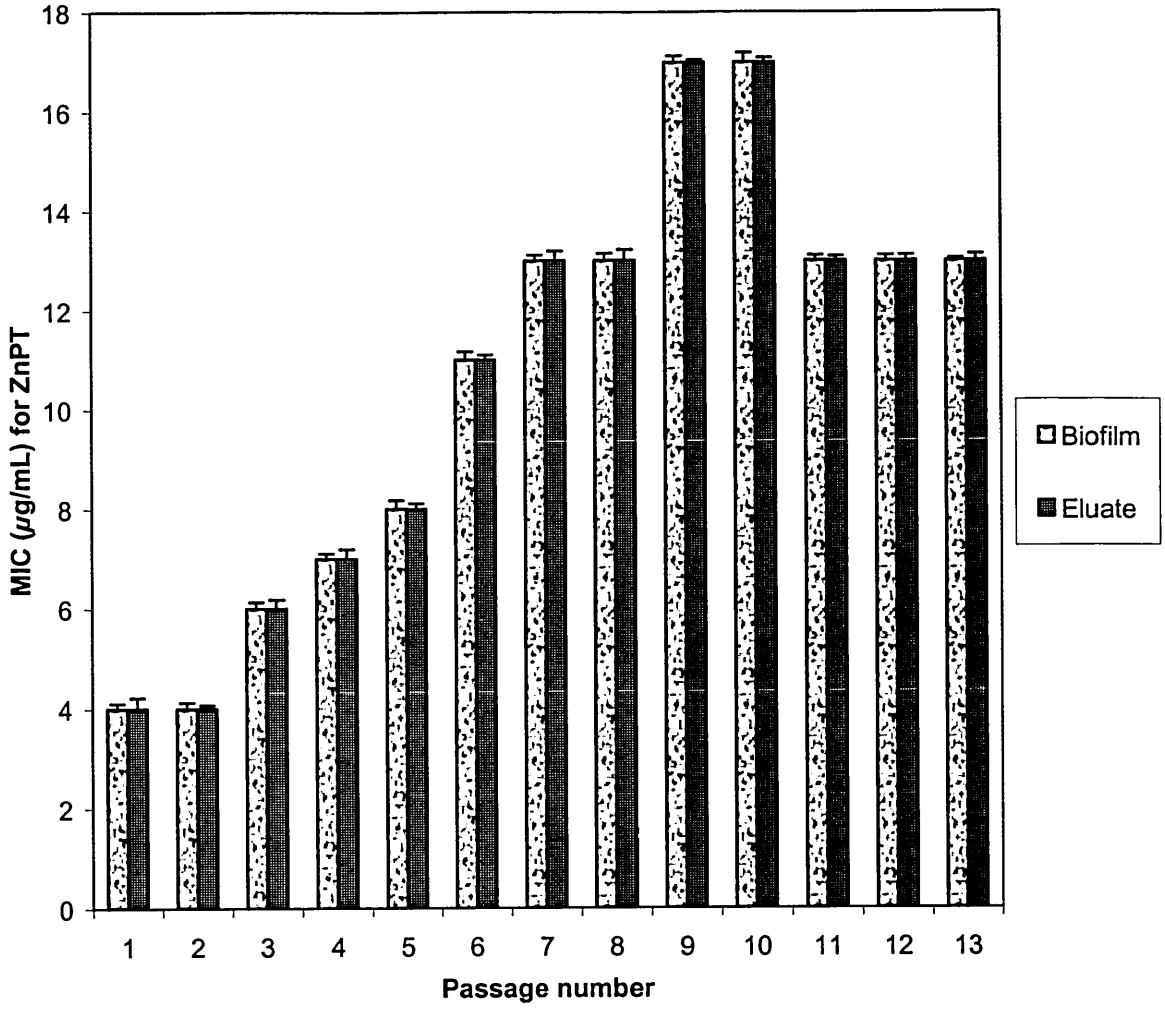


Figure 19: Increase in MIC for ZnPT against *Pseudomonas aeruginosa* PAO1 in CDM as a function of passage number. The error bars are calculated as the standard deviation of the individual data points. n = 3 replicates. Passages 11, 12 and 13 were performed without the addition of biocide.

In comparing MIC values for biofilm (adherent) and eluate cells with those obtained from planktonic cells exposed to ZnPT (previous work performed by this group; Abdel Malek *et al.*, 2002), the initial MIC for planktonic cells in CDM was 4.5 μg mL^{-1}. The MIC value continued to increase gradually with every successive passage in the presence of biocide, until Passage 10 (12.75 μg mL^{-1}) where an increase of 2.8-fold was observed. When the cells were grown in biocide-free medium the MIC values began to decrease and by Passage 13 the MIC was 9.75 μg mL^{-1} (**Table 9**).

From the previous comparison, one can demonstrate that the susceptibility of the biofilm-derived (adherent) cells and their offspring (eluate cells) was markedly greater than that of cells of planktonic origin (17 μg mL^{-1} for biofilm/eluate cells and 12.75 μg mL^{-1} for planktonic cells). For both types of growth the MIC values did not revert to the initial value. This suggests that the induced biocide resistance is largely irreversible.

Exposure to Sodium Pyrithione (NaPT)

There was a difference in MIC values between biofilm and eluate cells in the first two passages, but after that the MIC values remained the same throughout the successive passages (**Figure 20**). The initial MIC for the cells exposed to NaPT was 48 μg mL^{-1} for adherent cells and 40 μg mL^{-1} for eluate cells. This is considerably higher than the initial MIC against ZnPT (4 μg mL^{-1} for both adherent and eluate cells) indicating that ZnPT demonstrates a greater activity towards *Ps. aeruginosa* PAO1 than NaPT. For the next four successive passages in the presence of biocide, there was a gradual increase in MIC values. At Passage 6, the MIC value decreased to 51 μg mL^{-1} for both eluate and biofilm cells. For the following passages (Passages 7 to 10) there was no increase in the MIC value. Immediately following the biocide exposure passages, the cells were passaged in biocide-free medium and the MIC value did not decrease remaining at 51 μg mL^{-1}.

For planktonic cells, the initial MIC against NaPT when the cells were cultured in CDM was 54 μg mL^{-1}(Abdel Malek *et al.* 2002). The MIC values gradually increased up to Passage 10, at which point the cells that were deemed 'resistant' and the corresponding MIC was 115 μg mL^{-1}. However, upon removing the cells from the presence of the biocide, the MIC began to decrease and at Passage 13 the MIC was 75 μg mL^{-1} (**Table 9**).

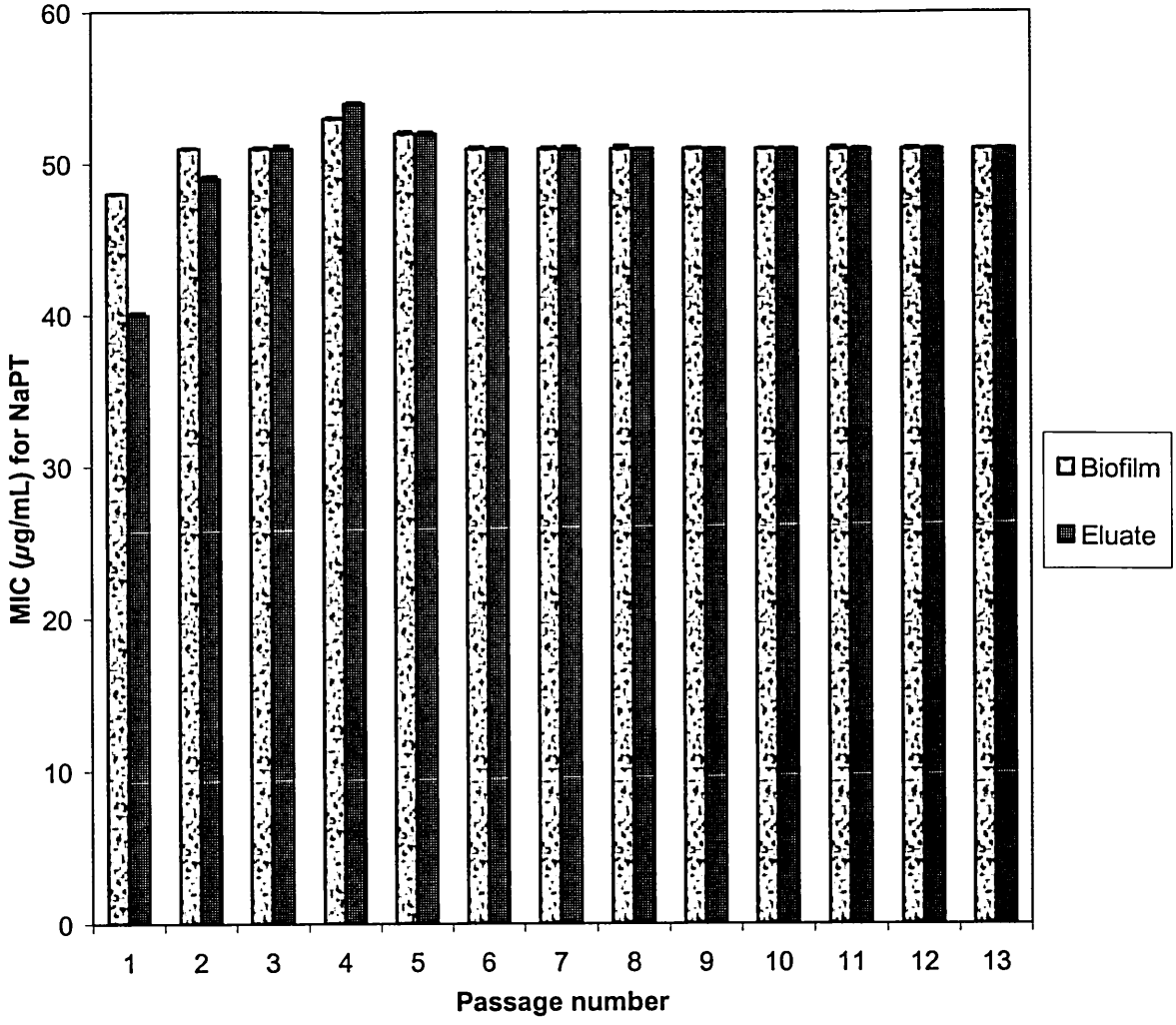


Figure 20: Increase in MIC for NaPT against *Pseudomonas aeruginosa* PAO1 in CDM as a function of passage number. The error bars are calculated as the standard deviation of the individual data points. n = 3 replicates. Passages 11, 12 and 13 were performed without the addition of biocide.

Exposure to Cetrimide

Cells grown in CDM and exposed to Cetrimide (**Figure 21**) exhibited a considerable difference in MIC values between biofilm and eluate cells. Newly divided daughter populations were generally more sensitive than their heterogeneous donor cultures. This suggests some sensitisation of the cells at or shortly after division (Evans *et al.*, 1990b). The initial MIC was 18 μg mL^{-1} and 14 μg mL^{-1} for biofilm and eluate cells respectively. At Passage 2, the MIC value was 24 μg mL^{-1} for biofilm cells and 19 μg mL^{-1} for eluate cells. This is an increase of 1.33-fold for the adherent cells and 1.36-fold for the eluate cells from their initial MIC values. The MIC value gradually increased with each progressive exposure to the biocide, until Passage 10 at which point the MIC was 56 μg mL^{-1} for both adherent and eluate cells. This is an increase of 3.11-fold for adherent cells and 4-fold for eluate cells from their initial MICs. The difference in MIC value between eluate and adherent cells was observed until Passage 8, at which point the MIC was 52 μg mL^{-1} for both adherent and eluate cells. Following the biocide-exposure passages the cells were passaged in biocide-free medium, and immediately the MIC value decreased to 51 μg mL^{-1}. This value remained the same for the 2 successive passages in biocide-free medium and there was no difference in MIC values between biofilm and eluate cells. Wright *et al.* (1997) demonstrated that MICs for cells taken from the biofilm and immediately exposed to antibiotic are the same as MICs for the wild type.

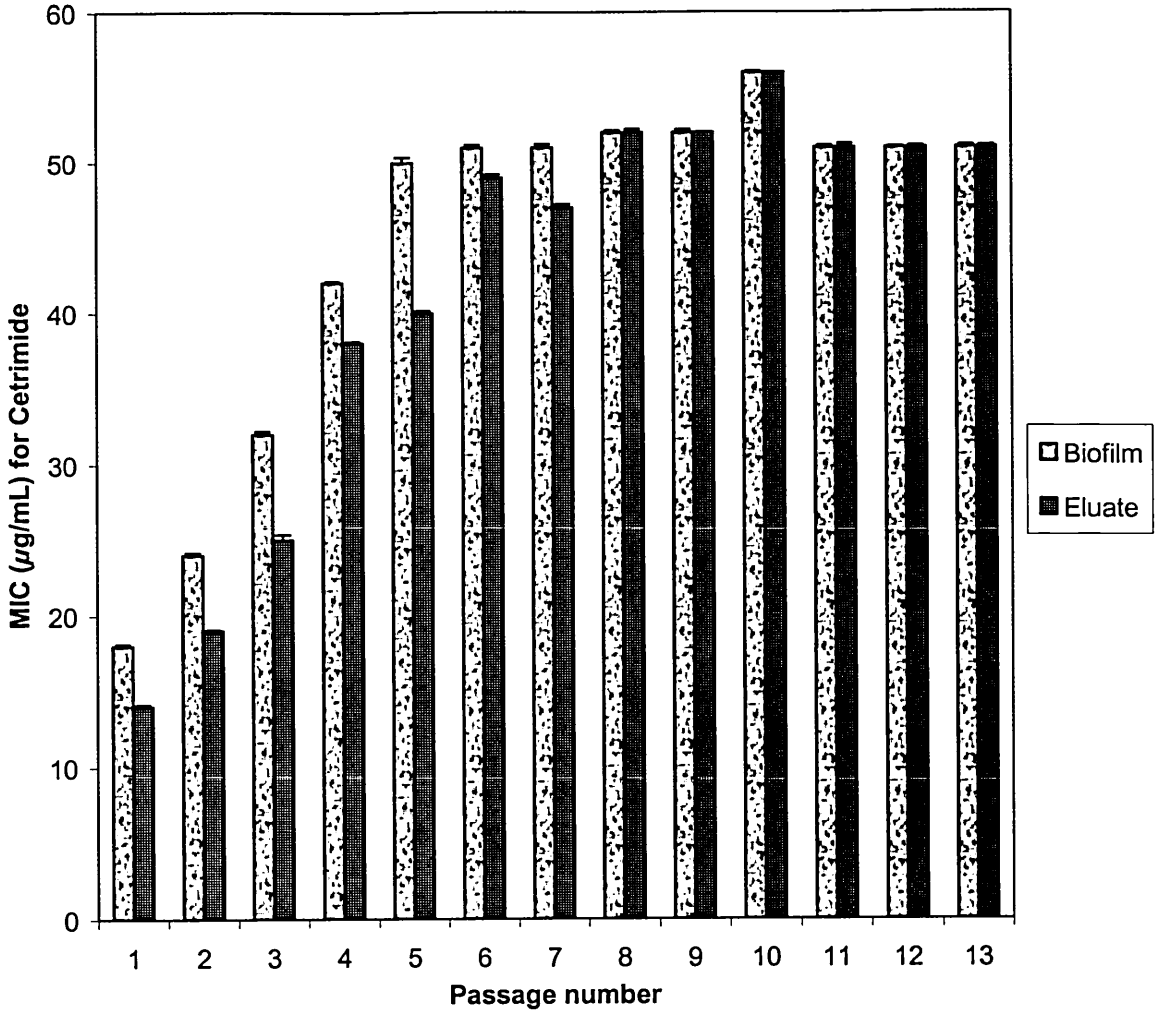


Figure 21: Increase in MIC for Cetrimide against *Pseudomonas aeruginosa* PAO1 in CDM as a function of passage number. The error bars are calculated as the standard deviation of the individual data points. n = 3 replicates. Passages 11, 12 and 13 were performed without the addition of biocide.

For planktonic cells, the initial MIC against cetrimide in CDM was 15 μg mL^{-1}. The MIC continued to increase up to Passage 10, the MIC was 130 μg mL^{-1}, an increase of 8.67-fold. The cells were then cultured in biocide-free medium; at Passage 13 the MIC had decreased to 90 μg mL^{-1} (**Table 9**). The MIC values for planktonic cells and those for biofilm cells demonstrated that sessile bacteria and their daughter cells were less resistant to cetrimide than their planktonic counterparts, which was unexpected. This is thought to be due to the higher numbers of bacterial cells in planktonic cultures (10^9 CFU mL^{-1}) in comparison to those numbers, which had been harvested, on sacrifice, from the Sorbarod Filter (i.e. 10^7 to 10^8 CFU mL^{-1}) (**Figure 12**, Chapter 3).

Exposure to Benzisothiazolone (BIT)

The initial MIC value for biofilm and eluate cells exposed to BIT when grown in CDM was 2 μg mL^{-1} (**Figure 22**). There was no difference in MIC value between adherent and eluate cells in Passage 1, but a significant difference in this value between both types of cells was observed in the following passages. Where the MICs continued to increase in a step-wise fashion, until at Passage 10 the MIC had increased to 34 μg mL^{-1} for the adherent cells and 20 μg mL^{-1} for the eluate cells, an increase from the initial MIC of 17-fold for the adherent cells and 10-fold for the eluate cells. The cells were then passaged in biocide-free medium, and a significant decrease in MIC was observed at 13 μg mL^{-1} for both types of cells (Passage 11). The MIC value for the biofilm cells deceased to 10 μg mL^{-1} at Passage 13.

For planktonic cells (Winder *et al.*, 2000), the initial MIC for the cells exposed to BIT cultured in CDM was 5.73 μg mL^{-1}. The MIC values gradually increased up to Passage 10, the cells that were then deemed "resistant" and the corresponding MIC was 9.07 μg mL^{-1}. This is an increase of 1.58-fold. Following the biocide-exposure passages the cells were then passaged in biocide-free medium. The MIC values immediately began to decrease, at Passage 13 the MIC had decreased to 6.4 μg mL^{-1} (**Table 9**). By comparing the MIC values for planktonic cells and biofilm cells, one can demonstrate the high resistance of sessile growth to BIT in comparison for that of planktonic growth.

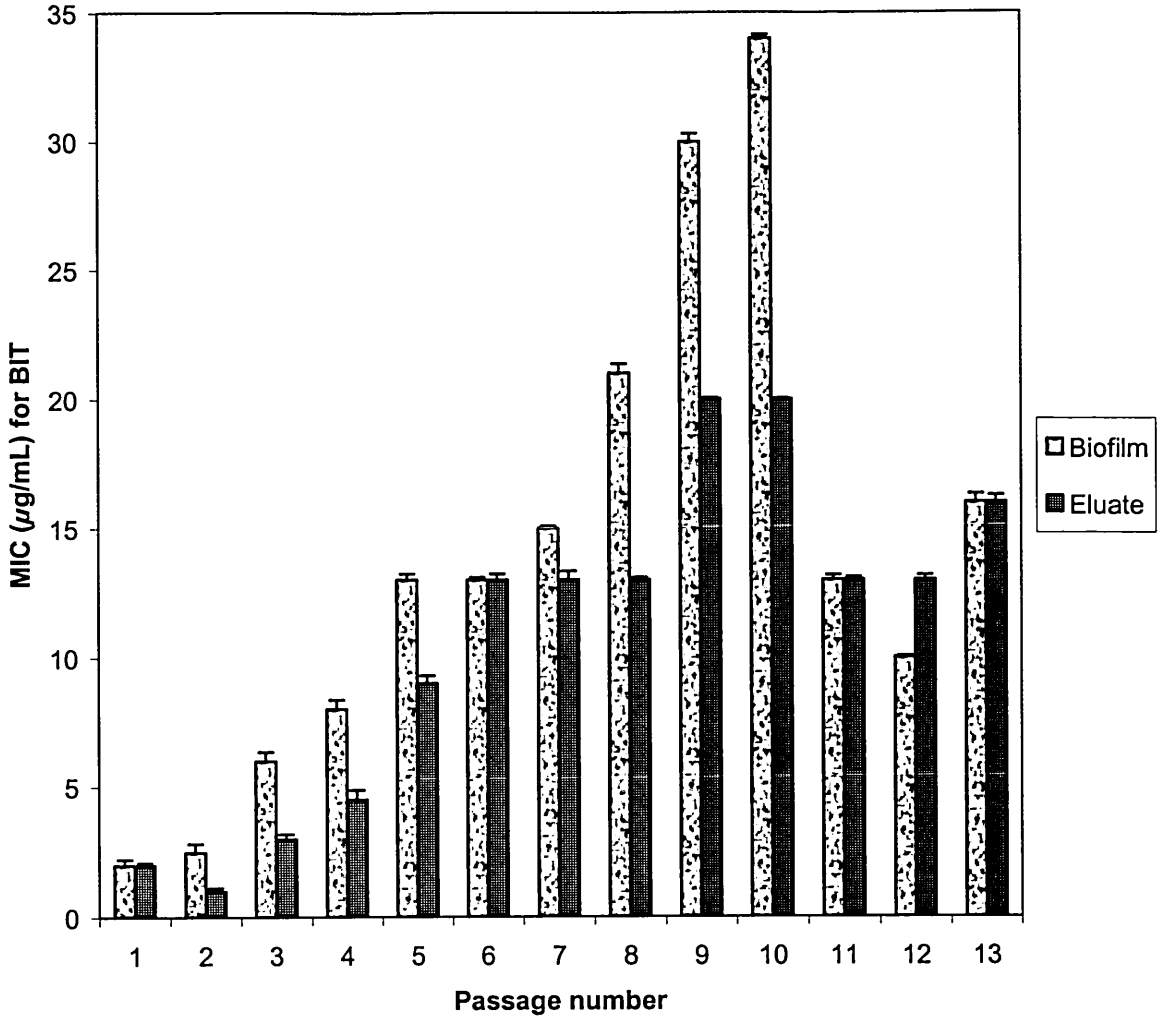


Figure 22: Increase in MIC for BIT against *Pseudomonas aeruginosa* PAO1 in CDM as a function of passage number. The error bars are calculated as the standard deviation of the individual data point. n = 3 replicates. Passages 11, 12 and 13 were performed without the addition of biocide.

Table 9: Summary of the average MICs for *Ps. aeruginosa* PAO1 biofilm, eluate and planktonic cells in CDM when exposed to the four Biocides (from three different studies). n = 3 replicates.

Biocide	Type of Cells	MIC (μg ml $^{-1}$) of passage number		
		1	**10**	**13**
BIT	Biofilm	2	34	16
	Eluate	2	20	16
	Planktonic*	5.73	9.07	6.4
ZnPT	Biofilm	4	17	13
	Eluate	4	17	13
	Planktonic**	4.5	12.75	9.75
NaPT	Biofilm	48	51	51
	Eluate	40	51	51
	Planktonic**	54	115	75
Cetrimide	Biofilm	18	56	51
	Eluate	14	56	51
	Planktonic**	15	130	90

* = results from Winder *et al.* (2000); ** = results from Abdel Malek *et al.* (2002).

Conclusions

The induction of resistance to the three biocides (ZnPT, Cetrimide and BIT) was observed to occur in a step-wise fashion. This would indicate, that the process of adaptation to these biocides is a phenotypic process. This supports the third mechanism of reduced biofilm susceptibility, which was outlined in the introduction. That is, some of the cells in a biofilm adopt a distinct, and relatively protected, biofilm phenotype (Cochran *et al.*, 2000a). However, upon removal of the presence of the biocides the MIC value decreased. Therefore, this indicates that the biofilm resistance development process is phenotypic. A phenotypic adaptation is an alteration, which demonstrates a change in response to environmental stimuli, but does not necessarily require a genome alteration. Although the MIC value decreased, this value did not revert to the original, initial value. This suggests that the induced biocide resistance is largely irreversible. There was no difference in the MIC value between adherent and eluate cells for ZnPT, but this was not the case with cetrimide and BIT. For BIT, there was immediate difference in MIC value between both types of cells and this continued for the whole series of passages. The possible explanation for this phenomenon is due to quenching of BIT by the presence of accessible thiol groups (-SH) in the EPS that surrounds the biofilm. The mode of action of BIT is to react oxidatively with accessible thiol groups (Fuller *et al.*, 1985; Collier *et al.*, 1990a). Hence, the presence of -SH in EPS will result in a greater MIC for biofilm cells than that for eluate cells. In the case of cetrimide, there was a difference in MIC value between adherent and eluate cells in the early passages (1 to 7), but in the later passages (8 to 13) the MIC value remained the same for both eluate and adherent cells. This indicates that variations in the exact mechanisms of resistance development are occurring between biocide treatments. For NaPT, there was no induced resistance in biofilm or eluate cells

after 10 passages. However, results from this group showed that planktonic cells passaged against NaPT gave an approximately doubling of MIC after 10 passages (Abdel Malek *et al.*, 2002). This suggests that the activity of NaPT was quenched by biofilm structure in these experiments. This result suggests that biofilm exposure to NaPT does not result in the development of tolerance or resistance under the experimental parameters used here. Therefore, it was decided to cease investigation of the action of this biocide at this point. However, the outer membrane profiles of NaPT-exposed biofilm and eluate cells were subsequently observed by SDS-PAGE. The induction of resistance towards thiomersal was not possible in CDM. This may be due to the combined stress effect of growing the cells in a nutrient limited medium and to biocide exposure. This result could open the door to many novel approaches to study and investigate the resistance mechanism for this compound.

The Sorbarod biofilm is a relatively thick biofilm; therefore, there is an associated transport limitation, which would support the first two hypotheses of reduced biofilm susceptibility discussed in Chapter one. From this discussion, one can suggest that biofilms are probably protected by multiple resistance mechanisms (Mah & O'Toole, 2001). However, these results elucidate several previously undescribed phenomena associated with biofilm growth. In particular, the differential MICs observed for BIT-exposed biofilm and eluate cells suggest an intimate involvement of the biofilm EPS or the biofilm phenotype in the development of resistance towards biocides. These results suggest several further avenues of investigation, in order to develop our understanding of these phenomena. One such investigation may involve the testing and observation of EPS / biocide interactions. Another route may include the observation of biocide effects upon biofilms exhibiting altered biofilm phenotypes.

Investigation of Cross-Resistance

The intense use and misuse of antibiotics are undoubtedly the major forces associated with the high numbers of resistant pathogenic and commensal bacteria worldwide (Barbosa & Levy, 2000). The extensive use of cationic biocides could lead to the selection of staphylococcal strains showing resistance to both antibiotics and biocides (Russell *et al.*, 1998). Dance *et al.* (1987) isolated a strain of *Proteus mirabilis*, responsible for a hospital outbreak that was resistant to chlorhexidine (CHX) and antibiotics. Also, Stickler *et al.* (1983) observed resistance to CHX, quaternary ammonium compounds (QACs) and at least five antibiotics of Gram-negative bacteria, isolated from urinary tract infections, and proposed that the widespread use of CHX was responsible for selecting antibiotic-resistant strains (Russell, 1995).

The MIC of the previously induced resistant cultures was obtained in order to determine whether cross-resistance occurs between the two-pyrithione biocides, BIT and the membrane active control, Cetrimide. The induced resistant cultures (Passage 10) for both biofilm and eluate cells (against the four biocides) were maintained on CDM gradient plates (Chapter 2). The most resistant colonies on these plates will be the ones growing in the region with the highest biocidal concentration. These colonies were resuspended in 0.9% (w/v) sterile normal saline (25 mL) and used as an inoculum for MIC determination (after observing the OD at 470 nm). The MIC was determined against the other three biocides (**Tables 10 & 11**) by tube dilution method (Bloomfield, 1991). This was performed for both sessile and eluate cells, in triplicate.

Table 10: MICs of *Ps. aeruginosa* PAO1 resistant biofilm cultures in cross-resistant study. n = 3 replicates.

Cultures resistant towards	**MIC (μg mL^{-1}) of original biocide at passage number**		**MIC (μg mL^{-1}) to test biocides cultures (at Passage 10)**			
	1	**10**	**ZnPT**	**NaPT**	**Cet.**	**BIT**
ZnPT	4	17	nd	78	38	12
NaPT	48	51	9	nd	12	6
Cetrimide	18	56	5	52	nd	7
BIT	2	34	10	66	18	nd

nd = not done.

Table 11: MICs of *Ps. aeruginosa* PAO1 resistant eluate cultures in cross-resistant study. n = 3 replicates.

Cultures resistant towards	MIC (μg mL^{-1}) of original biocide at passage number		MIC (μg mL^{-1}) to test biocide cultures (at Passage 10)			
	1	10	ZnPT	NaPT	Cet.	BIT
ZnPT	4	17	nd	72	34	13
NaPT	40	51	8	nd	16	7
Cetrimide	14	56	4	62	nd	7
BIT	2	20	12	56	18	nd

nd = not done.

The results obtained from the cross-resistance experiments were variable (**Tables 10 & 11**). For ZnPT, there were marked increases in the MICs against the three biocides (NaPT, Cetrimide and BIT) for both biofilm and eluate cells. This indicates that the induced tolerance developed against ZnPT is transferable to the other biocides, despite a different mode of actions. These results are similar to those obtained from work on planktonic cultures (Abdel Malek *et al.*, 2002), which showed that ZnPT exhibited cross-resistance with both NaPT and Cetrimide.

For NaPT, it was a different story. The induced resistance to this biocide did not yield resistance towards the other three biocides (ZnPT, Cetrimide and BIT). This was the case for both adherent and eluate cells. From this observation, one can suggest that the resistance mechanism is only a membrane associated one and that there is no internal resistance mechanism. In comparing these results with those from the planktonic study, there was only an observed cross-resistance between NaPT and Cetrimide. The MIC did not show any increase (from the original, pre-exposure MIC value for NaPT) when tested against ZnPT.

For the positive control, Cetrimide, the cross-resistance was variable. A significant increase in Cetrimide initial MIC was observed against NaPT. This was observed with both biofilm and eluate cells. There was no cross-resistance between Cetrimide and ZnPT or between Cetrimide and BIT (sessile and eluate cells). This suggests that the membrane action was not impaired by the resistance mechanism. The planktonic results showed the same trend is that there was an observed cross-resistance between Cetrimide and NaPT, but this was not the case between Cetrimide and ZnPT.

Finally, the story for BIT was similar to that for ZnPT. There was an observed cross-resistance between BIT and the other three biocides (ZnPT, NaPT and Cetrimide) for both adherent and eluate cells. Although the mechanism of action is different between the

isothiazolones and the pyrithiones, there was a marked cross-resistance between both types of biocides. These results indicate that the pyrithione biocides have similar resistance profiles in *Ps. aeruginosa* PAO1 to those exhibited by the isothiazolones (BIT). This is both an unexpected result and also, an indication as to the general non-specific nature of the resistance developed by *Ps. aeruginosa* biofilms upon exposure to these distinct groups of biocides. One would generally expect that resistance towards one chemically distinct group of biocides, particularly if they have a distinct specific target mechanism, would not lead to resistance towards a chemically dissimilar group, with a distinctly different target mechanism. However, this has been exampled by the work of Winder *et al.* (2000) and Abdel Malek *et al.* (2002), who showed that planktonic cells of *Ps. aeruginosa* PAO1 developed resistance towards both the isothiazolone group and the pyrithione group *via* similar passage mechanisms. They also showed that the outer membrane profiles of the exposed cells responded in markedly similar fashions. The observations of this project suggest that the resistance developed towards both groups of biocides is a general, non-specific resistance mechanism, as result of this general cross-resistance.

The Extracellular Polymeric Substances in *Pseudomonas aeruginosa* PAO1 biofilms

Pseudomonas aeruginosa responds to favourable nutrient conditions by adhering to available surfaces, and, by binary fission, develops mature biofilms (Costerton *et al.*, 1995). Electron microscopy demonstrated that *Ps. aeruginosa* adheres to surfaces within 4 hours and that after 24 hours at 37°C it begins to form adherent microcolonies and the first stages of a biofilm on the surface of medical catheters (Sagripanti & Bonifacino, 2000). In biofilms, *Ps. aeruginosa* is nearly 500 times more resistant to antimicrobial agents than are bacteria in suspension (Costerton *et al.*, 1995). As mentioned earlier in this chapter, one of the primary mechanisms associated with the resistance of biofilms to antimicrobial agents relates to the presence of highly hydrated, polyanionic matrices of extracellular polymeric substances (EPS) (Hodges *et al.*, 1991; Anwar *et al.*, 1992a; Hoyle *et al.*, 1992b). These encase the cells growing at the surface and provide protection from external aggression by reacting chemically with, or binding, biocides and providing a diffusion barrier (Brown *et al.*, 1977; Brown *et al.*, 1988; Lawrence *et al.*, 1994).

The precise manner by which EPS protect the cells is unclear, but the presence of bound extracellular enzymes, such as β-lactamases, within the glycocalyx may reinforce its action as a diffusion barrier (Bolister *et al.*, 1991) with respect to some antibiotics, and its molecular sieving properties are enhanced through binding of divalent cations, such as Ca^{2+}, from the environment (Hoyle *et al.*, 1992a). It has also been proposed that the glycocalyx provides intrinsic protective effects against antimicrobial agents, which are additional to those associated with its diffusion and charge related properties (Hodges & Gordon, 1991; Wood *et al.*, 1996).

These extracellular polymeric substances, known collectively as glycocalyces, may

adhere to the bacterial cell wall to form the capsule or they may diffuse into the extracellular milieu to form the so-called slime layer (Dickinson and Bisno, 1993). The EPS are predominant components of biofilms (Wuertz *et al.*, 2001). In a typical biofilm, 95% of the mass is water and 5% is dry material, however, up to 90% of biofilm organic carbon is EPS material (Characklis and Marshall, 1990; Zhang & Bishop, 2001). Because EPS is a key constituent of a biofilm, biofilm properties such as density, porosity, strength, elasticity, frictional resistance, thermal conductivity, and metabolic activity may be greatly influenced by EPS composition and quantities (Zhang *et al.*, 1999). EPS is believed to have many important functions in biofilm attachment and activated sludge floc formation (Characklis and Marshall, 1990). These extracellular polymeric substances contain polysaccharides, proteins (including enzymes), DNA, lipids and uronic acids (Goodwin & Forster, 1985; Horan & Eccles, 1986; Wuertz *et al.*, 2001) and are believed to play a substantial role in the sorption of both organic and inorganic substances in biofilms.

The extracellular polysaccharide alginate has been widely associated with chronic *Ps. aeruginosa* infections in the cystic fibrosis lung (Govan & Deretic, 1996). The polysaccharide alginate was first isolated from marine microalgae at the end of the 19th century, but it was approximately 80 years later that a bacterial source (*Ps. aeruginosa*) of the polysaccharides was identified (Linker & Jones, 1966). The bacterial alginates, like their algal counterparts, are formed from irregular sequences of D-mannuronic acid and L-guluronic acid residues (Evans & Linker, 1973), but differ from the algal material in being heavily acetylated on many of the D-mannuronosyl residues (Davidson *et al.*, 1977; Boyd & Chakrabarty, 1995; Sutherland, 2001).

Alginate biosynthesis and biofilm formation are triggered by a number of environmental factors including high osmolarity (Zielinski *et al.*, 1992), nitrogen or

phosphate limitation (DeVault *et al.*, 1989) and ethanol (DeVault *et al.*, 1990). Environmental stress yields alginate-forming clones of varying stability, synthesizing very large amounts of polymer. Davies *et al.* (1993) suggested that the increase in alginate production, which they observed on solid media in comparison with planktonic growth of *Ps. aeruginosa*, was due to activation of the *algR1* gene product by adhesins, which triggered alginate synthesis. In the case of alginate complex regulatory systems are involved in the control of expression and production of the respective polysaccharides (Chitnis & Ohman, 1993). The biosynthetic gene for alginate *algD* is controlled through three regulatory genes-*algR1*, *algR2* and *algR3*. Similar to the *algD* promoter, the *algC* promoter has been shown to be activated by environmental signals, such as high osmolarity, and this activation is dependent on the presence of a response regulator protein, AlgR1 (Zielinski *et al.*, 1992). The term "activation" is used in this study to refer to an increase in promoter activity observed in response to an environmental signal which results in up-expression of the gene under the promoter's control (Davies *et al.*, 1993). Thus, in bacterial alginate synthesis in *Ps. aeruginosa*, the sigma factor RpoS (σ^S) exhibited function as a general stress response regulator (Suh *et al.*, 1999).

The aim of this study was to test EPS, which was separated from *Ps. aeruginosa* biofilm cells, against Ellmans assay (for the presence of thiol groups) and to use a spectrometric analysis for observing the chemical reaction of BIT with EPS.

The Extraction of EPS

Biofilm cultures of *Ps. aeruginosa* PAO1 were grown within Sorbarod filter plugs, which were perfused with medium (CDM) (Hodgson *et al.*, 1995; Chapters 1 & 3). These were run for 48 hours (steady state). The Sorbarod filters were sacrificed in 0.9% (w/v)

sterile normal saline and the resultant cell suspensions were then centrifuged (IEC Centra-4B; at 5000 rpm for 30 minutes) to collect the pellets. The EPS extraction method was according to Brown and Lester (1980), which used EDTA as an extracting agent (This method was explained in detail in Chapter 2).

Testing the EPS

Ellmans Assay for the Presence of Thiol Groups

Isothiazolone biocides (such as BIT, MIT and CMIT) are strongly antagonised by exogenous thiol-containing agents and are thought to interact oxidatively with accessible thiols, such as glutathione, within the cell (Fuller *et al.*, 1985; Collier *et al.*, 1990a). Therefore, the presence of thiols quenches the activity of these biocides. Fuller *et al.* (1985) demonstrated chemical reactions between BIT and glutathione and proposed a mechanistic pathway for such interaction (**Figure 5**). There are three major sources of accessible thiols within cells, thiol containing proteins, free cysteine, and the major non-protein thiol, glutathione (GSH), which is likely to be the major interactive thiol in the cells (Owens and Hartman, 1986; Collier *et al.*, 1991).

The Ellmans assay (Ellman, 1964) can be performed in order to estimate the concentration of thiols in EPS and hence, determine the magnitude by which the BIT activity may be quenched by a particular medium or substance. Ellmans reagent (5,5-dithiobis (2-nitro benzoic acid); DTNB) reacts with thiol containing agents. This reaction can be detected by the developing of yellow coloration, which can be assayed at E_{412nm}. The assay determines the glutathione equivalency of the reading that is proportional to the total thiol content. The absorbencies at 412 nm are plotted against known concentrations of glutathione to construct a standard curve. The test samples are compared against these

standards.

Preparation of Reagents

Tubes were prepared as outline in **Table 12**. The buffer (0.1M NaH_2PO_4 (5.5 mL) and 0.1M Na_2HPO_4 (94.5 mL), pH 8.0) was the first to be added, followed by distilled water and glutathione stock (0.05g L^{-1}) or test sample. The stock DTNB (30.8 mg in 100 mL deionised water) was the final component to be added. Tubes 1 to 11 used as samples for the standard curve. Tubes 12 to 15 are the test samples. The tubes were vortexed (Rotamix, Hook & Tucker) and incubated at room temperature for 15 minutes and the optical density at 412 nm was determined.

Table 12: Preparation of Tubes in the Ellmans Assay. n = 3 replicates.

Tube number	Buffer (mL)	DTNB (mL)	GSH (0.05g/L) (µL)	Water (mL)	EPS (mL)	Mean Optical Density (412 nm)
1	1	1	1000	2.0	-	0.473
2	1	1	900	2.1	-	0.449
3	1	1	800	2.2	-	0.382
4	1	1	700	2.3	-	0.336
5	1	1	600	2.4	-	0.285
6	1	1	500	2.5	-	0.242
7	1	1	400	2.6	-	0.187
8	1	1	300	2.7	-	0.141
9	1	1	200	2.8	-	0.097
10	1	1	100	2.9	-	0.040
11	1	1	0	3.0	-	0
12	1	1	-	-	3.0	0.01
13	1	1	-	2.7	0.3	0
14	1	1	-	2.97	0.03	0
15	1	1	-	2.997	0.003	0

- = no addition; tubes 1 to 11 = standard curve; tubes 12 to 15 = test samples; tube 11= used as a reagent blank.

After constructing the standard curve (**Figure 23**), the absorbency of tube 12 (0.01) was extrapolated on the graph and the glutathione (GSH) concentration was equal to 0.000276 g L^{-1}. Taking into account the dilution factor, this is equal to 0.00138 g L^{-1}, which is equivalent to 1.38 μg mL^{-1}. Using the value of 1.38 μg mL^{-1} and assuming that all the thiol present in the EPS is GSH, it is possible to determine this value as a concentration of GSH of $2.1x10^{-6}$ mmol L^{-1}. The concentration of BIT at the lowest MIC exhibited against *Ps. aeruginosa* PAO1 is $1.32x10^{-5}$ mmol L^{-1}. Hence, it is possible to estimate that the quenching effect of thiol in biofilm EPS is equivalent to approximately 16% of the applied biocide, which is equivalent to 0.32 μg mL^{-1}.

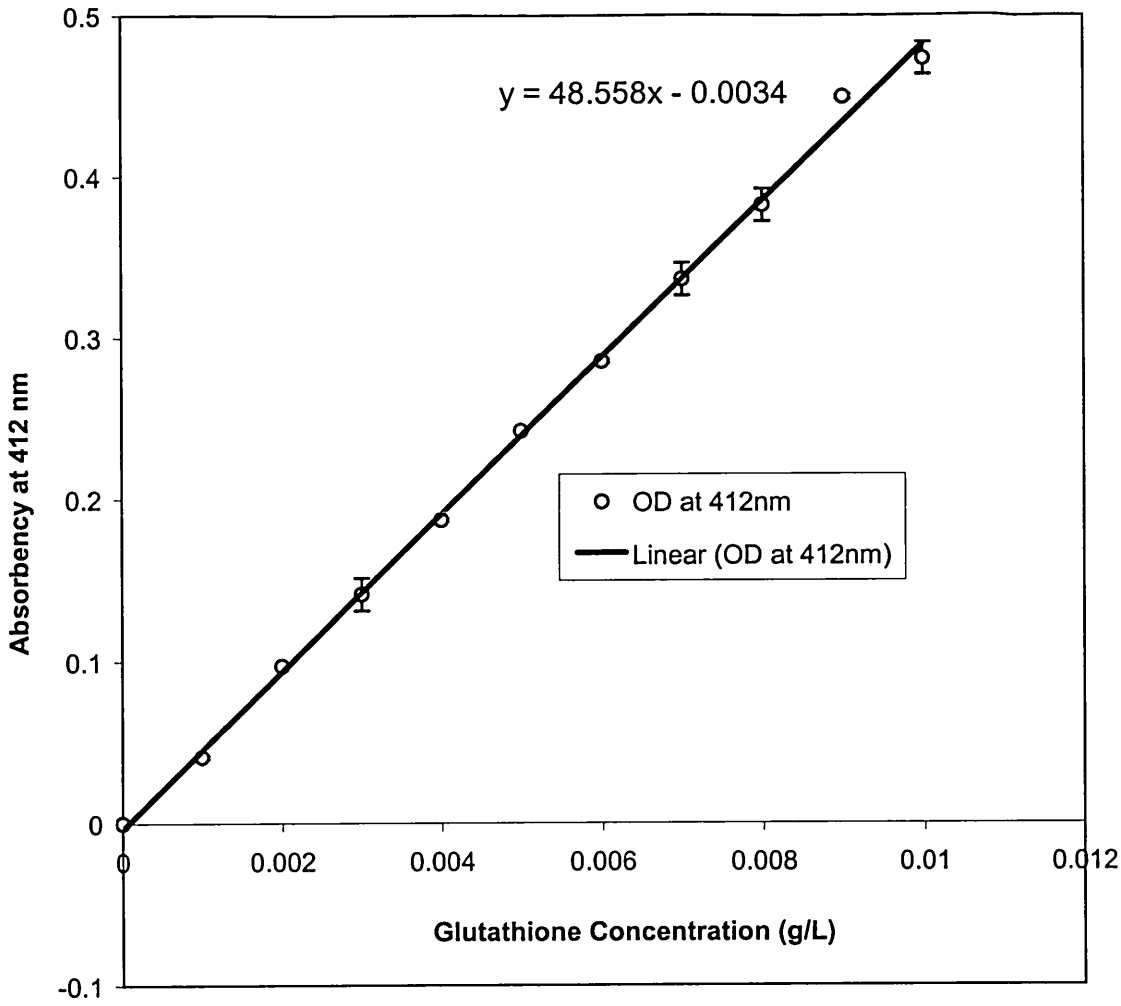


Figure 23: The standard curve for the estimation of accessible thiol groups (Ellmans assay). Error bars are calculated as the standard deviation of the individual data points. n = 3 replicates.

Table 13: Comparison of tube dilution MICs determined for Benzisothiazolone (BIT) in CDM, taking into account quenching by accessible thiol-groups (GSH) in the EPS of the biofilm cells.

Passage number	**MIC ($\mu g\ mL^{-1}$)**		
	Biofilm Cells	**Biofilm Cells minus Quenching Factor (Equivalent to 0.32 $\mu g\ mL^{-1}$)**	**Eluate Cells**
1	2	1.68	2
2	2.5	2.18	1
3	6	5.68	3
4	8	7.68	4.5
5	13	12.68	9
6	13	12.68	13
7	15	14.68	13
8	21	20.68	13
9	30	29.68	20
10	34	33.68	20
11	13	12.68	13
12	10	9.68	13
13	16	15.68	16

Table 13 demonstrates the difference between MIC values for biofilm cells before and after subtracting the quenching factor (equivalent to 0.32 $\mu g\ mL^{-1}$) and those for eluate cells. As a result of this subtraction, the MIC for biofilm cells in the first passage was lower than that for eluate cells. In the following passages, the MIC values for biofilm cells remained higher than those for the eluate cells. When the cells were grown in biocide-free medium (Passages 11 to 13), there were differences in the MICs between biofilm and eluate cells but the values for the eluates were greater than those for the biofilms.

The results from **Tables 12** and **13** indicate that the EPS contains significant concentration of thiol groups. Therefore, the interaction between BIT and those accessible thiols could be partially explained the observed difference in MICs between biofilm and eluate cells.

Spectral Analysis of BIT-EPS Mixtures

In the present study, spectral measurements for GSH : BIT and EPS : BIT mixtures were made up at pH 8.0 in phosphate buffer (66.66 mM; Sørensen's phosphate. This buffer was made up from two solutions: solution A (1/15M KH_2PO_4) and solution B (1/15M Na_2HPO_4). The final volume was achieved by adding: x mL (solution A) + (100-x) mL (solution B); Geigy & Basle, 1962). The experiment was divided into two parts. In part one, solutions of 100 μM BIT and 100 μM GSH were prepared and were mixed together in quartz cuvettes, at a molar ratio of 1:1. In part two, solutions of EPS and BIT were mixed together at molar ratios 1:1 and 3:1 using a fixed BIT concentration (100 μM). An EPS solution was prepared as described above (Chapter 2). For part one, spectra in the 220 nm to 380 nm range were determined using an ultra-violet visible spectrometer (Perkins Elmer, Model Lambda 2, England) (**Figure 24**). For part two, the spectra range was between 180 nm and 380 nm (**Figure 25**).

Figure 24 showed that BIT has an absorption maximum at 318 nm (a). When BIT was mixed with GSH (b), the E_{318nm} peak diminished as BIT was lost and peaks appeared at E_{270nm} and E_{340nm}. These had been identified by Fuller (1986) to correspond to uBIT (uncoupled BIT; III, **Figure 5**; Chapter 1).

Figure 25 showed that the peak for EPS (in the absence of BIT) was at 218 nm (a). After mixing EPS with BIT at a molar ratio 3:1 (b), the E_{218nm} peak diminished and a new peak appeared at 318 nm (which is the peak for BIT itself). When the molar ratio had changed to 1:1 (EPS : BIT (c)), the BIT peak (E_{318nm}) diminished and new peaks appeared in the spectra at 270 nm and 340 nm. These are the same peaks that appeared in GSH : BIT reaction. However, the absorbencies for the two reactions are different. For the BIT :

GSH reaction, the absorbency at 270 nm was 0.875 and that at 340 nm was 0.24. For the BIT : EPS reaction, the absorbencies were 0.505 and 0.135 (at 270 nm and 340 nm, respectively). This result suggests that the EPS, which was extracted from *Ps. aeruginosa* biofilm cells, contains non-protein thiol (as was demonstrated from Ellmans assay). As previously mentioned (Chapter 1), the main composition of the EPS is carbohydrate and the protein represents a small percentage of the chemical composition. This explains the differences in the absorbencies between the two reactions. Therefore, the presence of accessible thiol within the EPS will probably be a major target for BIT before it enters the cells. The reaction between BIT and GSH within the EPS of *Ps. aeruginosa* biofilm cultures explained the difference in MIC between eluate and sessile cells when those were passaged in the presence of BIT (**Figure 22**). The MIC for the biofilm cells was higher than that for the eluate cells in the whole passaging process, because BIT was quenched by EPS before it entered the cells.

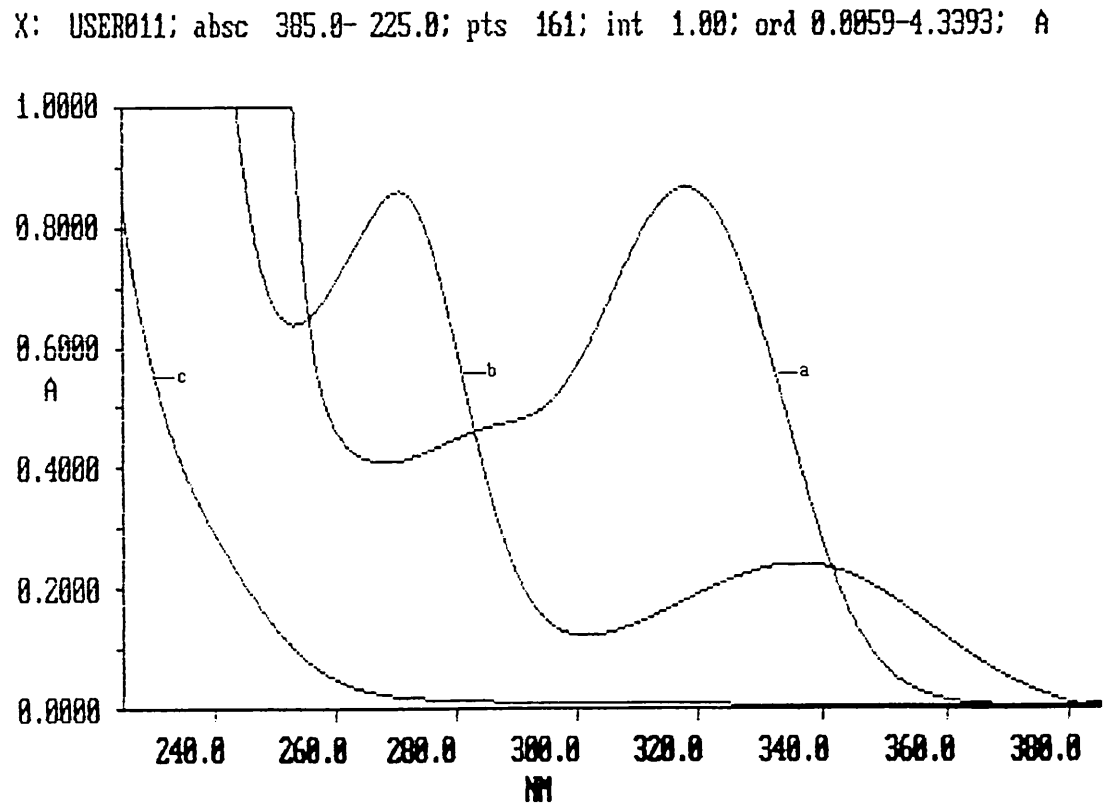


Figure 24: Absorption spectra determined for (a) BIT (100 μM) (b) BIT : GSH (1:1) (c) GSH (100 μM) at pH 8.0; all spectra were determined in 66.66 mM phosphate buffer at 25°C.

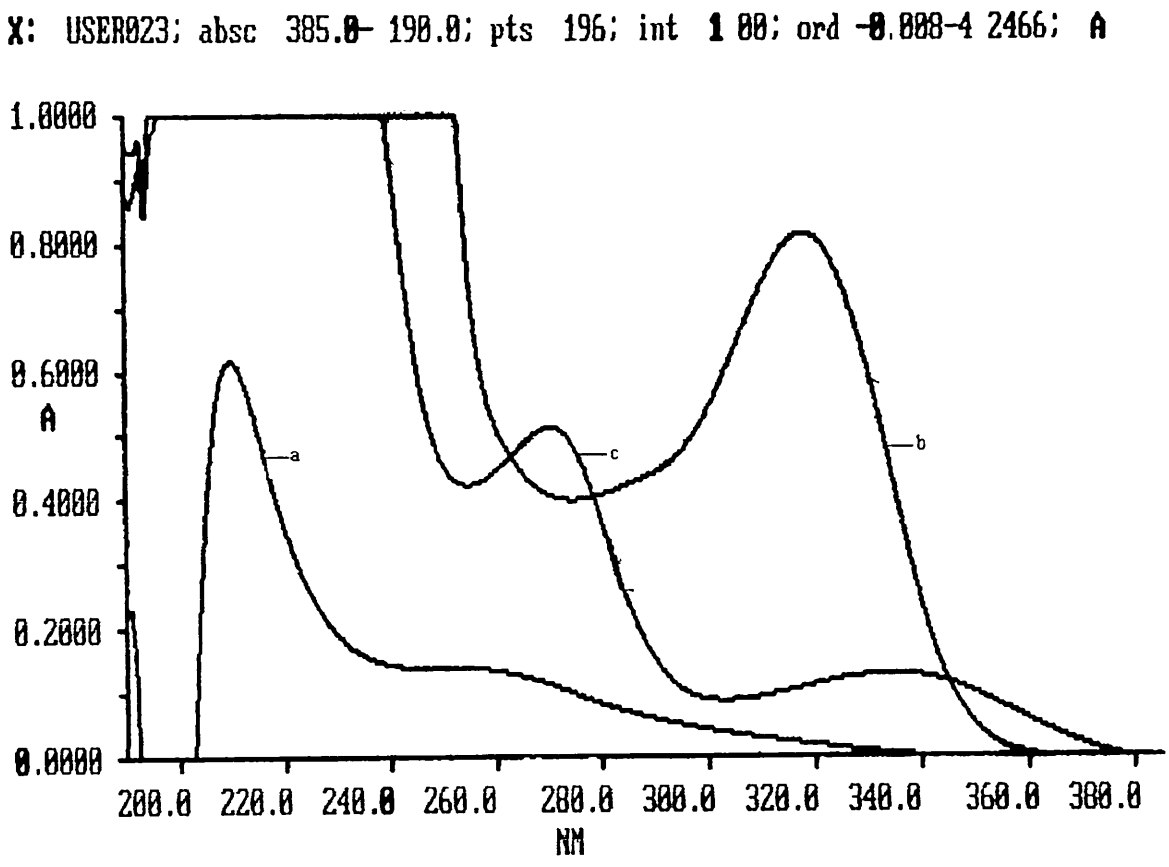


Figure 25: Absorption spectra determined for (a) EPS (b) EPS : BIT (3:1) (c) EPS : BIT (1:1) at pH 8.0; all spectra were determined in 66.66 mM phosphate buffer at 25°C.

Chapter Five:

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

Introduction

Pseudomonas aeruginosa strains are generally less susceptible to a number of antibiotics than other Gram-negative bacteria. Furthermore, many clinical isolates of *Ps. aeruginosa* show elevated levels of this intrinsic resistance (Li *et al.*, 1994). This property is now recognized to result mainly from the activity of broadly specific antibiotic efflux systems (Poole *et al.*, 1993; Nikaido, 1996; Li & Poole, 1999), in conjunction with low outer membrane permeability (Angus *et al.*, 1982; Yoshimura & Nikaido, 1982; Nikaido, 1994). Lipopolysaccharide (LPS) is a characteristic component of the outer membrane of *Ps. aeruginosa* and all Gram-negative bacteria (Nikaido & Vaara, 1985). Alterations in the outer membrane proteins (OMPs) and the integrity of the LPS influence the permeability of the outer membrane to antimicrobial agents (Giordano *et al.*, 1993). For example, the outer membrane of *Ps. aeruginosa* was shown to have a 100 to 400-fold greater reduction in permeability to glucose-6-phosphate, fructose-6-phosphate and ρ-nitrophenylphosphate compared with that of *Escherichia coli* (Yoshimura & Nikaido, 1982). This observation indicates that the outer membrane of *Ps. aeruginosa* is an effective permeability barrier against many hydrophilic compounds and the low permeability is most easily explained by the outer membrane proteins forming pores or porin channels (Nikaido *et al.*, 1991), which prevent entry of molecules with a molecular weight greater than 350 to 400 D.

Ps. aeruginosa contains a limited number of OMPs present in very high numbers. For example, OprF is present at 10^5 copies per cell (Kragelund *et al.*, 1996). Most OMPs of this bacterium are specific for certain substrates (e.g. for basic amino acids (OprD), or for phosphate (OprP); Hancock *et al.*, 1990). Hence, these porins permit entry of structural analogues; an example of which is OprD, which is the protein by which the antibiotic

imipenem enters through the outer membrane of *Ps. aeruginosa* (Nikaido, 1992). This microorganism has only one general uptake porin, OprF, exhibiting a low rate of solute transport (Nikaido, 1992). Therefore, it is suggested that the majority of solutes cross the outer membrane *via* specific OMPs. However, even in bacteria such as *Ps. aeruginosa*, which has an outer membrane of exceptionally low permeability, the periplasmic concentrations of many antibiotics reach 50% of their external concentrations within 20 seconds (Greenway & England, 1999a). Thus, the presence of the outer membrane alone cannot explain the extent of intrinsic resistance. Recent studies have shown that multiple drug efflux pumps, sometimes with unusually broad specificity, act as major factors to create the general intrinsic resistance of Gram-negative bacteria to natural and synthetic antimicrobial agents (Nikaido, 1994). The first evidence of multiple drug resistance efflux pumps (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).

Efflux transporter proteins are located in the bacterial cytoplasmic membrane. These transporter proteins are generally linked to the outer membrane channels by accessory proteins situated in the periplasmic space (Green & England, 1999a). Three efflux systems have been described in *Ps. aeruginosa* (Masuda *et al.*, 1995), encoded by the MexA-MexB-OprM (Poole *et al.*, 1993; Li *et al.*, 1995), MexC-MexD-OprJ (Poole *et al.*, 1996a) and MexE-MexF-OprN (Kohler *et al.*, 1997) operons. The MexA-MexB-OprM efflux system has been demonstrated to contribute to the high intrinsic antibiotic resistance of this microorganism (Li *et al.*, 1994; Li *et al.*, 1995; Poole *et al.*, 1996b). Li *et al.* (1994) suggested that the efflux system is an important factor (in addition to the low outer membrane permeability) in *Ps. aeruginosa* strains resistant to tetracycline, chloramphenicol, and fluoroquinolones. Also, Li

and Poole (1999) emphasized the importance of the MexA-MexB-OprM efflux system in organic solvent tolerance.

For many years, it has been recognized that bacteria, which appear susceptible to antimicrobial agents when growing under optimum laboratory conditions, are not necessarily amenable to therapy under slow growth infectious conditions (Cozens *et al.*, 1986; Gristina *et al.*, 1987). The influence of nutrient limitation and hence, slow growth rate of various harmful bacteria, has been extensively studied in chemostat culture and also within biofilms (Finch & Brown, 1975; Gilbert & Brown, 1978; Gilbert & Brown, 1980; Brown *et al.*, 1990). *Ps. aeruginosa* grows as biofilms in nature, in foreign-device associated infections and chronic lung infection in patients with cystic fibrosis (CF), and undergoes phenotypic changes with respect to typability, exoenzyme secretion, antibiotic resistance, serum sensitivity and colony morphology that may result from changes in the outer membrane (OM) components (Costerton *et al.*, 1987).

Giwercman *et al.* (1992) used polyacrylamide gel electrophoresis (PAGE) to analyse LPS from biofilm and planktonically grown monoagglutinable (118) and polyagglutinable (258 and 15703) strains of *Ps. aeruginosa* isolated from cystic fibrosis patients with chronic pulmonary infections. Their study showed an apparent change in LPS sub-unit components of the bacteria when grown as biofilms, which may reflect changes in the outer membrane structure. Other studies (using SDS-PAGE) demonstrated significant differences between the protein profiles of *Staphylococcus aureus* biofilm and eluted populations relative to planktonic controls (Hodgson *et al.*, 1995). These differences include the repression of a 48 kD protein and increased expression of 21 kD protein. Further experiments confirmed that these proteins neither corresponded to heat shock proteins (Qoronfleh *et al.*, 1990) nor to proteins expressed under oxygen limitation. Therefore, these changes in protein composition

are probably related directly to growth as a biofilm.

Although MDR pumps play a major role in the intrinsic resistance of *Ps. aeruginosa* to a number of antimicrobial agents (Ma *et al.*, 1994), De Kievit *et al.* (2001) revealed that the expression of the four well-characterized (MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY) MDR pumps is not increased in *Ps. aeruginosa* biofilms. This suggests that the other factors, such as decreased membrane permeability and/or alteration of antimicrobial targets, must be responsible for the innate resistance to antibiotics by this population of bacteria.

Experimental Approach

Outer Membrane Protein (OMP) Preparations

There were two different methods for OMP preparation, one for the biofilm cells and another for the eluate cells. Biofilm OMPs were prepared for SDS-PAGE and 2D-PAGE analysis according to Hodgson *et al.* (1995). Eluate OMPs were prepared for the same analyses by the method of Pugsley *et al.* (1986). These are explained in detail in Chapter 2.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE is probably the most widely used technique for analysing mixtures of proteins. In this technique, proteins are reacted with the anionic detergent, sodium dodecylsulphate (SDS, or sodium Lauryl sulphate), to form negatively charged complexes. The amount of SDS bound by a protein, and therefore the charge on the complex, is roughly proportional to its size. Commonly, about 1.4g SDS is bound per 1g protein, although there are exceptions to this rule (Deyl, 1979). The proteins are generally denatured and solubilised by their binding of SDS and the complex forms a prolate elipsoid or rod of a length roughly

proportionate to the protein's molecular weight. Thus, proteins of either acidic or basic pI form negatively charged complexes that can be separated on the basis of differences in charges and sizes by electrophoresis through a sieve-like matrix of polyacrylamide gel (Smith, 1984).

The system of buffers used in the gel system (described in Chapter 2) is that of Laemmli (1970). A polyacrylamide gel of slab shape is used in this technique. This form allows simultaneous electrophoresis of more than one sample and thus, is ideal for comparative purposes.

Preparation of Separating Gel Mixture and Stacking Gel Mixture, were undertaken as described in detail in Chapter 2. Outer membrane protein profiles were obtained using a Powerpack 300 (constant current of 30 mA for 1.5 h) and then stained with Coomassie brilliant blue R250 or silver staining (these steps are described in detail in Chapter 2).

The Analysis of OMP Profiles

The first stages in analysing the gels were performed using Phoretix imaging analysis software (non-Linear Dynamics Ltd, Newcastle upon Tyne, England). Then the molecular weights and Rf (Retardation factor) values of all the outer membrane proteins in the samples were estimated manually. This was performed in order to observe any possible differences between the sensitive and resistant cultures for both biofilm and eluate cells.

There are four basic steps in analysing a gel using the Phoretix system. The first step is to create a lane profile, such that the system can identify the lanes and individual proteins. The system can efficiently resize a lane and grimace a band (Grimacing is the name designated by Phoretix for straightening bands). The second step is to subtract the background from lane profile, such that the system distinguishes the protein bands. The third step is to detect the

peaks of the profile. Peak detection is the process by which the system decides which parts of the lane profile are protein bands. Once the protein peaks are detected the edges of the protein can be identified (step four), such that the system knows when the protein begins and ends on the image (edge detection).

Once those preliminary stages of analysis had been established, the molecular weights and Rf values were determined manually. Lane1 in each gel demonstrates the standard molecular weights (M.Wt.) markers for that gel. These M.Wt. Markers are given below:

Protein	**M.Wt. (Daltons)**	**Rf**
Phosphorylase b	94000	0.16
Albumin	67000	0.26
Ovalbumin	43000	0.41
Carbonic anhydrase	30000	0.57
Trypsin inhibitor	20100	0.71
α-lactalbumin	14400	0.80

By plotting the molecular weights against the Rf values, a calibration curve was constructed (**Figure 26**). However, Rf values for these standard molecular weights slightly vary from one gel to another. Therefore, a standard calibration curve was constructed for each gel. This curve was used to determine the molecular weights of all the proteins on that gel. The Rf for each band was estimated according to **Equation 5:**

Rf = distance migrated by protein/distance migrated by dye **(Equation 5)**

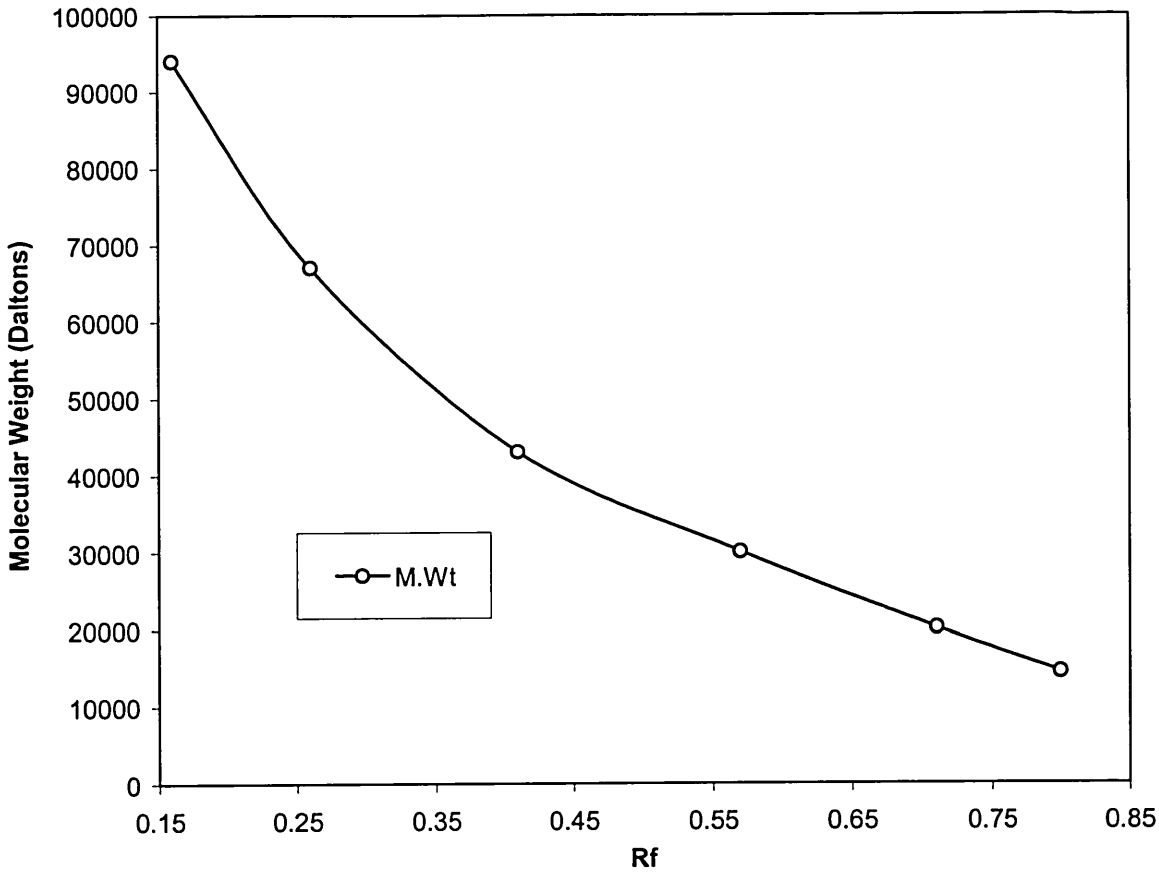


Figure 26: The standard calibration curve generated by plotting the molecular weight of the low range molecular weight standards vs. the distance migrated from the interface of the stacking and separating gels in centimeters.

Results & Discussion

Upon the exposure of *Ps. aeruginosa* PAO1 biofilm and eluate cells to all four biocides (BIT, ZnPT, NaPT & Cetrimide), there were some distinct variations in the number of observed outer membrane proteins (OMPs) between the planktonic cells and biofilm/eluate resistant cells.

OMPs shifts in BIT exposed cells

An outer membrane protein of 38 kD was present in planktonic cells, but disappeared from Passages 1, 3, 5, 7 and 10 (biofilm cells) and then reappeared in Passage 13 (**Figure 27**). A chemical alteration in the OMPs can be observed from the same figure. A 46 kD OMP appeared in planktonic unexposed cells. For the purpose of this study, OMP shifts or movements indicating the absence and/or presence of protein in any given lane coupled with the corresponding presence and/or absence of protein of similar molecular weight has been assumed to indicate a small molecular weight change in a single protein rather than the coupled disappearance / appearance of two proteins. In the resistant Passages (1, 3, 5, 7 & 10) the molecular weight of this OMP altered to 47 kD; once the cells were cultured in BIT-free medium, the molecular weight changed again to 46 kD. The same story was observed for proteins at both 22 kD and 17 kD (**Table 14**). An outer membrane protein in the range of between 30 kD and 30.7 kD appeared in the resistant biofilm passages, then disappeared completely from Passage 13. This suggests that the induction of this OMP is due to biocide exposure. For the eluate cells, different OMPs were observed to appear and disappear (**Figure 28**). A 23.5 kD OMP appeared in planktonic control cells disappeared from Passages 1, 3, 10 and 12 and then reappeared in Passage 13. A chemical alteration for an 81 kD OMP was

observed in the resistant passages (78 kD in Passages 1, 3 & 10). In passage 12 and 13, the molecular weight altered again to 81 kD (**Table 15**).

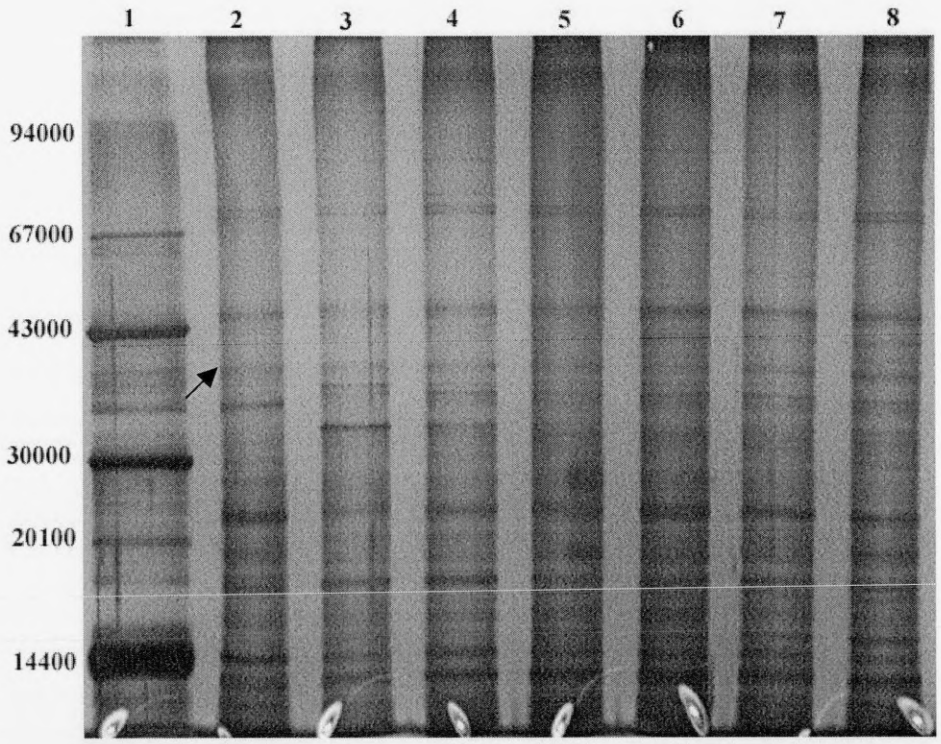


Figure 27: Outer membrane protein profile of *Pseudomonas aeruginosa* PAO1 biofilm cells cultured in CDM and exposed to BIT. Lane 1: molecular weights markers; Lane 2: planktonic control cells (1.1×10^{-3} mg/well); Lane 3: Passage 1 cells (1.03×10^{-3} mg/well); Lane 4: Passage 3 cells (1.06×10^{-3} mg/well); Lane 5: Passage 5 cells (1.05×10^{-3} mg/well); Lane 6: Passage 7 cells (1.00×10^{-3} mg/well); Lane 7: Passage 10 cells (1.05×10^{-3} mg/well); Lane 8: Passage 13 cells (1.05×10^{-3} mg/well). The arrow indicates the 38 kD OMP.

Table 14: Measurement Data indicating Band Number, Molecular Weight and Rf value for Outer Membrane Proteins of *Ps. aeruginosa* PAO1 Biofilm Cells exposed to BIT and cultured in CDM (band no: band number; M.Wt.: molecular weight; Rf: retardation factor; Co: corresponding protein to lane 2; N: novel protein).

Lane 1 (molecular weight markers)				Lane 2 (planktonic cells)				Lane 3 (Passage 1)			
Band no.	MWt (kD)	Rf	Co.	Band no.	MWt	Rf	Co.	Band no.	MWt	Rf	Co.
2	94	0.14	-	1	46	0.41	-	2	47	0.40	1
3	67	0.28	-	2	38	0.49	-	3	36	0.51	3
4	43	0.43	-	3	33	0.54	-	4	30.7	0.57	N
7	30	0.58	-	4	22	0.70	-	5	24.5	0.66	N
9	20.1	0.73	-	5	17	0.80	-	6	22.5	0.69	4
11	14.4	0.89	-	6	14	0.90	-	7	17.1	0.79	5
								8	14.4	0.89	6
Lane 4 (Passage 3)				Lane 5 (Passage 5)				Lane 6 (Passage 7)			
2	70	0.26	N	2	47	0.40	1	2	70	0.26	N
3	47	0.40	1	3	30	0.58	N	3	47	0.40	1
4	35.2	0.52	3	4	25.7	0.64	N	4	42	0.44	N
5	30.8	0.57	N	5	17.1	0.79	5	5	30	0.58	N
6	22.5	0.69	4					6	25.5	0.65	N
7	17.1	0.79	5					7	23.1	0.68	4
8	14.4	0.89	6					8	17.1	0.79	5
Lane 7 (Passage 10)				Lane 8 (Passage 13)							
2	68.1	0.27	N	2	70	0.26	N				
3	47	0.4	1	3	46	0.41	1				
4	35.2	0.52	3	4	41.1	0.45	N				
5	30	0.58	N	5	38	0.49	2				
6	22.5	0.69	4	6	34.1	0.53	3				
7	17.5	0.78	5	7	22	0.70	4				
				8	18.8	0.75	N				
				9	17	0.80	5				

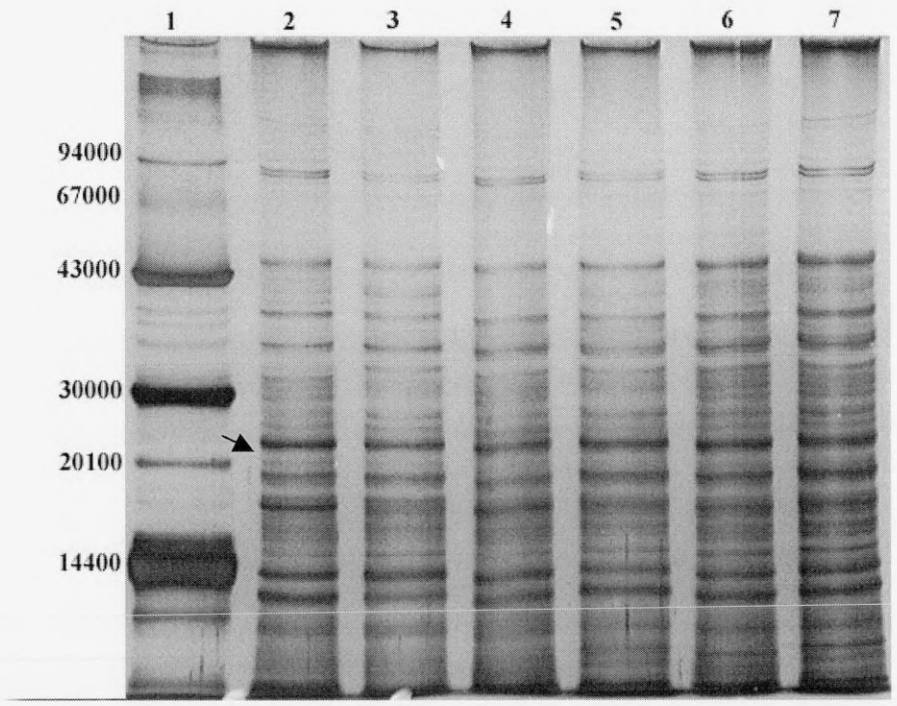


Figure 28: Outer membrane protein profile of *Pseudomonas aeruginosa* PAO1 eluate cells cultured in CDM and exposed to BIT. Lane 1: molecular weights markers; Lane 2: planktonic control cells (1.104 x 10^{-3} mg/well); Lane 3: Passage 1 cells (1.20 x 10^{-3} mg/well); Lane 4: Passage 3 cells (1.404 x 10^{-3} mg/well); Lane 5: Passage 10 cells (1.44 x 10^{-3} mg/well); Lane 6: Passage 12 cells (1.11 x 10^{-3} mg/well); Lane 7: Passage 13 cells (1.212 x 10^{-3} mg/well). The arrow indicates the 23.5 kD OMP.

Table 15 (Part 1): Measurement Data indicating Band Number, Molecular Weight and Rf value for Outer Membrane Proteins of *Ps. aeruginosa* PAO1 Eluate Cells exposed to BIT and cultured in CDM (band no: band number; M.Wt.: molecular weight; Rf: retardation factor; Co: corresponding protein to lane 2; N: novel protein).

Lane 1 (molecular weight markers)				Lane 2 (planktonic cells)				Lane 3 (Passage 1)			
Band no.	MWt (kD)	Rf	Co.	Band no.	MWt	Rf	Co.	Band no.	MWt	Rf	Co.
1	94	0.16	-	1	81	0.20	-	2	78	0.21	1
2	67	0.26	-	2	49	0.34	-	3	47	0.35	2
3	43	0.36	-	3	39	0.42	-	4	41.6	0.39	N
4	30	0.55	-	4	36	0.47	-	5	39	0.42	3
5	20.1	0.71	-	5	33.8	0.50	-	6	36	0.47	4
6	14.4	0.80	-	6	25.6	0.62	-	7	33.8	0.50	5
				7	23.5	0.65	-	8	31.5	0.53	N
				8	22.7	0.67	-	9	28.8	0.57	N
				9	20.1	0.71	-	10	27.5	0.59	N
				10	17.7	0.75	-	11	25.6	0.62	6
				11	13	0.82	-	12	22.7	0.67	8
								13	20.1	0.71	9
								14	18	0.74	10
								15	15.8	0.78	N
								16	13	0.82	11
Lane 4 (Passage 3)				Lane 5 (Passage 10)				Lane 6 (Passage 12)			
1	78	0.21	1	1	78	0.21	1	1	81	0.20	1
2	47	0.35	2	2	49	0.34	2	2	49	0.34	2
3	39	0.42	3	3	42.4	0.38	N	3	44	0.37	N
4	36	0.47	4	4	39	0.42	3	4	40	0.41	3
5	33	0.51	5	5	36.8	0.46	4	5	36.8	0.46	4
6	25.6	0.62	6	6	33.8	0.50	5	6	33.8	0.50	5
7	22.7	0.67	8	7	31.5	0.53	N	7	29.2	0.56	N
8	20.1	0.71	9	8	29.2	0.56	N	8	26	0.61	6
9	17.7	0.75	10	9	26	0.61	6	9	23	0.66	8
10	13.8	0.81	11	10	23	0.66	8	10	21	0.70	9
				11	21	0.70	9	11	17.7	0.75	10
				12	19	0.73	10	12	16	0.77	N
				13	16	0.77	N	13	13.8	0.81	11
				14	14.4	0.80	11				

Table 15 (Part 2): Measurement Data indicating Band Number, Molecular Weight and Rf value for Outer Membrane Proteins of *Ps. aeruginosa* PAO1 Eluate Cells exposed to BIT and cultured in CDM (no: band number; M.Wt.: molecular weight; Rf: retardation factor; Co: corresponding protein to lane 2; N: novel protein).

Lane 7 (Passage 13)			
Band no.	MWt (kD)	Rf	Co.
2	84	0.19	N
3	81	0.20	1
4	61.5	0.28	N
5	51	0.33	2
6	44	0.37	N
7	40	0.41	3
8	37	0.45	4
9	34.8	0.49	5
10	31.5	0.53	N
11	29.2	0.56	N
12	27	0.60	N
13	23.5	0.65	7
14	21.7	0.69	9
15	19	0.73	10
16	16	0.77	N
17	14.4	0.80	11

OMPs shifts in ZnPT exposed cells

When biofilm cells were exposed to ZnPT (**Figure 29**) an OMP (17.8 kD) appeared in planktonic control cells, disappeared from Passages 1, 4, 9, 10 and 11, and then reappeared in passage 13 (**Table 16**). **Figure 30** demonstrates the outer membrane protein profiles *Ps. aeruginosa* eluate cells exposed to ZnPT. OMPs at 86.8 kD, 59.2 kD and 39 kD appeared in planktonic, unexposed, cells and totally disappeared from eluate cells (from Passage 1 to Passage 13); thus, these proteins are planktonic specific OMPs. However, an OMP in the range of between 17.8 kD and 18.5 kD, only appeared in the eluate cells. This OMP was not observed in planktonic cells (**Table 17**). Two outer membrane proteins, 40.9 kD and 31 kD, were observed in lane 2 (planktonic control cells), disappeared from the resistant passages (1, 4, 9 & 10) and once the cells were cultured in biocide free medium, both OMPs appeared again (Passages 13).

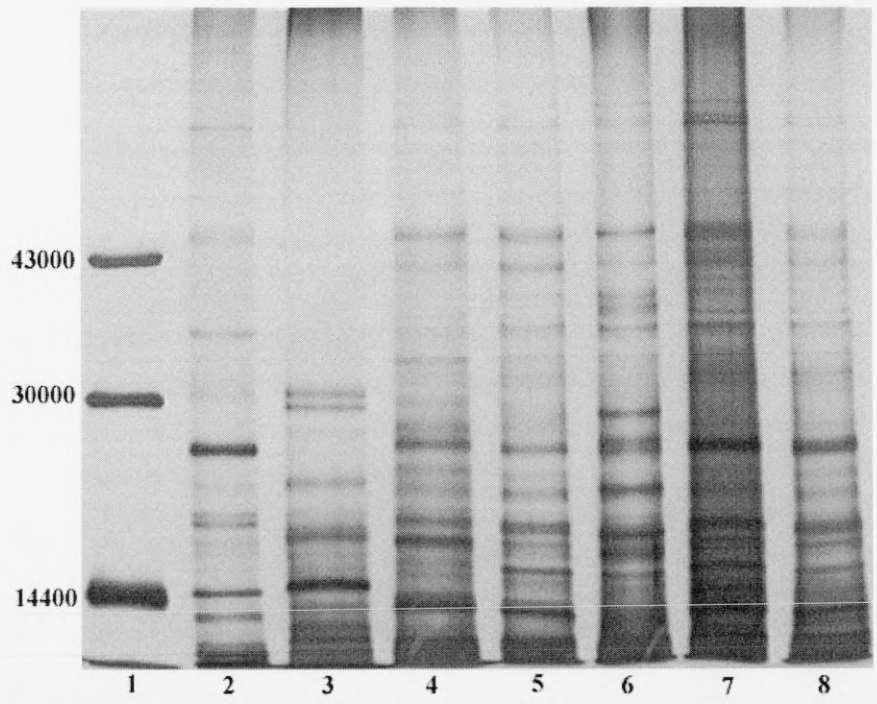


Figure 29: Outer membrane protein profile of *Pseudomonas aeruginosa* PAO1 biofilm cells cultured in CDM and exposed to ZnPT. Lane 1: molecular weights markers; Lane 2: planktonic control cells (1.10 x 10^{-3} mg/well); Lane 3: Passage 1 cells (1.18 x 10^{-3} mg/well); Lane 4: Passage 4 cells (1.07 x 10^{-3} mg/well); Lane 5: Passage 9 cells (1.071 x 10^{-3} mg/well); Lane 6: Passage 10 cells (1.22 x 10^{-3} mg/well); Lane 7: Passage 11 cells (1.035 x 10^{-3} mg/well); Lane 8: Passage 13 cells (1.28 x 10^{-3} mg/well).

Table 16 (Part 1): Measurement Data indicating Band Number, Molecular Weight and Rf value for Outer Membrane Proteins of *Ps. aeruginosa* PAO1 Biofilm Cells exposed to ZnPT and cultured in CDM (band no: band number; M.Wt.: molecular weight; Rf: retardation factor; Co: corresponding protein to lane 2; N: novel protein).

Lane 1 (molecular weight markers)				Lane 2 (planktonic cells)				Lane 3 (Passage 1)			
Band no.	MWt (kD)	Rf	Co.	Band no.	MWt	Rf	Co.	Band no.	MWt	Rf	Co.
1	43	0.40	-	2	85.5	0.19	-	1	28.8	0.59	5
2	30	0.58	-	3	50	0.35	-	2	27.7	0.61	N
3	14.4	0.85	-	4	34.8	0.50	-	3	25	0.64	N
				5	28.8	0.59	-	4	19.6	0.72	7
				6	22.9	0.67	-	5	16	0.80	10
				7	19.6	0.72	-	6	14.4	0.87	11
				8	17.8	0.77	-				
				9	16.9	0.78	-				
				10	15.9	0.81	-				
				11	13.8	0.88	-				
Lane 4 (Passage 4)				Lane 5 (Passage 9)				Lane 6 (Passage 10)			
1	85.5	0.19	2	1	85.5	0.19	2	1	85.5	0.19	2
2	50	0.35	3	2	50	0.35	3	2	50	0.35	3
3	44	0.39	N	3	43	0.40	N	3	44	0.39	N
4	37.8	0.47	N	4	39.5	0.44	N	4	39.5	0.44	N
5	32	0.54	N	5	35.9	0.49	4	5	38	0.46	N
6	28	0.60	5	6	32	0.54	N	6	35.9	0.49	4
7	25	0.64	N	7	22.9	0.67	6	7	32.8	0.53	N
8	23.6	0.66	6	8	20.1	0.71	7	8	26.8	0.62	5
9	52.8	0.70	N	9	18.8	0.74	N	9	23.6	0.66	6
10	19	0.73	7	10	16.6	0.79	9	10	19	0.73	7
11	16.9	0.78	9	11	15.9	0.81	10	11	16	0.80	9
12	15.9	0.81	10	12	14.7	0.85	N	12	15	0.83	10
13	14.9	0.84	N	13	14.4	0.87	11	13	14	0.86	11

Table 16 (Part 2): Measurement Data indicating Band Number, Molecular Weight and Rf value for Outer Membrane Proteins of *Ps. aeruginosa* PAO1 Biofilm Cells exposed to ZnPT and cultured in CDM (no: band number; M.Wt.: molecular weight; Rf: retardation factor; Co: corresponding protein to lane 2; N: novel protein).

Lane 7 (Passage 11)				Lane 8 (Passage 13)			
Band no.	MWt (kD)	Rf	Co.	Band no.	MWt (kD)	Rf	Co.
2	101	0.15	N	1	85.5	0.19	N
3	88	0.18	2	2	50	0.35	3
4	52	0.34	3	3	44	0.39	N
5	44	0.39	N	4	37.8	0.47	N
6	37.8	0.47	N	5	36.6	0.48	4
7	36.6	0.48	4	6	33.5	0.52	N
8	30.9	0.56	N	7	30.9	0.56	N
9	25.8	0.63	N	8	22.9	0.67	6
10	22.9	0.67	6	9	20.1	0.71	7
11	19	0.73	7	10	18.8	0.74	8
12	16.9	0.78	9	11	16.6	0.79	9
13	14.7	0.85	N	12	15.9	0.81	10
				13	14.7	0.85	N

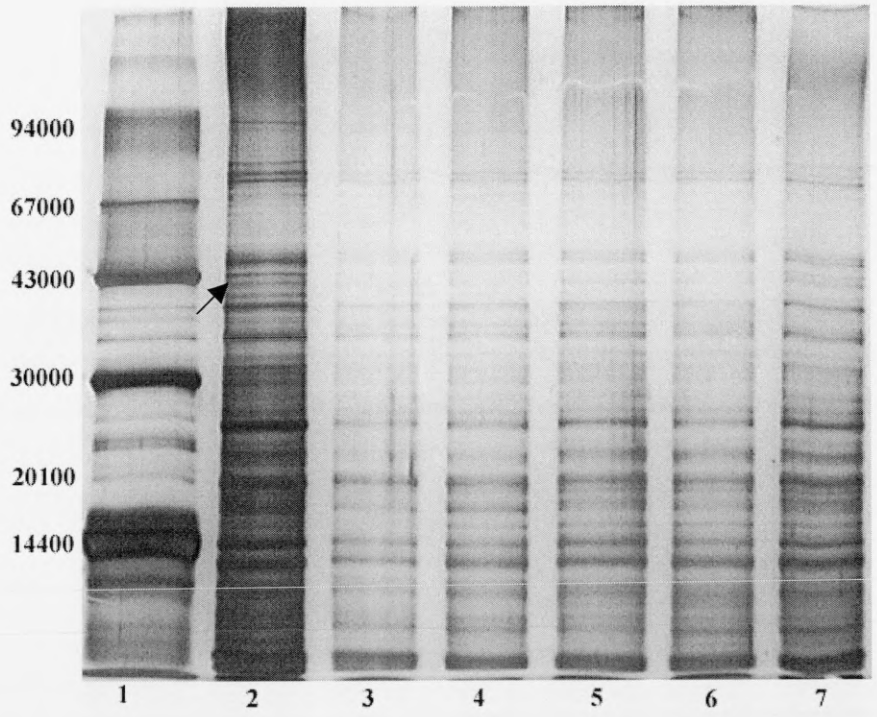


Figure 30: Outer membrane protein profile of *Pseudomonas aeruginosa* PAO1 eluate cells cultured in CDM and exposed to ZnPT. Lane 1: molecular weights markers; Lane 2: planktonic control cells (1.104 x 10^{-3} mg/well); Lane 3: Passage 1 cells (1.15 x 10^{-3} mg/well); Lane 4: Passage 4 cells (1.485 x 10^{-3} mg/well); Lane 5: Passage 9 cells (1.40 x 10^{-3} mg/well); Lane 6: Passage 10 cells (1.10 x 10^{-3} mg/well); Lane 7: Passage 13 cells (1.40 x 10^{-3} mg/well). The arrow indicates the 40.9 kD OMP.

Table 17 (Part 1): Measurement Data indicating Band Number, Molecular Weight and Rf value for Outer Membrane Proteins of *Ps. aeruginosa* PAO1 Eluate Cells exposed to ZnPT and cultured in CDM (band no: band number; M.Wt.: molecular weight; Rf: retardation factor; Co: corresponding protein to lane 2; N: novel protein).

Lane 1 (molecular weight markers)				Lane 2 (planktonic cells)				Lane 3 (Passage 1)			
Band no.	MWt (kD)	Rf	Co.	Band no.	MWt	Rf	Co.	Band no.	MWt	Rf	Co.
1	94	0.18	-	2	86.8	0.20	-	2	38	0.46	10
2	43	0.41	-	3	70	0.26	-	3	34	0.51	11
3	30	0.57	-	4	63.9	0.29	-	4	27.9	0.60	13
4	20.1	0.72	-	5	59.2	0.31	-	5	25.5	0.63	14
5	14.4	0.80	-	6	44	0.40	-	6	21.9	0.68	15
				7	41.9	0.42	-	7	20.8	0.71	16
				8	40.9	0.43	-	8	17.8	0.75	N
				9	39	0.45	-	9	14.4	0.80	17
				10	38	0.46	-				
				11	34	0.51	-				
				12	31	0.55	-				
				13	26.1	0.62	-				
				14	25.5	0.63	-				
				15	21.9	0.68	-				
				16	20.1	0.72	-				
				17	14.4	0.80	-				

Table 17 (Part 2): Measurement Data indicating Band Number, Molecular Weight and Rf value for Outer Membrane Proteins of *Ps. aeruginosa* PAO1 Eluate Cells exposed to ZnPT and cultured in CDM (band no: band number; M.Wt.: molecular weight; Rf: retardation factor; Co: corresponding protein to lane 2; N: novel protein).

Lane 4 (Passage 4)				Lane 5 (Passage 9)				Lane 6 (Passage 10)			
Band no.	MWt (kD)	Rf	Co.	Band no.	MWt (kD)	Rf	Co.	Band no.	MWt (kD)	Rf	Co.
3	65.9	0.28	4	2	65.9	0.28	4	2	65.9	0.28	4
4	44	0.40	6	3	45.9	0.39	6	3	45.9	0.39	6
5	38	0.46	10	4	38	0.46	10	4	38	0.46	10
6	35.5	0.49	N	5	35.5	0.49	N	5	35	0.50	11
7	34	0.51	11	6	35	0.50	11	6	26.9	0.61	13
8	25.5	0.63	14	7	32.7	0.53	N	7	25.5	0.63	14
9	21.9	0.68	15	8	30	0.57	12	8	22.8	0.67	15
10	20.1	0.72	16	9	28.4	0.59	13	9	20.8	0.71	16
11	17.8	0.75	N	10	25.5	0.63	14	10	17.8	0.75	N
12	16	0.78	N	11	22.8	0.67	15	11	16.5	0.77	N
13	14.4	0.80	17	12	20.8	0.71	16	12	14.4	0.80	17
				13	18.5	0.74	N				
				14	16.5	0.77	N				
				15	14.4	0.80	17				

Lane 7 (Passage 13)			
2	63.9	0.29	4
3	45.9	0.39	6
4	40.9	0.43	8
5	38	0.46	10
6	35	0.50	11
7	31	0.55	12
8	28.4	0.59	13
9	25.5	0.63	14
10	22.8	0.67	15
11	20.8	0.71	16
12	18.5	0.73	N
13	16.5	0.77	N
14	14.4	0.80	17

OMPs shifts in Cetrimide exposed cells

An OMP with a molecular weight of 36.5 kD appeared in *Ps. aeruginosa* planktonic cells (**Figure 31**). When exposing biofilm cells to cetrimide, this OMP did not disappear from the first passage. In the following passages (6, 8, 10 & 11), it was not possible to observe this protein. At Passage 13, the 36.5 kD OMP reappeared. Another OMP in the range of between 33.1 kD and 34 kD appeared in biofilm cells. This OMP was not observed in planktonic control cells (**Table 18**). For the eluate cells (**Figure 32**) two outer membrane proteins with molecular weights of 40.8 kD and 40 kD appeared in planktonic, unexposed cells. Both OMPs disappeared from the resistant passages (1, 8 & 10) and at Passage 12, the 40 kD OMP appeared again, but it was not until Passage 13 that the 40.8 kD reappeared. A 19 kD OMP was observed in planktonic cells disappeared from the resistant passages and then reappeared in Passages 12 and 13 at 18.6 kD. A planktonic specific OMP (42.4 kD) was observed in planktonic cells and disappeared completely from biofilm cells. On the other hand, an OMP with a molecular weight of 30.8 kD appeared in Passages 1, 8 and 10. At Passage 12, this protein appeared as a 30 kD OMP before it disappeared completely from Passage 13. This outer membrane protein could be the same OMP, which was observed when exposing biofilm cells to BIT (30 kD to 30.7 kD) (**Table 19**).

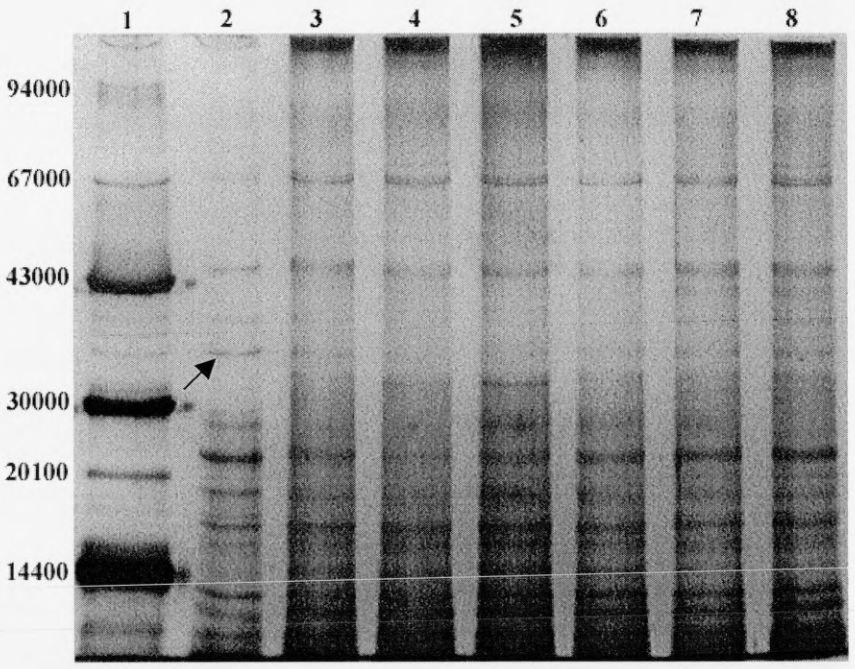


Figure 31: Outer membrane protein profile of *Pseudomonas aeruginosa* PAO1 biofilm cells cultured in CDM and exposed to Cetrimide. Lane 1: molecular weights markers; Lane 2: planktonic control cells (1.10 x 10^{-3} mg/well); Lane 3: Passage 1 cells (1.145 x 10^{-3} mg/well); Lane 4: Passage 6 cells (1.116 x 10^{-3} mg/well); Lane 5: Passage 8 cells (1.147 x 10^{-3} mg/well); Lane 6: Passage 10 cells (1.125 x 10^{-3} mg/well); Lane 7: Passage 11 cells (1.114 x 10^{-3} mg/well); Lane 8: Passage 13 cells (1.126 x 10^{-3} mg/well). The arrow indicates the 36.5 kD OMP.

Table 18 (Part 1): Measurement Data indicating Band Number, Molecular Weight and Rf value for Outer Membrane Proteins of *Ps. aeruginosa* PAO1 Biofilm Cells exposed to Cetrimide and cultured in CDM (band no: band number; M.Wt.: molecular weight; Rf: retardation factor; Co: corresponding protein to lane 2; N: novel protein).

Lane 1 (molecular weight markers)				Lane 2 (planktonic cells)				Lane 3 (Passage 1)			
Band no.	MWt (kD)	Rf	Co.	Band no.	MWt	Rf	Co.	Band no.	MWt	Rf	Co.
1	94	0.09	-	1	47.5	0.36	-	1	69.2	0.22	N
2	67	0.24	-	2	36.5	0.49	-	2	49	0.35	1
3	43	0.40	-	3	29	0.60	-	3	36.5	0.49	2
4	30	0.57	-	4	24.5	0.65	-	4	34	0.53	N
5	20.1	0.71	-	5	20.1	0.71	-	5	24.5	0.65	4
6	14.4	0.84	-	6	17	0.76	-	6	20.1	0.71	5
								7	17	0.76	6
								8	14.8	0.83	N
Lane 4 (Passage 6)				Lane 5 (Passage 8)				Lane 6 (Passage 10)			
1	69.2	0.22	N	1	87.5	0.12	N	1	67.8	0.23	N
2	47.5	0.36	1	2	67.8	0.23	N	2	46.2	0.37	1
3	33.1	0.54	N	3	47.5	0.36	1	3	34	0.53	N
4	28	0.61	3	4	33.1	0.54	N	4	29	0.60	3
5	24.5	0.65	4	5	29	0.60	3	5	24.5	0.65	4
6	19.5	0.72	5	6	23.9	0.66	4	6	20.1	0.71	5
7	17	0.76	6	7	20.1	0.71	5	7	17	0.76	6
				8	17	0.76	6	8	14.8	0.83	N
				9	14.8	0.83	N				

Table 18 (Part 2): Measurement Data indicating Band Number, Molecular Weight and Rf value for Outer Membrane Proteins of *Ps. aeruginosa* PAO1 Biofilm Cells exposed to Cetrimide and cultured in CDM (band no: band number; M.Wt.: molecular weight; Rf: retardation factor; Co: corresponding protein to lane 2; N: novel protein).

Lane 7 (Passage 11)				Lane 8 (Passage 13)			
Band no.	MWt (kD)	Rf	Co.	Band no.	MWt (kD)	Rf	Co.
1	67.8	0.23	N	1	69.2	0.22	N
2	47.5	0.36	1	2	47.5	0.36	1
3	34	0.53	N	3	36.5	0.49	2
4	28	0.61	3	4	34	0.53	N
5	24.5	0.65	4	5	28	0.61	3
6	17	0.76	6	6	24.5	0.65	4
				7	17	0.76	6

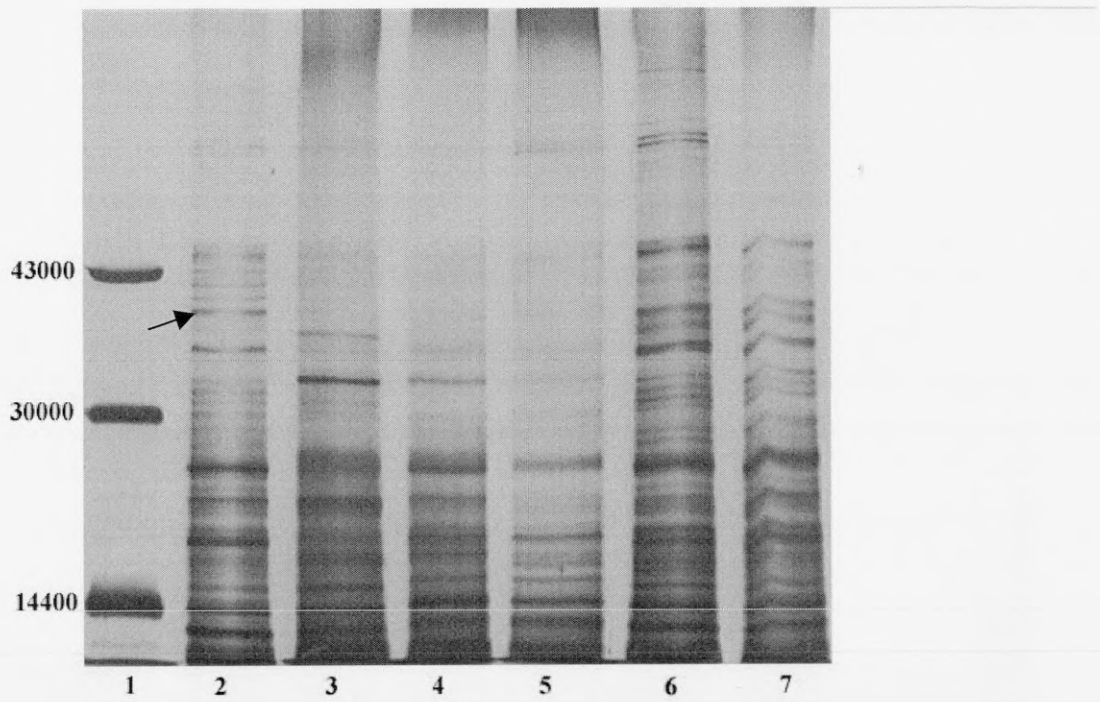


Figure 32: Outer membrane protein profile of *Pseudomonas aeruginosa* PAO1 eluate cells cultured in CDM and exposed to Cetrimide. Lane 1: molecular weights markers; Lane 2: planktonic control cells (1.104 x 10^{-3} mg/well); Lane 3: Passage 1 cells (1.140 x 10^{-3} mg/well); Lane 4: Passage 8 cells (1.152 x 10^{-3} mg/well); Lane 5: Passage 10 cells (1.152 x 10^{-3} mg/well); Lane 6: Passage 12 cells (1.160 x 10^{-3} mg/well); Lane 7: Passage 13 cells (1.144 x 10^{-3} mg/well). The arrow indicates the 40 kD OMP.

Table 19 (Part 1): Measurement Data indicating Band Number, Molecular Weight and Rf value for Outer Membrane Proteins of *Ps. aeruginosa* PAO1 Eluate Cells exposed to Cetrimide and cultured in CDM (band no: band number; M.Wt.: molecular weight; Rf: retardation factor; Co: corresponding protein to lane 2; N: novel protein).

Lane 1 (molecular weight markers)				Lane 2 (planktonic cells)				Lane 3 (Passage 1)			
Band no.	MWt (kD)	Rf	Co.	Band no.	MWt	Rf	Co.	Band no.	MWt	Rf	Co.
1	43	0.41	-	1	81.6	0.20	-	1	81.6	0.21	1
2	30	0.57	-	2	48.6	0.37	-	2	48.6	0.37	2
3	14.4	0.80	-	3	46	0.39	-	3	36.8	0.49	N
				4	42.4	0.42	-	4	34	0.52	7
				5	40.8	0.44	-	5	30.8	0.56	8
				6	40	0.45	-	6	21.9	0.69	11
				7	35	0.51	-	7	17.5	0.75	13
				8	29	0.58	-	8	12.9	0.84	15
				9	26.5	0.62	-				
				10	25	0.64	-				
				11	21	0.70	-				
				12	19	0.73	-				
				13	17.5	0.75	-				
				14	14	0.81	-				
				15	12.9	0.84	-				

Table 19 (Part 2): Measurement Data indicating Band Number, Molecular Weight and Rf value for Outer Membrane Proteins of *Ps. aeruginosa* PAO1 Eluate Cells exposed to Cetrimide and cultured in CDM (band no: band number; M.Wt.: molecular weight; Rf: retardation factor; Co: corresponding protein to lane 2; N: novel protein).

Lane 4 (Passage 8)				Lane 5 (Passage 10)				Lane 6 (Passage 12)			
Band no.	MWt (kD)	Rf	Co.	Band no.	MWt (kD)	Rf	Co.	Band no.	MWt (kD)	Rf	Co.
1	48.6	0.37	2	1	78.8	0.21	1	1	94	0.16	N
2	34	0.52	7	2	48.6	0.37	2	2	88	0.18	N
3	30.8	0.56	8	3	35	0.51	7	3	85	0.19	1
4	26.5	0.62	9	4	30.8	0.56	8	4	50	0.36	2
5	21.9	0.69	11	5	27.2	0.61	9	5	44.8	0.40	3
6	18	0.74	13	6	21.9	0.69	11	6	40	0.45	6
7	14.4	0.80	14	7	17.5	0.75	13	7	37.7	0.48	N
				8	14.4	0.80	14	8	35	0.51	7
				9	12.9	0.84	15	9	31.5	0.55	N
								10	30	0.57	8
								11	28.4	0.59	N
								12	25	0.64	10
								13	21.9	0.69	11
								14	18.6	0.72	12
								15	18	0.74	13
								16	15	0.79	N
								17	14	0.81	14

Lane 7 (Passage 13)			
1	53.5	0.34	2
2	40.8	0.44	5
3	40	0.45	6
4	38.5	0.47	N
5	32.4	0.54	N
6	26.5	0.62	9
7	23.2	0.67	10
8	18.6	0.72	12
9	15.9	0.78	N
10	14	0.81	14

OMPs shifts in NaPT exposed cells

The OMP profiles of biofilm cells exposed to NaPT are given in **Figure 33** and the measurement data performed manually is given in **Table 20**. As is the case with the other three biocides, there were some variations in the number of outer membrane proteins in the planktonic, control cells and resistant biofilm cells. A 21.9 kD OMP appeared in planktonic cells, disappeared from Passage 1 until Passage 10 and then reappeared in Passages 11 and 13 as a 21 kD OMP. An OMP with a molecular weight of 12 kD was observed in planktonic cells (Lane 2), disappeared from Passages 1, 3, 6 and 10 and then reappeared in Passage 11. At Passage 13, this OMP appeared with a molecular weight of 12.8 kD. Another OMP (28 kD) appeared in planktonic cells then disappeared completely from biofilm (exposed and unexposed) cells. A biofilm specific OMP with a molecular range of between 33.1 kD and 34.2 kD was observed in each biofilm passage, but there was no sign of this OMP in planktonic cells. **Figure 34** demonstrates the OMP profiles of eluate cells exposed to NaPT. Unlike biofilm cells, there were not many differences between the planktonic, unexposed cells and the resistant eluate cells. The only OMP shift, observed in planktonic cells, disappeared from the resistant passages (1, 4, 8 & 10) and then reappeared in Passage 13, having a molecular weight of 39.2 kD (**Table 21**).

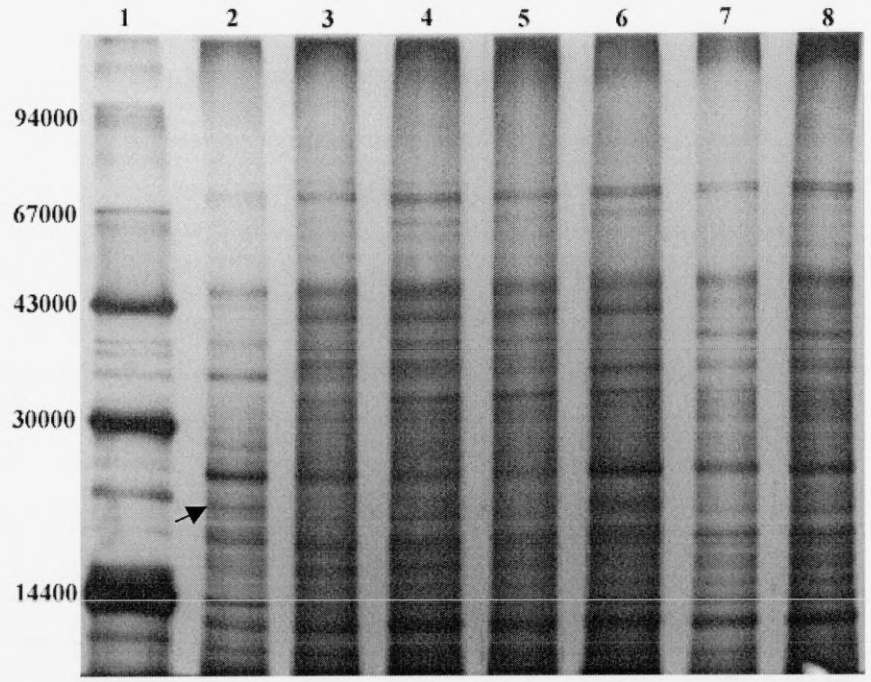


Figure 33: Outer membrane protein profile of *Pseudomonas aeruginosa* PAO1 biofilm cells cultured in CDM and exposed to Sodium Pyrithione (NaPT). Lane 1: molecular weights markers; Lane 2: planktonic control cells (1.10 x 10^{-3} mg/well); Lane 3: Passage 1 cells (1.176 x 10^{-3} mg/well); Lane 4: Passage 3 cells (1.144 x 10^{-3} mg/well); Lane 5: Passage 6 cells (1.156 x 10^{-3} mg/well); Lane 6: Passage 10 cells (1.160 x 10^{-3} mg/well); Lane 7: Passage 11 cells (1.170 x 10^{-3} mg/well); Lane 8: Passage 13 cells (1.150 x 10^{-3} mg/well). The arrow indicates the 21.9 kD OMP.

Table 20 (Part 1): Measurement Data indicating Band Number, Molecular Weight and Rf value for Outer Membrane Proteins of *Ps. aeruginosa* PAO1 Biofilm Cells exposed to NaPT and cultured in CDM (band no: band number; M.Wt.: molecular weight; Rf: retardation factor; Co: corresponding protein to lane 2; N: novel protein).

Lane 1 (molecular weight markers)				Lane 2 (planktonic cells)				Lane 3 (Passage 1)			
Band no.	MWt (kD)	Rf	Co.	Band no.	MWt	Rf	Co.	Band no.	MWt	Rf	Co.
1	94	0.16	-	1	70	0.24	-	1	76.8	0.21	N
2	67	0.26	-	2	47	0.38	-	2	70	0.24	1
4	43	0.41	-	3	44.2	0.40	-	3	48.5	0.37	2
8	30	0.57	-	4	39.8	0.45	-	4	43	0.41	3
9	20.1	0.71	-	5	35.8	0.50	-	5	37	0.48	5
10	14.4	0.80	-	6	30.9	0.56	-	6	33.1	0.53	6
				7	28	0.60	-	7	25.5	0.64	8
				8	25.5	0.64	-	8	18.9	0.74	10
				9	21.9	0.69	-	9	15.9	0.78	N
				10	18.9	0.74	-				
				11	12	0.83	-				
Lane 4 (Passage 3)				Lane 5 (Passage 6)				Lane 6 (Passage 10)			
1	70	0.24	1	1	70	0.24	1	1	72	0.23	1
2	65	0.27	N	2	48.5	0.37	2	2	67	0.26	N
3	47	0.38	2	3	43	0.41	3	3	48.5	0.37	2
4	43	0.41	3	4	33.1	0.53	6	4	44.2	0.40	3
5	39.8	0.45	4	5	25.5	0.64	8	5	32.8	0.49	5
6	37	0.48	5	6	19	0.73	10	6	31.4	0.52	6
7	33.1	0.53	6					7	25.5	0.64	8
8	25.5	0.64	8								
9	18.9	0.74	10								

Table 20 (Part 2): Measurement Data indicating Band Number, Molecular Weight and Rf value for Outer Membrane Proteins of *Ps. aeruginosa* PAO1 Biofilm Cells exposed to NaPT and cultured in CDM (band no: band number; M.Wt.: molecular weight; Rf: retardation factor; Co: corresponding protein to lane 2; N: novel protein).

Lane 7 (Passage 11)				Lane 8 (Passage 13)			
Band no.	MWt (kD)	Rf	Co.	Band no.	MWt (kD)	Rf	Co.
1	72	0.23	1	1	72	0.23	1
2	50.8	0.36	2	2	58.4	0.31	N
3	40.8	0.44	4	3	50.8	0.36	2
4	37	0.48	5	4	40.8	0.44	4
5	34.2	0.52	6	5	36.6	0.49	5
6	26	0.63	8	6	34.2	0.52	6
7	21	0.70	9	7	26	0.63	8
8	19	0.73	10	8	21	0.70	9
9	16.9	0.77	N	9	19	0.73	10
10	14.4	0.80	N	10	16.9	0.77	N
11	12	0.83	11	11	14.4	0.80	N
				12	12.8	0.82	11
				13	10.8	0.85	12

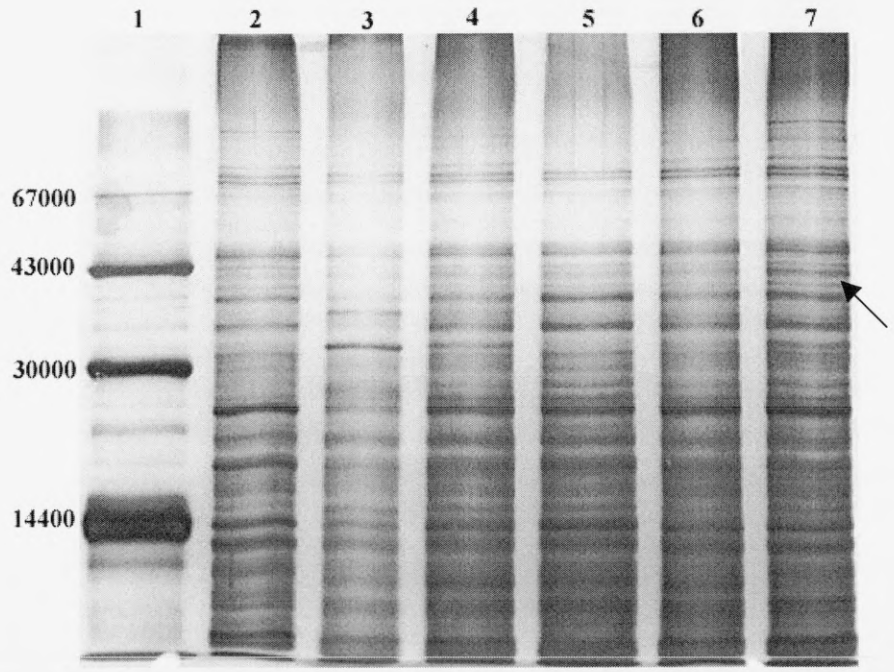


Figure 34: Outer membrane protein profile of *Pseudomonas aeruginosa* PAO1 eluate cells cultured in CDM and exposed to Sodium Pyrithione (NaPT). Lane 1: molecular weights markers; Lane 2: planktonic control cells (1.104×10^{-3} mg/well); Lane 3: Passage 1 cells (1.125×10^{-3} mg/well); Lane 4: Passage 4 cells (1.144×10^{-3} mg/well); Lane 5: Passage 8 cells (1.150×10^{-3} mg/well); Lane 6: Passage 10 cells (1.115×10^{-3} mg/well); Lane 7: Passage 13 cells: (1.140×10^{-3} mg/well). The arrow indicates the 39.2 kD OMP.

Table 21 (Part 1): Measurement Data indicating Band Number, Molecular Weight and Rf value for Outer Membrane Proteins of *Ps. aeruginosa* PAO1 Eluate Cells exposed to NaPT and cultured in CDM (band no: band number; M.Wt.: molecular weight; Rf: retardation factor; Co: corresponding protein to lane 2; N: novel protein).

Lane 1 (molecular weight markers)				Lane 2 (planktonic cells)				Lane 3 (Passage 1)			
Band no.	MWt (kD)	Rf	Co.	Band no.	MWt	Rf	Co.	Band no.	MWt	Rf	Co.
1	67	0.25	-	1	94	0.16	-	1	72	0.23	N
3	43	0.38	-	2	74.8	0.22	-	2	69.5	0.24	3
5	30	0.57	-	3	67	0.25	-	3	44.9	0.37	4
7	14.4	0.80	-	4	43	0.38	-	4	40.5	0.41	5
				5	41.5	0.40	-	5	37.8	0.45	7
				6	39.2	0.43	-	6	36.5	0.47	N
				7	37.8	0.45	-	7	35.2	0.49	8
				8	43.4	0.50	-	8	32.5	0.53	9
				9	31	0.55	-	9	30	0.57	10
				10	30	0.57	-	10	26.8	0.61	11
				11	27.7	0.60	-	11	24.8	0.64	12
				12	24.8	0.64	-	12	21	0.69	13
				13	21	0.69	-	13	18	0.74	14
				14	18	0.74	-	14	16	0.77	15
				15	15	0.79	-	15	13.7	0.82	16
				16	13.7	0.82	-	16	12.9	0.84	17
				17	12.5	0.85	-	17	11.5	0.87	18
				18	11.5	0.87	-				

Table 21 (Part 2): Measurement Data indicating Band Number, Molecular Weight and Rf value for Outer Membrane Proteins of *Ps. aeruginosa* PAO1 Eluate Cells exposed to NaPT and cultured in CDM (band no: band number; M.Wt.: molecular weight; Rf: retardation factor; Co: corresponding protein to lane 2; N: novel protein).

Lane 4 (Passage 4)				Lane 5 (Passage 8)				Lane 6 (Passage 10)			
Band no.	MWt (kD)	Rf	Co.	Band no.	MWt (kD)	Rf	Co.	Band no.	MWt (kD)	Rf	Co.
1	94	0.16	1	1	74.8	0.22	2	1	90	0.17	1
2	72	0.23	N	2	72	0.23	N	2	81.1	0.20	N
3	69.5	0.24	3	3	65	0.26	3	3	74.8	0.22	2
4	63	0.27	N	4	44.9	0.37	4	4	72	0.23	N
5	44.9	0.37	4	5	32	0.39	N	5	67	0.25	3
6	42	0.39	N	6	41.5	0.40	5	6	45.6	0.36	4
7	41.5	0.40	5	7	37.8	0.45	7	7	41.5	0.40	5
8	37.8	0.45	7	8	35.2	0.49	8	8	38.8	0.44	7
9	35.2	0.49	8	9	31.9	0.54	9	9	35.2	0.49	8
10	32.5	0.53	9	10	30	0.57	10	10	31.9	0.54	9
11	30	0.57	10	11	27.7	0.60	11	11	27.7	0.60	11
12	27.7	0.60	11	12	26	0.62	N	12	24.8	0.64	12
13	26	0.62	N	13	24.8	0.64	12	13	21	0.69	13
14	24.8	0.64	12	14	22.5	0.67	N	14	18	0.74	14
15	21	0.69	13	15	21	0.69	13	15	11	0.88	18
16	18	0.74	14	16	18.8	0.73	14				
17	15.5	0.78	15	17	15.5	0.78	15				
18	14	0.81	16	18	14	0.81	16				
19	12.9	0.84	17	19	12.9	0.84	17				
				20	11.5	0.87	18				

Table 21 (Part 3): Measurement Data indicating Band Number, Molecular Weight and Rf value for Outer Membrane Proteins of *Ps. aeruginosa* PAO1 Eluate Cells exposed to NaPT and cultured in CDM (band no: band number; M.Wt.: molecular weight; Rf: retardation factor; Co: corresponding protein to lane 2; N: novel protein).

Lane 7 (Passage 13)			
Band no.	MWt (kD)	Rf	Co.
1	98	0.14	N
2	90	0.17	1
3	81.1	0.20	N
4	74.8	0.22	2
5	72	0.23	N
6	67	0.25	3
7	54	0.31	N
8	45.6	0.36	4
9	41.5	0.40	5
10	39.2	0.43	6
11	38.8	0.44	7
12	35.2	0.49	N
13	31.9	0.54	9
14	27.7	0.60	11
15	26	0.62	N
16	24.8	0.64	12
17	21	0.69	13
18	18	0.74	14
19	15.5	0.78	15
20	14	0.81	16
21	12.5	0.85	17
22	11	0.88	18

From the previous commentary, it is clear that there are significant variations in the OMP profiles between biofilm and eluate cells following their exposure to the four biocides. This result was expected from the differences in MIC values between the passages for each biocide (**Figures 19, 20, 21 & 22**; Chapter 4). However, an outer membrane protein in the range of 36.5 kD and 41 kD was observed to have disappeared following the exposure of biofilm cells to BIT (38 kD) and Cetrimide (36.5 kD) and the eluate cells to ZnPT (41 kD) and NaPT (39.2 kD). The slight variations in the observed molecular weight of this OMP may be due to differences in scanning (as mentioned earlier) as this analysis was performed manually. Therefore, any slight variation will affect the Rf value and thus, the molecular weight assignation. This OMP reappeared when the cells were passaged in biocide-free medium. This implies that the loss of this protein is a phenotypic adaptation related to direct presence of the biocide and not a genotypic adaptation.

In comparing these results with the ones obtained from passaging planktonic cells in the presence of BIT, Winder *et al.* (2000) observed an outer membrane protein with a molecular weight of 36.577 kD in control unexposed planktonic cells. This OMP disappeared after the first passage in the presence of BIT and once the cells were cultured in biocide free medium (CDM), the OMP reappeared. This protein is designated T-OMP and it was first observed by Brözel and Cloete (1994). Brözel and Cloete (1994) demonstrated the presence of T-OMP in *Ps. aeruginosa* PAO1 sensitive cells. After exposing the cells to Kathon™ (a mixture of 1.15% CMIT and 0.35% MIT) the T-OMP disappeared (Chapter 1). Abdel Malek *et al.* (2002) observed T-OMP in sensitive planktonic cells before their exposure to the pyrithione biocides. The onset and the development of resistance to these biocides were accompanied by the loss of T-OMP from the outer membrane profiles of those cells. When the cells were passaged in the absence of pyrithione, the T-OMP reappeared. Winder *et al.* (2000) and

Abdel-Malek *et al.* (2002) used an imaging software package to estimate the molecular weights of proteins, which may explain the observed difference in the molecular weight of this OMP (T-OMP) between this study and those previous studies.

Experimental Approach

Two-Dimensional Polyacrylamide Gel Electrophoresis

Since O'Farrell (1975) introduced the improved technique for high-resolution two-dimensional polyacrylamide gel electrophoresis (2-D PAGE), the electrophoretic separation of proteins has become one of the main methods for the fractionation and characterisation of all types of proteins at both analytical and preparative levels. This form of separation depends not only on the molecular weights of polypeptides, but also on their overall charge, which in turn depends on the amino acid composition of the protein, the presence or absence of detergents and the pH of the solution. In this technique, the proteins are separated in the first dimension by isoelectric focusing according to their isoelectric point and in the second dimension according to molecular weight by SDS electrophoresis. Consequently, this system has sufficient resolution to separate individual proteins as discrete spots on the gel.

There are two different ways for performing the first-dimensional stage (isoelectric focusing; IEF). The first way is by pre-running the first dimensional gel (tube gel) without the sample to establish the pH gradient, this is known as the pre-formed stage. In the second way, there is no pre-running since a stable pH gradient is not formed; thus samples are loaded directly onto the tube-gels and the pH gradient established during the isoelectric focusing stage (the non-equilibrium pH gradient electrophoresis technique; NEPHGE; O'Farrell, 1977). The second dimension remains the same as that originally developed by Laemmli (1970). By

combining IEF or NEPHGE in the first dimension with sodium dodecyl sulphate (SDS) gel electrophoresis in the second dimension, this procedure (IEF-SDS) or (NEPHGE-SDS) can resolve over 1000 proteins.

Preparation of Glass Tubes for the First Dimensional Stage, Preparation of Tube-Gel (First Dimension Protein Separation), Isoelectric Focusing of Protein Samples and Preparation of Slab Gel (Second Dimension Protein Separation) were performed as described in Chapter 2.

The Analysis of OMP Profiles

Figure 35 illustrates the two-dimensional electrophoretic protein pattern of 2-D SDS-PAGE Standards separated according to their molecular weights and pIs (isoelectric points). The IEF range is between pH 3.5 and pH 10; the pI for each protein was estimated manually and compared to the standard pIs (**Table 22**) in order to determine the molecular weight for each spot. After estimating the molecular weight for all the proteins on the standard gel, the Rf value for each standard protein was calculated according to **Equation 4** (**Table 23**). By plotting the molecular weights (Daltons) against Rf values, a calibration curve was constructed (**Figure 36**). For the sample gels (**Figures 37, 38, 39 & 40**) the Rf value was estimated from **Equation 5** and then extrapolated onto the calibration curve, thus the molecular weight was determined for that protein. The individual numbered proteins (on each sample gel) and their corresponding estimated molecular weights, Rf and pI (analysed manually) are shown in **Tables 24 & 25**.

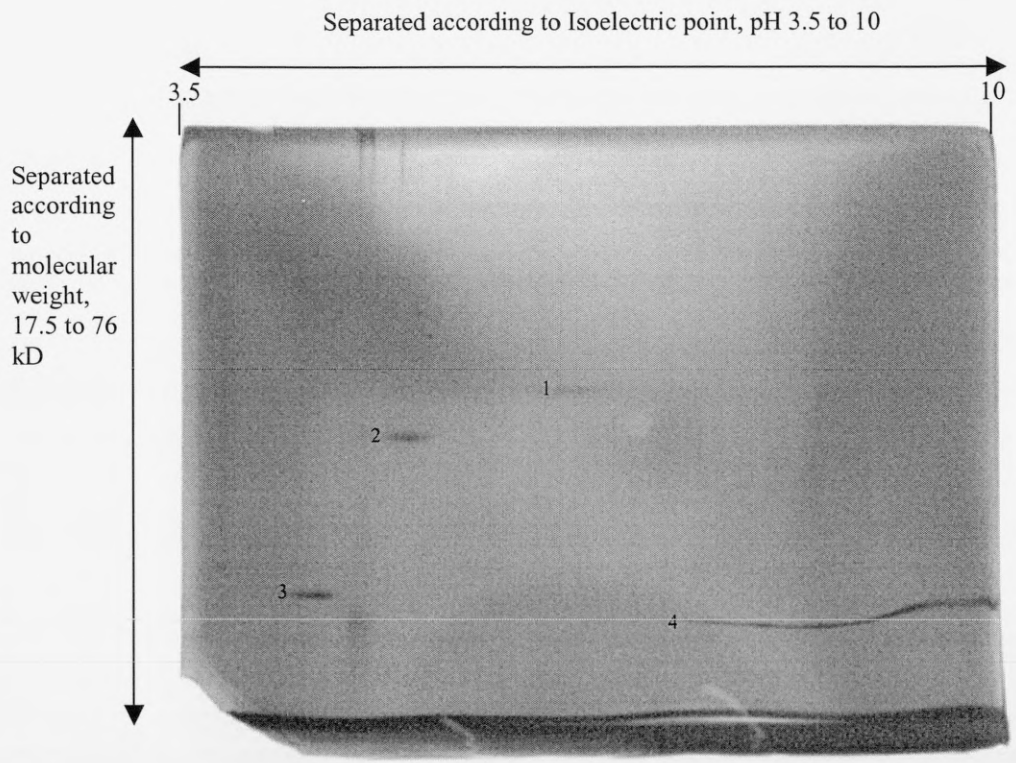


Figure 35: Two-dimensional electrophoretic protein pattern of 2-D SDS-PAGE Standards separated according to their molecular weights and pIs.

Table 22: Bio-Rad Standard Molecular Weights and Isoelectric points (pI).

Protein	**Molecular Weight (Daltons)**	**pI (pH)***
Conalbumin	76,000	6.0; 6.3; 6.6
Albumin	66,200	5.4; 5.6
Actin	43,000	5.0; 5.1
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	36,000	8.3-8.5
Carbonic anhydrase	31,000	5.9; 6.0
Trypsin inhibitor	21,500	4.5
Myoglobin	17,500	7.0

* = Reference to Bio-Rad.

Table 23: Measurement data indicating Protein number, Molecular weight, Retardation factor and Isoelectric point of the proteins identified from the Two-Dimensional SDS-PAGE Standard Proteins Gel (protein no.: protein number; M.Wt.: molecular weight; Rf: retardation factor; pI: isoelectric point).

Protein no.	M.Wt. (Daltons)	Rf	PI (pH)
1	76000	0.44	6.7
2	43000	0.53	5
3	21500	0.79	4.4
4	17500	0.84	7.15

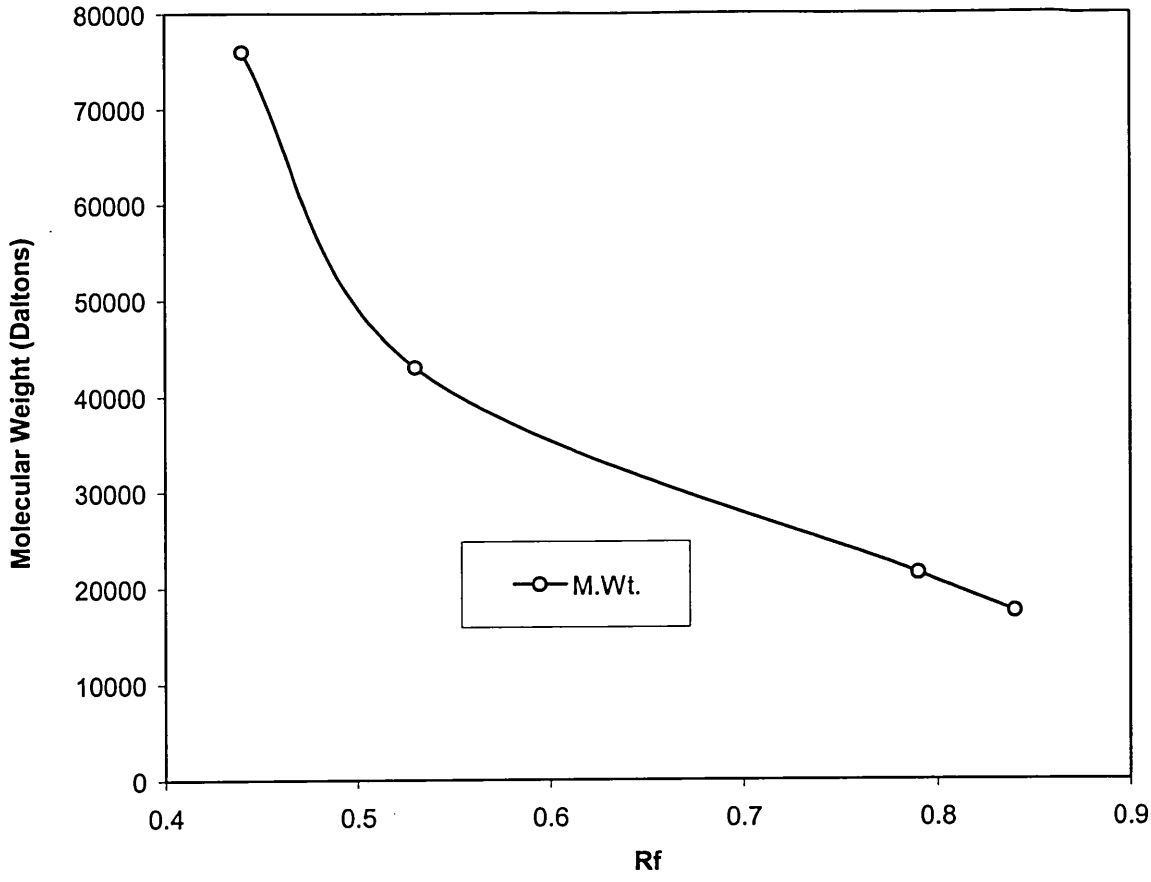


Figure 36: Calibration curve generated by plotting the molecular weights of 2D-PAGE Standards in Daltons vs Rf values.

Results & Discussion

The outer membrane profiles of resistant biofilm and eluate cultures were analysed by triangulation method against proteins from the sensitive (biofilm and eluate) cells (**Tables 24** and **25**). 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. The most prominent difference between biofilm sensitive gel (**Figure 37**) and biofilm resistant gel (**Figure 38**) is the appearance of various proteins with high molecular weights (>76000 Daltons) and also the observation of OMPs with alkaline isoelectric points. This suggests that some other mechanisms of resistance are being switched on in these cells to account for the increased resistance towards BIT. An outer membrane protein with a molecular weight of 30.4 kD (pI 9.7) appeared in biofilm resistant cells, but there was no sign of this OMP in biofilm sensitive cells. This protein could be the same OMP (30 to 30.7 kD), which appeared upon the exposure of biofilm cells to BIT in SDS-PAGE gels (Passage 7, **Figure 27**). A 47.8 kD OMP (pI 4.5) appeared in biofilm sensitive cells. The molecular weight of this OMP altered to 49.9 kD in biofilm resistant cell (pI 4.5), this could be considered to be a chemical alteration resultant from passaging the cells in a medium contains BIT. A similar image was demonstrated from **Figure 27** (SDS-PAGE gel); an outer membrane protein with a molecular weight of 46 kD appeared in control cells. In biofilm resistant passages, the molecular weight altered to 47 kD and once again the molecular weight of this OMP changed to 46 kD when the cells were cultured in BIT-free medium.

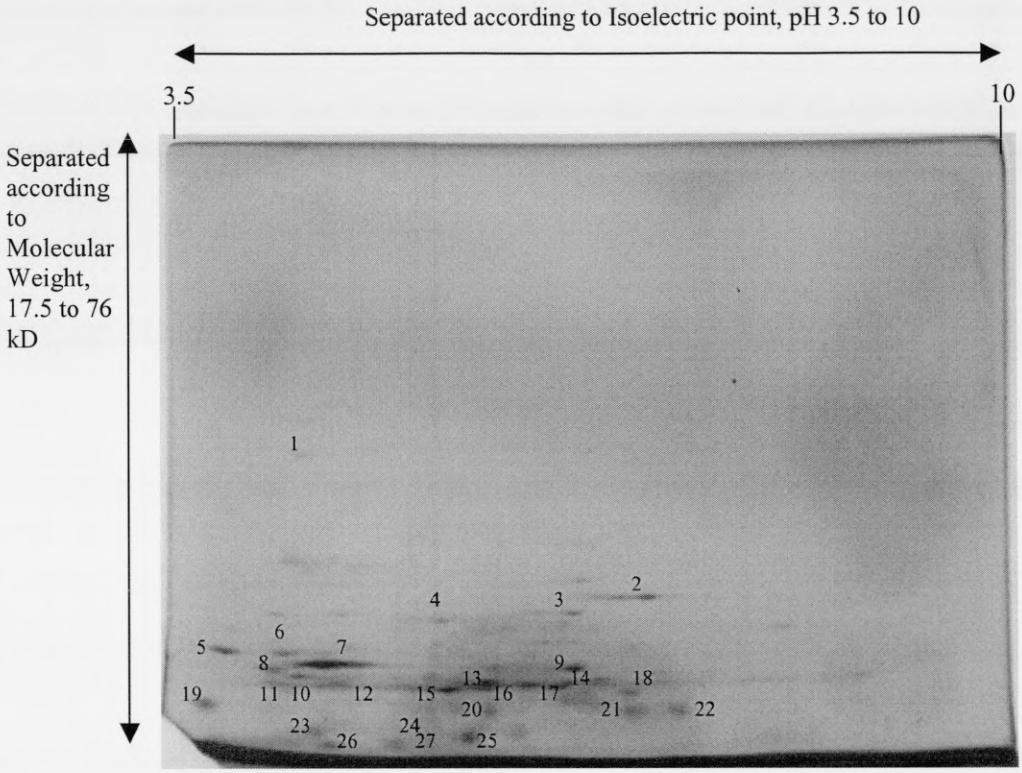


Figure 37: Two-dimensional gel electrophoresis for outer membrane protein (OMP) profiles of *Pseudomonas aeruginosa* PAO1 biofilm sensitive cells in CDM. The OMPs are separated by the isoelectric point (reading left to right) and molecular weight (top to bottom). Protein concentration: 1.125×10^{-3} mg/gel.

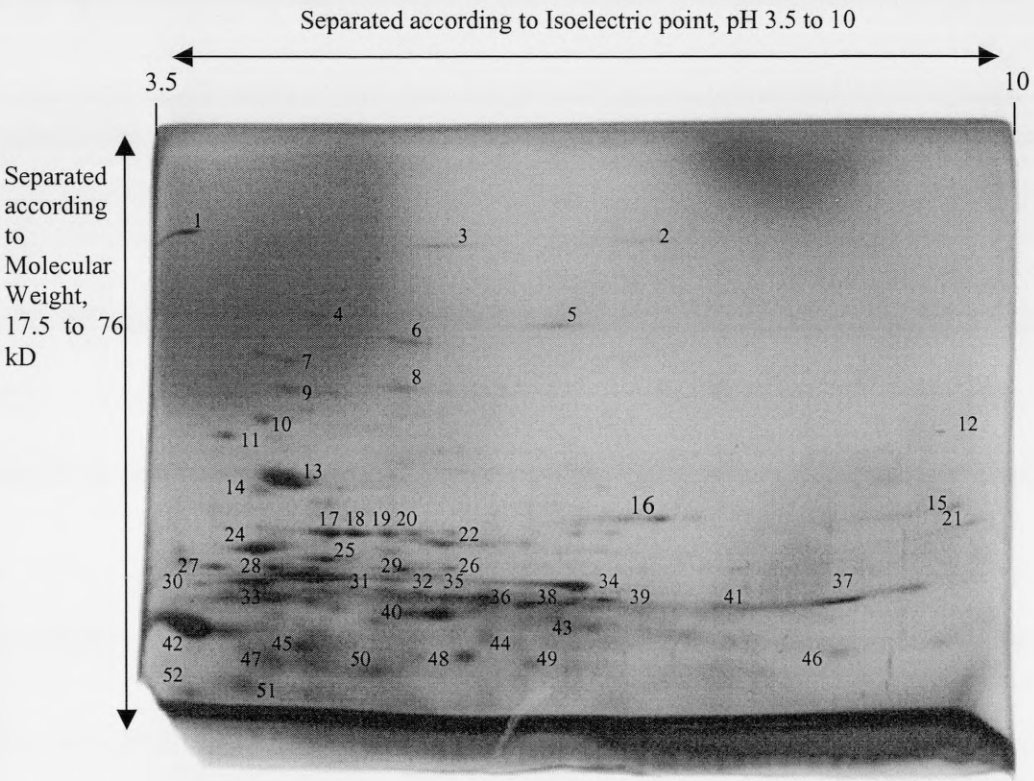


Figure 38: Two-dimensional gel electrophoresis for outer membrane protein (OMP) profiles of *Pseudomonas aeruginosa* PAO1 biofilm resistant cells exposed to BIT and cultured in CDM. The OMPs are separated by the isoelectric point (reading left to right) and molecular weight (top to bottom). Protein concentration: 1.58 x 10^{-3} mg/gel.

Table 24 (Part 1): Measurement data indicating Protein number, Molecular weight, Retardation factor and Isoelectric point of the proteins identified from the Two-Dimensional gel analysis of *Ps. aeruginosa* PAO1 sensitive biofilm cells and compared against biofilm resistant cells (Passage 7: BIT) (protein no.: protein number; M.Wt.: molecular weight; Rf: retardation factor; pI: isoelectric point; Co.: corresponding protein to **Figure 37**; N: novel protein; nc: not calculated).

Figure 37 (sensitive biofilm cells)					**Figure 38** (resistant biofilm cells)				
Protein no.	MWt (kD)	Rf	pI (pH)	Co.	Protein no.	MWt (kD)	Rf	pI (pH)	Co.
1	47.8	0.51	4.50	-	1	nc	0.18	3.75	N
2	24.2	0.74	7.20	-	2	nc	0.2	7.20	N
3	22.6	0.77	6.70	-	3	nc	0.21	5.70	N
4	21.8	0.78	5.60	-	4	nc	0.32	4.70	N
5	18.8	0.82	3.90	-	5	nc	0.34	6.60	N
6	18	0.83	4.39	-	6	nc	0.38	5.45	N
7	17.5	0.84	4.80	-	7	nc	0.40	4.55	N
8	16.9	0.85	4.20	-	8	76	0.44	5.40	N
9	16.3	0.86	6.55	-	9	65	0.45	4.55	N
10	15.9	0.87	4.45	-	10	49.9	0.50	4.30	N
11	15.5	0.88	4.25	-	11	43	0.53	4.00	N
12	15.5	0.88	4.65	-	12	43	0.53	9.55	N
13	15.5	0.88	5.95	-	13	35.8	0.60	4.50	N

Table 24 (Part 2): Measurement data indicating Protein number, Molecular weight, Retardation factor and Isoelectric point of the proteins identified from the Two-Dimensional gel analysis of *Ps. aeruginosa* PAO1 sensitive biofilm cells and compared against biofilm resistant cells (Passage 7: BIT) (protein no.: protein number; M.Wt.: molecular weight; Rf: retardation factor; pI: isoelectric point; Co.: corresponding protein to **Figure 37**; N: novel protein).

Figure 37 (sensitive biofilm cells)					**Figure 38** (resistant biofilm cells)				
Protein no.	MWt (kD)	Rf	pI (pH)	Co.	Protein no.	MWt (kD)	Rf	pI (pH)	Co.
14	15.5	0.88	6.88	-	14	33.2	0.63	4.25	N
15	14.8	0.89	5.70	-	15	30.4	0.66	9.70	N
16	14.8	0.89	6.20	-	16	29.2	0.67	7.40	N
17	14.8	0.89	6.44	-	17	27.7	0.69	4.80	N
18	14.5	0.90	7.04	-	18	27	0.69	5.01	N
19	13.2	0.92	3.70	-	19	27.7	0.69	5.20	N
20	13.2	0.92	5.95	-	20	27.7	0.69	5.45	N
21	13.2	0.92	7.03	-	21	27	0.70	9.80	N
22	12.8	0.93	7.45	-	22	26.2	0.71	5.70	N
23	11.5	0.96	4.63	-	23	25.7	0.72	4.20	N
24	11	0.97	5.55	-	24	25	0.73	3.62	N
25	11	0.97	5.70	-	25	25	0.73	4.70	N
26	10.8	0.98	4.70	-	26	24.2	0.74	5.70	N
27	10.8	0.98	5.20	-	27	23.5	0.75	3.90	N

Table 24 (Part 3): Measurement data indicating Protein number, Molecular weight, Retardation factor and Isoelectric point of the proteins identified from the Two-Dimensional gel analysis of *Ps. aeruginosa* PAO1 sensitive biofilm cells and compared against biofilm resistant cells (Passage 7: BIT) (protein no.: protein number; M.Wt.: molecular weight; Rf: retardation factor; pI: isoelectric point; Co.: corresponding protein to **Figure 37**; N: novel protein).

Figure 37 (sensitive biofilm cells)					**Figure 38** (resistant biofilm cells)				
Protein no.	MWt (kD)	Rf	pI (pH)	Co.	Protein no.	MWt (kD)	Rf	pI (pH)	Co.
					28	23.5	0.75	4.28	N
					29	23	0.76	5.32	N
					30	22.6	0.77	3.80	N
					31	22.6	0.77	4.50	N
					32	22.6	0.77	5.60	N
					33	21.8	0.78	4.20	N
					34	21.8	0.78	6.77	3
					35	20.5	0.80	5.69	N
					36	20.5	0.80	6.00	N
					37	20.5	0.80	8.80	N
					38	19.5	0.81	6.30	N
					39	19.5	0.81	7.00	N
					40	18.8	0.82	5.60	N
					41	18	0.83	7.80	N

Table 24 (Part 4): Measurement data indicating Protein number, Molecular weight, Retardation factor and Isoelectric point of the proteins identified from the Two-Dimensional gel analysis of *Ps. aeruginosa* PAO1 sensitive biofilm cells and compared against biofilm resistant cells (Passage 7: BIT) (protein no.: protein number; M.Wt.: molecular weight; Rf: retardation factor; pI: isoelectric point; Co.: corresponding protein to **Figure 37**; N: novel protein).

Figure 37 (sensitive biofilm cells)					**Figure 38** (resistant biofilm cells)				
Protein no.	MWt (kD)	Rf	pI (pH)	Co.	Protein no.	MWt (kD)	Rf	pI (pH)	Co.
					42	16.9	0.85	3.72	N
					43	16.3	0.86	6.85	14
					44	15.9	0.87	6.00	13
					45	15.5	0.88	4.60	12
					46	14.5	0.90	8.70	N
					47	13.8	0.91	4.30	11
					48	13.8	0.91	5.78	15
					49	13.2	0.92	6.40	N
					50	12.8	0.93	5.20	27
					51	12	0.95	4.20	N
					52	11.6	0.96	3.72	19

Figure 39 illustrates the outer membrane protein profile of sensitive eluate cells. The number of proteins on this gel is small and only two proteins (protein numbers 3 & 4) are clearly visible and there are faint marks in the position of the other three (protein numbers 1, 2 & 5). In comparing eluate sensitive cells with eluate resistant cells (**Figures 39 & 40**, respectively), it is possible to illustrate a marked difference between the two gels. As is the case in biofilm resistant cells, it is possible to identify a greater number of proteins in the resistant eluate cells compared to those in sensitive eluate cells (**Table 25**). Hence, this may be an indication that significant changes occurred in the protein profiles once the cells were passaged in a medium containing the biocide. An outer membrane protein with a molecular weight of 23.5 kD (pI 4.4) was present in biofilm control cells. This protein disappeared from biofilm resistant cells and could be the same OMP, which appeared in control cells (SDS-PAGE gel, **Figure 28**), disappeared from eluate resistant passages and then reappeared in passage 13. A possible chemical alteration was observed in these gels and a 22.6 kD OMP (pI 5.4) appeared in eluate control cells. In eluate resistant cells, the molecular weight of this OMP altered to 24.2 kD (pI 5.4). For 19.5 kD (pI 3.95) and 18.8 kD (pI 4.8) OMPs, the molecular weights and the isoelectric points of the two proteins altered in eluate resistant cells to 21.8 kD, pI 3.85 and 16.3 kD, pI 4.7, respectively.

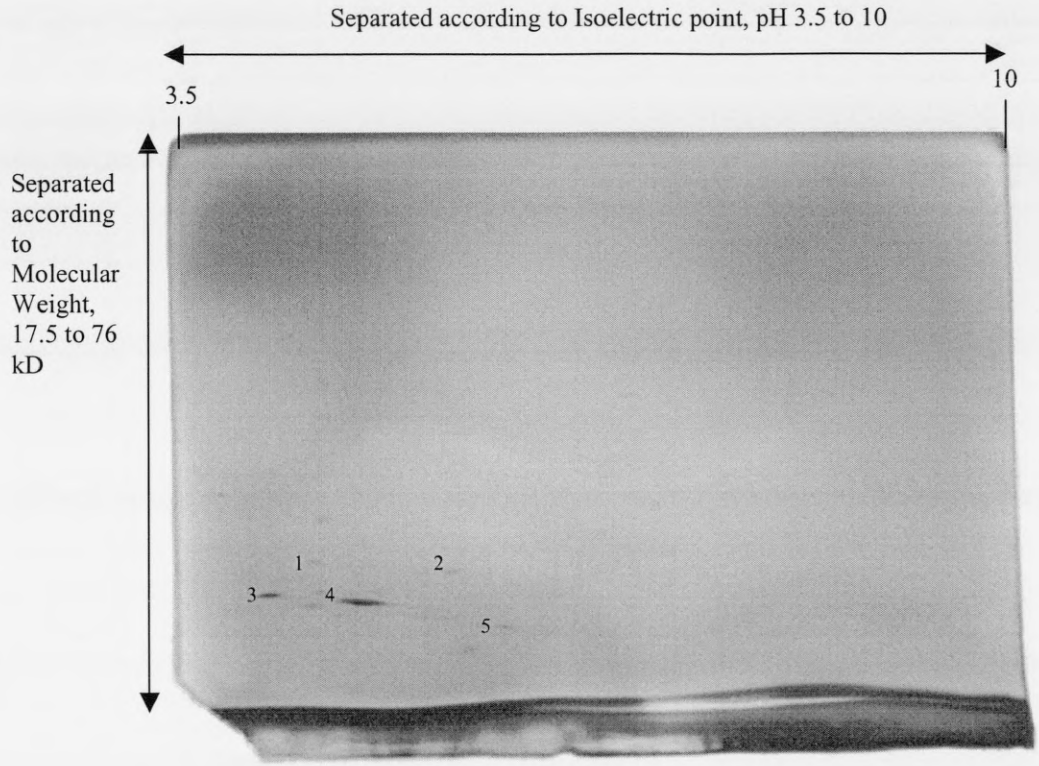


Figure 39: Two-dimensional gel electrophoresis for outer membrane protein (OMP) profiles of *Pseudomonas aeruginosa* PAO1 eluate sensitive cells in CDM. The OMPs are separated by the isoelectric point (reading left to right) and molecular weight (top to bottom). Protein concentration: 1.89×10^{-3} mg/gel.

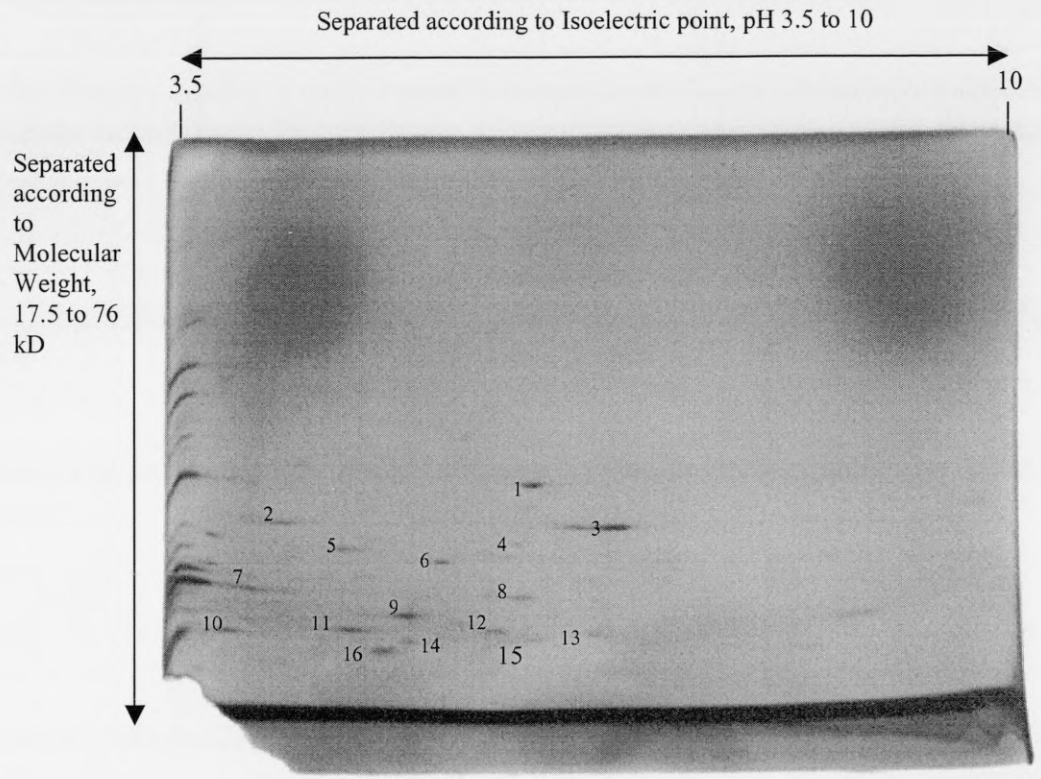


Figure 40: Two-dimensional gel electrophoresis for outer membrane protein (OMP) profiles of *Pseudomonas aeruginosa* PAO1 eluate resistant cells exposed to BIT and cultured in CDM. The OMPs are separated by the isoelectric point (reading left to right) and molecular weight (top to bottom). Protein concentration: 2.01 x 10^{-3} mg/gel.

Table 25 (Part 1): Measurement data indicating Protein number, Molecular weight, Retardation factor and Isoelectric point of the proteins identified from the Two-Dimensional gel analysis of *Ps. aeruginosa* PAO1 sensitive eluate cells and compared against eluate resistant cells (Passage 7: BIT) (protein no.: protein number; M.Wt.: molecular weight; Rf: retardation factor; pI: isoelectric point; Co.: corresponding protein to **Figure 39**; N: novel protein).

Figure 39 (sensitive eluate cells)					**Figure 40** (resistant eluate cells)				
Protein no.	MWt (kD)	Rf	pI (pH)	Co.	Protein no.	MWt (kD)	Rf	pI (pH)	Co.
1	23.5	0.75	4.40	-	1	34.9	0.61	6.20	N
2	22.6	0.77	5.40	-	2	29.2	0.67	4.10	N
3	19.5	0.81	3.95	-	3	28.5	0.68	6.85	N
4	18.8	0.82	4.80	-	4	26.2	0.71	6.10	N
5	15.9	0.87	5.90	-	5	25.7	0.72	4.65	N
					6	24.2	0.74	5.40	2
					7	21.8	0.78	3.85	3
					8	20.5	0.80	6.05	N
					9	17.5	0.84	5.15	N
					10	16.3	0.86	3.75	N
					11	16.3	0.86	4.70	4
					12	15.9	0.87	5.82	5
					13	15.9	0.87	6.65	N

Table 25 (Part 2): Measurement data indicating Protein number, Molecular weight, Retardation factor and Isoelectric point of the proteins identified from the Two-Dimensional gel analysis of *Ps. aeruginosa* PAO1 sensitive eluate cells and compared against eluate resistant cells (Passage 7: BIT) (protein no.: protein number; M.Wt.: molecular weight; Rf: retardation factor; pI: isoelectric point; Co.: corresponding protein to **Figure 39**; N: novel protein).

Figure 39 (sensitive eluate cells)					**Figure 40** (resistant eluate cells)				
Protein no.	MWt (kD)	Rf	pI (pH)	Co.	Protein no.	MWt (kD)	Rf	pI (pH)	Co.
					14	15.5	0.88	5.20	N
					15	15.5	0.88	6.20	N
					16	14.5	0.90	4.95	N

Conclusions

There were significant variations between biofilm and eluate cells when passaged in the presence of biocide. However, the sub-minimum inhibitory concentrations of BIT, Cetrimide (biofilm cells), ZnPT, NaPT and (eluate cells) appear to inhibit the presence of outer membrane proteins in the range of 36.5 and 41 kD, which was suggested earlier in this Chapter to be the T-OMP. Once, the presence of biocides is removed, this protein reappeared. This suggests that this is a phenotypic adaptation brought about as a direct result of the presence of biocide. A phenotypic adaptation is an alteration, which demonstrates a change in response to environmental stimuli, but does not necessarily require a genome alteration. In the 2-D gel analysis, it was not possible to identify the T-OMP in biofilm sensitive cells. However, the 2-D gels illustrate the induction of new proteins in biofilm and eluate resistant cells, and alterations in protein movement within the cells. Two proteins, 30.4 kD (biofilm cells) and 23.5 kD (eluate cells) appeared in resistant cells, but it was not possible to demonstrate their presence in sensitive cells (biofilm and eluate, respectively). These OMPs could be the same proteins that appeared upon the exposure of biofilm (30 to 30.7 kD) and eluate (23.5 kD) cells to BIT, in SDS-PAGE gels. It is also evident that other proteins may be lost from the cells following the induction of resistance. The overall conclusion is that there are a combination of mechanisms, which may be occurring within the cells and hence, contributing to the observed resistance.

Chapter Six:

Induction of Tolerance towards Benzisothiazolone (BIT) in Binary Biofilms (*Pseudomonas aeruginosa* PAO1 and *Escherichia coli*)

Introduction

Many studies have focused on adhesion and biofilm formation by planktonic monocultures (e.g. *Ps. aeruginosa*; Costerton *et al.*, 1995). However, under natural conditions, true monospecies biofilms are comparatively rare and in most natural and industrial environments, biofilms are complex communities (Skillman *et al.*, 1999). Although prominent in nature, much less information is known about mixed population biofilms. Bacteria do not have uniform colonization and physiological properties (Fletcher, 1991), a feature that enables them to utilize different ecological niches. Therefore, one would predict that increasing species diversity of planktonic bacterial communities would lead to increased species diversity and overall cell density within biofilms (Whiteley *et al.*, 2001a). The resultant biofilms may be thicker and more stable than monospecies biofilms and this could further influence their susceptibly to antimicrobials (Bourion & Cerf, 1996). A two species system is simple enough to allow quantitative analysis of interactions and *in situ* speciation (Banks & Bryers, 1991; Siebel & Characklis, 1991; Stewart *et al.*, 1997).

Escherichia coli is a Gram-negative bacterium that exhibits great tolerance towards many antimicrobial agents. Vaara (1993) reported that antibiotics of natural origin showed that >90% lacked activity against *E. coli*. If *E. coli* cells are exposed to antibiotics, (such as chloramphenicol or tetracycline) at concentrations slightly greater than their minimum inhibitory concentration (MIC), resistant derivatives are observed, which not only occur at high frequencies but which also give cross resistance to other antibiotics. This is the so-called multiple antibiotic resistance (*mar*) phenotype (George & Levy, 1983a & b). In addition, physicochemical reactions between biocide and non-living components of the biofilm can also result in an apparent cellular resistance (Morton *et al.*, 1998). Chapman *et al.* (1993) found

that *E. coli* cells unable to produce glutathione were more sensitive to electrophilic biocides such as formaldehyde and 2-bromo-2-nitropropane-1,3-diol, whereas there was little difference in response to surface active biocides, such as benzalkonium chloride.

When *E. coli* encounters environmental stress conditions, such as nutrient limitation and/or the presence of antimicrobial agents, growth rate slows down and there is a marked alteration in gene expression. This change in gene expression is essential for the long-term survival of bacteria and is, in part, mediated by alternative sigma factors (Greenway & England, 1999b). One such sigma factor, that appears to be a key transcriptional factor directing gene expression during stress conditions and slow growth, is the stationary phase σ^s factor (Lange & Hengge-Aronis, 1994).

Microorganisms such as *E. coli* may live a lifestyle described as "*feast or famine*" (Schlessinger & Schaechter, 1989) and thus, the bacterium has evolved so that it is able to survive both extremes, which may follow each other in rapid succession. One well-characterized way in which *E. coli* responds to nutrient deprivation (and other stress conditions), is by producing the "stringent response" (Stent & Brenner, 1961; Alfoldi *et al.*, 1962). Work by Sarubbi *et al.* (1988) has shown that in *E. coli*, growth rate is an inverse linear function of the intracellular ppGpp concentration, which led to the suggestion that ppGpp has a significant role in growth rate control and gene expression during slow growth in bacteria. Rodionov *et al.* (1995) attributed penicillin tolerance of *E. coli* to the induction of the stringent response and an increase in ppGpp concentration. Tuomanen (1986) reported that the *relA* gene product appeared to be involved with the induction of phenotypic tolerance to β-lactam antibiotics in *E. coli*.

From the previous demonstration, one can observe a possible link between the induction of ppGpp and the activation of σ^s. Gentry *et al.* (1993) have previously shown that ppGpp activates σ^s synthesis by positively regulating *rpoS* expression. Greenway and England (1999b) proposed a regulatory cascade, whereby the presence of an antimicrobial agent leads to an increased level of ppGpp, which in turn causes an increased expression of *rpoS*, culminating in production of σ^s. This then leads to altered gene expression of intrinsic resistance determinants.

Another link between *rpoS* and biofilms was recently identified (Adams & McLean, 1999). Adams and McLean (1999) studied *E. coli* biofilm formation in chemostats and compared strains with and without the *rpoS* gene. *E. coli* cells that lack *rpoS* are unable to form normal biofilms, whereas planktonic cells are apparently unaffected by the absence of this σ factor. This result strongly suggested that conditions that elicit a slowing of bacterial growth, such as nutrient limitation or build up of toxic metabolites, are conducive to the formation of biofilms, at least for this microorganism (*E. coli*) (Donlan, 2000). A slowdown in growth rate would not be deleterious for cell metabolism and would actually benefit the bacterium by decreasing antimicrobial uptake. Evans *et al.* (1991) examined growth-rate-related effects under controlled growth conditions for planktonic cultures and biofilms of *Ps. aeruginosa* and *E. coli*. The general observation was that the sensitivities of both the planktonic and biofilm cells towards ciprofloxacin increased with increasing growth rate, thus supporting the suggestion that the slow growth rate of biofilm cells protects the cells from antimicrobial action.

The up-regulation of the *mar* operon results in a multidrug-resistant phenotype in *E. coli* planktonic cultures. However, Maira-Litrán *et al.* (2000a) did not support the idea that the

mar operon is up-regulated in biofilms, as the level of *mar* was lower in biofilms compared with the level seen in equivalent stationary-phase cultures grown in batch. Furthermore, this same group made use of *mar*- and *acrAB*-deleted strains to determine if the resistance of *E. coli* to ciprofloxacin was affected by the loss of these loci (*AcrAB* is the efflux pump, which is thought to be responsible for the multidrug resistant phenotype in *E. coli* planktonic cells), (Maira-Litrán *et al.*, 2000b). Loss of *mar* and *acrAB* did not adversely affect the *E. coli* biofilms, but constitutive expression of *acrAB* did provide a certain level of protection against ciprofloxacin.

The primary aim of this study was to induce resistance in *E. coli* biofilms against BIT. This was followed by the determination of the effect of species interactions on biofilm formation and the investigation of the susceptibility of component species towards BIT in binary biofilms (*Ps. aeruginosa* and *E. coli*). The reason for choosing BIT as a selected biocide, to induce resistance in *E.* coli and in binary biofilms, is that this was the only biocide to exhibit differences in MICs between sessile and eluate cells (**Figure 22**, Chapter 4).

Experimental Approach

Test Cultures

Sterile CDM, 25 mL in a 100 mL Erlenmeyer flask, was inoculated with a pure culture of *E. coli* ATCC 10000 from a streak plate. The flask was incubated overnight at 37°C, 180 osc min^{-1} in an orbital incubator (Gallenkamp INA-305). A fresh aliquot of CDM was inoculated with 250 µL of an overnight culture of *E. coli*. This was performed in triplicate. The cultures were incubated at 37°C, 180 osc min^{-1} in an orbital incubator (Gallenkamp INA-305).

Assay for Growth

At hourly intervals an aliquot (1 mL) of culture was removed aseptically from each flask and the optical density was measured at 470 nm using a spectrophotometer (Novaspec II LKB). The sample was aseptically replaced in the respective flask. 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 OD at 470nm of this dilution was observed and the volume multiplied by 10 to give the actual optical density.

This experiment was performed for *Ps. aeruginosa* and *E. coli*, simultaneously in separate flasks, to obtain a specific CDM, which is suitable for the optimal and non-advantageous growth of both microorganisms. In order to achieve this, CDM was modified by increasing the concentration of nitrogen (the concentration of nitrogen (NH_4Cl) in solution A (CDM) was 0.0187 M; in MCDM the concentration was modified to 0.0280 M) and decreasing the concentration of carbon (solution C: the concentration was decreased from 1 M to 0.66 M). These modifications in the concentrations of carbon and nitrogen will make the doubling times for both bacteria closer to each other in this modified CDM.

Results & Discussion

Figure 41 shows the growth curves in modified CDM for *Ps. aeruginosa* and *E. coli*. For *Ps. aeruginosa*, the lag phase in this medium was short, lasting one hour only. The exponential phase lasted between 2 and 12 hours after inoculation. Mid-exponential phase was at 7 hours after inoculation. The doubling time in this CDM was 288 minutes (4.8 hours), which is less than the doubling time for this microorganism in non-modified CDM (8 hours;

Figure 11, Chapter 3).

The growth of *E. coli* in this modified medium was considerably slower than that for *Ps. aeruginosa* in the same medium. The cells in this medium continued in the lag phase up to 5 hours. Although *E. coli* cells needed more time to prepare for their proliferation, the exponential phase was quicker (than that for *Ps. aeruginosa*) lasted from the sixth hour to the twelfth hour. Therefore, mid-exponential phase was not observed until the ninth hour after inoculation. The generation time of *E. coli* in modified CDM was calculated to be 162 minutes (2.7 hours).

Comparing the growth curves of both bacteria, there were slight variations in the duration of lag and exponential phases. However, as mentioned earlier, the aim from modifying CDM was to make the generation times for both microorganisms closer to one another. In non-modified CDM (Dinning, 1995), the doubling time for *E. coli* was calculated to be 228 minutes (3.8 hours), and that for *Ps. aeruginosa* was 480 minutes (8 hours; **Figure 11**, Chapter 3). In addition, the maximum OD_{470nm} (stationary phase) for the two microorganisms were nearly the same (0.44 for *Ps. aeruginosa* and 0.45 for *E. coli*) and this is important as it indicates the growth potential of the modified CDM (MCDM). By these modifications, MCDM was developed, which supports the impartial growth of both *Ps. aeruginosa* and *E. coli* binary biofilm cultures.

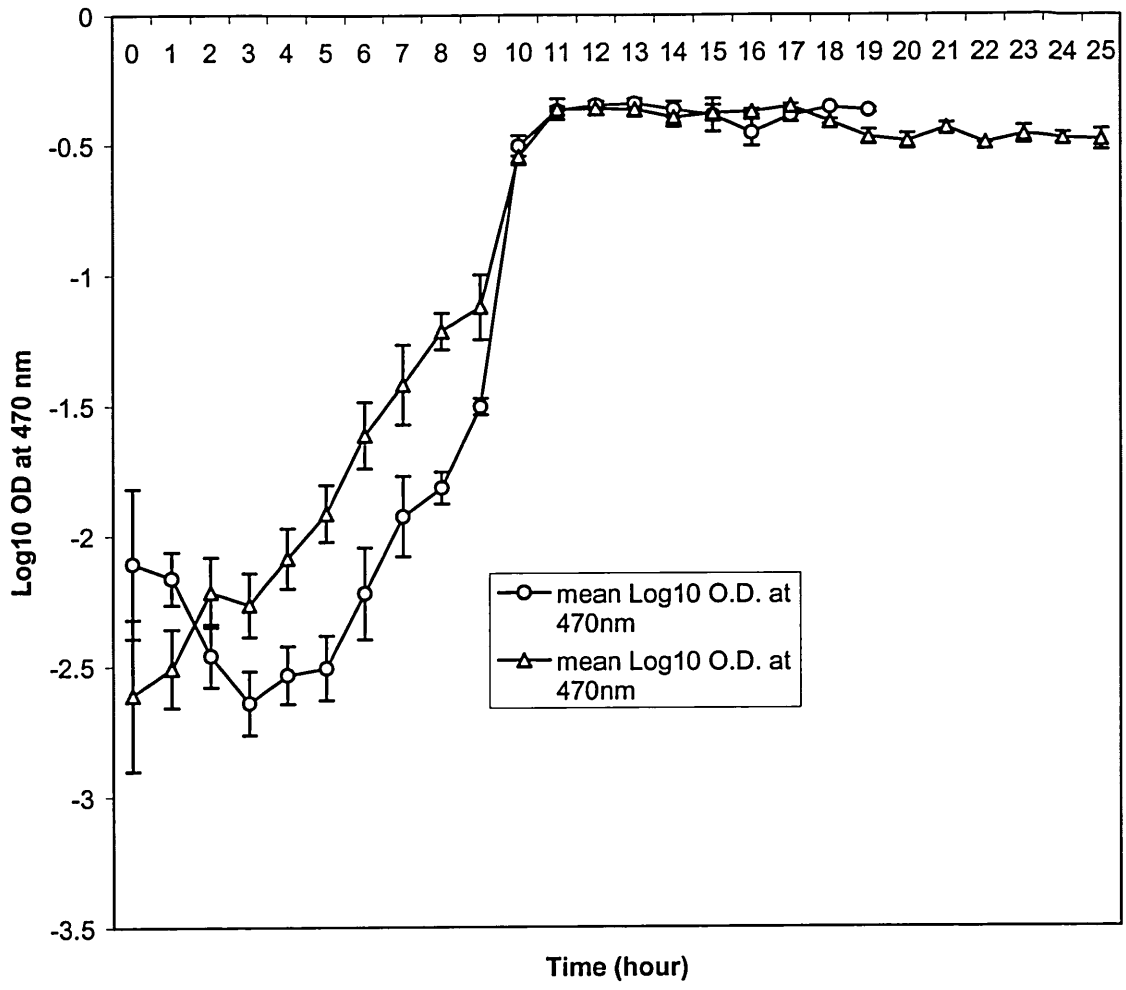


Figure 41: The Observation of Growth of *Pseudomonas aeruginosa* PAO1 and *Escherichia coli* ATCC 10000 in separate modified CDM (MCDM). Error bars are calculated as the standard deviation of the individual data points. n = 3 replicates. (o) $\log_{10}$ OD at 470 nm for *E. coli*; (Δ) $\log_{10}$ OD at 470 nm for *Ps. aeruginosa*.

The Control Experiment for *E. coli* Biofilm and MIC Determination

Establishing *E. coli* biofilm on a Sorbarod filter was similar to that for *Ps. aeruginosa* PAO1 biofilm (Hodgson *et al.*, 1995; Chapters 1, 3 & 4). The Sorbarod filter was pre-wetted with 5 mL of 0.9% (w/v) sterile normal saline then inoculated with mid-logarithmic phase culture (10 mL) from a syringe dropwise onto the Sorbarod. The rubber plunger seal was withdrawn from a sterile, disposable 2 mL syringe. The syringe was introduced into PVC tubing containing the Sorbarod and a sterile, disposable needle (0.8 x 40 mm) inserted through the rubber seal. Media inlet tubing was attached *via* the needle and sterile modified CDM was delivered into the unit. Triplicates of these units were established and placed within a 37°C incubator. Numbers of cells eluted from the Sorbarod filters were estimated with time by performing viable counts until achieving the steady-state value. At 56 hours, after reaching steady-state, Sorbarod filters were removed and stored at -18°C for later analysis. Since steady-state had been achieved in both the biofilm and the eluate populations, growth rate can be calculated from a knowledge of the elution-rate. The logarithm of the viable counts was calculated and plotted against time to establish the growth curve of *E. coli* biofilm (**Figure 42**).

The previous experiment was repeated for MIC determination. After achieving the steady-state (48 hours), the Sorbarod filters were sacrificed (the adherent cells were removed and resuspended in 0.9% (w/v) sterile normal saline). The MIC against BIT was determined according to the tube dilution method (Bloomfield, 1991; Chapter 3) using a standard inoculum (100 μL in 10 mL; inoculum size: 10^9 CFU mL^{-1}). This was performed for both biofilm and eluate cells, **Table 26**.

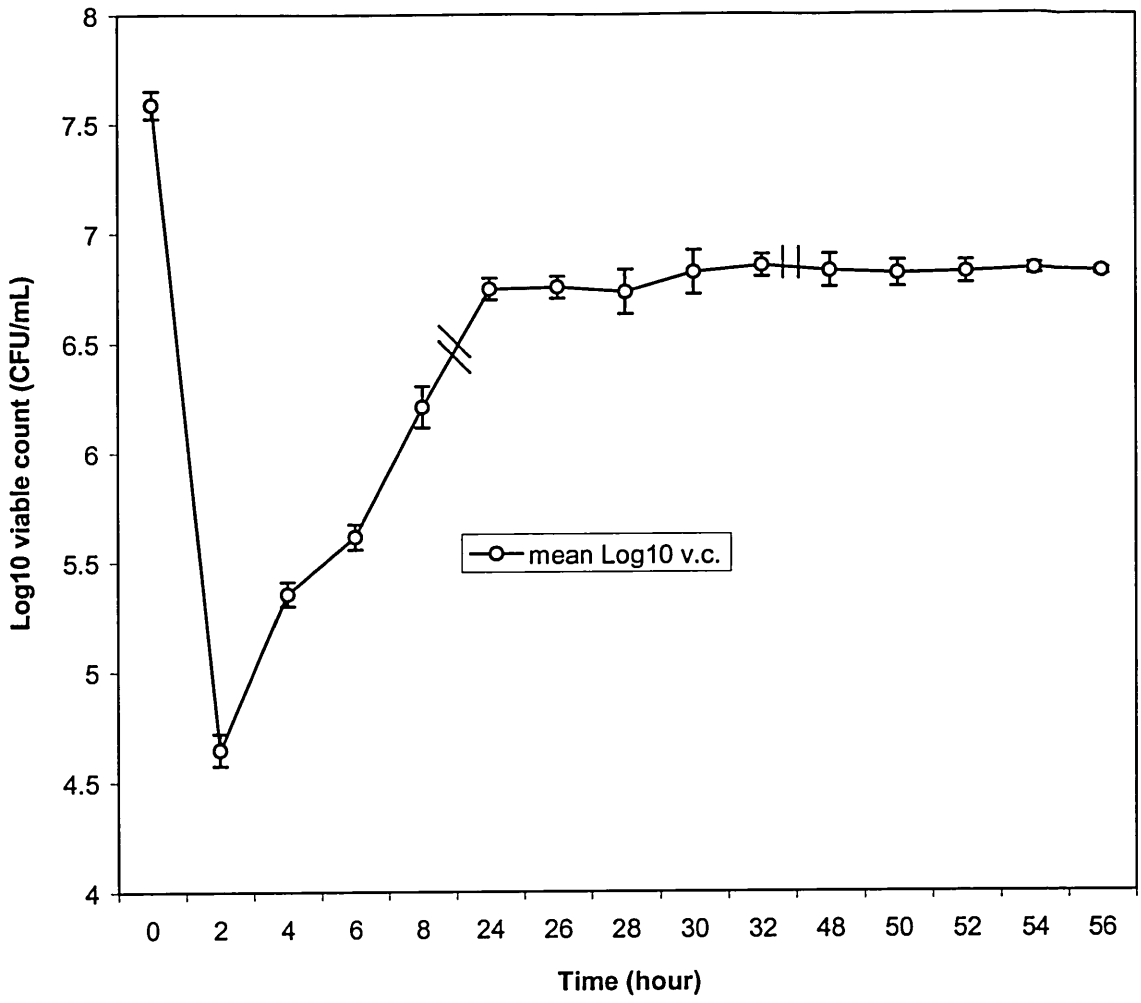


Figure 42: Graph of Mean *Escherichia coli* ATCC 10000 Biofilm Eluate Counts over Time. Error bars are calculated as the standard deviation of the individual data points. n = 3 replicates. (o) $\log_{10}$ viable count (CFU mL^{-1}).

Table 26: MIC values for *E. coli* against BIT in modified CDM. n = 3 replicates.

Type of Cells	Screening MIC ($\mu g\ mL^{-1}$)		Exact MIC ($\mu g\ mL^{-1}$)	
	19h	**48h**	**19h**	**48h**
Biofilm	8	8	5	5
Eluate	4	4	4	4

The Control Experiment for Binary Biofilm and MIC Determination

In order to establish a binary biofilm, the Sorbarod model was used (Hodgson *et al.*, 1995; Chapter 1, 3 & 4). The Sorbarod filter was inoculated with mid-logarithmic phase of *E. coli* culture (10 mL). This was perfused with MCDM at 37°C. The *E. coli* biofilm was run for 48 hours (steady-state) before adding an aliquot (10 mL) of mid-logarithmic phase of *Ps. aeruginosa* to the Sorbarod filter. This was performed in order to give *E. coli* a *'head start'* in establishing itself on the Sorbarod filter and building the first monolayer of colonies before adding *Ps. aeruginosa* to the community. This system was run for 126 hours, in order to achieve the steady-state for both microorganisms. This was performed in triplicate. The eluate culture was collected at two-hour intervals and viable counts were performed for each eluate sample. The colonies were counted for each plate and the logarithm of viable counts was calculated and plotted against time to construct the growth curve of the binary biofilm (**Figure 43**).

Viable cell number

An aliquot (100 μL) of eluate culture was aseptically removed and used for the preparation of serial dilutions in the range of 10^{-1} to 10^{-10}. Aliquots (100 μL) were spread plated onto sterile Chromogenic *E. coli*/Coliform (Oxoid LTD., Basingstoke, Hampshire, England) agar plates in triplicates. The plates were incubated at 37°C for 48 hours and the subsequent colonies were counted (Chapter 2). Using the Chromogenic *E. coli*/Coliform medium was essential in this experiment to differentiate between both types of bacteria. *Ps. aeruginosa* gives straw colonies while *E. coli* gives purple colonies (**Figure 7**, Chapter 2).

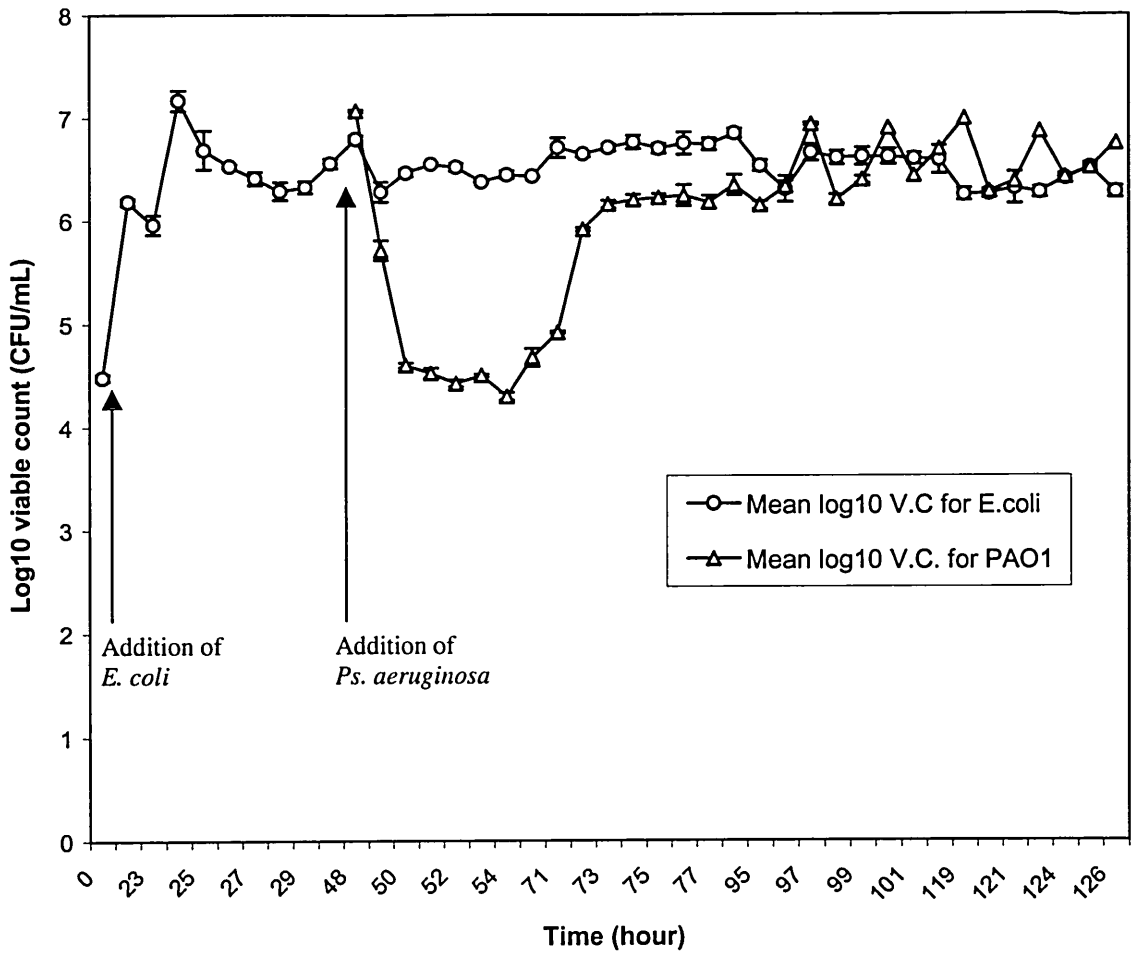


Figure 43: Graph of Mean Binary Biofilm Eluate Counts for *Pseudomonas aeruginosa* PAO1 and *Escherichia coli* ATCC 10000 over Time. Error bars are calculated as the standard deviation of the individual data points. n = 3 replicates. (o) $\log_{10}$ viable count (CFU mL^{-1}) for *E. coli*; (Δ) $\log_{10}$ viable count (CFU mL^{-1}) for *Ps. aeruginosa*.

The first trial to run this experiment was performed by adding both bacteria (*Ps. aeruginosa* and *E. coli*) on the Sorbarod filter, simultaneously. The resultant binary biofilm was characterized by the dominance of *Ps. aeruginosa*. This may be partially explained by the extensive piliation of *Ps. aeruginosa*. Prat and Kolter (1998) revealed that the presence of type I pili is essential for the initial attachment of *E. coli* on abiotic surfaces, but no role in facilitating the development of mature biofilm had been documented. Unlike type I pili in *E. coli*, *Ps. aeruginosa* type IV pili are not essential for the initial attachment to abiotic surface (O'Toole & Kolter, 1998). However, it was suggested that type IV pili and type IV pili-mediated twitching motility in *Ps. aeruginosa* play a role in biofilm development. O'Toole and Kolter (1998) proposed that type IV pili have a direct role in stabilizing interactions with the abiotic surface and/or in the cell-to-cell interactions required to form a microcolony. Type IV pili-mediated twitching motility may also be necessary for cells to migrate along the surface to form the multicell aggregates characteristic of the wild-type strain. Therefore, both bacteria have specific type of pili. However, the role that these surface structures play is different in each bacterium and under various environmental conditions.

Results & Discussion

The relative concentrations of *Ps. aeruginosa* and *E. coli* in binary biofilms (as determined by observation of eluate cell numbers) (**Figure 43**) could not be predicted from their relative abundance in monospecies communities (**Figure 12** (Chapter 3) & **42**). The overall effect of growth conditions (monoculture and binary) on population size was shown to be significant, indicating interactions between *Ps. aeruginosa* and *E. coli* in binary culture. If no interactions were occurring between these two species, binary biofilm numbers would

equal the sum of *Ps. aeruginosa* and *E. coli* monoculture biofilms. Differences in Log_{10} counts between binary biofilms (6.34 for *Ps. aeruginosa* and 6.84 for *E. coli*) and those for monospecies biofilms (*Ps. aeruginosa*: 7.18 and *E. coli*: 6.80) indicate that binary biofilms are not simply a result of the addition of monoculture values. Therefore, *E. coli* has a negative effect on *Ps. aeruginosa* populations. The numbers of *Ps. aeruginosa* decreased when grown in binary cultures, whereas the numbers of *E. coli* in the same culture did not alter significantly. This observation once again reinforces the concept that planktonic population composition may not accurately predict the biofilm population (James *et al.*, 1995; Whiteley *et al.*, 2001a).

MIC Determination

The binary control experiment was repeated. At 126 hours, the Sorbarod filters were sacrificed and the MIC against BIT was determined for both the attached and eluate cells as a mixed population (**Table 27**). MIC determination was according to the tube dilution method (Bloomfield, 1991; Chapter 4).

Table 27: MIC values for binary biofilm mixed populations against BIT in modified CDM. n = 3 replicates.

Type of cells	Screening MIC ($\mu g\ mL^{-1}$)		Exact MIC ($\mu g\ mL^{-1}$)	
	19h	48h	19h	48h
Biofilm	8	8	5	5
Eluate	8	8	5	5

Using the tube dilution method to determine the MIC values for both binary biofilms and monospecies *E. coli* biofilms illustrates the similarities between the two sets of results (**Table 26 & 27**). For *E. coli* monospecies biofilms, the MIC was 5 $\mu g\ mL^{-1}$ (biofilm cells) and 4 $\mu g\ mL^{-1}$ (eluate cells). For the binary cells, the MIC was 5 $\mu g\ mL^{-1}$ for both biofilm and eluate cells. Therefore, this method did not differentiate between the MIC for *E. coli* and that for *Ps. aeruginosa* and thus, the need for another adequate method for MIC determination for both bacteria was necessary (e.g. the gradient plate method, using Chromogenic *E. coli* /Coliform medium).

The Induction of Resistance

The induction of resistance in *E. coli*/binary biofilms to benzisothiazolone was performed according to the method previously described by Brözel and Cloete (1994) (Chapters 1 & 4). This study was similar to that performed for *Ps. aeruginosa* biofilm (Chapter 4). The Sorbarod model was established as described earlier in this chapter (*E. coli* and binary biofilm control experiment). This was perfused with modified CDM, which was prepared to contain a quarter concentration of biocide of the previously established MIC (MIC/4). The eluate culture was collected for the last hour (the 48 hour for *E. coli* biofilm and the 126 hour for binary biofilm) and the biofilm filter was sacrificed to remove the adherent cells (in 10 mL, 0.9% (w/v) sterile normal saline). Aliquots (100 µL) from the eluate and the biofilm culture were spread plated onto sterile Chromogenic *E. coli*/Coliform agar. This was performed in order to separate *Ps. aeruginosa* (straw) colonies from those for *E. coli* (purple). For MIC determination, a single colony from each bacterium was inoculated onto the surface of sterile Chromogenic *E. coli*/Coliform gradient plate (this was performed in triplicates). The plates were incubated at 37°C for 48 hours (Chapter 2). The point at which growth ceased on the surface of the agar was used to indicate the approximate MIC (Passage 1). The colonies growing in this region (the region with the greatest biocidal concentration) are the most tolerant and therefore, were used to inoculate the next passage. This process was repeated until 4 successive passages had been performed in the presence of increasing MIC/4 of BIT. This was the point at which the cells were deemed to be sufficiently resistant (Passage 5) for our experimental purposes. Although the MICs may continue to increase with further exposure to biocides, at this point an aliquot (10 mL) of the final cultures in the presence of biocide was inoculated into new Sorbarod filter, which was perfused with a fresh modified

CDM in the absence of biocide. The MIC was redetermined by the gradient plate method (Passage 6). This was repeated until two successive passages had been performed in the absence of BIT.

Passaging of *E. coli* biofilms

The initial pre-exposure MIC value against BIT when grown in modified CDM was 5 $\mu g\ mL^{-1}$ and 4 $\mu g\ mL^{-1}$ (biofilm and eluate cells, respectively; Passage 0). There was a marked difference between biofilm and eluate MICs during the whole passaging process. Within 48 hours of exposure to the biocide, the MIC had decreased to a value of 1.88 $\mu g\ mL^{-1}$ for biofilm cells and 0.98 $\mu g\ mL^{-1}$ for eluate cells, Passage 1 (**Figure 44**). In the following passages the MIC started to increase in a step-wise fashion until Passage 4, where the MIC values were 8.85 $\mu g\ mL^{-1}$ and 3.5 $\mu g\ mL^{-1}$ (biofilm and eluate cells, respectively). Passage 5 demonstrated a marked increase in the MIC value for both biofilm (47.79 $\mu g\ mL^{-1}$) and eluate cells (40.85 $\mu g\ mL^{-1}$). This is an increase of 9.6-fold for the adherent cells and 10.2-fold for the eluate cells over the initial, pre-exposure value. At Passage 6, which is the first passage without the addition of biocide, the MIC decreased immediately to 26.97 $\mu g\ mL^{-1}$ for the adherent cells and 16.20 $\mu g\ mL^{-1}$ for the eluate cells. Within the two successive passages in biocide-free medium, the MIC showed a marked decrease and at Passage 8, the MIC was 4.96 $\mu g\ mL^{-1}$ (biofilm cells) and 3.95 $\mu g\ mL^{-1}$ (eluate cells). These results are summarized in **Table 28**.

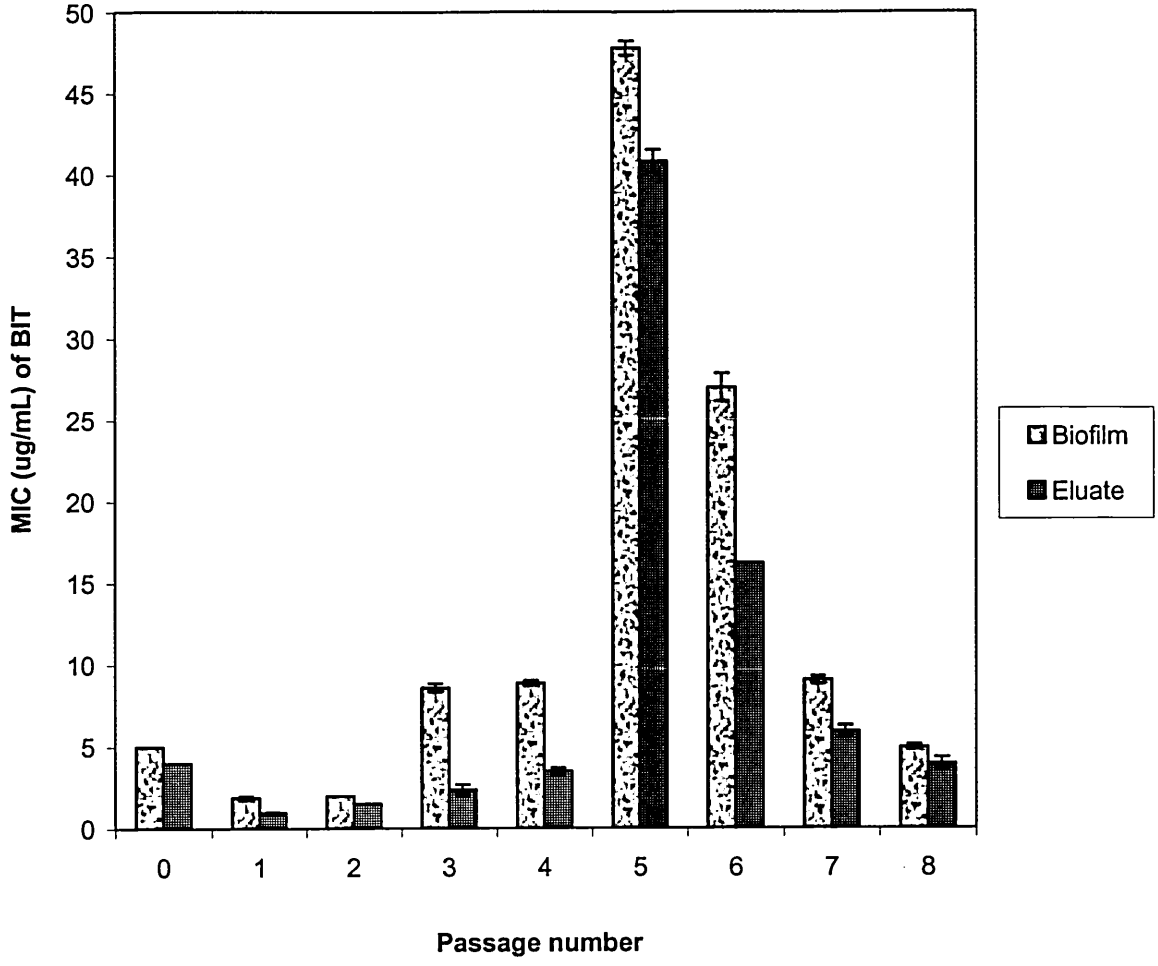


Figure 44: Increase in MIC for BIT against *Escherichia coli* ATCC 10000 in MCDM as a function of passage number. Error bars are calculated as the standard deviation of the individual data points. n = 3 replicates. Passages 6, 7 and 8 were performed without the addition of biocide.

Table 28: Summary of the average MICs for *E. coli* biofilms in modified CDM when exposed to BIT. The figures in the brackets indicate the fold-increase in MICs for Passage 5 and the fold-decrease in MICs for Passage 8. n = 3 replicates.

Type of cells	**MIC (μg mL^{-1}) of passage number (n-fold increase in brackets)**		
	0	**5**	**8**
Biofilm	5	47.79	4.96
	(-)	(9.6)	(-)
Eluate	4	40.85	3.95
	(-)	(10.2)	(-)

The previous results demonstrated a complete reversion to the original pre-exposure MIC (Passage 0, **Figure 44**) for both type of cells. This suggests that the induced tolerance is unstable and the developing tolerance was the result of a phenotypic adaptation rather than a mutation.

Passaging of Binary Biofilms

By using the gradient plate method to calculate the MIC value, it was possible to differentiate between the MIC for *Ps. aeruginosa* and that for *E. coli.* Starting with *Ps. aeruginosa*, there was a considerable difference between the biofilm and the eluate MIC values (as was the case in *Ps. aeruginosa* monospecies biofilm when exposed to BIT; **Figure 22**, Chapter 4). The original MIC was 5 μg mL^{-1} for both adherent and eluate cells. For

Passage 1, there was a marked increase in the MIC value and that was 37.76 μg mL^{-1} (biofilm cells) and 26.88 μg mL^{-1} (eluate cells). This is an increase of 7.6-fold for the biofilm cells and 5.4-fold for the eluate cells (**Figure 45**). After Passage 1, the MICs continued to increase gradually with every successive passage in the presence of biocide. At Passage 5, the MIC had increased to 66.11 μg mL^{-1} for the adherent cells and 62.16 μg mL^{-1} for the eluate cells, an increase of 13.2-fold for the sessile cells and 12.4-fold for the eluate cells from their initial, pre-exposure, MIC values. However, upon removing the cells from the presence of biocide the MIC began to decrease. At Passage 8, the MIC had decreased to 41.60 μg mL^{-1} (biofilm cells) and 38.93 μg mL^{-1} (eluate cells).

In comparing these results with the ones obtained from the passaging process of *Ps. aeruginosa* monospecies biofilms against BIT (**Figure 22**, Chapter 4), the MIC value for the first passage was 2 μg mL^{-1} (for both types of cells), whereas the initial MIC in binary biofilms was 37.76 μg mL^{-1} and 26.88 μg mL^{-1} (biofilm and eluate cells, respectively). This indicates that BIT demonstrates a greater activity towards *Ps. aeruginosa* as a monospecies biofilm than that as a binary biofilm. For *Ps. aeruginosa* monospecies biofilm, the MIC values increased in a step-wise fashion, and at Passage 10, the MIC had increased to 34 μg mL^{-1} for the adherent cells and 20 μg mL^{-1} for the eluate cells. This is an increase from the initial MIC of 17-fold and 10-fold for the biofilm and the eluate cells, respectively. However, in binary biofilms, there was a 13-fold (biofilm cells) and 12-fold (eluate cells) increase in the MIC value, from the initial, pre-exposure value after only 5 successive passages in the presence of biocide. Therefore, the induction of resistance in *Ps. aeruginosa* binary biofilms was faster than that in monospecies *Ps. aeruginosa* biofilms.

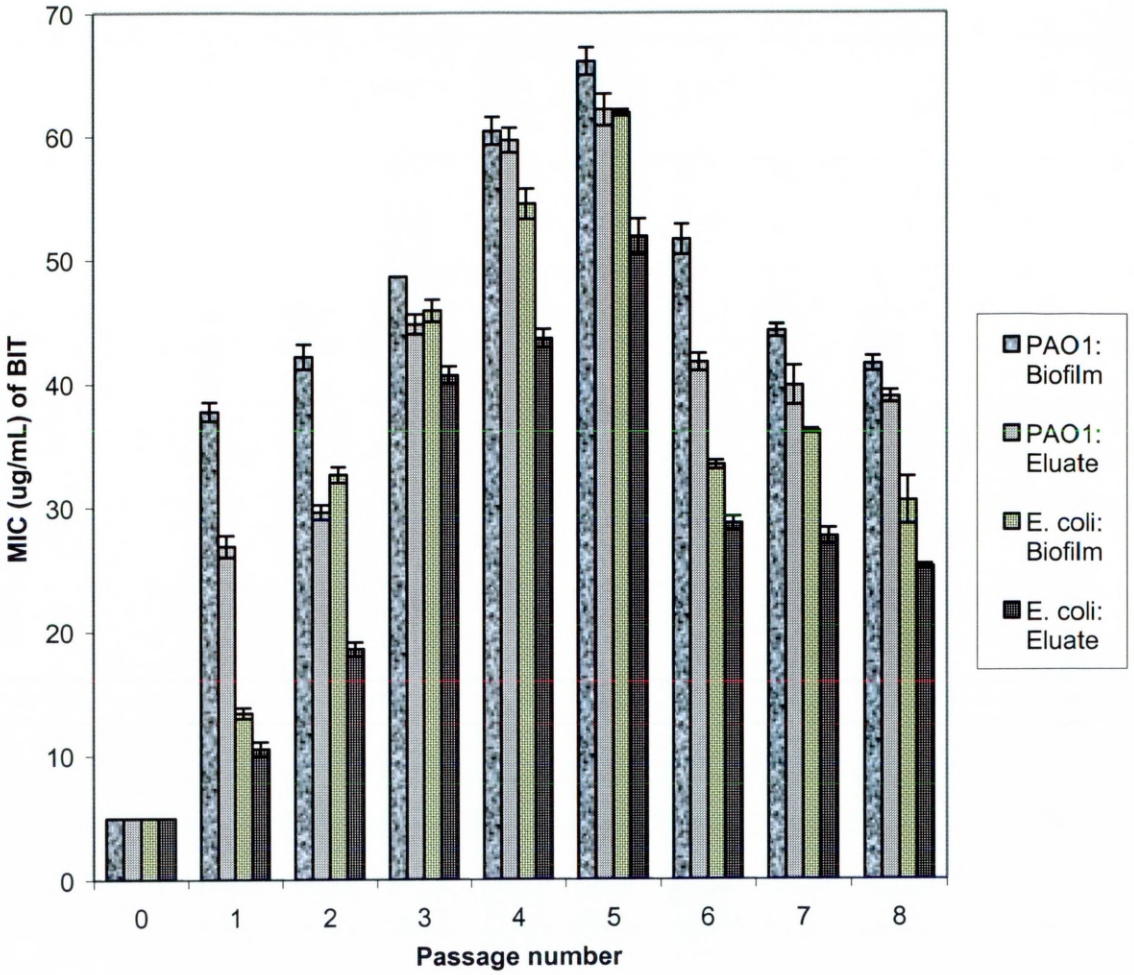


Figure 45: Increase in MIC for BIT against binary biofilms (*Pseudomonas aeruginosa* PAO1 and *Escherichia coli* ATCC 10000) in MCDM as a function of passage number. Error bars are calculated as the standard deviation of the individual data points. n = 3 replicates. Passages 6, 7 and 8 were performed without the addition of biocide.

For *E. coli*, the initial, pre-exposure, value was 5 μg mL^{-1} (biofilm and eluate cells). There was a marked difference in MIC values between adherent and eluate cells in each passage. At Passage 1, the MIC increased to 13.44 μg mL^{-1} (biofilm cells) and 10.56 μg mL^{-1} (eluate cells). In the following passage, there was a considerable increase in MIC values: 32.64 μg mL^{-1} for the sessile cells and 18.56 μg mL^{-1} for the eluate cells. For each successive passage, with the presence of increasing concentration of biocide, the MIC continued to increase in a step-wise fashion. At Passage 5, the MIC increased to 61.91 μg mL^{-1} (biofilm cells) and 52.29 μg mL^{-1} (eluate cells). This is an increase of 12.4-fold and 10.5-fold adherent and eluate cells, respectively. The cells were then passaged in biocide-free medium and within the first 126 hours a significant decrease in MIC was observed and by Passage 8, the MIC had fallen to 30.54 μg mL^{-1} and 25.20 μg mL^{-1} (biofilm and eluate cells, respectively).

For *E. coli* monospecies biofilms, the MICs for the first passage were 1.88 μg mL^{-1} and 0.98 μg mL^{-1} (biofilm and eluate cells, respectively) (**Figure 44**), which were significantly lower than those for *E. coli* binary biofilm at the same passage (13.44 μg mL^{-1} (biofilm cells) and 10.56 μg mL^{-1} (eluate cells)). At Passage 5, the MICs were 47.79 μg mL^{-1} (biofilm cells) and 40.85 μg mL^{-1} (eluate cells). These were less than the ones obtained from the fifth passage in binary biofilms. This suggests that being part of a binary biofilm will provide a significant protection against BIT. Therefore, the species composition of binary biofilms is important in determining the survival of component species to biocide.

At Passage 8, the MIC for both bacteria (in binary biofilms) did not revert to the original, pre-exposure, value. It is possible that further passaging in biocide-free media could result in a complete reversion to the original, pre-exposure value (Passage 0). However, this was not done in these experiments due to both time constraints and that the mechanism and

nature of reversion of resistance was not a primary aim in these experiments. However, the observation of a gradual loss in resistance suggests that the induced resistance is unstable. This also indicates that the induction of resistance in binary biofilms (as was the case in monospecies biofilms) is a result of a phenotypic adaptation rather than a mutation. This phenotypic adaptation may be due to alterations in the outer membrane profiles of monospecies and binary biofilms and eluate cells as a result of being passaged in the presence of biocide. This possibility will be further examined in Chapter seven.

Ellmans assay for the presence of Thiol groups

1,2-benzisothiazolin-3-one (BIT) chemically reacts with thiol containing protein/enzyme cellular components and is particularly active against metabolizing cells (Fuller *et al.*, 1985; Collier *et al.*, 1990a). Therefore, the presence of thiols in media quenches the activity of this biocide. As previously mentioned (Chapter 4), the Ellmans assay (Ellman, 1964) can be performed in order to estimate the concentration of thiols in medium and hence, determine the magnitude by which the biocide activity may be quenched. As a result of using Chromogenic *E. coli*/Coliform gradient plate method to determine the MIC for *E. coli* and binary biofilms, it was necessarily to apply Ellmans assay in order to estimate the thiol concentration in this medium. This medium was dissolved in distilled water and then filtered through a 0.22 μm pore size cellulose acetate filter (Merck Eurolab Ltd, Lutterworth). The agar was left on the surface of the filter and the suspension containing the nutrients was used for the Ellmans assay. This procedure resulted in the separation of the agar from the nutrients and was thought to be desirable, as the agar should have no available thiol groups for biocide interactions, but may have interfered with the Ellmans assay. This assay was performed as described in Chapter 4. The tubes were prepared as outline in **Table 29**. A standard curve was constructed (**Figure 23**, Chapter 4). The accessible thiol-groups (as glutathione equivalency) contained in the medium can be estimated from the graph. The absorbency of tube 12 was extrapolated on the graph, and the thiol concentration was equal 0.0028 g L^{-1}. Taking into account the dilution factor, this is equal 0.014 g L^{-1}, which is equivalent to 2.13 x 10^{-5} mmol L^{-1} of GSH. Therefore, the activity of BIT in the Chromogenic *E. coli*/Coliform medium was quenched by a factor of 3.2 μg mL^{-1}. Hence, this must be taken into account when estimating the MIC value for both *E. coli* and binary (biofilm and eluate) cells against benzisothiazolone.

Table 29: Preparation of tubes in the Ellmans assay. n = 3 replicates.

Tube number	Buffer (mL)	DTNB (mL)	GSH (0.05 g L^{-1}) (µL)	Water (mL)	*Medium (mL)	Mean OD (412nm)
1	1	1	1000	2.0	-	0.474
2	1	1	900	2.1	-	0.451
3	1	1	800	2.2	-	0.382
4	1	1	700	2.3	-	0.336
5	1	1	600	2.4	-	0.285
6	1	1	500	2.5	-	0.242
7	1	1	400	2.6	-	0.187
8	1	1	300	2.7	-	0.141
9	1	1	200	2.8	-	0.097
10	1	1	100	2.9	-	0.041
11	1	1	0	3.0	-	0
12	1	1	-	-	3.0	0.132
13	1	1	-	2.7	0.3	0.021
14	1	1	-	2.97	0.03	0.002
15	1	1	-	2.997	0.003	0

* medium = Chromogenic *E. coli*/Coliform medium; - = no addition; Tubes 1 to 11 = standard curve; Tubes 12 to15 = test samples; Tube 11 = used as a reagent blank.

Table 30: The average MICs (in Chromogenic *E. coli*/Coliform media) for *E. coli* biofilms when exposed to BIT, taking into account quenching by accessible thiol-groups.

Passage number	MIC ($\mu g\ mL^{-1}$)			
	Biofilm	**Biofilm - QF (Equivalent to 3.2 $\mu g\ mL^{-1}$)**	**Eluate**	**Eluate - QF (Equivalent to 3.2 $\mu g\ mL^{-1}$)**
1	1.88	-1.32	0.98	-2.22
2	2.0	-1.20	1.52	-1.68
3	8.57	5.37	2.37	-0.83
4	8.85	5.65	3.5	0.30
5	47.79	44.59	40.85	37.65
6	26.97	23.77	16.20	13.0
7	9.04	5.84	5.92	2.72
8	4.96	1.76	3.92	0.72

QF = Quenching factor.

Table 31: The average MICs (in Chromogenic *E. coli*/Coliform media) for both species in binary biofilms when exposed to BIT, taking into account quenching by accessible thiol groups (PAO1 = *Ps. aeruginosa* PAO1; QF = quenching factor).

Passage number	Bacteria	MIC ($\mu g\ mL^{-1}$)			
		Biofilm	**Biofilm - QF (Equivalent to 3.2 $\mu g\ mL^{-1}$)**	**Eluate**	**Eluate - QF (Equivalent to 3.2 $\mu g\ mL^{-1}$)**
1	PAO1	37.76	34.56	26.88	23.68
	E. coli	13.44	10.24	10.56	7.36
2	PAO1	42.18	38.98	29.6	26.4
	E. coli	32.64	29.44	18.56	15.36
3	PAO1	48.64	45.44	44.80	41.6
	E. coli	45.88	42.68	40.70	37.5
4	PAO1	60.43	57.23	59.69	56.49
	E. coli	54.51	51.31	43.66	40.46
5	PAO1	66.11	62.91	62.16	58.96
	E. coli	61.91	58.71	52.29	49.09
6	PAO1	51.63	48.43	41.73	38.53
	E. coli	33.44	30.24	28.70	25.5
7	PAO1	44.25	41.05	39.85	36.65
	E. coli	36.16	32.96	27.65	24.45
8	PAO1	41.60	38.4	38.93	35.73
	E. coli	30.54	27.34	25.20	22.0

Table 30 indicates that *E. coli* may be considered as a susceptible bacterium towards BIT. However, within the binary biofilm (**Table 31**), *E. coli* benefited from the presence of *Ps. aeruginosa*, a more BIT-resistant species, and there was a marked increase in MIC values (in each passage). The same observation was found by Whiteley *et al.* (2001a), who demonstrated that the resistance of *Pseudomonas putida* increased significantly against betadine in binary cultures (*Ps. putida* and *Vogesella indigofera*). Therefore, the resistant bacterial components of binary culture biofilms may protect the more susceptible bacteria.

Conclusions

The results above suggest that it is possible to establish nutrient controlled binary biofilms of *E. coli* and *Ps. aeruginosa* in the Sorbarod model and that these biofilms may be subsequently passaged towards biocide tolerance or resistance. A differential MIC was observed for BIT against both *E. coli* and *Ps. aeruginosa* biofilm and eluate cells. It was suggested that this difference was the result of exogenous thiol present in the chromogenic *E. coli*/Coliform medium gradient plates used to determine MIC. Investigation of the thiol concentration of this medium revealed that, whilst thiol was present, it was not available in sufficient concentrations to cause the overall difference observed.

Chapter Seven:

The Analysis of the Outer Membrane Protein Profiles of Monospecies Biofilms (*Escherichia coli* ATCC 10000) and Binary Biofilms (*Pseudomonas aeruginosa* PAO1 and *Escherichia coli*) following Exposure to Biocidal Agents

Introduction

The double-membrane system of Gram-negative bacteria forms an efficient barrier, which is instrumental in maintaining a stable milieu in the cytoplasm. Only a few low-molecular weight substances are able to cross the barrier unassisted from the exterior (Nikaido, 1992). On the other hand, the Gram-negative cell envelope also forms a formidable obstacle, which proteins destined for the cell exterior have to cross (Kjærgaard *et al.*, 2000).

As an interface between the environment and the interior of the cell, the bacterial cell envelope plays an important role in facilitating responses to change. The outer membranes of Gram-negative bacteria are in close contact with the environment and contain a number of major proteins present in high copy number. Some of these proteins are highly regulated in response to growth, nutrient, and environmental conditions (Overbeeke & Lugtenberg, 1980; Kaufmann *et al.*, 1994). An altered expression of outer membrane proteins (OMPs) implies a change in the outer most cell surface, which may have direct effects on adhesion. In fact, several OMPs have been suggested to be involved in adhesion of bacteria to various surfaces (Zhao *et al.*, 1996; Prigent-Combaret *et al.*, 1999). In *Escherichia coli*, OmpR has been shown to regulate the production of curli, which are involved in colonization of inert surfaces (Vidal *et al.*, 1998). The outer membrane protein, Ag43, facilitates both cell-surface and cell-cell contacts when cells are grown on minimal medium, but apparently plays no role when cells are grown on rich medium (O'Toole *et al.*, 2000; Danese *et al.*, 2000). Ag43 was reported to consist of two equivocal protein subunits, α and β, with apparent molecular masses of about 50 and 53 kD, respectively (Owen *et al.*, 1987; Caffrey & Owen, 1989). The α subunit is attached to the cell surface *via* interaction with the β subunit, which is an integral outer membrane component. Hasman *et al.* (1999) indicated that Ag43-Ag43 interaction is

responsible for the cell aggregation observed in Ag43-expressing *E. coli*. Further work by Kjærgaard *et al.* (2000) demonstrated that Ag43 is required for interspecies cell aggregation (between *E. coli* and *Ps. fluorescens*). This same group also investigated the presence of Ag43 in *Ps. fluorescens*; the results of their study suggested that Ag43 is indeed fully functional in this strain. Therefore, the expression of Ag43 is not functionally restricted to *E. coli*.

Although type 1 fimbriae in *E. coli* do not facilitate initial adhesion, they stabilize the contact of the cell with hydrophobic surfaces (Otto *et al.*, 1999a) and, following initial attachment, they interact with the surface more than non-fimbriated cells do (Otto *et al.*, 1999b). Otto *et al.* (2001) suggested that physical interactions between type 1 fimbriae and the surface are part of a surface-sensing mechanism in which protein turnover may contribute to the observed change in composition of outer membrane proteins. This change alters the surface characteristics of the cell envelope and may thus influence adhesion. This group used two-dimensional (2-D) polyacrylamide gel electrophoresis to demonstrate that attachment of *E. coli* to abiotic surfaces leads to alteration in the composition of outer membrane proteins. Among those altered proteins was the OmpA, which is one of the major proteins in the outer membrane of *E. coli* (Sugawara *et al.*, 1996). This OMP forms small nonspecific diffusion channels with low permeability (Nikaido, 1992). It is also required for structural integrity of the outer membrane and the generation of a normal cell shape. This protein is involved in cell-cell interactions (Anthony *et al.*, 1994) and invasion of *E. coli* (Prasadarao *et al.*, 1996). Otto *et al.* (2001) identified OmpA as two mass isoforms with sizes of approximately 30 and 40 kD.

Experimental Approach

Outer Membrane Protein (OMP) Preparations

The preparations of OMPs for SDS-PAGE were performed as described in detail in Chapter 2.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

This technique was performed as described in detail in Chapter 2.

The Analysis of OMP Profiles

The first stages in analysing the gels were performed using Phoretix imaging analysis software (non-Linear Dynamics Ltd, Newcastle upon Tyne, England). Then the molecular weights and Rf (Retardation factor) values of all the outer membrane proteins in the samples were estimated manually. This was performed in order to observe any possible differences between the sensitive and tolerant cultures for both biofilm and eluate cells. All these steps were described in detail in Chapter 5. **Figures 46** and **47** show the outer membrane protein profiles of *Escherichia coli* ATCC 10000 biofilm and eluate cells, respectively. The measurement data for these two gels (indicating Band number, molecular weight and Rf value of the protein bands) are given in **Tables 30** and **31**, respectively. **Figures 48** and **49** illustrate the outer membrane protein profiles of binary biofilm and eluate cells, respectively. The measurement data for these two gels are given in **Tables 32** and **33.**

Results & Discussion

In *E. coli* biofilm cells (**Figure 46**), two outer membrane proteins with a molecular weight of 35.2 kD and 26 kD were present in control, unexposed cells, disappeared from resistant cells (Passages 1, 3, 4 & 5) and once the cells were cultured in biocide free medium, both OMPs reappeared. A 30.8 kD OMP appeared in control cells; as a result of exposing the cells to BIT, the molecular weight of this protein altered to 30 kD. When the cells were passaged without the addition of biocide, the molecular weight decreased to 29.2 kD (**Table 32**).

For the eluate cells (**Figure 37**), a 23 kD outer membrane protein appeared in control cells, disappeared from resistant passages and then reappeared in Passages 7 and 8. However, the molecular weight of this OMP slightly altered to 22.5 kD. Another OMP with a molecular weight range of between 32.6 and 33.1 kD was observed in resistant eluate cells. Although the cells were cultured in biocide-free medium (Passages 7 & 8), this OMP did not disappear. A chemical alteration in the molecular weights for some of the outer membrane proteins, which were present in both sensitive and resistant cells, was observed too. A 37.3 kD OMP appeared in eluate sensitive cells. After exposing the cells to BIT, the molecular weight altered to 38 kD. An OMP with a molecular weight of 26.9 kD appeared in control, unexposed cells. In Passages 1, 3, 5 and 7 the molecular weight altered to 27.5 kD and at Passage 8, this value slightly increased to 27.9 kD (**Table 33**).

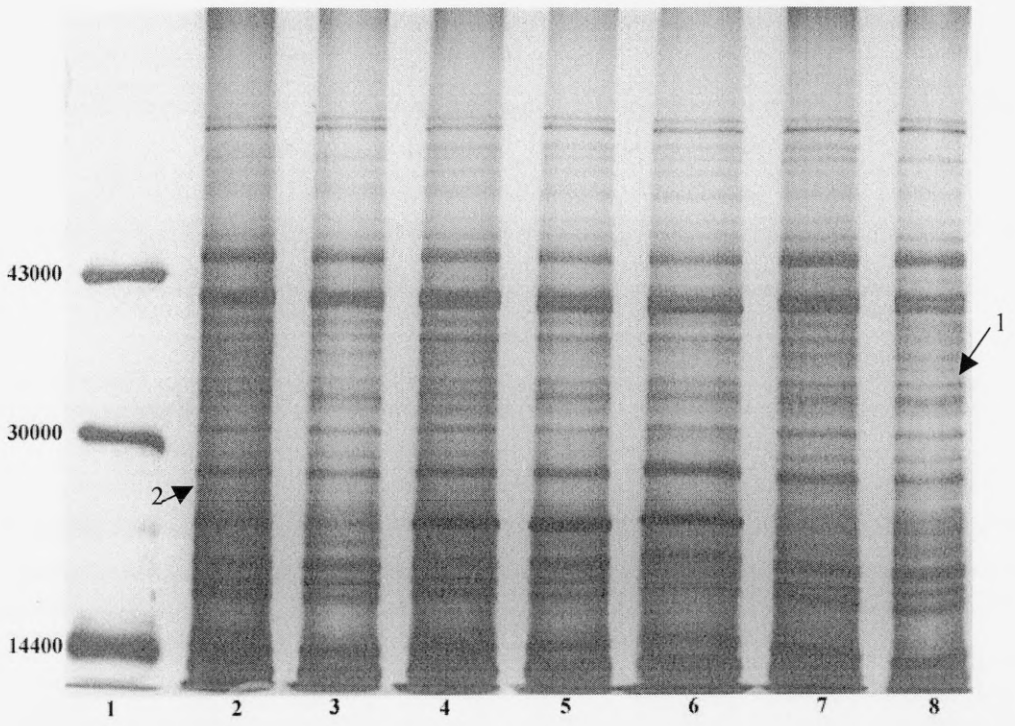


Figure 46: Outer membrane protein profile of *Escherichia coli* ATCC 10000 biofilm cells cultured in modified CDM and exposed to BIT. Lane 1: molecular weights markers; Lane 2: biofilm control cells (1.15 x 10^{-3} mg/well); Lane 3: Passage 1 cells (1.13 x 10^{-3} mg/well); Lane 4: Passage 3 cells (1.16 x 10^{-3} mg/well); Lane 5: Passage 4 cells (1.15 x 10^{-3} mg/well); Lane 6: Passage 5 cells (1.14 x 10^{-3} mg/well); Lane 7: Passage 7 cells (1.15 x 10^{-3} mg/well); lane 8: Passage 8 cells (1.15 x 10^{-3} mg/well). Arrow (1) indicates the 35.2 kD OMP; arrow (2) indicates the 26 kD OMP.

Table 32 (Part 1): Measurement data indicating Band number, Molecular weight and Retardation factor for Outer Membrane Proteins of *E. coli* biofilm cells exposed to BIT and cultured in modified CDM (band no.: band number; M.Wt.: molecular weight; Rf: retardation factor; Co: corresponding protein to lane 2; N: novel protein).

Lane 1 (molecular weight markers)				Lane 2 (control biofilm cells)				Lane 3 (Passage 1 cells)			
Band no.	MWt. (kD)	Rf	Co	Band no.	MWt. (kD)	Rf	Co	Band no.	MWt. (kD)	Rf	Co
1	43.0	0.35	-	1	105.0	0.12	-	1	96.5	0.15	2
2	30.0	0.57	-	2	96.5	0.15	-	2	91.0	0.17	N
3	14.4	0.85	-	3	94.0	0.16	-	3	83.0	0.20	4
				4	83.0	0.20	-	4	77.8	0.22	N
				5	72.5	0.24	-	5	72.5	0.24	5
				6	61.5	0.28	-	6	59.0	0.29	6
				7	56.4	0.30	-	7	53.6	0.31	7
				8	48.0	0.33	-	8	45.5	0.34	8
				9	40.5	0.39	-	9	41.6	0.37	N
				10	38.8	0.42	-	10	40.5	0.39	9
				11	37.5	0.44	-	11	38.8	0.42	10
				12	36.2	0.46	-	12	37.5	0.44	11
				13	35.2	0.48	-	13	36.2	0.46	12
				14	33.5	0.51	-	14	34.0	0.50	14
				15	31.8	0.54	-	15	32.9	0.52	N
				16	30.8	0.56	-	16	30.0	0.57	16
				17	27.0	0.62	-	17	28.2	0.60	N
				18	26.0	0.64	-	18	27.0	0.62	17
				19	23.8	0.68	-	19	23.2	0.69	19
				20	22.0	0.71	-	20	22.0	0.71	20
				21	20.4	0.74	-	21	20.4	0.74	21
				22	18.0	0.78	-	22	18.9	0.77	N
				23	15.0	0.84	-	23	18.0	0.78	22
								24	14.4	0.85	23

Table 32 (Part 2): Measurement data indicating Band number, Molecular weight and Retardation factor for Outer Membrane Proteins of *E. coli* biofilm cells exposed to BIT and cultured in modified CDM (band no.: band number; M.Wt.: molecular weight; Rf: retardation factor; Co: corresponding protein to lane 2; N: novel protein).

Lane 4 (Passage 3 cells)				Lane 5 (Passage 4 cells)				Lane 6 (Passage 5 cells)			
Band no.	MWt. (kD)	Rf	Co	Band no.	MWt. (kD)	Rf	Co	Band no.	MWt. (kD)	Rf	Co
1	105.0	0.12	1	1	94.0	0.16	3	1	94.0	0.16	3
2	96.5	0.15	2	2	91.0	0.17	N	2	88.5	0.18	N
3	91.0	0.17	N	3	85.9	0.19	N	3	83.0	0.20	4
4	85.9	0.19	N	4	80.5	0.21	4	4	80.5	0.21	N
5	80.5	0.21	4	5	67.0	0.26	N	5	75.0	0.23	5
6	77.8	0.22	N	6	59.0	0.29	6	6	67.0	0.26	N
7	70.0	0.25	5	7	53.6	0.31	7	7	56.4	0.30	7
8	59.0	0.29	6	8	45.5	0.34	8	8	43.0	0.35	N
9	53.6	0.31	7	9	39.1	0.41	10	9	39.9	0.40	N
10	45.5	0.34	8	10	38.0	0.43	N	10	37.0	0.45	11
11	39.9	0.40	N	11	37.0	0.45	11	11	36.2	0.46	12
12	38.8	0.42	10	12	36.2	0.46	12	12	33.5	0.51	14
13	37.0	0.45	11	13	34.0	0.50	14	13	32.2	0.53	15
Lane 4 (Passage 3 cells)				Lane 5 (Passage 4 cells)				Lane 6 (Passage 5 cells)			
Band no.	MWt. (kD)	Rf	Co	Band no.	MWt. (kD)	Rf	Co	Band no.	MWt. (kD)	Rf	Co
14	34.0	0.50	14	14	32.9	0.52	N	14	30.0	0.57	16
15	32.9	0.52	N	15	31.8	0.54	15	15	27.0	0.62	17
16	31.8	0.54	15	16	30.0	0.57	16	16	23.2	0.69	19
17	30.0	0.57	16	17	26.5	0.63	17	17	21.0	0.73	21
18	28.2	0.60	N	18	23.2	0.69	19	18	18.9	0.77	N
19	27.0	0.62	17	19	20.4	0.74	21	19	15.0	0.84	23
20	23.2	0.69	19	20	17.5	0.79	22				
21	20.4	0.74	21	21	14.4	0.85	23				
22	19.5	0.76	N								
23	18.0	0.78	22								
24	14.4	0.85	23								

Table 32 (Part 3): Measurement data indicating Band number, Molecular weight and Retardation factor for Outer Membrane Proteins of *E. coli* biofilm cells exposed to BIT and cultured in modified CDM (band no.: band number; M.Wt.: molecular weight; Rf: retardation factor; Co: corresponding protein to lane 2; N: novel protein).

Lane 7 (Passage 7 cells)				Lane 8 (Passage 8 cells)			
Band no.	MWt. (kD)	Rf	Co	Band no.	MWt. (kD)	Rf	Co
1	88.5	0.18	N	1	91.0	0.17	N
2	83.0	0.20	4	2	88.5	0.18	N
3	77.8	0.22	N	3	77.8	0.22	N
4	75.0	0.23	5	4	75.0	0.23	5
5	67.0	0.26	N	5	67.0	0.26	N
6	56.4	0.30	7	6	56.4	0.30	7
7	51.0	0.32	8	7	51.0	0.32	8
8	43.0	0.35	N	8	43.0	0.35	N
9	39.9	0.40	N	9	41.0	0.38	9
10	38.0	0.43	N	10	39.9	0.40	N
11	36.2	0.46	12	11	38.0	0.43	N
12	35.2	0.48	13	12	36.2	0.46	12
13	33.5	0.51	14	13	35.2	0.48	13
14	31.8	0.54	15	14	34.0	0.50	14
15	29.2	0.58	16	15	32.9	0.52	N
16	27.5	0.61	17	16	31.8	0.54	15
17	26.0	0.64	18	17	29.2	0.58	16
18	23.2	0.69	19	18	27.0	0.62	17
19	29.5	0.76	N	19	26.0	0.64	18
20	17.0	0.80	22	20	24.9	0.66	19
21	13.6	0.86	23	21	22.0	0.71	20
				22	20.4	0.74	21
				23	19.5	0.76	N
				24	17.5	0.79	22
				25	16.5	0.81	N
				26	12.5	0.88	N

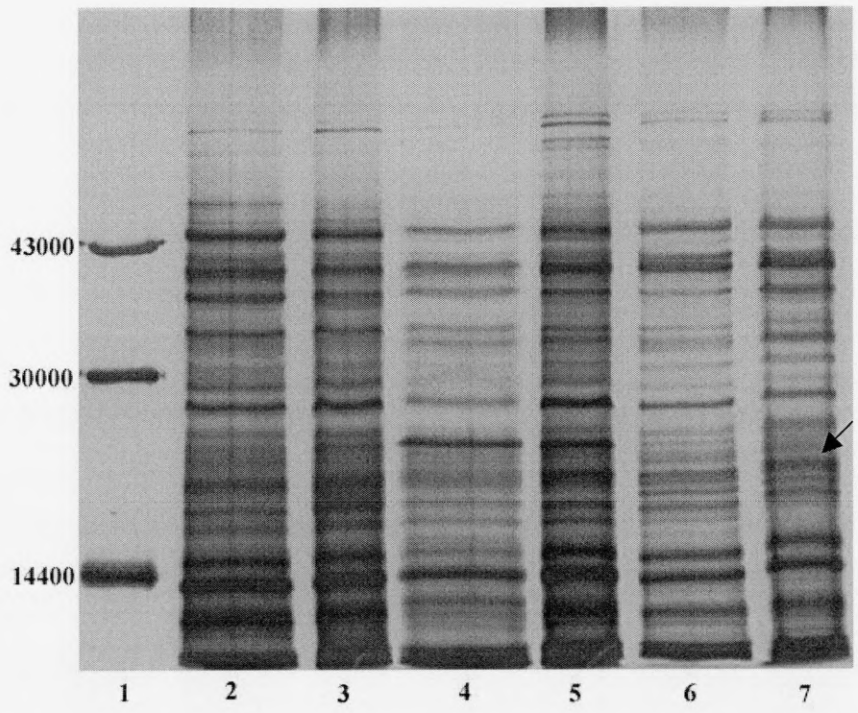


Figure 47: Outer membrane protein profile of *Escherichia coli* ATCC 10000 eluate cells cultured in modified CDM and exposed to BIT. Lane 1: molecular weights markers; Lane 2: eluate control cells (1.34 x 10^{-3} mg/well); Lane 3: Passage 1 cells (1.38 x 10^{-3} mg/well); Lane 4: Passage 3 cells (1.44 x 10^{-3} mg/well); Lane 5: Passage 5 cells (1.44 x 10^{-3} mg/well); Lane 6: Passage 7 cells (1.44 x 10^{-3} mg/well); Lane 7: Passage 8 cells (1.39 x 10^{-3} mg/well). Arrow indicates the 23 kD OMP.

Table 33 (Part 1): Measurement data indicating Band number, Molecular weight and Retardation factor for Outer Membrane Proteins of *E. coli* eluate cells exposed to BIT and cultured in modified CDM (band no.: band number; M.Wt.: molecular weight; Rf: retardation factor; Co: corresponding protein to lane 2; N: novel protein).

Lane 1 (molecular weight markers)				Lane 2 (control eluate cells)				Lane 3 (Passage 1 cells)			
Band no.	MWt. (kD)	Rf	Co	Band no.	MWt. (kD)	Rf	Co	Band no.	MWt. (kD)	Rf	Co
1	43.0	0.38	-	1	84.1	0.20	-	1	89.0	0.18	N
2	30.0	0.57	-	2	77.1	0.23	-	2	84.1	0.20	1
3	14.4	0.88	-	3	70.0	0.26	-	3	77.1	0.23	2
				4	58.8	0.31	-	4	65.5	0.28	N
				5	47.5	0.36	-	5	56.5	0.32	4
				6	40.1	0.42	-	6	47.5	0.36	5
				7	37.3	0.46	-	7	40.9	0.41	6
				8	33.8	0.51	-	8	38.0	0.45	7
				9	30.0	0.57	-	9	33.8	0.51	8
				10	28.8	0.59	-	10	33.1	0.52	N
				11	26.9	0.63	-	11	30.5	0.56	9
				12	24.9	0.67	-	12	28.8	0.59	10
				13	23.0	0.71	-	13	27.5	0.62	11
				14	20.8	0.75	-	14	24.9	0.67	12
				15	19.0	0.79	-	15	24.0	0.69	N
				16	17.5	0.82	-	16	21.5	0.74	14
				17	15.5	0.86	-	17	19.4	0.78	15
				18	13.4	0.90	-	18	18.0	0.81	16
								19	15.5	0.86	17
								20	13.4	0.90	18

Table 33 (Part 2): Measurement data indicating Band number, Molecular weight and Retardation factor for Outer Membrane Proteins of *E. coli* eluate cells exposed to BIT and cultured in modified CDM (band no.: band number; M.Wt.: molecular weight; Rf: retardation factor; Co: corresponding protein to lane 2; N: novel protein).

Lane 4 (Passage 3 cells)				Lane 5 (Passage 5 cells)				Lane 6 (Passage 7 cells)			
Band no.	MWt. (kD)	Rf	Co	Band no.	MWt. (kD)	Rf	Co	Band no.	MWt. (kD)	Rf	Co
1	86.5	0.19	N	1	91.0	0.17	N	1	91.0	0.17	N
2	58.8	0.31	4	2	86.5	0.19	N	2	89.0	0.18	N
3	49.8	0.35	5	3	81.9	0.21	1	3	49.8	0.35	5
4	40.9	0.41	6	4	77.1	0.23	2	4	40.1	0.42	6
5	38.0	0.45	7	5	70.0	0.26	3	5	38.0	0.45	7
6	34.6	0.50	8	6	61.2	0.30	4	6	33.8	0.51	8
7	32.6	0.53	N	7	49.8	0.35	5	7	32.6	0.53	N
8	30.5	0.56	9	8	40.9	0.41	6	8	30.5	0.56	9
9	28.8	0.59	10	9	38.0	0.45	7	9	28.4	0.60	10
10	27.5	0.62	11	10	33.8	0.51	8	10	27.5	0.62	11
11	24.4	0.68	12	11	33.1	0.52	N	11	24.9	0.67	12
12	21.5	0.74	14	12	30.5	0.56	9	12	23.0	0.71	13
13	19.9	0.77	15	13	28.8	0.59	10	13	21.9	0.73	N
14	18.5	0.80	16	14	27.5	0.62	11	14	20.5	0.76	14
15	16,0	0.85	17	15	23.9	0.69	N	15	19.4	0.78	15
16	14.0	0.89	18	16	21.9	0.73	N	16	15.5	0.86	17
				17	19.4	0.78	15	17	14.0	0.89	18
				18	18.5	0.80	16				
				19	16.0	0.85	17				
				20	14.4	0.88	18				

Lane 7 (Passage 8 cells)			
1	91.0	0.17	N
2	89.0	0.18	N
3	79.5	0.22	2
4	61.2	0.30	4
5	49.8	0.35	5
6	40.9	0.41	6
7	38.0	0.45	7
8	34.6	0.50	8
9	33.1	0.52	N
10	31.0	0.55	9
11	29.4	0.58	10
12	27.9	0.61	11
13	25.5	0.66	12
14	23.9	0.69	N
15	22.5	0.72	13
16	20.5	0.76	14
17	17.0	0.83	16
18	15.0	0.87	17

In binary biofilm (mixed) cells (**Figure 48**) a 30.4 kD OMP appeared in sensitive cells, disappeared from Passages 1 and 3, reappeared in Passage 4 and at Passage 5, this OMP disappeared again. Once the cells were passaged in biocide-free medium, this protein reappeared. Another OMP with a molecular weight of 27.4 kD appeared in control, unexposed cells. When the cells were exposed to BIT, this OMP disappeared from Passage 1, reappeared in Passage 3 as a 28 kD OMP, before it disappeared again from Passages 4, 5 and 7. At Passage 8, this protein reappeared with a molecular weight of 28 kD (**Table 34**).

Figure 49 illustrates binary eluate cells; two outer membrane proteins with a molecular weight of 63.9 and 51.2 kD were present in sensitive cells. Following the exposure to BIT, both OMPs disappeared and there was no sign for these two proteins even after removing the biocide from the medium (Passages 6 & 8). A 25.5 kD OMP appeared in control unexposed cells, disappeared from resistant passages and reappeared in Passages 6 and 8. Another OMP with a molecular weight of 32 kD was observed in sensitive eluate cells. When the cells were passaged in modified CDM (which contains BIT), this OMP disappeared from Passage 1, reappeared in Passage 4 before it disappeared completely from Passage 5. Once the cells were cultured in biocide-free medium, this OMP reappeared with a molecular weight of 32 kD (**Table 35**).

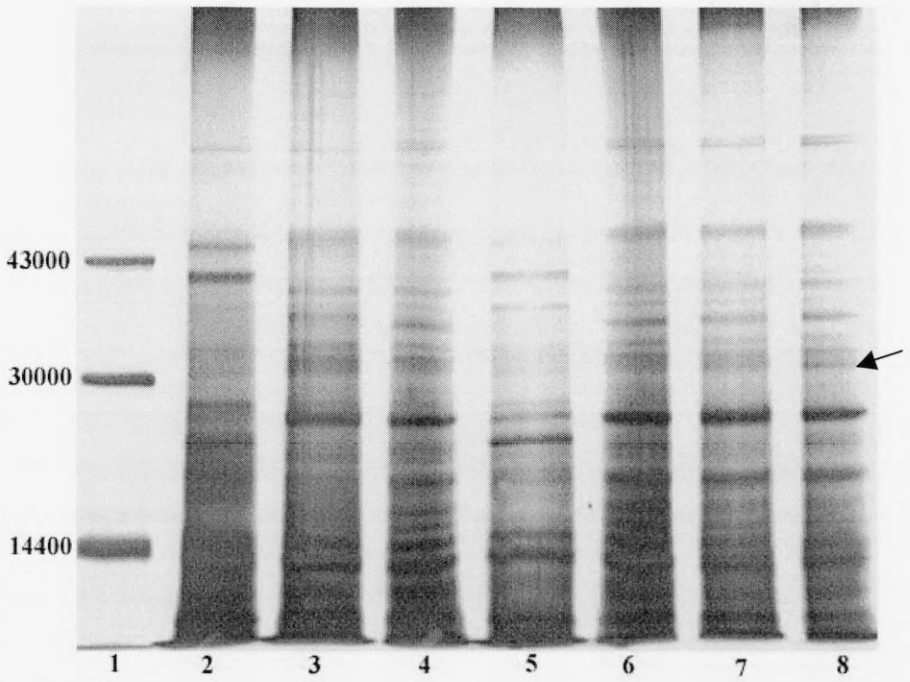


Figure 48: Outer membrane protein profile of binary biofilm cells cultured in modified CDM and exposed to BIT. Lane 1: molecular weights markers; Lane 2: biofilm control cells (1.1 x 10^{-3} mg/well); Lane 3: Passage 1 cells (1.06 x 10^{-3} mg/well); Lane 4: Passage 3 cells (1.04 x 10^{-3} mg/well); Lane 5: Passage 4 cells (1.06 x 10^{-3} mg/well); Lane 6: Passage 5 cells (1.05 x 10^{-3} mg/well); Lane 7: Passage 7 cells (1.04 x 10^{-3} mg/well); lane 8: Passage 8 cells (1.05 x 10^{-3} mg/well). Arrow indicates the 30.4 kD OMP.

Table 34 (Part 1): Measurement data indicating Band number, Molecular weight and Retardation factor for Outer Membrane Proteins of Binary biofilm cells exposed to BIT and cultured in modified CDM (band no.: band number; M.Wt.: molecular weight; Rf: retardation factor; Co: corresponding protein to lane 2; N: novel protein).

Lane 1 (molecular weight markers)				Lane 2 (control biofilm cells)				Lane 3 (Passage 1 cells)			
Band no.	MWt. (kD)	Rf	Co	Band no.	MWt. (kD)	Rf	Co	Band no.	MWt. (kD)	Rf	Co
1	43	0.40	-	1	71.0	0.24	-	1	71.0	0.24	1
2	30	0.57	-	2	64.0	0.27	-	2	46.9	0.37	3
3	14.4	0.81	-	3	45.1	0.38	-	3	39.0	0.44	N
				4	41.0	0.42	-	4	36.0	0.48	5
				5	36.8	0.47	-	5	33.0	0.52	6
				6	33.0	0.52	-	6	31.8	0.54	N
				7	30.4	0.56	-	7	25.1	0.63	N
				8	27.4	0.60	-	8	21.8	0.68	9
				9	23.0	0.66	-	9	20.0	0.71	10
				10	20.0	0.71	-	10	14.4	0.81	11
				11	14.4	0.81	-	11	13.0	0.84	12
				12	13.0	0.84	-				

Lane 4 (Passage 3 cells)				Lane 5 (Passage 4 cells)				Lane 6 (Passage 5 cells)			
Band no.	MWt. (kD)	Rf	Co	Band no.	MWt. (kD)	Rf	Co	Band no.	MWt. (kD)	Rf	Co
1	68.8	0.25	1	1	68.8	0.25	1	1	71.0	0.24	1
2	53.5	0.33	N	2	45.1	0.38	3	2	64.0	0.27	2
3	45.1	0.38	3	3	41.0	0.42	4	3	54.6	0.32	N
4	38.2	0.45	N	4	38.2	0.45	N	4	46.9	0.37	3
5	36.8	0.47	5	5	36.8	0.47	5	5	40.0	0.43	4
6	35.3	0.49	N	6	32.5	0.53	6	6	38.2	0.45	N
7	32.5	0.53	6	7	30.4	0.56	7	7	36.8	0.47	5
8	31.8	0.54	N	8	25.1	0.63	N	8	35.3	0.49	N
9	28.0	0.59	8	9	23.0	0.66	9	9	33.0	0.52	6
10	25.1	0.63	N	10	20.0	0.71	10	10	31,8	0.54	N
11	22.5	0.67	9	11	18.2	0.74	N	11	25.1	0.63	N
12	19.5	0.72	10	12	15.0	0.80	11	12	22.5	0.67	9
13	17.25	0.76	N	13	14.0	0.82	12	13	19.5	0.72	10
14	16.1	0.78	N	14	11.5	0.87	N	14	17.25	0.76	N
15	14.4	0.81	11					15	16.1	0.78	N
16	13.0	0.84	12					16	14.4	0.81	11
17	11.5	0.87	N					17	13.0	0.84	12
								18	11.0	0.88	N

Table 34 (Part 2): Measurement data indicating Band number, Molecular weight and Retardation factor for Outer Membrane Proteins of Binary biofilm cells exposed to BIT and cultured in modified CDM (band no.: band number; M.Wt.: molecular weight; Rf: retardation factor; Co: corresponding protein to lane 2; N: novel protein).

Lane 7 (Passage 7 cells)				Lane 8 (Passage 8 cells)			
Band no.	MWt (kD)	Rf (cm)	Co	Band no.	MWt (kD)	Rf (cm)	Co
1	71.0	0.24	1	1	71.0	0.24	1
2	54.6	0.32	N	2	64.0	0.27	2
3	46.9	0.37	3	3	54.6	0.32	N
4	40.0	0.43	4	4	48.2	0.36	3
5	39.0	0.44	N	5	41.0	0.42	4
6	36.8	0.47	5	6	39.0	0.44	N
7	35.3	0.49	N	7	37.5	0.46	5
8	33.0	0.52	6	8	35.3	0.49	N
9	30.4	0.56	7	9	33.0	0.52	6
10	25.1	0.63	N	10	31.8	0.54	N
11	22.5	0.67	9	11	30.4	0.56	7
12	19.5	0.0.72	10	12	28.0	0.59	8
13	17.25	0.76	N	13	25.1	0.63	N
14	16.1	0.78	N	14	22.5	0.67	9
15	14.4	0.81	11	15	20.0	0.71	10
16	13.0	0.84	12	16	17.8	0.75	N
				17	16.1	0.78	N
				18	14.4	0.81	11
				19	13.0	0.84	12

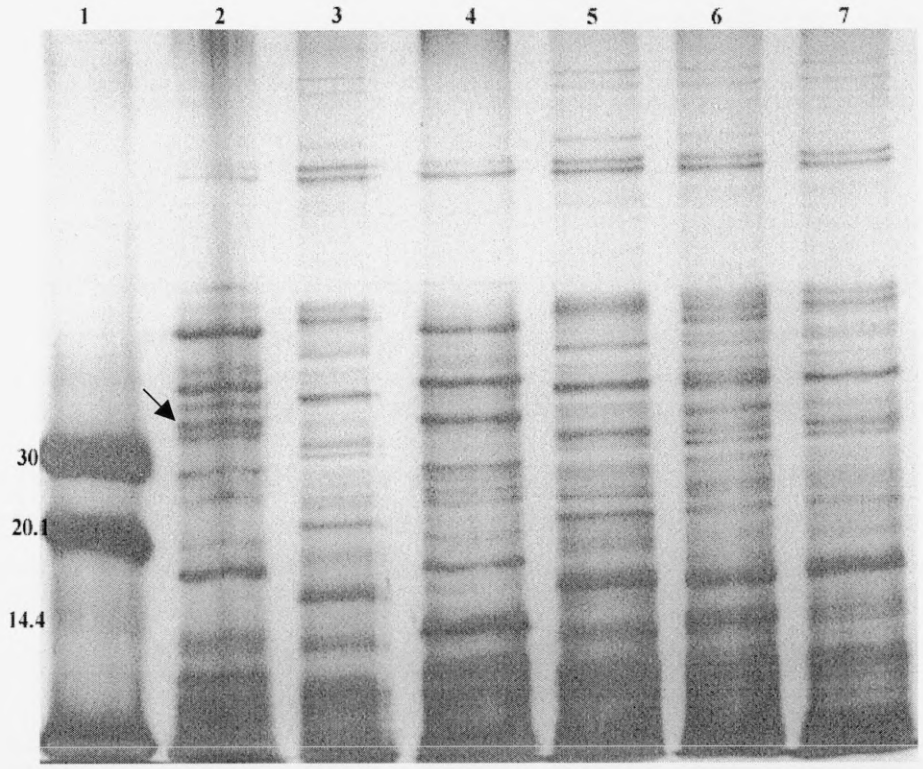


Figure 49: Outer membrane protein profile of binary eluate cells cultured in modified CDM and exposed to BIT. Lane 1: molecular weights markers; Lane 2: eluate control cells (1.104 x 10^{-3} mg/well); Lane 3: Passage 1 cells (1.10 x 10^{-3} mg/well); Lane 4: Passage 4 cells (1.104 x 10^{-3} mg/well); Lane 5: Passage 5 cells (1.14 x 10^{-3} mg/well); Lane 6: Passage 6 cells (1.11 x 10^{-3} mg/well); Lane 7: Passage 8 cells (1.12 x 10^{-3} mg/well). The arrow indicates the 32 kD OMP.

Table 35 (Part 1): Measurement data indicating Band number, Molecular weight and Retardation factor for Outer Membrane Proteins of Binary eluate cells exposed to BIT and cultured in modified CDM (band no.: band number; M.Wt.: molecular weight; Rf: retardation factor; Co: corresponding protein to lane 2; N: novel protein).

Lane 1 (molecular weight markers)				Lane 2 (control eluate cells)				Lane 3 (Passage 1 cells)			
Band no.	MWt. (kD)	Rf	Co	Band no.	MWt. (kD)	Rf	Co	Band no.	MWt. (kD)	Rf	Co
1	30.0	0.60	-	1	79.0	0.25	-	1	96.1	0.15	N
2	20.1	0.70	-	2	77.5	0.26	-	2	92.4	0.17	N
3	14.4	0.82	-	3	63.9	0.41	-	3	80.4	0.24	1
				4	51.2	0.43	-	4	77.5	0.26	2
				5	47.0	0.46	-	5	74.0	0.28	N
				6	39.0	0.52	-	6	48.5	0.45	N
				7	36.4	0.54	-	7	47.0	0.46	5
				8	33.8	0.56	-	8	41.8	0.50	N
				9	32.0	0.58	-	9	37.8	0.53	7
				10	25.5	0.64	-	10	33.8	0.56	8
				11	22.5	0.67	-	11	30.8	0.59	N
				12	18.8	0.73	-	12	27.6	0.62	N
				13	16.5	0.77	-	13	26.5	0.63	N
				14	12.9	0.85	-	14	24.4	0.65	N
								15	20.1	0.70	N
								16	19.2	0.72	12
								17	17.0	0.76	13
								18	14.8	0.81	N
								19	11.8	0.87	14

Table 35 (Part 2): Measurement data indicating Band number, Molecular weight and Retardation factor for Outer Membrane Proteins of Binary eluate cells exposed to BIT and cultured in modified CDM (band no.: band number; M.Wt.: molecular weight; Rf: retardation factor; Co: corresponding protein to lane 2; N: novel protein).

Lane 4 (Passage 4 cells)				Lane 5 (Passage 5 cells)				Lane 6 (Passage 6 cells)			
Band no.	MWt. (kD)	Rf	Co	Band no.	MWt. (kD)	Rf	Co	Band no.	MWt. (kD)	Rf	Co
1	75.5	0.27	2	1	92.4	0.17	N	1	90.2	0.18	N
2	72.5	0.29	N	2	88.8	0.19	N	2	88.8	0.19	N
3	44.5	0.48	N	3	78.8	0.25	1	3	72.5	0.29	N
4	39.0	0.52	6	4	74.0	0.28	N	4	70.8	0.30	N
5	35.0	0.55	8	5	72.5	0.29	N	5	65.8	0.33	N
6	32.0	0.58	9	6	65.8	0.33	N	6	44.5	0.48	N
7	23.4	0.66	11	7	45.8	0.47	5	7	41.8	0.50	N
8	21.0	0.69	N	8	39.0	0.52	6	8	37.8	0.53	7
9	19.6	0.71	N	9	35.0	0.55	7	9	35.0	0.55	8
10	17.5	0.75	13	10	32.8	0.57	8	10	32.0	0.58	9
11	15.5	0.79	N	11	28.8	0.61	N	11	28.8	0.61	N
12	11.8	0.87	14	12	26.5	0.63	N	12	25.5	0.64	10
				13	22.0	0.68	11	13	24.4	0.65	N
				14	19.6	0.71	N	14	22.0	0.68	11
				15	18.0	0.74	12	15	20.1	0.70	N
				16	16.1	0.78	13	16	18.0	0.74	12
				17	14.4	0.82	N	17	16.5	0.77	13
				18	11.2	0.88	N	18	13.6	0.83	14
								19	11.2	0.88	N

Lane 7 (Passage 8 cells)			
1	90.2	0.18	N
2	87.0	0.20	N
3	70.8	0.30	N
4	69.0	0.31	N
5	45.8	0.47	5
6	43.0	0.49	N
7	37.8	0.53	6
8	35.0	0.55	8
9	32.0	0.58	9
10	28.8	0.61	N
11	25.5	0.64	10
12	24.4	0.65	N
13	22.0	0.68	11
14	19.6	0.71	N
15	18.0	0.74	12
16	16.1	0.78	13
17	14.4	0.82	N
18	11.2	0.88	N

From the commentary above, it is obvious that there are some variations in the outer membrane profiles between *E. coli* (biofilm and eluate) cells and binary (biofilm and eluate) cells. However, an outer membrane protein in the range of between 23 and 26 kD was observed to have disappeared following the exposure of *E. coli* biofilm and eluate cells to BIT. The slight variations in the observed molecular weight of this OMP, as mentioned in Chapter 5, may be due to variations in scanning and exact estimation of the Rf values, which were determined manually in this study. Once the cells were passaged in biocide-free medium (modified CDM), this OMP reappeared. This outer membrane protein could be the same OMP, which appeared in control *Ps. aeruginosa* cells (23.5 kD) (**Figure 42**, Chapter 5). After exposing biofilm cells to BIT, the 23.5 kD protein disappeared and once the biocide was removed, this OMP reappeared (Passage 13). For binary biofilm and eluate cells, another OMP with a molecular weight of between 30.4 and 32 kD appeared in control, unexposed cells, disappeared from resistant cells and then reappeared after the removal of BIT from the medium. Therefore, the outer membrane protein profile of binary (biofilm and eluate) cells was not similar to that of *Ps. aeruginosa* nor to that of *E. coli*. Some OMPs were similar between the *Ps. aeruginosa* and *E. coli* (biofilm and eluate cells). However, it was not possible to observe the T-OMP (36.5 to 41 kD) in *E. coli* control cells. This suggests that in the range of between 23 kD and 26 kD in monospecies biofilms (*Ps. aeruginosa* and *E. coli*) is the outer membrane protein, which is responsible for causing tolerance towards BIT. Growing the two bacteria on the same Sorbarod filter as a binary biofilm may affect the outer membrane profiles of both microorganisms, which may subsequently explain the alteration in the molecular weight of this OMP (30.4 to 32 kD). This suggests that *E. coli* and binary (biofilm and eluate) cells respond to the presence of biocide by some internal reorganization of metabolic processes, which utilizes both the energy and materials normally used in the

manufacture and operation of this OMP for some other, more essential purpose.

Experimental Approach

Two-Dimensional Polyacrylamide Gel Electrophoresis

This technique was performed as described in detail in Chapter 2.

The Analysis of OMP Profiles

Analysis of sample gels (**Figures 50, 51, 52 & 53**) was performed manually as previously described in Chapter 5. The Rf value for each protein was estimated from **Equation 5** (Chapter 5) and then extrapolated onto the calibration curve (**Figure 36**, Chapter 5). Thus the molecular weight was determined for that protein. The individual numbered proteins (on each sample gel) and their corresponding estimated molecular weights, Rf and isoelectric points (analysed manually) are shown in **Tables 36 & 37.**

Results & Discussion

Figure 50 demonstrates the outer membrane proteins of *E. coli* sensitive biofilm cells separated by two-dimensional gel electrophoresis according to molecular weights and isoelectric points. A comparison between the outer membrane protein profile of biofilm sensitive and tolerant cells is shown in **Table 36.** The individual numbered proteins (for **Figures 50 & 51**) and their corresponding estimated molecular weights, Rf values and isoelectric points (analysed manually) are given in this table. The number of proteins in the tolerant biofilm cells gel was far more than that in the control (unexposed) cells gel. This

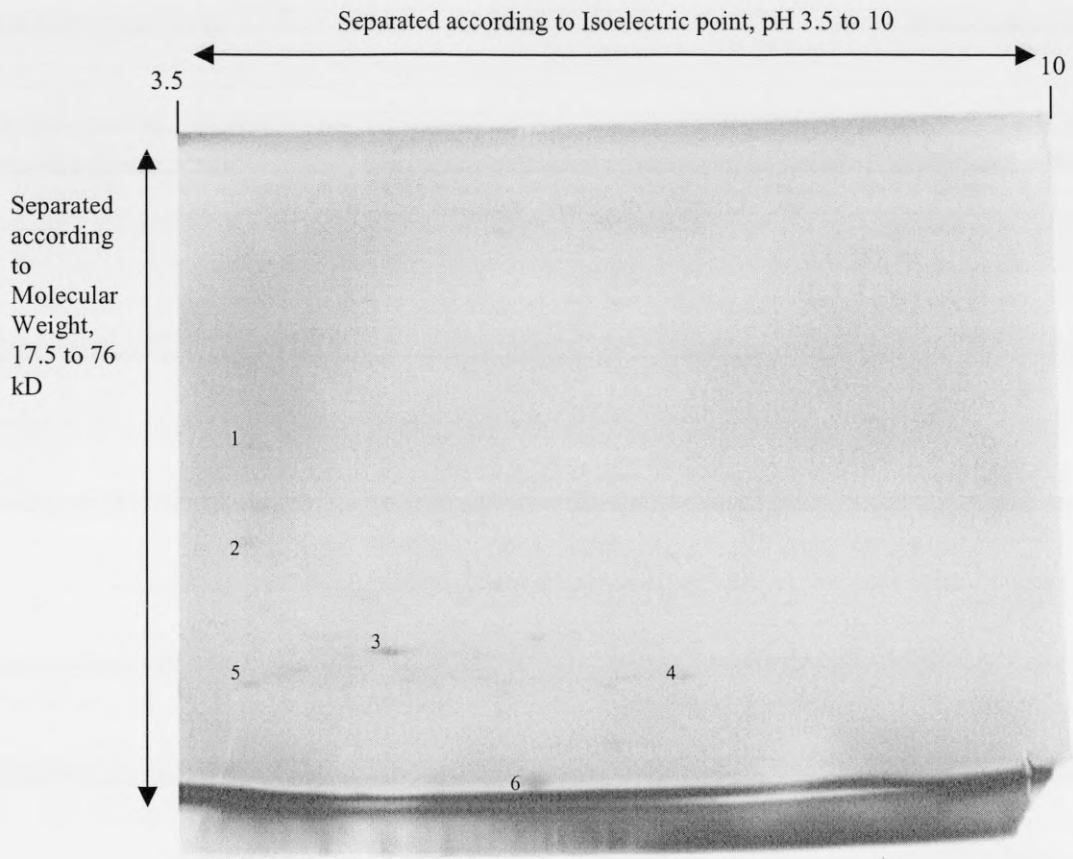


Figure 50: Two-dimensional gel electrophoresis for outer membrane protein (OMP) profiles of *Escherichia coli* ATCC 10000 sensitive biofilm cells in modified CDM. The OMPs are separated by the isoelectric point (reading left to right) and molecular weight (top to bottom). Protein concentration: 1.125×10^{-3} mg/gel.

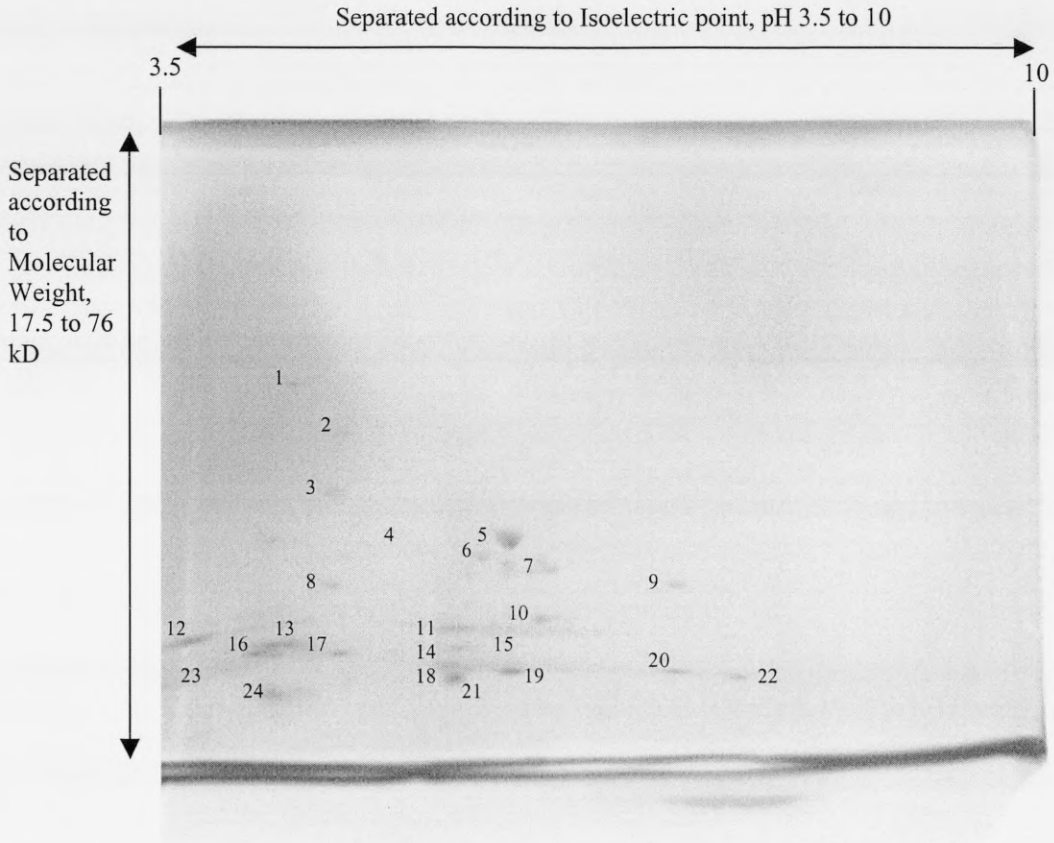


Figure 51: Two-dimensional gel electrophoresis for outer membrane protein (OMP) profiles of *Escherichia coli* ATCC 10000 resistant biofilm cells exposed to BIT and cultured in modified CDM. The OMPs are separated by the isoelectric point (reading left to right) and molecular weight (top to bottom). Protein concentration: 1.10×10^{-3} mg/gel.

Table 36: Measurement Data indicating Protein number, Molecular weight, Retardation factor and Isoelectric point of the proteins identified from the Two-Dimensional gel analysis of *E. coli* resistant biofilm cells (exposed to BIT and cultured in modified CDM) and compared to sensitive biofilm cells (protein no.: protein number; M.Wt.: molecular weight; Rf: retardation factor; pI: isoelectric point; Co: corresponding protein to sensitive cells (**Figure 50**); N: novel protein).

Figure 50 (sensitive biofilm cells)					**Figure 51** (resistant biofilm cells)				
Protein no	M.Wt. kD	Rf	pI (pH)	Co.	Protein no.	M.Wt. kD	Rf	pI (pH)	Co.
1	64.6	0.47	4.00	-	1	90.8	0.40	4.50	N
2	36.25	0.61	3.99	-	2	60.8	0.48	4.88	N
3	22.1	0.78	5.05	-	3	39.5	0.57	4.80	N
4	19.6	0.81	7.32	-	4	33.6	0.64	5.05	N
5	18.9	0.82	4.05	-	5	32.9	0.65	6.10	N
6	6.0	0.98	6.22	-	6	30.5	0.68	5.90	N
					7	28.8	0.70	6.39	N
					8	27.0	0.72	4.80	N
					9	27.0	0.72	7.33	N
					10	23.0	0.77	6.33	N
					11	21.5	0.79	5.70	N
					12	20.6	0.80	3.77	N
					13	19.6	0.81	4.49	N
					14	18.9	0.82	5.65	N
					15	18.9	0.82	6.13	N
					16	18.0	0.83	4.31	N
					17	18.0	0.83	4.82	N
					18	17.5	0.84	5.62	N
					19	16.6	0.85	6.13	N
					20	15.6	0.86	7.30	4
					21	14.8	0.87	5.70	N
					22	14.8	0.87	7.75	N
					23	14.5	0.875	3.53	N
					24	13.2	0.89	4.40	N

suggests that the presence of biocide induced the cells to switch on some mechanism of tolerance, which may account for the increased resistance towards BIT. There is only a small amount of proteins present in both sensitive and resistant biofilm cells. A 19.6 kD OMP (pI 7.32) is the only corresponding protein, which appears in tolerant biofilm cells gel as a 15.6 kD OMP (pI 7.30).

For *E. coli* eluate gels, there were significant differences between the sensitive and tolerant cells (**Figures 52 & 53**, respectively). Unlike biofilm cells, it is possible to identify a greater number of proteins in sensitive eluate cells than those tolerant to BIT. There are some proteins in the tolerant cells gel with similar molecular weights as those in the sensitive cells gel. However, the isoelectric points are different. The only two proteins, which appear to be present in both sensitive and tolerant cells, are protein numbers 8 and 11 (sensitive gel) and these are corresponding to protein numbers 6 and 9, respectively (tolerant gel). The remaining proteins are completely novel in their molecular weights and isoelectric points (**Table 37**).

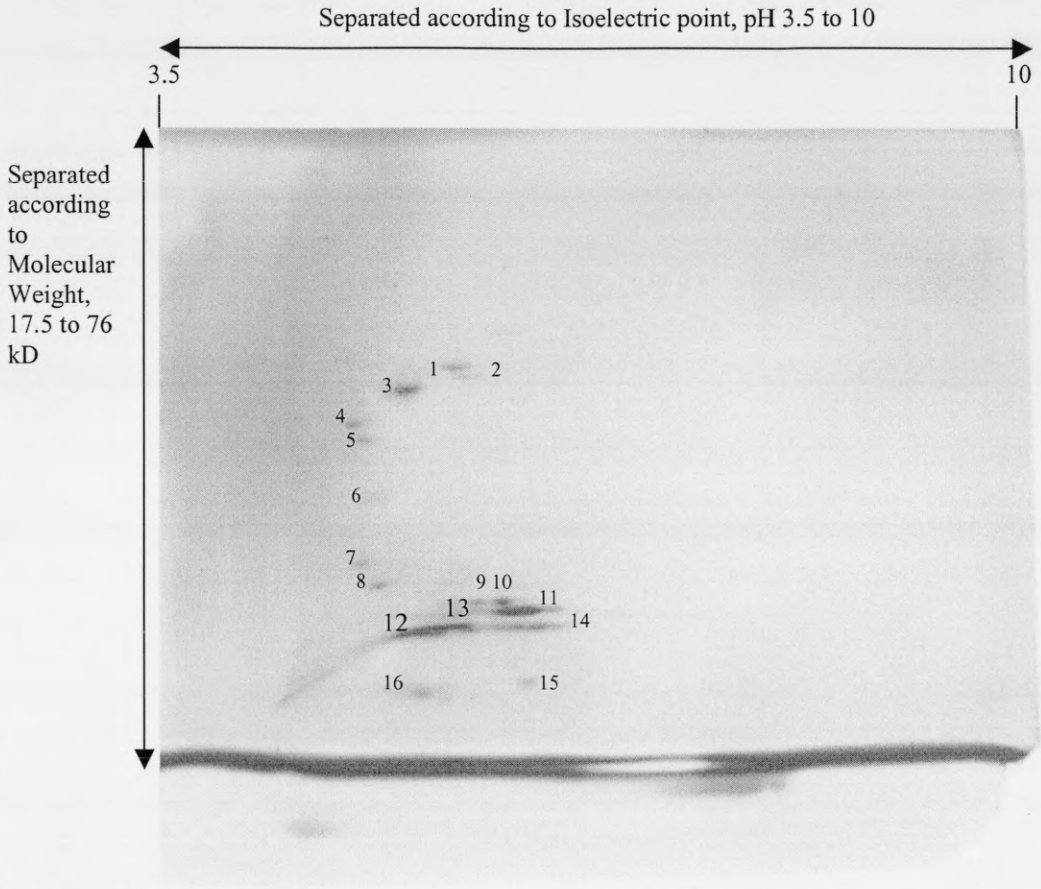


Figure 52: Two-dimensional gel electrophoresis for outer membrane protein (OMP) profiles of *Escherichia* coli ATCC 10000 sensitive eluate cells in modified CDM. The OMPs are separated by the isoelectric point (reading left to right) and molecular weight (top to bottom). Protein concentration: 1.125×10^{-3} mg/gel.

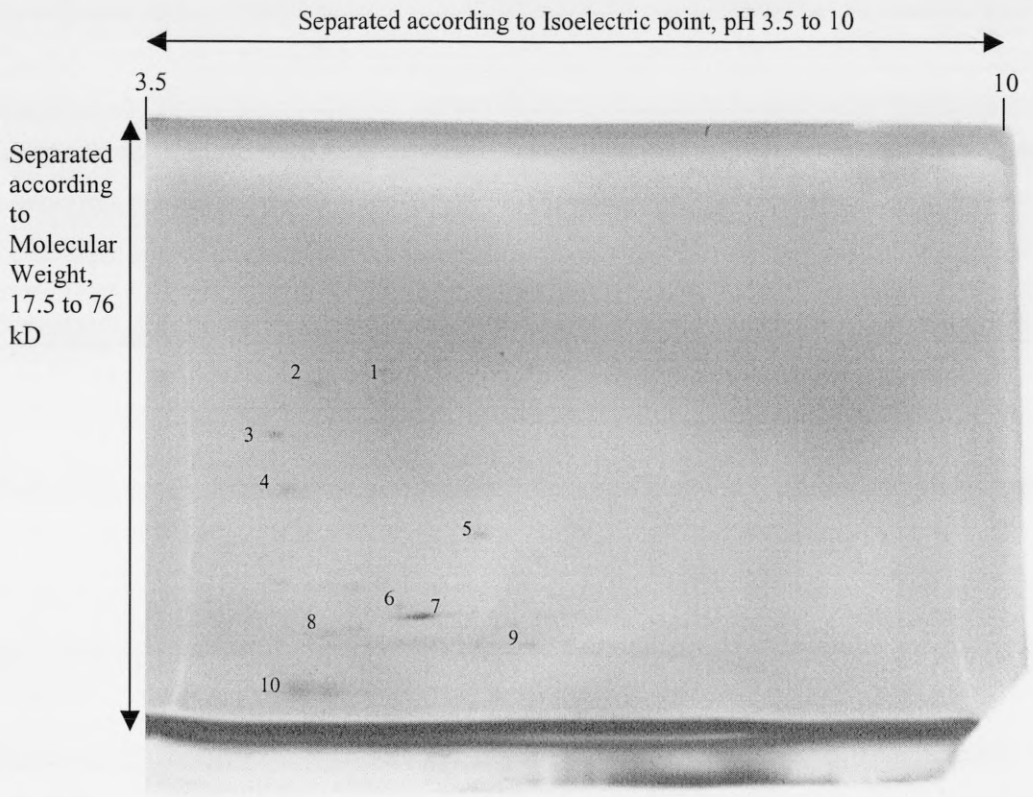


Figure 53: Two-dimensional gel electrophoresis for outer membrane protein (OMP) profiles of *Escherichia coli* ATCC 10000 resistant eluate cells exposed to BIT and cultured in modified CDM. The OMPs are separated by the isoelectric point (reading left to right) and molecular weight (top to bottom). Protein concentration: 1.12×10^{-3} mg/gel.

Table 37: Measurement Data indicating Protein number, Molecular weight, Retardation factor and Isoelectric point of the proteins identified from the Two-Dimensional gel analysis of *E. coli* resistant eluate cells (exposed to BIT and cultured in modified CDM) and compared to sensitive eluate cells (protein no.: protein number; M.Wt.: molecular weight; Rf: retardation factor; pI: isoelectric point; Co: corresponding protein to sensitive cells (**Figure 52**); N: novel protein).

Figure 52 (sensitive eluate cells)					**Figure 53** (resistant eluate cells)				
Protein no	M.Wt. kD	Rf	pI (pH)	Co.	Protein no.	M.Wt. kD	Rf	pI (pH)	Co.
1	98.5	0.38	5.75	-	1	98.5	0.38	5.30	N
2	90.8	0.40	5.90	-	2	90.8	0.40	4.78	N
3	83.0	0.42	5.32	-	3	64.6	0.47	4.40	N
4	64.6	0.47	4.98	-	4	41.25	0.55	4.52	N
5	53.6	0.50	5.00	-	5	35.5	0.62	5.93	N
6	37.9	0.59	5.08	-	6	26.2	0.73	5.33	8
7	29.6	0.69	5.00	-	7	24.6	0.75	5.51	N
8	26.2	0.73	5.18	-	8	23.0	0.77	4.90	N
9	24.6	0.75	5.95	-	9	21.5	0.79	6.30	11
10	24.6	0.75	6.1	-	10	15.6	0.86	4.70	N
11	23.0	0.77	6.29	-					
12	20.6	0.80	5.45	-					
13	20.6	0.80	5.73	-					
14	20.6	0.80	6.39	-					
15	13.2	0.89	6.29	-					
16	12.5	0.90	5.50	-					

Conclusions

The results obtained from SDS-PAGE and 2-D-PAGE analysis illustrated significant differences between biofilm and eluate cells, on one hand and sensitive and tolerant cells, on the other hand. Some outer membrane proteins appeared in both sensitive and resistant cells, whilst others appeared with alterations in their molecular weights. It is also evident that some OMPs were lost from the cells following the induction of resistance. Others appeared as novel proteins in the resistant cells. These results indicate a complex series of internal metabolic and external outer membrane associated changes induced by the presence of a sub-MIC of BIT. Such changes are indicative of BIT acting at a diversity of sites internal to the bacterial cell. Therefore, this suggests that the development of biocide tolerance in *E. coli* monospecies biofilms and in binary biofilms is supported by the induction of multiple mechanisms of tolerance and cannot be explained by the appearance and disappearance of a single protein.

The Microscopic Analysis of Biofilms

In order to gain a greater insight into the ecology of the microorganisms that exist in biofilms, it is necessary not only to be able to isolate them by traditional culture methods, but also to have some understanding of the way in which these individual microorganisms interact *in situ* (Surman *et al.*, 1996). Microscopic examination of entire biofilms enables us to increase our understanding of the spatial organization that occurs within them and on the surfaces supporting their development (Lawrence *et al.*, 1991). Structural studies of microbial biofilms and their formation have been performed by using light microscopy to examine wet mounts (Bakke & Olsson, 1986), by using transmission and scanning electron microscopy (Costerton *et al.*, 1987; Kellogg, 1989) and by developing conceptual models (Costerton *et al.*, 1987).

The **Scanning Electron Microscope (SEM)**, in which an electron beam is scanned across the surface of a specimen, is used primarily for viewing surface details rather than the internal structures of microorganisms (producing a pseudo-three-dimensional image). Magnification in a SEM is not achieved through lenses, as is the case in the light microscope. Rather, magnification in the SEM is determined by the ratio of the length of the scan across the specimen surface to the length of the scan of the cathode ray tube (CRT) (Atlas, 1988). If the electron beam scans 100 nm across the specimen and the corresponding image displayed on a CRT screen has a length of 100 mm, then the magnification will be 1,000,000x (100 mm divided by 100 nm). If the scan across the specimen is only 10 nm and the CRT screen is the same 100 mm, the magnification is 10,000,000x (100 mm divided by 10 nm). Commonly, a magnification of 10,000 to 100,000x with a resolution of 1 to 10 nm is achieved (Atlas, 1988).

Experimental Approach

Preparation of Specimens for the SEM

The goal of specimen preparation is to retain the natural shape of the living microorganism when it is viewed in the SEM and the minimization of artefacts. Biofilm samples were prepared from steady state biofilms (48 hr for *Ps. aeruginosa* and *E. coli* monospecies biofilms and 77 hr for binary biofilms) perfused with CDM and modified CDM (for *Ps. aeruginosa* and *E. coli*/binary biofilm, respectively) with or without selected biocide (BIT) addition. In order to expose and then view surface layers, the biofilm samples were dissected and mounted on aluminium stubs using double-sided carbon tabs. Samples were frozen at a very low temperature (-195°C) in liquid nitrogen then mounted in an Oxford cryofracturing unit under vacuum and sectioned using a mounted axe-blade (freeze fractured). The fractured specimen was then etched, that is, some of the ice was allowed to evaporate (the temperature was raised to -95°C), raising the surface layer of the specimen. Once more, the temperature was lowered to -195°C then the specimen was coated with 30 nm of gold-palladium (Au/Pd) to improve its characteristics as a target for the electron beam of the SEM. Coating was accomplished with a Cressington 208 HR sputter coater. This device ensures even coverage with a thin film of metal by vaporizing the metal under vacuum and depositing it on the specimen. Proper coating of the specimen minimizes electronic "*charging*" of the surface, a serious problem in scanning electron microscopy that can produce an intense emission of electrons that precludes the achievement of contrast and the viewing of surface detail. Surface charge occurs because biological specimens (such as biofilms) have non-conducting surfaces (Little *et al.*, 1991; Darkin *et al.*, 2001). Coating with a metal produces a conductive surface that permits dissipation of the electrons without charging the surface.

Following metallic coating, the samples were mounted and viewed at 5 kV in a Hitachi S-4700 field emission scanning electron microscope.

The Electroscan Corporation (Wilmington, MA) had introduced a new development in SEM technology: the **Environmental Scanning Electron Microscope (ESEM)**. This instrument uses a unique secondary electron detector capable of forming high-resolution images at pressures in the range of 0.1 to 20 torr. At these relatively high pressures, specimen charging is dissipated into the gaseous environment of the specimen chamber, enabling direct observation of uncoated, non-conductive specimens (Little *et al.*, 1991; Darkin *et al.*, 2001). ESEM allows intact biofilms to be examined in a naturally, fully hydrated state at high magnifications enabling detailed analysis (Surman *et al.*, 1996). Because of this ability to visualize hydrated samples, the shrinkage and artefacts seen with other SEM methods do not occur (Sutton *et al.*, 1994). However, as with other forms of electron microscopy, the electron beam causes damage to the biological samples. When untreated biofilms are imaged in the ESEM, this damage occurs after a relatively short time (a few minutes) (Bakke & Olsson, 1986).

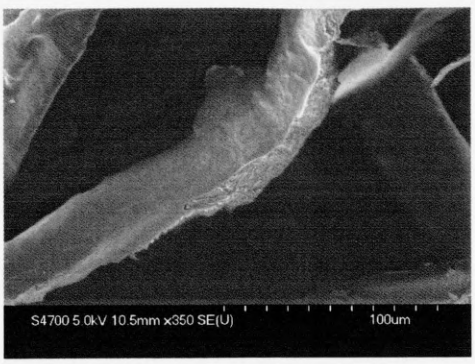
Sterilized razors were used to make incisions into the biofilm sample. A centimeter square sample was cut and then freely mounted (using a sterile forceps) upon the specimen holder stud and immediately placed within the specimen chamber of the ESEM for examination. Excess water in the specimen was sublimed off by evacuation to reveal the surface structures. Within the chamber low energy secondary electrons, from the beam impact area on the specimen surface, are accelerated towards the detector electrode by a moderate electric field. The secondary electron detector is based upon the principle of gas ionization. Successive collisions of the ambient gas molecules liberates more free electrons, resulting in a proportional cascade of current within the gas phase, where positive ions serve to effectively

neutralize the destructive build-up of excess electron charge at the specimen (Little *et al.*, 1991; Surman *et al.*, 1996; Darkin *et al.*, 2001).

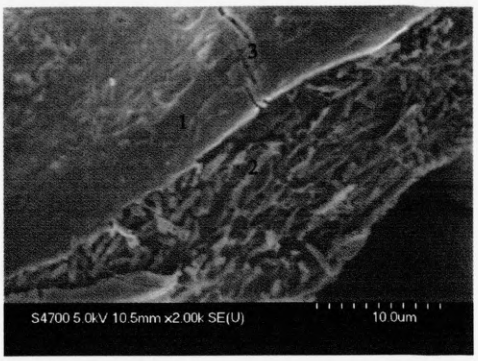
The objective of this study was to utilize SEM and ESEM techniques to analyse the biofilm architecture of monospecies bacterial biofilms (*Ps. aeruginosa* PAO1 and *E. coli* ATCC 10000) and binary bacterial biofilms and to illustrate any differences in the morphology, composition and physiology between control biofilms and the ones treated with BIT (tolerant biofilms).

Results & Discussion

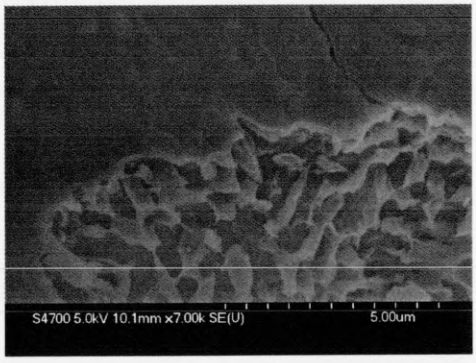
Figures 54a to **54c** demonstrate SEM images of *Ps. aeruginosa* PAO1 control biofilms grown on a Sorbarod filter. The images are a series of plates of the same area on the biofilm. **Figure 54a** illustrates a Sorbarod fibre encapsulated with biofilm and by increasing the magnification to 2000 times, a pseudo-three-dimensional view is obtained (**Figure 54b**). In this image, one can observe the EPS covering the whole biofilm, and bacterial cells embedded within the exopolymer matrix. There is a small fissure in the EPS, which is probably caused by sample preparation (dehydration events). When the magnification is increased to 7000 times, the spatial arrangement of the cells is clearly demonstrated. The cells are attached to each other and to the matrix *via* extracellular materials. At 11000 times, a closer view of the EPS fissure is given; the bacterial cells are organised along side the fissure. The individual cells connected with each other *via* extracellular materials are illustrated in **Figure 54e**. This series of images represents the biofilms architecture, which is a combination of the spatial arrangement of the cells, EPS and the extracellular spaces.



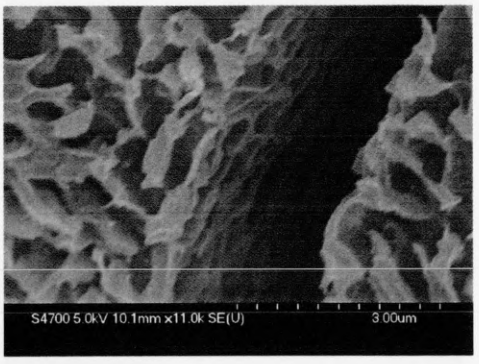
(a)



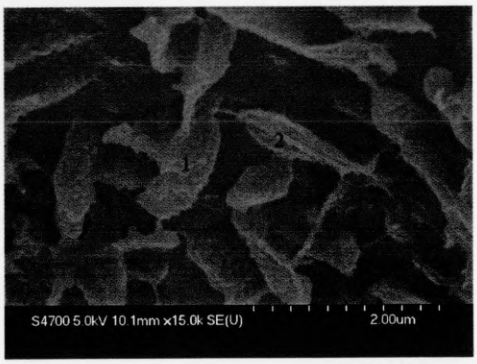
(b)



(c)



(d)

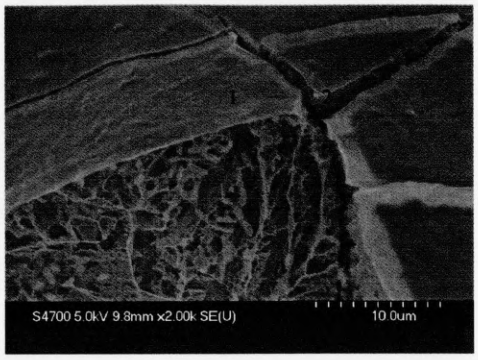


(e)

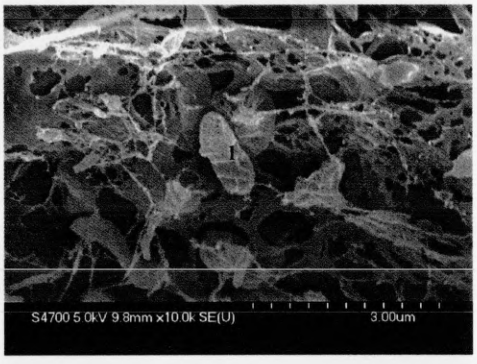
Figure 54: Scanning electron microscope images of a 48h *Ps. aeruginosa* PAO1 control biofilm grown on Sorbarod filters. **(a)** Sorbarod fibre encapsulated by biofilm (350x). **(b)** EPS (1) is covering the biofilm and bacterial cells (2) are embedded in the exopolymer matrix; a fissure (3) in the EPS (2000x). **(c)** The magnification is increased to 7000x exhibiting the torn edge of a biofilm. **(d)** The bacterial cells are organized along side the EPS fissure (11000x). **(e)** Two bacterial cells (1 & 2) connected to each other *via* extracellular materials (15000x).

Figures 55a to **55c** illustrate *Ps. aeruginosa* biofilm treated with 1.25 $\mu g\ mL^{-1}$ BIT. At 2000 times, which is the same magnification as in **Figure 54b** (control biofilm), the cells are deeply embedded in the EPS. After increasing the magnification to 10000 times (**Figure 55d**) fewer cells are observed, compared to control biofilm results (**Figure 54d**), and the bacterial EPS shows structural dehydration resulting in the disruption of the pseudo-3-dimensional structure of the biofilm. Comparing **Figure 54e** (control biofilm) with **Figure 55c** (resistant biofilm), one can observe the striking difference in the number of bacterial cells between the two images. The cells are still held together *via* extracellular materials.

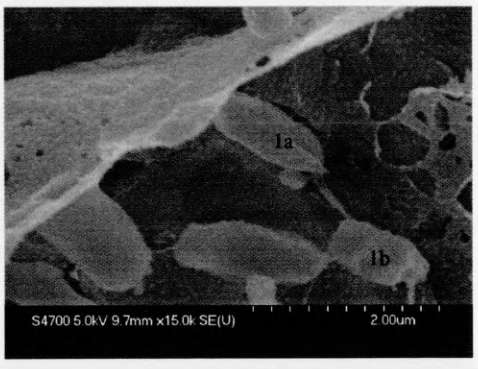
Figure 56a illustrates a SEM image of binary control biofilm (*Ps. aeruginosa* and *E. coli* growing on the same Sorbarod filter). The image indicates a monolayer of cells with conspicuous extracellular material associated with the bacterial cells. Micrograph (**Figure 56b**) reveals the change in biofilm structure following BIT treatment. Fewer cells are present and the spatial arrangement of the binary cells is completely disrupted. Comparing binary resistant biofilm (**Figure 56b**) with *Ps. aeruginosa* resistant biofilm (**Figure 56c**), one can demonstrate the significant effect of BIT on binary biofilm relative to that on *Ps. aeruginosa* biofilm. *Ps. aeruginosa* biofilm treated with BIT is actually more similar to binary control biofilm than to the tolerant, exposed one. However, the number of bacterial cells observed in **Figure 56a** appears to be far more than that in **Figure 56c**.



(a)

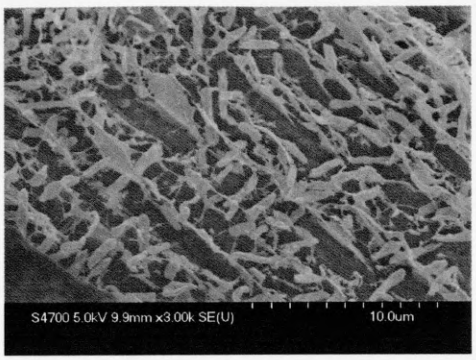


(b)

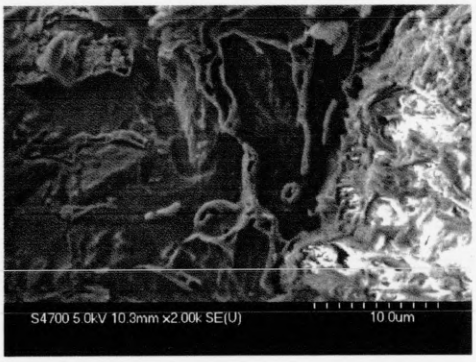


(c)

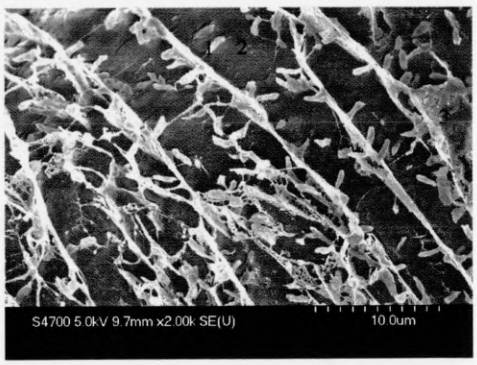
Figure 55: SEM images of 48h *Ps. aeruginosa* PAO1 biofilm treated with BIT grown on Sorbarod filters. **(a)** A pseudo-three dimensional view for the biofilm (2000x), which is fully covered by EPS (1). Note the fissure (2) within the EPS. **(b)** An observed reduction in the number of bacterial cells (1) and the EPS (2) show structural dehydration due to BIT treatment (10000x). **(c)** Two bacterial cells (1a & 1b) connected *via* extracellular materials (15000x).



(a)



(b)

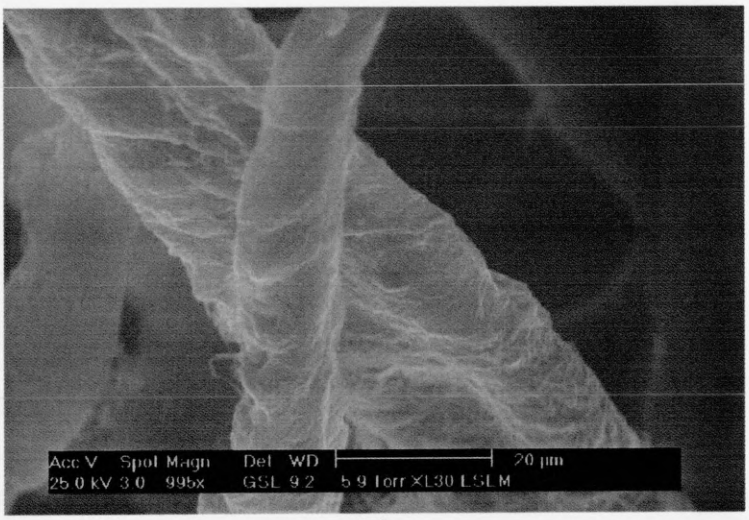


(c)

Figure 56: (a) Scanning electron micrograph of a 77h binary control biofilm grown on a Sorbarod filter (3000x). The image shows a monolayer of cells with conspicuous EPS associated with these cells. **(b)** SEM image of binary biofilm treated with BIT for 77h (2000x). Note the complete loss of spatial arrangement of cells and coagulation of EPS. **(c)** SEM image of *Ps. aeruginosa* PAO1 monospecies biofilm grown on Sorbarod fibre and treated with BIT for 48h (2000x). The spatial arrangement of cells is still observed. Fewer cells (1) embedded in the matrix (2) and the EPS (3) is associated with these cells.

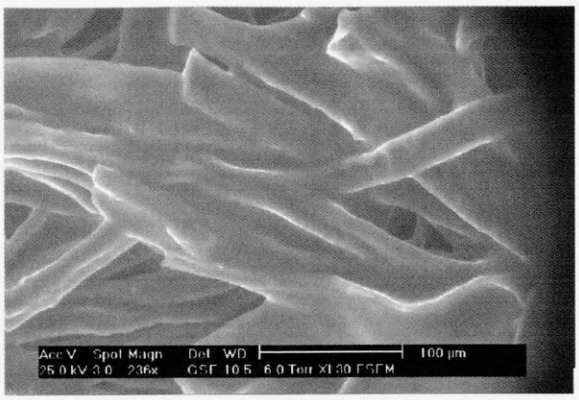
Figure 57a is an ESEM image of twisting Sorbarod fibres. The same fibres are encapsulated with control binary biofilm (**Figure 57b**). Micrograph (**Figure 57c**) shows the same area at a greater magnification (311x). EPS is covering the whole biofilm and it is not possible to distinguish any individual bacterial cells. Increasing the magnification to 622x, (**Figure 57d**) exhibits the gelatinous appearance of the wet biofilm. The environmental scanning electron micrographs of control binary biofilm demonstrate the true hydrated nature of the biofilm. Micrograph (**Figure 58a**) illustrates another area in the binary control biofilm, which shows the biofilm sheet (2) running between two encapsulated fibres (1a & 1b). There is a fissure in the biofilm sheet (3), which is an artefact due to sample preparation. **Figure 58b** shows EPS strand at a greater magnification (2505x).

Environmental scanning electron micrograph (**Figure 59a**) shows two different types of fibres; (1) is an un-encapsulated fibre, whereas fibre (2) is an encapsulated one, colonized by *E. coli* monospecies biofilm. Fibre B is shown in greater magnification in **Figure 59b**. The fibre is fully covered by the biofilm, but no individual bacterial cells can be distinguished. Micrograph (**Figure 59c**) illustrates a SEM surface image of an *E. coli* biofilm treated with BIT for 48 hours. The micrograph shows individual bacterial cells (1) fixed within the exopolymer matrix. There are gaps (2) in the EPS caused by SEM treatment (considered as artefacts). The topography of *E. coli* biofilm surface treated with BIT is completely different than that for binary biofilm (**Figure 59d**).

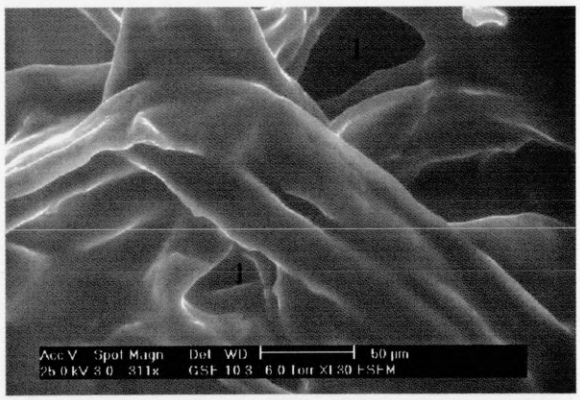


(a)

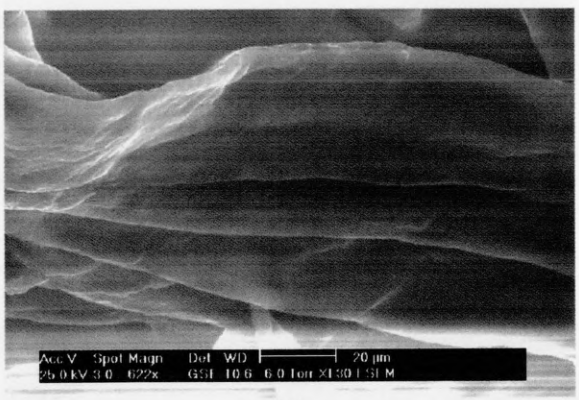
Figure 57 (Part 1): (a) Environmental scanning electron (ESEM) image of uninoculated Sorbarod fibres (995x).



(b)



(c)



(d)

Figure 57 (Part 2): (b), (c) & (d) ESEM of control binary biofilms grown on Sorbarod filters for 77h. The image demonstrates the true hydrated nature of the biofilm (236x, 311x & 622x, respectively). Note the presence of voids (1) in **Figure 57 (c)**.

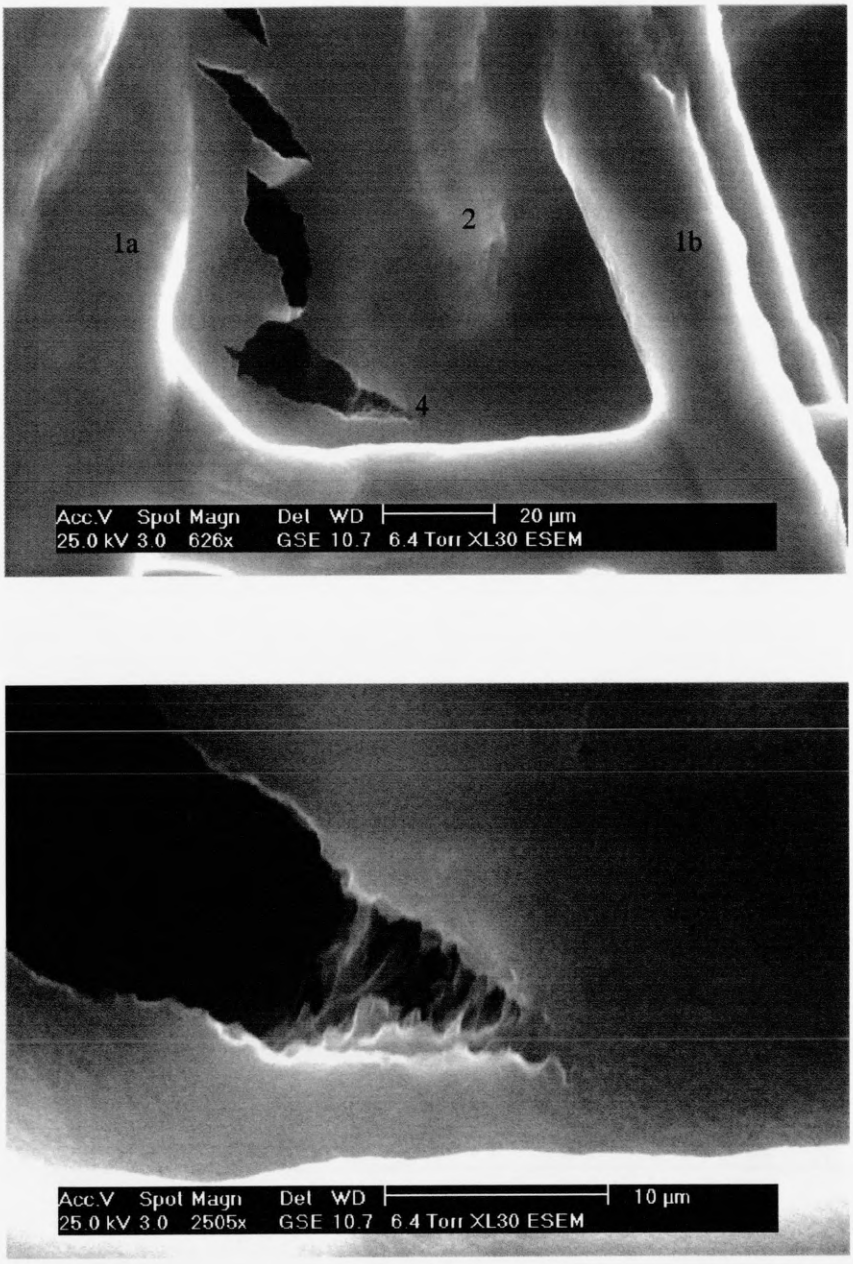
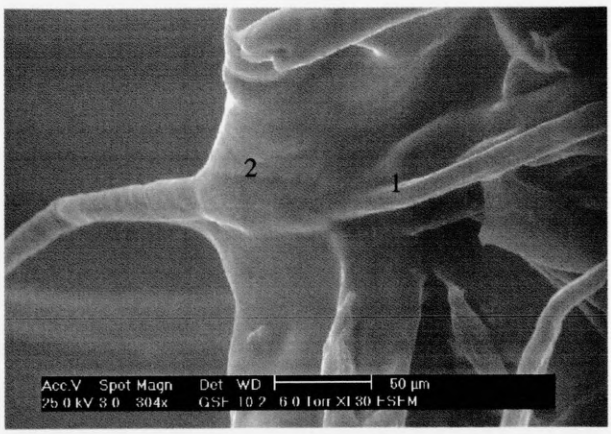
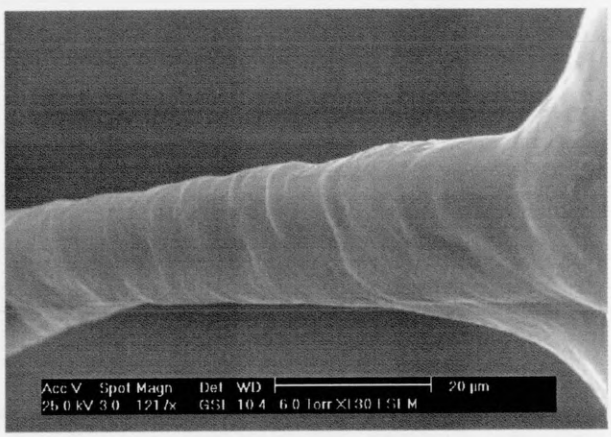


Figure 58: (a) Environmental scanning electron micrograph of a 77h control binary biofilm (626x). (1a & 1b) encapsulated fibres (2) biofilm sheet (3) fissure in biofilm sheet (4) EPS strands. **(b)** EPS strands at a greater magnification (2505x).

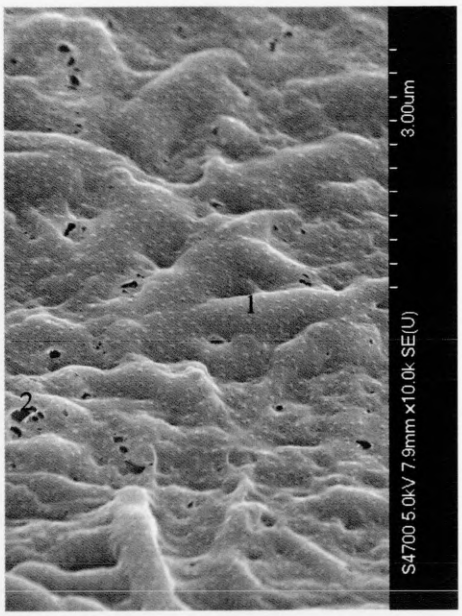


(a)

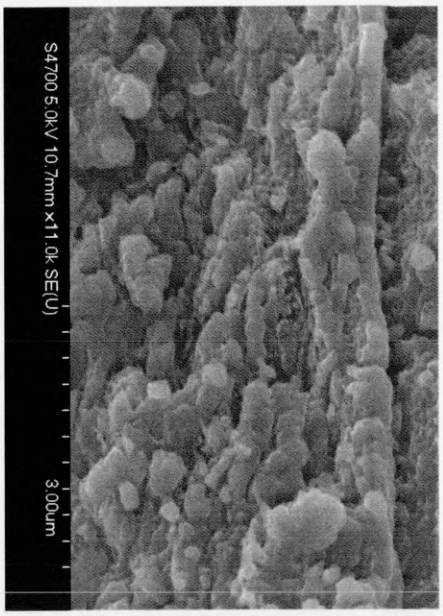


(b)

Figure 59 (Part 1): (a) ESEM image of *E. coli* control biofilm cultured in modified CDM for 48h (304x). (1) an un-encapsulated fibre (2) encapsulated fibre. **(b)** A Sorbarod fibre encapsulated by *E. coli* control biofilm (1217x).



(c)



(d)

Figure 59 (Part 2): **(c)** The SEM image shows the surface of *E. coli* biofilm treated with BIT for 48h (10000x). Note the individual encapsulated cells (1) and the gaps in EPS caused by sample preparation, considered as artefacts (2). **(d)** SEM image of 77h binary biofilm, treated with BIT (11000x). The image illustrates that the bacteria altered their morphology upon their exposure to the biocide. Note the coagulated surface of EPS.

Conclusions

Biofilms are not homogeneous in composition, but are complex matrices composed of microcolonies interspersed with channels allowing the movement of fluid and nutrients (Costerton *et al.*, 1995). Environmental microscopy allowed immediate visualization of the surface of the biofilm in its natural, hydrated viable state. The samples did not require extensive manipulation, fixation, dehydration and metal coating that the SEM samples required. However, it was impossible to image individual bacterial cells within the gel matrix. During samples treatments in SEM technique, much of the extracellular polymeric materials from the biofilms were dehydrated, decreasing the actual coverage by the biofilm of the surface and exposing bacterial cells. Comparing *Ps. aeruginosa* biofilm treated and untreated with BIT; the control biofilm revealed a greater number of bacterial cells and much lower amounts of EPS compared to that for the resistant biofilm. Exposing Binary biofilm cells to BIT caused the loss of the spatial arrangement of bacterial cells. The topography of the surface of *Ps. aeruginosa*, *E. coli* and binary biofilms is entirely distinct for each biofilm. Therefore, the SEM offered excellent resolution with the capacity to image complex shapes. Hence, each of the two techniques (SEM & ESEM) added a different dimension to our understanding of the spatial composition of biofilms. It is suggested that a combination of both techniques is required to overcome the problems of recognising artefacts and to give the most accurate picture of the true biofilm structure and organisation.

Chapter Eight:

Discussion and Conclusions

To prosper, every creature requires a measurement of safety from predators, ample nutrition (and water), and a place within a healthy community. It is apparent from the study of natural environments that bacteria are found most commonly associated with surfaces. These can be solid and inanimate interfaces (e.g. the surfaces of rocks in fluid media, pipelines used to transport fluids, the surfaces of medical implants such as pacemakers and catheters, etc) or softer animate interfaces (e.g. plant rootlets, intestinal tracts, etc) (Beveridge *et al.*, 1997). Rich, diverse microbial communities are found on teeth (i.e. dental plaque; Kolenbrander, 1993) as well as on the air-fluid interface of fresh water ponds. More recently, it has become apparent that those bacteria that inhabit the subsurface (whether the subsurface is in shallow sediments or soils, or in deep geological horizons) also prefer a "*surface mode of growth*" (Ghiorse & Wilson, 1988; Lawrence *et al.*, 1995; McLean *et al.*, 1996). Biofilms are found everywhere that life can exist. In fact, when cells exist in a biofilm, they can become 10 to 1000 times more resistant to the effects of antimicrobial agents (Evans & Holmes, 1987; Mah & O'Toole, 2001) than when they are free-living (planktonic).

The failure of biofilms to respond to antibiotics may arise through a number of mechanisms. These include (i) physical or chemical diffusion barriers to antimicrobial penetration into the biofilm, (ii) slow growth of the biofilm owing to nutrient limitation, (iii) activation of the general stress response and (iv) the emergence of a biofilm-specific phenotype (Foley and Gilbert, 1996; Morton *et al.*, 1998; Mah & O'Toole, 2001). Each of these mechanisms contributes to biofilm resistance and therefore, it is possible to suggest that multiple resistance mechanisms operate in concert within the biofilm community (Stewart, 2001).

The objective of this study was to induce tolerance in monospecies and binary biofilms (*Pseudomonas aeruginosa* PAO1 and *Escherichia coli* ATCC 10000) and to observe any variations in the outer membrane protein profiles between sensitive biofilm and eluate cells and those exposed to selected biocides. This was undertaken in order to explain the development of tolerance in biofilms towards antimicrobial agents.

Discussion

The exposure of *Pseudomonas aeruginosa* PAO1 biofilms to selected biocides (Benzisothiazolone (BIT), Thiomersal, Zinc Pyrithione (ZnPT), Sodium Pyrithione (NaPT) and Cetrimide).

The idea of inducing resistance in *Pseudomonas aeruginosa* PAO1 was previously mentioned by Brözel and Cloete (1994) and the Sorbarod filter for establishing a biofilm was previously developed by Hodgson *et al.* (1995). However, passaging biofilm cells growing on Sorbarod filters against a selected number of biocides has not been performed before. Therefore, combining the two methods is a novel approach for better understanding of the phenomena associated with biofilm tolerance. Biofilms of *Pseudomonas aeruginosa* PAO1 were passaged in the presence of sub-MICs of selected biocides (ZnPT, NaPT, BIT, Thiomersal and Cetrimide). In all cases both the biofilm and eluate cells exhibited increased MICs after each passage. After 10 passages in increasing MIC/4, there was an overall increase of 17-fold and 10-fold for biofilm and eluate cells respectively (in the case of BIT), 3.1-fold for adherent cells and 4-fold for eluate cells (in the case of Cetrimide) and 4.25-fold for both biofilm and eluate cells (in the case of ZnPT) from their initial MICs. However, once the cells were passaged in biocide-free medium, the MIC began to decrease immediately, but this value

did not return to the initial, pre-exposure MIC.

During the induction of tolerance towards ZnPT, there was no difference in the MIC values between biofilm and eluate cells (in the whole passaging process). For Cetrimide, there was a considerable difference in those values between adherent and eluate cells in the early Passages (1 to 7); after that the MIC stayed the same for both types of cells (Passages 8 to 13). However, in the case of BIT, a marked difference in the MIC value between adherent and eluate cells was observed in almost every passage. This difference might be due to quenching of BIT by the presence of accessible thiol groups (-SH) in the EPS that surrounds the biofilm. Fuller *et al.* (1985) suggested that cellular thiol groups are major target for BIT. The initial reaction of BIT with thiols results in the formation of S-(2-carbamoylphenylthio)-y-L-glutamyl-L- cysteinyl-glycine (Collier *et al.*, 1990b). Further reaction with thiols results in the formation and subsequent release of oxidized thiol-dimers and a reduced, ring-opened form of BIT (mercaptobenzamide; Collier *et al.*, 1990b). The mercaptobenzamide reacts further with BIT resulting in biocide dimers (Collier *et al.*, 1990b). Due to BIT mode of action, the presence of thiol in EPS will result in greater MIC for biofilm cells than that for eluate cells. Chapman *et al.* (1993) found that *E. coli* cells unable to produce glutathione were more sensitive to electrophilic biocides such as isothiazolones, whereas there was little difference in response to surface-active biocides such as benzalkonium chloride. In order to investigate this phenomenon a spectral analysis of BIT-EPS mixtures was performed. The results demonstrated the presence of non-protein thiol in the EPS of *Ps. aeruginosa* biofilm. Hence, BIT reacted oxidatively with those thiol groups, which partially explains the difference in MIC between eluate and biofilm cells when they were passaged in the presence of BIT.

The induction of resistance towards NaPT demonstrated a slightly different pattern than that of the previous biocides. The initial MIC values for the sessile cells and their

offspring in CDM was 48 μg mL^{-1} for adherent cells and 40 μg mL^{-1} for eluate cells. This is considerably higher than the initial MIC against ZnPT (4 μg mL^{-1} for both adherent and eluate cells) indicating that ZnPT demonstrates a greater activity towards *Ps. aeruginosa* PAO1 than NaPT. ZnPT interacts more strongly with the bacterial membrane phospholipid phosphatidylethanolamine than NaPT (hydrogen bonding), suggesting interactions with the orthophosphoric acid group and ammonium groups of the phospholipid head group (Dinning *et al.*, 1998b). There was no marked increase in MIC values throughout the passaging process. Following the biocide-exposure passages, the cells were passaged in biocide-free medium. Although the medium was free from any biocidal traces in three successive passages (11 to 13), the MIC value did not decrease. From the previous observations, one can demonstrate that the activity of NaPT was quenched by the biofilm structure. Dinning *et al.* (1998b) suggested the chelation of divalent metal cations present on the bacterial envelope by the pyrithiones, which would break down bacterial envelope configuration. The presence of such ions (Mg^{2+}, Mn^{2+} and Ca^{2+}) on the envelope maintains membrane integrity, the loss of which results in weakened binding of the Gram-negative outer membrane to the peptidoglycan layer *via* Braun's lipoprotein. The disrupted configuration of the bacterial cell envelope would then allow the passage of free pyrithione molecules across the envelope and into the cytosol. This in turn would result in the intracellular action of NaPT molecules and may allow them to interact with phospholipid head groups on the inner leaflet of the cytosolic membrane. However, this activity was quenched by the presence of EPS surrounding the biofilm. Biofilm exopolysaccharides can retain and capture nutrients, ions and other compounds and may be responsible for concentrating foci of extracellular factors of *Ps. aeruginosa* (Costerton *et al.*, 1987). In addition, biosorption of metals may occur in the EPS, and is thought to involve ion exchange reactions since EPS contains a high amount of negatively charged

functional groups like carboxyl, phosphate and sulphate groups (Wuertz *et al.*, 2001). Morton *et al.* (1998) stated that cationic biocides such as quaternary ammonium compounds (QACs) can adsorb to EPS molecules, thus reducing their concentration in the free aqueous phase where they exert their antimicrobial effects. On the other hand, Dinning *et al.* (1998b) suggested that the mode of action for the pyrithione biocides is not dissimilar to that of the QACs, which destabilize the ionic content of the bacterial membrane prior to disruption of the phospholipid bilayer.

Extended exposure of *Ps. aeruginosa* planktonic cells to NaPT, ZnPT, Cetrimide and BIT resulted in a gradual, stepwise increase in the observed MIC of these compounds up to Passage 10 where there was an overall increase of 2.8-fold (ZnPT), 2.1-fold (NaPT), 8.7-fold (Cetrimide) (Abdel Malek *et al.*, 2002) and 1.6-fold (BIT) (Winder *et al.*, 2000). Cultures from Passage 10 were then passaged on three further occasions in the absence of biocide. In those passages, all cultures exhibited a reduction in MIC as the cultures lost some of their tolerant properties.

From the previous comparison between MIC values for biofilm/eluate cells and those for planktonic cells, one can illustrate that the susceptibility of planktonic cells towards ZnPT was significantly greater than that of biofilm and eluate cells. However, cells from planktonic origin were more resistant against Cetrimide than their biofilm and eluate counterparts. It is possible that the higher numbers of bacterial cells in planktonic cultures (10^9) in comparison to those numbers, which had been harvested, on sacrifice, from the Sorbarod filter (10^7 to 10^8) may account for this unexpected result. Sessile growing bacterial cells were more resistant towards BIT in comparison to those growing in a planktonic situation. Therefore, the biofilm mode of growth protects the sessile bacteria from concentrations of antibiotics and biocides, which would swiftly kill planktonic cells (Hoyle and Costerton, 1991; O'Toole *et al.*, 2000).

Brooun *et al.* (2000) illustrated that cells librated from a biofilm into growth medium were considerably more resistant to tobramycin than planktonic cells. Therefore, this experiment suggests that cells become intrinsically more resistant when growing in the biofilm and retain part of this resistance even outside the biofilm.

It was not possible to induce resistance in biofilm and eluate cells towards thiomersal in CDM. Studies from planktonic cultures (Winder *et al.*, 2000) demonstrated the same result. Therefore, one can suggest that unlike NaPT, the biofilm structure was not the reason for quenching the activity of thiomersal. The inability of inducing a classical, gradual resistance in *Ps. aeruginosa* cells towards this biocide may be due to the combined stress effect of growing the cells in a nutrient limited medium and biocide exposure.

The resistance observations indicated that the resistance mechanism was dependent upon the presence of the biocide, but it was a gradual adaptation and not an instantaneous one, (i.e. it was not the result of single point mutation). When the cells were cultured in biocide-free medium the MIC decreased, indicating that the biocide presence was required for the mechanism of resistance. This type of resistance could be considered as phenotypic tolerance, which was first described by Tuomanen (1986) to describe the non-genetic resistance of bacteria to antibiotics. A major feature of the phenotypic tolerance was that the bacteria were growing slowly and many antibiotics, which are highly bactericidal for rapidly growing cells, are less effective on slowly growing cells (Cozens *et al.*, 1986). Although, the MIC decreased that value did not revert to the initial MIC. Therefore, it is imperative that a small proportion of the population be able to avoid the biocidal effect and ultimately be responsible for the survival and recovery of the community. Survivors of a sub-lethally treated biofilm might therefore possess gene modifications that could confer resistance towards the biocide (*biofilm phenotype*; Cochran *et al.*, 2000a). Following removal of an inimical stress, those survivors

would grow rapidly in the presence of nutrients released from their lysed community partners and the community would become restored (Brooun *et al.*, 2000). However, in this study, there is a possibility that further passaging in biocide-free media could result in a complete reversion to the initial MIC. This was not performed in these studies due to both time constraints and that the mechanism and nature of reversion of resistance was not a primary aim of these experiments. Hence, the observation of a small loss in resistance in all cases suggests that the resistance-reversal mechanism is a gradual, step-wise adaptation, similar to the mechanism for the development of resistance.

Cross-Resistance between antimicrobial compounds

Following the introduction of penicillin into human therapeutics in the 1940s and throughout the past 60 years, antibiotics have been used and misused (Foley & Gilbert, 1996). Developed originally to treat human infectious diseases, their properties in veterinary, animal and plant agriculture and aquaculture were applied soon thereafter. Broad use has created a strong selective pressure, which has consistently resulted in the survival and spread of resistant bacteria, providing an excellent example of Darwinian evolution (Caldwell *et al.*, 1997b). The emergence of resistance has revealed multiple and complex mechanisms by which resistance genes spread across the bacterial kingdom, with apparent disregard for species barriers. However, the bacterial evolutionary response has not been limited to the acquisition of resistance genes. Bacteria have also developed means for stabilizing the resistance phenotype, thus dashing initial hopes of reversing resistance by simply reducing antibiotic use (Barbosa & Levy, 2000).

There are similarities between antibiotic and biocide resistance, and Gram-negative bacteria that have developed resistance to biocides may also be insusceptible to some

antibiotics, possibly as a result of outer membrane changes (Russell, 2000; Russell, 2001). 2-Hydroxyphenylethers are a class of compounds that exhibit broad-spectrum antimicrobial activity. Triclosan is the most potent and widely used member of this class (Bhargava & Leonard, 1996; Chuanchuen *et al.*, 2001), and is used in hand soaps, lotions, toothpastes and oral rinses, as well as in fabrics and plastics (Travis, 2000). It was long thought to act as a non-specific biocide (Travis, 2000), but recent biochemical and genetic studies have shown that triclosan acts on a defined bacterial target in a fatty acid biosynthetic pathway, enoyl-acyl carrier protein (ACP) reductase (FabI) (McMurry *et al.*, 1998a; Hoang & Schweizer, 1999). Some bacteria possess triclosan-resistant enoyl-ACP reductase homologues (FabK), and to date *Ps. aeruginosa* is unique among Gram-negative bacteria in that it possesses both triclosan-sensitive and triclosan-resistant enzymes (Heath & Rock, 2000). Alterations in FabI active-site residues confer resistance to triclosan (McMurry *et al.*, 1998a). Of particular concern is that such amino acid changes, selected by exposure to triclosan, lead to cross-resistance with other antimicrobial agents (Heath *et al.*, 1999), including clinically useful front-line drugs, since some mutations leading to triclosan resistance in *Mycobacterium smegmatis* also cause resistance to isoniazid (McMurry *et al.*, 1999; Travis, 2000). Chuanchuen *et al.* (2001) demonstrated that exposure of triclosan-sensitive mutants of *Ps. aeruginosa* to triclosan confers an efflux-mediated resistance to ciprofloxacin. Moreover, triclosan is a substrate of multidrug efflux pumps in clinical and laboratory *E. coli* strains (McMurry *et al.*, 1998b). 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 selected biocides (ZnPT, NaPT, Cetrimide and BIT) may be resistant to the other biocides. The results of the cross-resistance study, investigating the possibility of cross-resistance between the induced resistant biofilm and eluate cultures of *Ps.*

aeruginosa towards ZnPT and BIT, indicate the emergence of cross-resistance with these two biocides. For both biocides, there were marked increases in the MICs of the cross-resistant test strains towards the other three test biocides over the original MIC. This indicates that the induced tolerance developed against ZnPT or BIT is transferable to the other biocides. However, the biochemical mode of action is different between the isothiazolones and the pyrithiones. These results illustrate that the resistance profiles exhibited by both the pyrithione and the isothiazolone biocides are similar in *Ps. aeruginosa* PAO1. These results are similar to those obtained from previous work on cultures of planktonic origin (Abdel Malek *et al.*, 2002), which showed that ZnPT exhibited cross-resistance with both NaPT and Cetrimide.

In the case of Cetrimide, there was observed cross-resistance between this biocide and NaPT for both biofilm and eluate cells. However, there was no increase in the original MIC values of the cross-resistant test strains (Cetrimide) towards ZnPT. This was observed with both biofilm and eluate cells. The planktonic results showed the same trend, in that there was a cross-resistance between Cetrimide and NaPT, but this was not the case between Cetrimide and ZnPT.

For NaPT, there was no observed cross-resistance between this biocide and the other three. This result was expected because it was not possible to induce resistance in *Ps. aeruginosa* biofilm or eluate cells towards NaPT, as was the case with the other three biocides.

The effect of nutrient limitation on *Pseudomonas aeruginosa* PAO1

When a bacterial cell culture becomes starved for a particular nutrient, it slows its growth. Transition from exponential to slow or no growth is generally accompanied by an increase in resistance to antibiotics. Slow growth of bacteria has been observed in mature

biofilms (Brown *et al.*, 1988; Wentland *et al.*, 1996). Because cells growing in biofilms are expected to experience some form of nutrient limitation, it has been suggested that this physiological change can account for the resistance of biofilms to antimicrobial agents. In order to eliminate this possibility from the current study, a nutrient limitation experiment was performed for both planktonic and biofilm *Ps. aeruginosa* PAO1 cells. For planktonic cultures, the growth rates of this bacterium under nutrient limitation (carbon and nitrogen) were significantly affected. Cultures were more responsive to carbon limitation than to nitrogen limitation. The K_S (Monod constant) was 1M for carbon and 1.43M for nitrogen, which suggests that *Ps. aeruginosa* PAO1 has a greater requirement for a carbon source than for a nitrogen source. For biofilm cultures, the eluate cell numbers decreased in a step-wise fashion with the decreasing concentrations of the nutrient. Growth in a biofilm may lead to improved viability, as it allows accessibility to nutrients that are within the surface or have absorbed to it and to the metabolic products of other microorganisms (Boyd and Chakrabarty, 1995). In response to stress, when essential cations or nutrients are required, anionic EPS would sequester them, increasing the gradients across the cell membranes (Harder and Dijkhuizen, 1983). Alternatively, excretion of the charged polymer may provide the driving force for importation of other charged ions (Wilkinson, 1958; Weiner *et al.*, 1995). Therefore, it is possible to conclude that the induction of resistance in *Ps. aeruginosa* PAO1 biofilm and eluate cells towards selected antimicrobial agents, was related to biocide challenge and not nutrient limitation.

The exposure of monospecies *Escherichia coli* ATCC 10000 and Binary biofilms (*Pseudomonas aeruginosa* PAO1 and *Escherichia coli* ATCC 10000) to Benzisothiazolone (BIT)

Genetic studies of single species biofilms have shown that they form in a series of steps (Watnick & Kolter, 1999), require intercellular signaling (Davies *et al.*, 1998) and demonstrate a profile of gene transcription that is distinct from that of planktonic cells (Prigent-Combaret *et al.*, 1999). However, in natural environments the biofilm is almost invariably a multispecies microbial community. Increased species diversity within the biofilm may provide spatial and temporal niches not available within the monoculture or may create microenvironments within the community (Korber *et al.*, 1993; Lens *et al.*, 1993; DeBeer *et al.*, 1994). In addition, species composition of binary or mixed biofilms is important in determining the survival of component species towards antimicrobial agents. *Pseudomonas fluorescens* has been shown to protect *Salmonella typhimurium* against chlorine (Leriche and Carpentier, 1995). Aldsworth *et al.* (1998) showed that *Salmonella typhimurium* can be protected from environmental stress by the presence of competing microorganisms, perhaps by reducing the impact of oxidative damage by consuming oxygen. Coliform survival is enhanced in mixed population biofilms (Camper *et al.*, 1996). Several studies have shown the impact of antimicrobial agents on biofilms (Costerton *et al.*, 1995). However, the induction of tolerance in *E. coli* and binary biofilm cultures (binary biofilm composed of equal numbers of *E. coli* and *Ps. aeruginosa* ; hereinafter, refered to as binary biofilms) towards BIT has not been performed before. This could be considered a novel and beneficial idea to enable our better understanding of the development of resistance in natural biofilms. Initial experiments investigating the development of resistance in *E. coli* and binary biofilms towards BIT were performed by repeated passage in MIC/4 of the biocide (Chapter 6). The gradient plate

method using Chromogenic *E. coli*/Coliform agar was used for MIC determination in order to differentiate between *Ps. aeruginosa* (straw coloured) and *E. coli* (purple coloured) colonies in binary biofilms.

Passaging of E. coli biofilms

During the induction of resistance in *E. coli* biofilm and eluate cells towards BIT in modified CDM, the original, pre-exposure MIC was 5 μg mL^{-1} (biofilm cells) and 4 μg mL^{-1} (eluate cells). At Passage 1 the MIC decreased to 1.88 μg mL^{-1} and 0.98 μg mL^{-1} (biofilm and eluate cells, respectively). Following the first exposure to MIC/4 of biocide, the MIC started to increase and by Passage 5 (the point at which the cells are deemed resistant), there was an overall increase of 9.6-fold for the adherent cells and 10.2-fold for the eluate cells. Upon removal of the cells from the presence of the biocide, the MIC immediately deceased and at Passage 8 the MIC was 4.96 μg mL^{-1} (biofilm cells) and 3.95 μg mL^{-1} (eluate cells).

The results obtained from passaging *E. coli* biofilm and eluate cells with BIT demonstrate a marked difference between biofilm MICs and those for eluate cells (as was the case in *Ps. aeruginosa* monospecies biofilm and eluate cells). In addition, a complete reversion to the original, pre-exposure MIC value (Passage 0) for both biofilm and eluate cells was observed. This suggests that the induced tolerance is unstable and that the developing tolerance was the result of a phenotypic adaptation rather than a mutation.

Passaging of Binary biofilms

The initial MIC for sensitive *Ps. aeruginosa* cells towards BIT during the induction of resistance was 5 μg mL^{-1} (for both biofilm and eluate cells). Following the initial exposure to the presence of the biocide, there was a marked difference in MIC value between both type of

cells and that value increased gradually with every successive passage in the presence of biocide. At Passage 5, the adherent and eluate cells exhibited an increase of 13.2-fold and 12.4-fold, respectively, over their original, pre-exposure MIC. Once the cells were removed from the presence of the biocide, the MIC immediately began to decrease, however, by Passage 8 the MICs did not return to their pre-exposure values.

Passaging *Ps. aeruginosa* as monospecies and binary biofilms yielded two different results. The initial MIC value for *Ps. aeruginosa* growing as a binary biofilm was much greater than that as a monospecies biofilm. This result indicates that BIT demonstrates a greater activity towards *Ps. aeruginosa* as a monospecies biofilm than when it is part of a binary biofilm. Following the first passage, there was a significant increase in MIC values (for both monospecies and binary biofilms). At Passage 10 (monospecies biofilms), there was an overall increase of 17-fold and 10-fold (biofilm and eluate cells, respectively) over the initial MIC. However, in binary biofilms there was a 13-fold (biofilm cells) and 12-fold (eluate cells) increase in the MIC from the original value after only 5 successive passages in the presence of biocide. Therefore, this suggests that the induction of tolerance in *Ps. aeruginosa* binary biofilms was faster than that in monospecies biofilms.

For *E. coli* cells growing as binary biofilms, the original MIC was 5 $\mu g\ mL^{-1}$ for both biofilm and eluate cells. Upon initial exposure of cells towards MIC/4 of BIT cultured in modified CDM, the MIC for biofim cells (13.44 $\mu g\ mL^{-1}$) was higher than that for eluate cells (10.56 $\mu g\ mL^{-1}$) and this was the case during the whole passaging process. For the following passages the MICs increased significantly and by Passage 5, there was an overall increase of 12.4-fold and 10.5-fold (biofilm and eluate cells, respectively). Once the cells were cultured in the absence of biocide, a significant decrease was observed, indicating that the mechanism of resistance was dependent upon the presence of the biocide.

In comparing these results with the ones obtained from passaging *E. coli* as a monospecies biofilms. It is obvious that the survival of *E. coli* upon exposure to BIT is enhanced by being part of a binary biofilm. *Ps. aeruginosa* provided a significant protection for *E. coli* against BIT. Whiteley *et al.* (2001a) illustrated that *Vogesella indigofera*, a betadine-resistant microorganism, enhanced the survival of *Pseudomonas putida*, a betadine-susceptible bacterium in mixed cultures. Therefore, resistant bacterial components of binary biofilms may protect more susceptible bacteria, again emphasizing that the induction of tolerance in binary biofilms towards a selected biocide is a complex phenomenon, influenced by community composition, biocide concentration and growth rate. As was the pattern in monospecies biofilms, the gradual adaptation in MIC values (for binary biofilms) indicates that the mechanism of resistance is not a mutational event, but a specific intracellular phenotypic mechanism.

The Biofilm Phenotype and its association with the development of antimicrobial resistance within biofilms

An emerging idea in the antimicrobial resistance field is that a biofilm-specific phenotype is induced in a sub-population of the community that results in the expression of active mechanisms to combat the detrimental effects of antimicrobial agents (Cochran *et al.*, 2000a; Maira-Litrán *et al.*, 2000a). Furthermore, it is possible that all or just a subset of these biofilm cells can express increased resistance to antimicrobial agents. Recent work has focused on the identification of genes that could contribute to this increased-resistance phenotype (Kuchma & O'Toole, 2000; Whiteley *et al.*, 2001b).

Many bacteria carry genes specifying multidrug resistance efflux pumps (MDRs). All MDRs share the unique functional property of discriminating between a fairly broad array of

chemically unrelated antimicrobials on one hand and all cellular substances on the other hand. Most toxins have to be fairly hydrophobic to cross the cell membrane, while cytoplasmic molecules have to be hydrophilic to stay within the cell. MDRs use this difference in polarity to discriminate between these two broad groups of compounds (Lewis *et al.*, 1997). In *Ps. aeruginosa* there are four known multidrug-efflux pumps (Chapter 1) and there are several other putative pumps that have been identified by the *Ps. aeruginosa* genome project (Whiteley *et al.*, 2001b). Brooun *et al.* (2000) suggested the importance of one of these pumps in the resistance to the antibiotic ofloxacin. Using strains of *Ps. aeruginosa* that either lacked or over expressed the MexAB-OprM pump, it was shown that at low concentrations of ofloxacin, biofilms lacking the pump were more susceptible to this drug than biofilms that over expressed the pump. However, for a different quinolone, ciprofloxacin, there was no difference. Another study by De Kievit *et al.* (2001) showed that the four characterized efflux pumps do not play a role in the antibiotic-resistant phenotype of *Ps. aeruginosa* biofilms.

In *E. coli*, the expression of multi-drug-resistance operons such as *mar* and efflux pumps such as *acrAB*, has been shown to be up-regulated by exposure to sub-effective concentrations of antibiotics, such as tetracycline and chloramphenicol (George & Levy, 1983a; Ma *et al.*, 1993) and to xenobiotics such as salicylates and triclosan (Alekshun & Levy, 1999). However, studies by Maira-Litrán *et al.* (2000b) showed that neither *mar* nor *acrAB* is induced by sub-lethal treatment of biofilms against ciprofloxacin. On the other hand, constitutive expression of *acrAB* protected the biofilm against low concentrations of ciprofloxacin. Therefore, as was the case with the *Ps. aeruginosa* studies, the question of whether induction of pumps is one of the key alterations conferring resistance to biofilm cells awaits further experimentation.

Another resistance mechanism that can be induced in biofilm cells is the alteration of the membrane-protein composition in response to antimicrobial agents. This change could result in decreased permeability of the cell to these compounds (Mah & O'Toole, 2001). A study by Prigent-Combaret *et al.* (1999) demonstrated that the expression of OmpC and three other osmotically regulated genes was increased in *E. coli* biofilm cells compared with planktonic cells. This result suggests that bacteria in a biofilm are living in an environment of increased osmotic stress. Thus, the environmental conditions within the biofilm can lead to alterations within the cell envelope that protect the bacteria from the detrimental effects of antimicrobial agents (Mah & O'Toole, 2001). The alterations of the OMPs in Gram-negative bacterial biofilms may not be the direct mechanism of resistance, but it is often an indication of some other resistance mechanism occurring within the biofilm cell. Therefore, the OMP profiles in the biofilm and eluate cells, resistant towards selected biocides, from this study were investigated to determine if any alterations were observed.

The Observations of OMPs shifts in *Pseudomonas aeruginosa* PAO1 biofilms

The OMP profiles of selected passages of *Ps. aeruginosa* biofilm and eluate cells exposed to MIC/4 of selected biocides (ZnPT, NaPT, Cetrimide and BIT) were investigated to determine whether any alterations could be related to the observed resistance to these biocides. An outer membrane protein in the range of 36.5 kD and 41 kD was observed to disappear in biofilm and eluate cultures exposed to MIC/4 concentrations of the selected biocides. There are slight variations in the estimated molecular weights of this OMP (Biofilm cells; BIT (38 kD), Cetrimide (36.5 kD). Eluate cells ; ZnPT (41 kD), NaPT (39.2 kD)), as this analysis was performed manually. Therefore, any slight variation will affect the Rf value and thus, the molecular weight assignations. Previous studies on planktonic cultures by Brözel

and Cloete (1994) demonstrated the presence of a mobile OMP at 35 kD, designated as T-OMP. Following the exposure of *Ps. aeruginosa* sensitive cells to Kathon™ (a mixture of 1.15% CMIT & 0.35% MIT), the T-OMP disappeared. Another study by Winder *et al.* (2000) illustrated the presence of an outer membrane protein with a molecular weight of 36.577 kD in control unexposed planktonic cells. As the cells were passaged in CDM containing BIT, the T-OMP disappeared. Similar studies on planktonic cultures passaged against the pyrithione biocides showed the presence of T-OMP in sensitive control cells. When the cells were exposed to the biocide (ZnPT, NaPT or Cetrimide), the T-OMP disappeared. However, Winder *et al.* (2000) and Abdel Malek *et al.* (2002) used an imaging software package to estimate the molecular weights of proteins, which may explain the observed difference in the molecular weight of this OMP (T-OMP) between the current study and these previous studies. Once the cells were cultured in biocide-free medium, T-OMP reappeared, while the passaged cultures still exhibited some residual tolerance towards those compounds. This suggests that while T-OMP appearance and disappearance are intimately linked to biocide susceptibility in *Ps. aeruginosa* PAO1, T-OMP itself is not the sole cause of this decreased susceptibility. This would also indicate that T-OMP is not an isothiazolone-specific OMP, as suggested by Brözel and Cloete (1994), but merely an OMP, the synthesis of which is adversely affected by the presence of biocides of differing mode of action (Abdel Malek *et al.*, 2002).

The Observations of OMPs shifts in *E. coli* and Binary biofilms

Investigating the outer membrane protein profiles of *E. coli* and binary biofilms illustrated some variations between both types of biofilms. However, an outer membrane protein in the range of between 23 and 26 kD was observed to have disappeared following the exposure of *E. coli* (biofilm and eluate) cells to BIT. This protein reappeared when the

resistant cells were passaged in the absence of biocide. For binary (biofilm and eluate) cells, an OMP with a molecular weight of between 30.4 and 32 kD appeared in control, unexposed cells and disappeared from resistant cells. It is possible to suggest that this is the same protein that appeared in *E. coli* biofilm and eluate cells. However, when both bacteria (*Ps. aeruginosa* and *E. coli*) were grown together on a single Sorbarod filter (as a binary biofilm) the molecular weight of this OMP altered to an average of 31.2 kD. After the removal of BIT from the medium, this protein reappeared. Hence, indicating that the disappearance / reappearance of this OMP is just a phenomenon related to the presence / absence of biocide and not the direct cause of resistance or tolerance.

Analyzing the OMP profiles of monospecies biofilms (*Ps. aeruginosa* and *E. coli*) and binary biofilms using the 2-D-PAGE did not yield evidence of the disappearance / reappearance of any specific proteins. However, **Tables 24, 25, 34** and **35** illustrate significant variations between biofilm and eluate cells, on one hand and sensitive and resistant cells, on the other hand. A small number of outer membrane proteins appeared in both sensitive and resistant cells, others appeared with alterations in their molecular weights or isoelectric points. It is also obvious that some OMPs were lost from the cells following the induction of resistance. Others appeared as novel OMPs in the resistant cells. Therefore, this suggests that the development of resistance in *Ps. aeruginosa* and *E. coli* monospecies biofilms and binary biofilms is accompanied by the induction of multiple mechanisms of resistance and cannot be explained by the disappearance and reappearance of a single protein.

Conclusions

The first aim of this study was to induce resistance in monospecies *Ps. aeruginosa* biofilms towards selected biocides (ZnPT, NaPT, Cetrimide, BIT & Thiomersal). The idea of induction of resistance in *Ps. aeruginosa* PAO1 to isothiazolone biocides was taken from a method previously described by Brözel and Cloete (1994) (Chapter 1). The mechanism of resistance to the selected biocides outlined above was a gradual adaptive process, dependent upon the presence of sub-MICs of the biocides. This was indicated from the MIC values obtained from the passaging process. The induction of resistance to the three biocides (ZnPT, Cetrimide and BIT) was seen to occur in a step-wise fashion. However, upon removal of the presence of the biocides the MIC values decreased, indicating that the biocide presence is required for the mechanism of resistance / tolerance to be exhibited. Following the induction of tolerance in *Ps. aeruginosa* monospecies biofilms, the second aim was to establish a binary biofilm under controlled growth rate and to induce tolerance in dual species (binary) biofilms towards BIT. As was the case with monospecies biofilms, the mechanism of resistance in binary biofilms towards this biocide was a gradual adaptive process dependent upon the presence of biocide. The physiological alterations of biofilm and eluate cells involved in biocide exposure were examined using contemporary methods and techniques, which in turn have led to an extension and improvement of our knowledge surrounding the development of resistance towards biocides. Concurrent shifts in OMPs were monitored, however, the results from these studies did not yield a striking disappearance / reappearance of any specific protein. However, there were significant differences in the OMP profiles between sensitive and resistant biofilm and eluate cells.

This project has yielded novel information and techniques regarding the use of

passage approaches to develop antimicrobials tolerance and resistance in both monospecies and binary species biofilms of medically important bacteria. The results from these experiments suggest that it is possible to use these models to investigate the results of environmental exposure of bacteria to sub-MICs of biocides and develop an understanding of their subsequent tolerance and resistance characteristics.

Suggestions for Further Work

- Investigations into the constitution and effects of bacterial EPS from both monospecies and binary species biofilms on the mode of action of the biocides selected in this project. This would include biochemical approaches to purify and separate out the constituents of EPS and the testing of these compounds at a chemical level.
- Further studies on the difference observed in the ability of ZnPT and NaPT to develop resistance in biofilm populations. This would include analysis of the action of EPS on NaPT and determination of the internal mode of action of NaPT in bacterial cells.
- Investigate the possibility of other multi-drug-resistance efflux pumps being induced in response to life in a biofilm. This would involve the use of metabolic inhibitors to investigate the role of these structures in biofilms exposed to biocides.
- Further studies must focus on identifying the genes (by using DNA microarrays), which are activated in biofilms following the exposure to antimicrobial agents. Identifying these genes will be of great use in the development of rapid screens for agents that block biofilm maintenance.
- Investigate a possible role of quorum sensing in the biocide tolerance developed by biofilms.

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