Studies on the Effects of Sub-Minimal Inhibitory Concentrations of Antibiotics on the Virulence Factors of Biofilm Bacteria

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Director of Studies

Abstract

Pseudomonas aeruginosa is a notorious nosocomial opportunist. Planktonic forms of this pathogen have been traditionally studied for its pathogenicity. Such studies have shown that sub-minimal inhibitory concentrations (sub-MICs) of antibiotics are able to negatively modulate pathogenicity. However, more recent findings suggest a biofilm basis of infection. In this study, monospecies and binary biofilms of *Pseudomonas aeruginosa* ATCC 15692 (PAO1) and *Escherichia coli* ATCC 10000 were investigated for their pathogenic potential using resistance and virulence as key pathogenic determinants, in the presence of sub-MICs of selected antibiotics (Ampicillin, Nalidixic acid and Streptomycin).

MICs of biofilms were observed to be at least 7-fold greater than those of the corresponding planktonic form of the same species (as judged from results obtained from MIC experiments). SDS-PAGE and 2D-PAGE analysis indicate alteration of outer membrane proteins (OMPs) within the envelope of the pathogen in sub-MIC antibiotic treated samples. The observed rearrangement of lipopolysaccharide (LPS; as observed in LPS gel experiments) may also contribute to the pathogens increased tolerance to antibiotics within the biofilm state.

While LPS changes may possibly help the biofilm bacteria escape host immune system *in vivo*, more direct evidence of increases in virulence of the pathogen comes from investigation of its secreted proteases and cytotoxins (leucocidin). Virulence-specific azocasein and micro-culture tetrazolium (MTT) assays against both monospecies and binary biofilms of *Pseudomonas aeruginosa* indicate significant increases in virulence potential of proteases and cytotoxins, respectively. These results were further substantiated in phase contrast microscopy images showing advanced stages of oncosis in tissue cultured mouse spleen myeloma (Sp2) cells treated with leucocidin isolated from *Ps. aeruginosa* treated with sub-MIC of ampicillin (8 $\mu g\ mL^{-1}$).

The results reported in this thesis provide evidence of observed increases in virulence and pathogenicity in biofilm cells of *Pseudomonas aeruginosa* in the presence of sub-MICs of selected antibiotics, *in vitro*. Although these findings are those of *in vitro* experiments, they may have significant implications regarding the usage and therapeutic control of antibiotics in clinical situations.

Dedication

This thesis is dedicated to His Majesty King Abdullah II of the Hashemite Kingdom of Jordan whose wisdom and leadership are a true gift to his people. I also dedicate this thesis to the people and land of my birth, Jordan.

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All praises is to Allah, I thank him for giving me the patience, good health, and the ability to complete my research.

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Figure 63: Schematic representation illustrating a possible biofilm model of pathogenesis in presence of sub minimal inhibitory concentrations of antibiotics (ampicillin, nalidixic acid and streptomycin). In the absence of antibiotics (Figure 63A), biofilm bacteria () secrete proteases () and leucocidins () having basal levels of virulence, and may be partly responsible for virulence. The ratio of damaged epithelial cells () to healthy epithelia () is low. Similarly the ratio of damaged leucocytes or white blood cells () to healthy ones ( ) is also basal. However, in the presence of antibiotics () (Figure 63B), although there is no significant increase in the levels of production of virulence factors (proteases and leucocidins), the activity of these determinants significantly increase causing damage to more cells.

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Chapter One: Introduction

Background and Significance

The Gram-negative human pathogen *Pseudomonas aeruginosa* is adept at infecting different tissues and organs. It is considered to be an opportunistic pathogen as it causes infections in individuals with neutropenia or whose normal immune status is compromised in some way. Although most infections caused by *Pseudomonas aeruginosa* are hospital acquired, community acquired infections in patients have also been reported (Arancibia, *et al.* 2002; Weingarten, *et al.* 2003). Recent epidemiological statistics on nosocomial infections (Table 1) indicate significant frequencies of occurrence of *Pseudomonas aeruginosa* infections (Jarvis 2003).

Table 1: Nosocomial infections attributed to *Pseudomonas aeruginosa*. Adapted from Jarvis (2003)

Site of Infection	Percentage caused by *Pseudomonas aeruginosa*	Rank relative to other bacterial causes
Respiratory tract	17%- 21%	1 or 2
Surgical site	11%- 15%	4
Urinary tract	10%- 13%	3
Blood stream	4%	5

During the 1960s when effective anti-pseudomonal antibiotics were unavailable, mortality rates due to *Pseudomonas* associated bacteraemia were as high as 90% (Schimpf, *et al.* 1974). With the subsequent development of effective anti-pseudomonal antibiotics, substantial improvement in treatment outcomes was observed. However, *Pseudomonas aeruginosa* still remains a threat to public health

with a high rate of morbidity and mortality ranging from 16% to 61% (Chatzinikolaou, *et al.* 2000; Maschmeyer & Braveny 2000).

Table 2: Antibiotics used in treatment of *Pseudomonas aeruginosa* infections
Adapted from Hauser and Sriram (2005)

Antibiotics	Class
Piperacillin sodium (Piperacil)	Extended spectrum penicillin
Ticarcillin disodium (Ticar)	Extended spectrum penicillin
Piperacillin and tazobactam sodium (Zosyn)	β-Lactam + β-Lactamase inhibitor combination
Ticarcillin and clauvulanate potassium (Timentin)	β-Lactam + β-Lactamase inhibitor combination
Ceftazidime	Cephalosporin
Cefepime (Maxipime)	Cephalosporin
Aztreonam (Azactam)	Monobactam
Imipenem-cilastatin (Primaxin)	Carbapenem
Meropenem (Merrem)	Carbapenem
Ciprofloxacin (Cipro)	Quinolone
Gatifloxacin (Tequin)	Quinolone
Gentamycin (Garamycin)	Aminoglycoside
Tobramycin (Nebcin)	Aminoglycoside
Amikacin sulphate (Amikin)	Aminoglycoside

A number of antibiotics are currently available (Table 2) to treat *Pseudomonas* infections. However, there is a growing concern about different strains of *Pseudomonas aeruginosa* developing resistance towards antibiotics currently employed. Multiple surveillance studies have demonstrated that antibiotic resistance is on the rise among nosocomial pathogens in significant proportions (Bonfiglio, *et al.* 1998; Chatzinikolaou, *et al.* 2000; Hauser & Sriram 2005; Henwood, *et al.* 2001;

Maschmeyer & Braveny 2000; Rotimi *et al* 1998; Van Eldere 2003). Susceptibility of *Pseudomonas aeruginosa* to anti-pseudomonal antibiotics is reported to have decreased over the years in European nations (Table 3). The issue of antibiotic resistance of *Pseudomonas aeruginosa* is further complicated by the fact that this bacterium exists in biofilms in the infective state. The biofilm mode of growth enables *Pseudomonas* to survive and thrive in hostile environments by colonizing cell surfaces and also induces the bacteria to increased levels of resistance towards antimicrobials.

Table 3: Decrease in the percent susceptibility rates of *Pseudomonas aeruginosa* as a function of time

Hauser & Sriram (2005); Van Eldere (2003)

Year / Antibiotics	1991	1993	1995	1997	1999	2003
Ciprofloxacin	87%	82%	84%	-	70%	71%
Amikacin	-	-	96%	91%	74%	71%
Cefepime	-	-	-	73%	64%	50%
Meropenem	-	-	-	-	86%	81%
Ceftazidime	-	-	95%	89%	78%	-
Piperacillin	-	93%	-	87%	74%	-

KEY:
- = Not determined

In the following sections, the issues of *Pseudomonas aeruginosa* antibiotic resistance within biofilms will be discussed further, in relation to virulence mechanisms exhibited by this organism. Introductory passages will summarise current knowledge on biofilms, antibiotic resistance in biofilms, virulence factors and virulence mechanisms of *Pseudomonas aeruginosa.* Subsequent to this general

introduction, the results obtained as part of this thesis are presented in Chapters 3 to 5. The conclusions and their implications are summarised and discussed in Chapter 6.

Pseudomonas aeruginosa pathogenesis

Humans as targets of *Pseudomonas aeruginosa* infections

Pseudomonas aeruginosa is capable of transiently colonizing skin and mucosal surfaces, but deficient in its ability to carry out initial steps of infection. Hence, the normal non-specific defenses of the human body are sufficient to prevent *Pseudomonas aeruginosa* from causing infections. However, the whole issue of infection takes a new turn when there is a serious breach in the body's non-specific defenses (e.g., burns, immunosuppressive therapy or wounds (Wenzel 1997).

Once the opportunist, *Pseudomonas aeruginosa*, overrides the primary defense systems, it may cause fatal systemic disease in the human body. The diseases caused by *Ps. aeruginosa* are diverse; urinary tract infections, respiratory tract infections, dermatitis, soft tissue infections, bone and joint infections, bacteraemia, gastrointestinal infections and a variety of systemic infections (in patients whose immune system is compromised, e.g., AIDS) and depends on the site of entry and colonization within the body. *Ps. aeruginosa* present on catheters or in irrigation solutions are a major cause of urinary tract infections (Appelgren, *et al.* 2002; Beckett, *et al.* 2001; Qarah, *et al.* 2004). Eye wounds or eye surgeries are responsible for corneal infections, while cardiac surgeries usually result in *Pseudomonas aeruginosa*-associated endocarditis. Chronic pseudomonal respiratory tract infections are common in cystic fibrosis patients and the use of contaminated respirators is often a cause for necrotizing *Ps. aeruginosa* pneumonia. Of the different nosocomial infections, *Ps. aeruginosa* infections account for 10% of blood stream infections, 8% of surgical wound infections (Wenzel 1997), 12% of urinary tract infections (Gordon,

et al 1998; Mandell, *et al* 1995) and 16% of pneumonia cases (Wenzel 1997). *Ps. aeruginosa* has also been associated with bacteraemia and currently accounts for 15% of all Gram-negative bacteraemias. Mortality rates attributed to this bacterium in hospital acquired infections (Table 4) and patients suffering from cystic fibrosis are high.

Table 4: Mortality rates attributed to *Ps. aeruginosa* in hospital acquired infections

Van Delden and Iglewski (1998)

Clinical condition	Mortality (%)	Study
Pneumonia and septicaemia among immunocompromised patients	30	(Fergie, *et al.* 1994)
Ventilator associated pneumonia in intubated patients	38	(Dunn & Wunderink 1995)
Burn unit outbreaks	60	(Richard, *et al.* 1994)
Death among AIDS population	50	(Mendelson, *et al* 1994)

The capacity of this organism to cause such high rates of illness and death is primarily due to a diverse range of virulence factors. *Ps. aeruginosa* possesses both cell-associated and extracellular virulence factors (Figure 1). Recent research has shown that the extracellular virulence factors of this bacterium are coordinated in a cell density dependent manner (Smith & Iglewski 2003; Zhu, *et al.* 2004). Thus, biofilms of *Pseudomonas aeruginosa* offer an excellent physiological model for *in vivo* bacterial cell density-dependent communication and may also act as *'hot spots'* for activation of virulence factors and emergence of organisms with increased virulence (Zhu, *et al.* 2004).

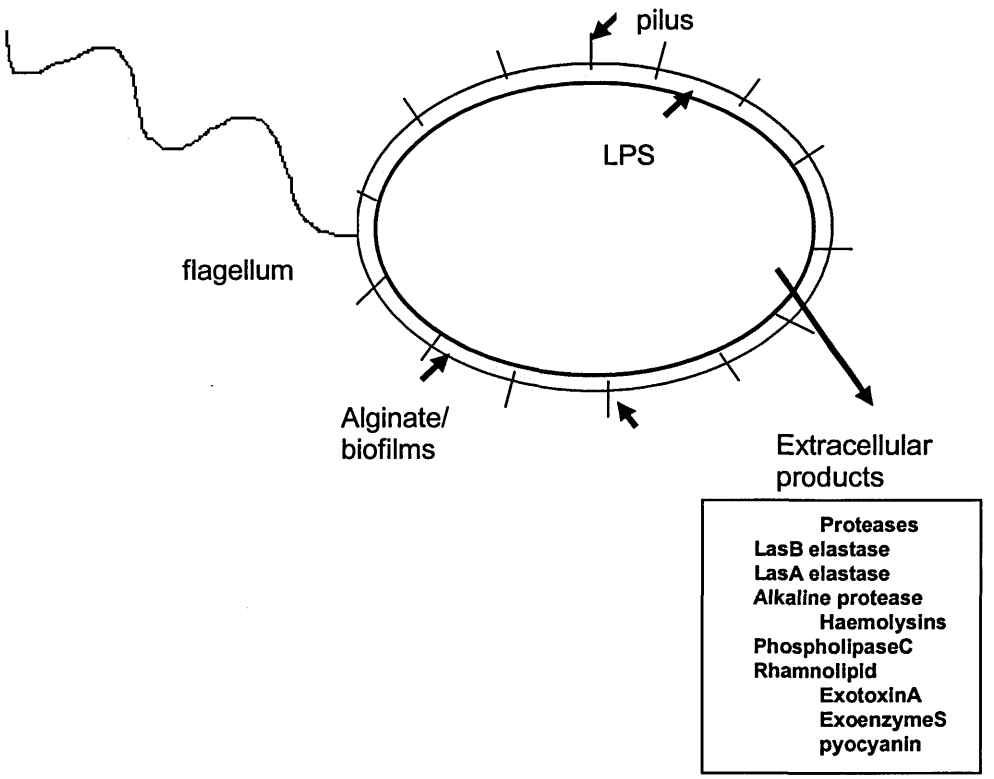


Figure 1: Cell associated and extracellular virulence factors of *Pseudomonas aeruginosa*. The cell associated virulence factors are flagellum, pilus, non-pilus adhesins and alginate matrix. The extracellular virulence factors include lipopolysaccharide (LPS) proteases, haemolysins, exotoxin A, exoenzyme S and pyocyanin. Adapted from Van Delden & Iglewski (1998).

Pseudomonas aeruginosa virulence factors

The virulence factors (determinants) that *Ps. aeruginosa* uses to colonize the immunosuppressed host are varied. Cell associated virulence factors include flagellum, pili, adhesins, alginate and lipopolysaccharide. Secreted or extracellular virulence factors include: haemolysins, proteases, lipases, Exotoxin A and cytotoxins; Exo S, Exo T, Exo U and Exo Y. The essential features of these virulence determinants are summarised in Table 5. The focus of this thesis is on three major virulence factors; lipopolysaccharide (LPS), proteases and cytotoxins (especially Leucocidin) of *Ps. aeruginosa* ATCC 15692 (PAO1).

Table 5: Characteristics of the virulence factors of *Ps. aeruginosa* (after Baltch & Smith 1994)		
Virulence factors	**Physical nature**	**Virulence mechanism**
Adhesins		
Pili	Protein polymer of 15kDa	Attachment to host cells; important for initial colonization events
Nonpili	Protein (?)	Adherence to mucins
Flagellum		
Serotype a and b	Protein polymer of 45kDa	Motility exhibited during chemotaxis or invasion
Iron acquisition		
Siderophores (Pyochelin and Pyoverdin)	Organic	Specifically bind iron (high affinity) help survive iron limited environments
Iron binding proteins	Outer membrane proteins	Uptake of siderophore- iron complex
Haemolysin	Glycolipid	Has a detergent like property. It is ciliostatic and enhances haemolytic property of phospholipase C
Phospholipase C		
Haemolytic phospholipase C	78 kDa protein	Degradation of phosphatidylcholine and sphingomyelin causing inflammation and stimulation of mucous production
Non-haemolytic phospholipase C	73 kDa protein	Degradation of phosphatidylcholine and sphingomyelin causing inflammation
Exotoxin A	66 kDa protein	Destrustion of tissues and macrophages due to ADP ribosylation of Elongation factor-2 and inhibition of protein synthesis
Alginate	Branched polymer of glucouronic and mannuronic acid	Physical barrier; also antiphagocytic and helps in the establishment of chronic infection
Lipopolysaccharide (LPS)	Outer membrane–bound lipids with extended polysaccharide chains	Endotoxin causing septic shock
Leucocidin	29kDa protein	Exotoxin damaging host leucocytes
Proteases	Approx. 29k kDa proteins	Proteolytic degradation of cellular function

Lipopolysaccharide (LPS)

Pseudomonas possesses outer membrane lipopolysaccharide (LPS) or endotoxin (Homma, *et al.* 1991). The cell envelope of *Ps. aeruginosa*, as with other Gram-negative bacteria, is composed of an external unit membrane (outer membrane, OM), a layer of peptidoglycan and an inner cytoplasmic membrane. The latter is mainly composed of phospholipids with randomly intercalated molecules of proteins (Schromm, *et al.* 2000). The outer membrane is an asymmetrical bilayer containing phospholipids on the inner side and lipopolysaccharide (LPS), as the major lipidic molecule, on the outer cell surface (Schromm, *et al.* 2000). The LPS of *Ps. aeruginosa* consists of three basic units: lipid A, core polysaccharide and O-specific side chain (Figure 2).

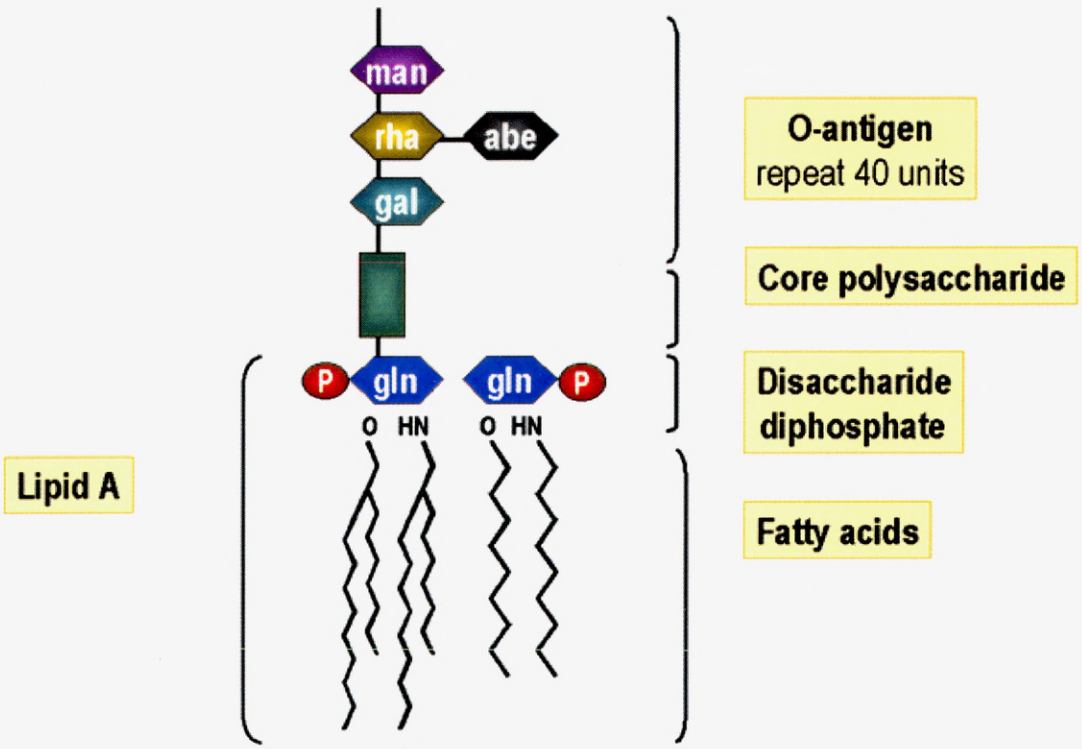


Figure 2: Structure of the Gram-negative lipopolysaccharide (LPS). The LPS has three basic units; O antigen (exterior to the membrane) composed of oligosaccharides (varies according to bacteria), the core polysaccharide containing several sugars (heptoses and ketodeoxyoctonic acid) and Lipid A (embedded in the outer leaflet of the membrane) made of β-hydroxyfatty acids. Image adapted from: (http:// www.med.sc.edu: 85/fox/lps.jpg) [Accessed on 02.03.2005]

The lipid A has five or six fatty acids linked to a backbone of diglucosamine phosphate. The core polysaccharide is covalently linked to lipid A and contains an unusual sugar, 2-keto-3-deoxyoctanoate (KDO), as well as a variety of heptose and hexose residues (Pier, *et al* 1995). The LPS of *Ps. aeruginosa* has a lower intrinsic toxicity compared with that of the enterobacteriaceae (Homma, *et al.* 1991). It plays a major role in protecting the cell from the complement-mediated bactericidal action of normal human serum (Pier, *et al* 1995). Antibodies specific for O antigens of *Ps. aeruginosa*, particularly the high molecular-weight O polysaccharide, can protect against mucosal surface colonization by *Ps. aeruginosa*, and this is achieved through circulating antibody alone rather than by induction of local antibodies (Pier, *et al* 1995).

Besides protection of the bacterial cell from the bactericidal activity of serum, the LPS is responsible for causing sepsis or septic shock in humans. The molecular arrangement and chemical structure of the Lipid A component of the LPS is said to be responsible for the biological (endotoxin) activity of LPS (Schromm, *et al.* 2000).

Proteases

Proteases are important extracellular proteolytic enzymes produced by *Ps. aeruginosa*; they liquefy gelatin, dissolve elastin and fibrin, and destroy collagen (Matsumoto 2004; Gerald, *et al* 1983). Recently some strains of *Ps. aeruginosa* were observed to degrade mucin. This activity has also been attributed to pseudomonal proteases (Aristoteli & Willcox 2003). *Ps. aeruginosa* isolates are known to produce proteases like elastase, alkaline protease, LasA protease, protease IV and a membrane protease, all of which may play important roles in the pathogenesis of *Ps. aeruginosa* (Matsumoto 2004).

The pseudomonal elastases are metal proteases with a molecular weight of 33 kDa. They are often called *LasB* proteases or pseudolysins. The elastases can cleave type I, type III and type IV collagens. Thus, they are able to cause sufficient damage (haemorrhage) to endothelial cells and destroy the basement membrane of blood vessels (Komori, *et al* 2001).

The alkaline protease is a major virulence factor produced by *Pseudomonas aeruginosa*. Like elastases, these proteases are also classified as metal proteases. They are 467 amino acids long as opposed to elastases, which are 301 amino acids in length. The alkaline proteases and the elastases have been reported to cleave components of the immune system. They are capable of degrading the human RANTES (Regulated upon Activation Normal T cells Expressed and Secreted); monocyte chemotatic protein- 1 (MCP-1), and epithelial neutrophil activating protein-78 (ENA-78) (Leidal, *et al.* 2003).

LasA protease (staphylolysin) produced by *Pseudomonas aeruginosa* is a metalloendopeptidase. It contains 182 amino acids and has a molecular weight of 20 kDa (Kessler, *et al.* 2004). LasA acts alongside elastase and alkaline protease to degrade elastin (Galloway 1991).

Recent studies (Caballero, *et al.* 2004) indicate the presence of a serine protease (251 amino acids in length) called protease IV, produced almost exclusively by *Pseudomonas aeruginosa*, and not other strains of the same genus. Purified protease IV protein is 26 kDa and is capable of cleaving the carboxyl side of lysine–containing peptides. Many biologically important proteins, including immunoglobulin, complement components, fibrinogen and plasminogen are digested by protease IV (Engel, *et al.* 1998).

Fricke *et al.* (1999) located a membrane proteinase called insulin-cleaving membrane proteinase (ICMP) on the outer membrane of the cell envelope in *Ps. aeruginosa.* It has been noted to cleave α and β fibrinogen chains (Fricke, *et al.* 1999).

Cytotoxins

Toxins produced by pathogenic bacteria play a major role in virulence of the bacteria. Secretory toxins (exotoxins) are capable of diffusion and cause lesions within the target host cell population. Cytotoxins are exotoxins produced by pathogens that can disrupt host cells and provide nutrients for the progress of infection (Popoff, 2005). *Pseudomonas aeruginosa* produces a number of cytotoxins. They include the CTX protein (initially referred to as leucocidin due to its lethal activity towards leucocytes) and type III secretory proteins; ExoS, ExoT, ExoU and ExoY (Barbieri & Sun 2004).

CTX is a 29 kDa polypeptide first described by Scharmann (1976a, 1976b). CTX exists in a cell-bound state, in the form of a 31 kDa precursor toxin (proCTX) with no, or only low, toxic activity. It is converted into a 29 kDa toxin by the proteolytic excision (endogenous protease (elastase) of *Ps. aeruginosa* and six other proteases with known specificity for peptide bonds) of 20 amino acids from the C terminus of proCTX, giving rise to a protein with high toxicity (Scharmann 1976a, 1976b, Scharmann, *et al* 1976).

CTX is considered to be a member of the pore forming toxin group. It induces selective leakage of low molecular weight substances from target cells and also forms ion-channels in planar lipid membranes. The pore-forming process triggered by CTX takes place in three steps: binding to the membrane, alteration of membrane structure, and release of cytoplasmic contents. These events lead to severe tissue inflammation and necrosis (Baltch, *et al.* 1985; Bishop, *et al.* 1987). Cytotoxin causes rapid

decrease of granulocyte function as demonstrated by phagocytosis, bactericidal activity and chemotaxis. However, cytotoxin has no effect on serum or complement (Baltch, *et al.* 1985). Its action is dependent on temperature, time, and concentration and completely inhibited by specific antibody.

The type III secretion system of *Ps. aeruginosa*, is part of the exoenzyme S regulon, which contains genes for secretion, translocation and regulation and effectors (Yahr, *et al.* 1997). Type III secretion is a contact dependent secretion and is triggered only when a pathogen comes into close contact with host cells. Four effector proteins of *Ps. aeruginosa* are secreted by the type III secretion pathway; ExoS, ExoT, ExoU and ExoY (Barbieri & Sun 2004).

ExoS and ExoT share 76% similarity in their protein primary structure. These effector proteins are bifunctional, as they contain Rho GTPase-Activating Protein (RhoGAP) domains at their N-terminus and ADP ribosylation domains at their C-terminus (Barbieri & Sun 2004). One of their mechanisms of cytotoxicity is due to their ability to rearrange actin cytoskeleton. RhoA, Rac and Cdc42 are members of the Rho family of proteins and control the assembly and organization of the actin cytoskeleton. RhoA stimulates the formation of stress fibers. Rac stimulates lamellopodia (membrane ruffling) and may be important for cell motility. Cdc42 stimulates the formation of microspikes, which are essential for cell polarity. The RhoGAP domain of ExoS and ExoT is responsible for the inactivation of Cdc42, Rac and RhoA. Such inactivation results in actin cytoskeleton rearrangement (Goehring, *et al.* 1999; Kazmierczak & Engel 2002, Kazmierczak, *et al* 2001). The C-terminus domain of ExoS and ExoT contains the ADP ribosyltransferase domain with which ADP ribosylates distinct host proteins, ultimately causing host cell damage. The ADP ribosyltransferase domain of ExoS was noted to target Ras and several Ras related proteins, disrupting cell signalling (Riese & Barbieri 2002; Riese, *et al.* 2002).

Alternately, ExoT targets the Crk-mediated phagocytosis pathway and prevents bacterial uptake by phagocytes (Sun & Barbieri 2003).

ExoU is a phospholipase, which is cytotoxic to epithelial cells and macrophages *in vitro* and *in vivo* (Sato, *et al.* 2003). ExoY is an adenylate cyclase, which causes actin cytoskeleton reorganisation by elevating intracellular cAMP levels in cultured mammalian cells (Yahr, *et al.* 1998).

The *Pseudomonas aeruginosa* infection model

Observations and evaluations of various bacterial virulence factors have shed light on how *Pseudomonas aeruginosa* is able to cause disease in a wide variety of human organs. Such observations have led to the proposal of a three-phase infection model; colonization, chronic infection and acute infection.

The primary step in the infection of *Pseudomonas aeruginosa* is colonization. However, for colonization to occur, the primary line of defense should be breached. Trauma, surgery, burns and indwelling devices have the capacity to cause such a breach in the primary line of defense. Additionally, alteration of immunologic defense mechanisms or use of broad-spectrum antibiotics may also result in colonization by *Ps. aeruginosa.* Cellular virulence factors like flagella (Feldman, *et al.,* 1998), type 4 pili (De Bentzmann, *et al.,* 1996) and several non-pilus adhesins may play potential roles in this process.

Once colonization is successfully accomplished, several extracellular virulence factors are secreted that cause extensive tissue damage. This results in the bacteria causing chronic and acute infections. Cell-to-cell signaling controls many of these extracellular virulence factors. Exotoxin A inactivates elongation factor 2, resulting in the cessation of protein biosynthesis and ultimately cell death (Wick, *et al.,* 1990). Exotoxin A may help the bacteria in its invasion process. Exoenzyme S

ribosylates GTP binding enzymes such as Ras (Iglewski, *et al.,* 1978) and causes tissue destruction and bacterial dissemination. Phospholipase C and rhamnolipid are two haemolysins, which break down lipids and lecithins and thus helps the bacteria invade tissues (De Kievit & Iglewski 2000).

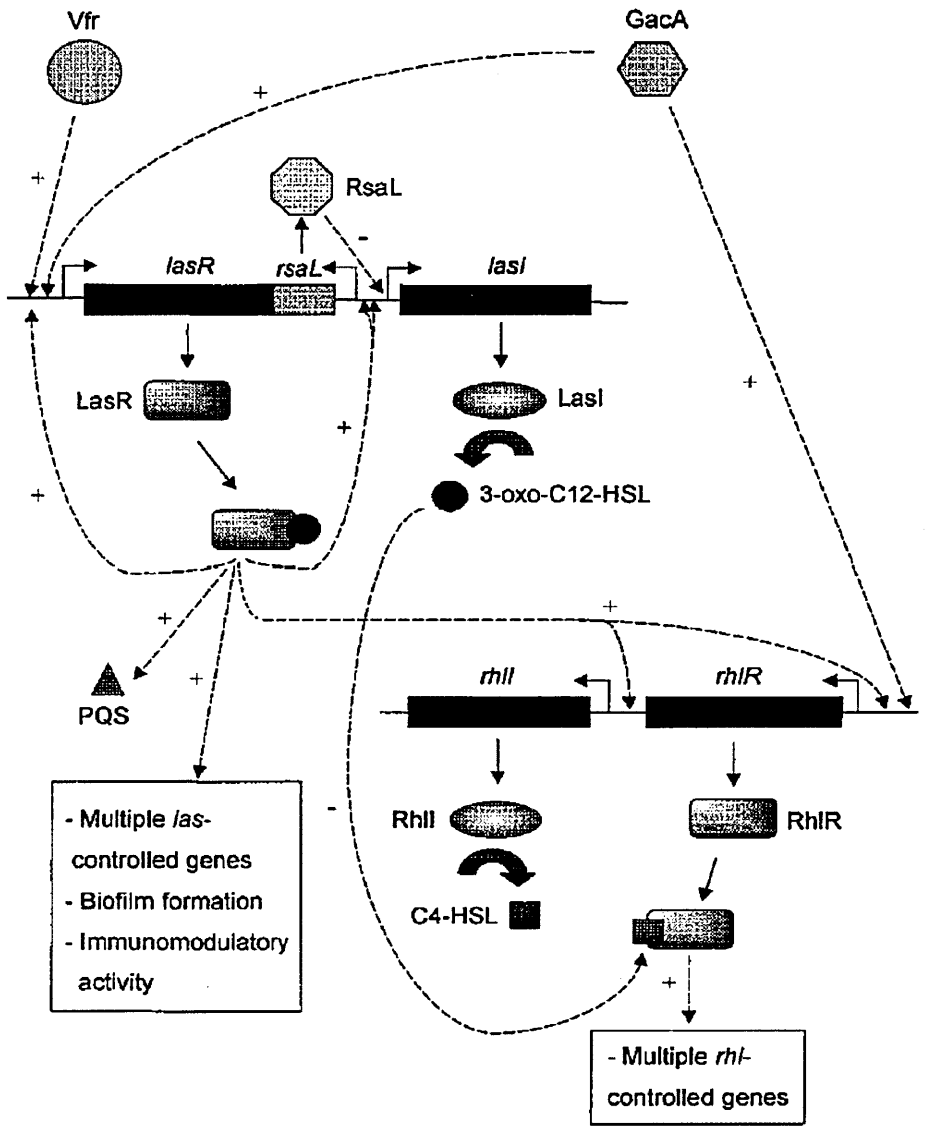


Figure 3: Quorum sensing circuit in *Pseudomonas aeruginosa*. The circuit is a hierarchical system wherein the *las* quorum sensing circuit controls the *rhl* circuit. The LasR/3-oxo-C12HSL (homoserine lactones) complex activates transcription of *rhlR* and 3-oxo-C12HSL blocks activation of *RhlR* by C4HSL (*N*-butyryl homoserine lactones). 3-oxo-C12HSL has some immunomodulatory activity and is also required for biofilm development. *GacA* and *Vfr* positively controls, the *las* system while *RsaL* negatively controls it. Both the *las* system and the *rhl* system regulate numerous virulence genes. Adapted from De Kievit and Iglewski, (2000). PQS: *Pseudomonas* quinolone signal.

Proteases produced by the bacterium, which include, *LasA* elastase, *LasB* elastase and alkaline protease, may also play a role in the invasion of tissues (De Kievit & Iglewski 2000).

Regulation of these extracellular virulence factors during acute and chronic infections is cell density dependent and therefore, quorum-sensing signaling mechanisms come into play during the infection process (Figure 3) (Latifi, *et al.*, 1996). *Ps. aeruginosa* signaling system is a hierarchical system, composed of two signaling systems, the *las* system and *rhl* system. Both these systems have their respective autoinducers, which activate transcriptional activator proteins. It is to be noted that though these autoinducers exhibit high specificity, in that they do not activate transcriptional activators of a different system, they are not totally independent of each other. The autoinducer-transcriptional activator (LasR/ 3-oxo-C12-HSL) complex of the *las* system is able to induce the expression of *rhlR*. This places the *las* system above the *rhl* system in the hierarchy (Latifi, *et al.*, 1996).

The *las* system controls the expression of LasB elastase and is probably involved with the production of other virulence factors like, Las protease and exotoxin A (Passador, *et al.,* 1993, Gambello, *et al.*, 1991). There are two genes involved in this system, *lasI* and *lasR*. The *lasI* gene leads to the synthesis of an autoinducer called 3-oxo-C12-HSL and the *lasR* codes for a transcriptional activator protein, LasR (Pearson, *et al.*, 1994). The LasR protein and the 3-oxo-C12-HSL form a complex, which binds to the *lasI* gene promoter and activates it.

The *lasI* gene promoter is linked to the expression of the autoinducer, resulting in an increased production of 3-oxo-C12-HSL and eventually the LasR / 3-oxo-C12-HSL complex. As a critical cell density is attained and concentration of LasR / 3-oxo-C12-HSL complex exceeds the threshold levels, virulence genes (*lasA*, *lasB*, *toxA*, *aprA*, etc) are activated. The *las* system has also been shown to activate secretory

pathway genes, *xcpP* and *xcpR* (Chapon, *et al.*, 1997; Smith & Iglewski 2003). The autoinducer 3-oxo-C12-HSL itself has immunomodulatory activity and hence, contributes to virulence (Telford *et al.*, 1998). *GacA* and *Vfr* are two genes that positively regulate the *las* signaling system. *Vfr* is required for the transcription of *lasR*, while *RsaL* is understood to inhibit transcription of the *lasI* gene (Reimann, *et al.* 1997, Albus, *et al.* 1997, Smith & Iglewski 2003).

The second signaling system in *Ps. aeruginosa* is called the *rhl* system as it controls the production of rhamnolipid. Analogous to the *las* system, the two genes involved in the *rhl* system are *rhlI* autoinducer synthase gene, which leads to the production of C4-HSL (*N*-butyryl homoserine lactone) and the *rhlR* gene, which codes for the transcriptional activator protein (Oschner & Reiser, 1995). The RhlR / C-4HSL complex is linked to the activation of *rhlAB* operon. As soon as a critical concentration of the autoinducer builds up, this operon is activated and a rhamnosyl-transferase that is required for the synthesis of rhamnolipid is produced. Other proteins that are linked to the rhl system are *LasB* elastase, *LasA* protease, pyocyanin, cyanide and alkaline protease (Pearson, *et al.,* 1997). There is some evidence that also indicates that the stationary phase sigma factor-*rpoS* (usually involved in regulating stress response genes) is controlled by this system (Latifi,*et al.*, 1996).

Pseudomonas aeruginosa **biofilms**

The medical importance of biofilms

The concept of natural existence of most bacteria as biofilms has evolved over the last three decades. (Costerton *et al.* 1995; Costerton *et al.* 1999; Lazazzera & Stanley 2004) defines the term biofilm as "*a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface.*" Biofilms can also develop on inert surfaces and dead tissues. In a medical context, medical devices, dead tissues like parts of a dead bone or even living tissues

can act as favourable surfaces for biofilm formation (Donlan 2001). The sedentary (biofilm) lifestyle of bacteria enables them to withstand host immune responses and also makes them much less susceptible to antibiotics compared to their planktonic counterparts. Bacterial biofilms act as bacterial sanctuaries where they are protected from hostile host environments (Donlan 2001).

If areas within the human body were to act as a '*home*' for biofilm bacteria, two conditions would have to be fulfilled,

(1) an overlying aqueous environment with a constant flow of nutrients and

(2) a surface where bacteria could attach and colonize (Bell 2001).

Microenvironments within the human body that satisfy these conditions are ubiquitous. For example, normal buccal movements cause a constant flow of saliva across the gingival crevices, which encourage bacteria to form oral biofilms at these sites.

A medical biofilm contains sessile bacteria that constantly release antigens into circulating blood and this eventually stimulates the production of corresponding antibodies. However, antibodies generated against biofilms have minimal effects against their target. The reason for this is that, rather than causing damage to the biofilms, immune complexes tend to damage host tissues immediately surrounding the biofilm. In the case of *Pseudomonas aeruginosa* biofilms, infections set in only when the host immune system is impaired, as the infection is opportunistic in nature. Hence, the immune response to *Pseudomonas* infections is minimal. As a result the only way to suppress the infection would be to resort to antibiotics (Costerton, *et al* 1999).

Antibiotic therapy is capable of reversing symptoms resulting from the adverse effects of planktonic bacteria that may detach from biofilms and could potentially act as the seed of infection. However, these antibiotics fail to inhibit or kill

the biofilm. This is perhaps why medical biofilm infections show recurring infections and elimination of infection is futile unless the sessile bacterial population is surgically removed (Costerton, *et al* 1999).

Current antimicrobial therapies have rarely been designed with microbial communities like biofilms in mind. Antimicrobials that are effective in eradicating planktonic bacteria may not be effective against their biofilm counterparts (Stewart & Costerton 2001). Biofilm characteristics that are important in the infectious disease process include;

1. Detachment of cells or aggregates from biofilms may result in urinary tract or blood stream infections.
2. Horizontal transfer of antibiotic resistance plasmids may occur within biofilms.
3. Reduction in antimicrobial susceptibilities of biofilm bacteria.
4. Endotoxin production by biofilm associated Gram-negative organisms and
5. Increased resistance shown by biofilms towards host immune system.

Hence, a fundamental shift in perspectives is required to battle microbes capable of forming biofilms and causing human disease (Donlan 2002).

Developmental stages of a biofilm

A biofilm is the culmination of a series of process (Figure 4) that beginswhen floating (planktonic) bacteria encounter a surface. Bacteria sense environmental conditions triggering the transition to life on a surface (O'Toole, *et al* 2000; Stoodley, *et al* 1999; Watnick & Kolter 1999). These environmental signals vary among organisms. For example, *Ps. aeruginosa* and *Ps. fluorescens* will form biofilms under almost any conditions that allow growth (O'Toole & Kolter, 1998b). On the other hand, some strains of *Escherichia coli* K-12 and *Vibrio cholerae* will not form

biofilms in minimal medium unless supplemented with amino acids (Pratt & Kolter 1998, Watnick & Kolter 1999). In contrast, *E. coli* O517:H7 is reported to make a biofilm only in low-nutrient media (Dewanti & Wong 1995). In addition to the nutritional content of the medium, other environmental cues that can influence biofilm formation include temperature, osmolarity, pH, iron availability and oxygen (O'Toole, *et al* 2000; Stoodley, *et al* 1999; Watnick & Kolter 1999).

Biofilm formation may occur by at least three mechanisms. The first is the redistribution of attached cells by surface motility (Dalton, *et al.* 1996). Studies on *Pseudomonas aeruginosa* suggest that type IV pili-mediated twitching motility plays a role in surface aggregation for this organism (O'Toole & Kolter 1998a). A second mechanism is from the binary division of attached cells (Heydorn, *et al.* 2000). As cells divide, daughter cells spread outward and upward from the attachment surface to form cell clusters, in a similar manner to colony formation on agar plates. The third mechanism of aggregation is the recruitment of cells from the bulk fluid to the developing biofilm (Tolker-Nielsen & Molin 2000). The relative contribution of each of these mechanisms will depend on the organisms involved, the nature of the surface being colonized and the physical and chemical conditions of the environment.

The physical, chemical and biological dynamics of the stages of biofilm development normally follow an ordered sequence of events. This involves attachment of the bacteria to a surface, growth and polysaccharide production, maturation and the import of media components, usually followed by detachment as the whole film '*sloughs off*' from its substratum (O'Toole, *et al* 2000).

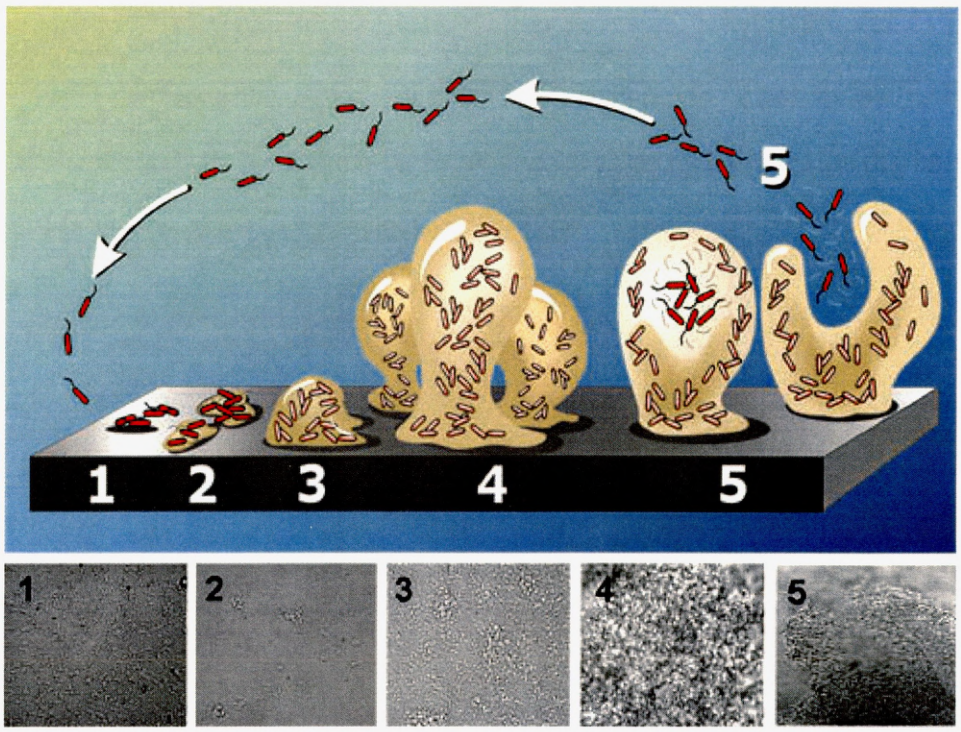


Figure 4: Diagram showing the development of a biofilm as a five-stage process. Stage 1: initial attachment of cells to the surface. Stage 2: production of extracellular polymeric substances (EPS) resulting in more firmly adhered "irreversible" attachment. Stage 3: early development of biofilm architecture. Stage 4: maturation of biofilm architecture. Stage 5: dispersion of single cells from the biofilm. The *bottom panel* (1-5) show each of the five stages of development represented by a photomicrograph of *Ps. aeruginosa* when grown under continuous-flow conditions on a glass substratum. (Adapted from Stoodley, *et al* 2002).

Bacterial attachment to surfaces

Attachment of the bacteria to a surface may occur as an active process, involving motility or chemotaxis by the bacteria, or due to passive encounter with a surface (Geesey 2001). Depending on the nature of the substrate, bacteria may show two types of attachment mechanisms. While initial attachment to an abiotic surface is mostly due to non-specific interactions (e.g., hydrophobic), adhesion to a living tissue is usually accomplished through specific molecular (lectin, ligand or adhesin) docking mechanisms. The process of attachment may be considered to take place in two phases: the docking phase and the locking phase (Dunne 2002). The docking phase involves initial attachment to the surface, largely based on attractive or repulsive forces and is largely reversible in nature. Electrostatic and hydrophobic forces

influence the attachment outcome at this stage. Although electrostatic interactions tend to favour repulsion (Geesey 2001; Jucker, *et al* 1996), as both charges of the surface and organism are negative, the events of the second phase, the locking phase, overcome the negative effects of electrostatic repulsion towards attachment. In the locking phase the loosely bound bacteria employ specific molecular mechanisms that will anchor it on the surface. For example, adhesins located on bacterial cell surface extensions like pili are interact with the surface and cause firm attachment by neutralising the effects of repulsive electrostatic forces (Dunne 2002).

Exopolysaccharides and receptor-specific ligands produced at this stage by organisms facilitate the process of locking the bacteria on the biotic / abiotic surface. At the end of the attachment stage, the organisms are firmly and irreversibly attached to the matrix. The attachment process involving the reversible and irreversible phases are indicated as stage 1 and stage 2 in Figure 4.

The forces involved in the docking and locking phases of bacterial adhesion have been extensively researched. Three types of forces interplay in the sorption of a microorganism on a substrate; electrostatic or electrodynamic forces, hydrodynamic or aerodynamic forces and forces due to external field such as gravitational, electric or magnetic fields (Ho, 1986). When the organism is in close proximity to the surface it is the electrostatic and electrodynamic forces that play the major role in the adhesion process. These forces dictate two possible states for adhesion viz. reversible and irreversible (based on the distance or proximity of the organism to the surface). When the bacterium is at a distance <1nm, the cells are said to be at a primary minimum and the adhesion is irreversible, while a distance >5 to 10nm from the surface, the bacterium is reversibly attached and the state is known to be secondary minimum. However, the transition from the reversible to the irreversible (docking to the locking) phases takes place over a distance range of 1 to 5nm from the surface, characterised

by very high repulsive forces (energy gap). This energy gap may be overcome if external forces are applied to the cell e.g. motility, turbulent flow, etc. (Strevett & Chen, 2003; Bos *et al.*, 1999).

The interplay of the attractive forces (mainly, Van der Waals forces) and repulsive electrostatic forces described above is incorporated into the DLVO theory elaborated by (Derjaguin & Landau, 1941; Verwey & Overbeek, 1948). Hence, according to this theory, the net force of interaction between a bacterial cell and a surface arises from a balance between the Van der Waals forces of attraction and electrostatic forces of repulsion. 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 fimbriae and pili. Flagella or flagellar driven motility was found to have a role in the initial cell-to-surface interactions for both *Pseudomonas aeruginosa* and *Escherichia coli* (Pratt & Kolter, 1998; O'Toole & Kolter, 1998a; Tolker-Nielsen & Molin, 2000).

More recently microbial surface thermodynamics has been explained according to the extended DLVO theory developed by Van Oss (2003). They showed that relatively short-range forces resulting from acid-base interactions also play a key role in addition to electrostatic and Van der Waal's forces (Van Oss 2003; Strevett & Chen, 2003; Bos *et al.*, 1999). Transition occurring within the initial attachment stage,

from the reversible to the irreversible stage may also be a genetically regulated event (Lazazzera *et al.*, 2004).

Microcolony formation

Pseudomonas aeruginosa mutants defective in surface attachment (*sad*, surface attachment defective) have been described (O'Toole & Kolter 1998b). Differential expression of *sad* genes causes downregulation of flagellum synthesis genes (Garrett, *et al* 1999) and upregulation of type IV pili genes, responsible for twitching motility. Twitching motility has been implicated in the aggregation of organisms into microcolonies. Microcolonies are a communities of bacterial cells three to five layers deep which develop following the initial attachment of bacterial cells to a surface (Davey & O'Toole 2000) (Figure 4). Extracellular polymeric substances (EPS) production is critical for the ongoing development of microcolonies after bacterial attachment to a surface. Studies on the model organism *Ps. aeruginosa* have shown that transcription of genes responsible for the production of alginate is a key development at this stage (Davies & Geesey 1995). The production of EPS results in the formation of 3-D biofilm structures and provides a safe haven for the bacteria to multiply (Costerton, *et al* 1999; Lazazzera, *et al.*, 2004).

Maturation of biofilm

Mature biofilms are thick heterogeneous mats of bacterial cells or complex structures containing water channels that may also allow nutrient influx and waste efflux (Davey & O'Toole 2000). Quorum sensing controls the differentiation of mature biofilms from the microcolony stage (De Kievet & Iglewski, 2000). The evidence for this lies in the relatively thin and undifferentiated biofilms of *LasI* mutants. *LasI* directs the synthesis of a quorum sensing molecule 3-

oxodecanoylhomoserine lactone and the observation that mature biofilms differentiate on the addition of this molecule *in vitro*, proves that quorum sensing plays a role in the development of mature biofilms (Costerton, *et al.* 1999; De Kievit & Iglewski, 2000).

The mature biofilm structure is encased in an EPS matrix and the final architecture is similar to the shape of a mushroom but much smaller. The shape of the mature biofilm may be the result of unregulated events (eg: shear forces) and regulated events (eg: depth and architecture of the biofilm). The depth of biofilms of *Pseudomonas* is regulated by the transcription factor *RpoS* (Heydorn, *et al.* 2002; Whiteley, *et al.* 2001a). Activation of *RpoS* in the biofilm community directs the organisms into stationary phase (Venturi 2003). This would signal a nutrient limitation within the biofilm community and cause a reduction in the depth of *Ps. aeruginosa* biofilms (Lazazzera, *et al.* 2004). In *Pseudomonas aeruginosa* biofilms, the architecture of the pillar structures and water channels are maintained by the production of rhamnolipid surfactant and its resulting surface tension reducing properties. Quorum sensing regulates the production of rhamnolipid surfactant in *Ps. aeruginosa* and mutant strains have been reported to form less complex biofilm structures (De Kievit, *et al.* 2001a).

Detachment and dispersal

The process of detachment has traditionally been considered as a passive behaviour dependant on fluid shear or starvation (Sauer, *et al.* 2004). While hydrodynamic shear properties may catalyse the detachment of biofilm clusters, the detachment process may also be a programmed event that bacteria employ to colonize new and favourable niches (Hall-Stoodley & Stoodley 2005). The detachment process may be triggered by chemical cues, similar to those that control biofilm maturation.

Alginate lyase, an enzyme that degrades alginate may play an important role in the sloughing of biofilms. In a recent report (Hall-Stoodley & Stoodley 2005), three different detachment and dispersal strategies were identified; (1) swarming dispersal, (2) clumping dispersal and (3) surface dispersal. In each of these strategies, dispersal may take place into an overlying fluid or over a solid surface. Additionally, dispersal may be self-propelled locomotion (swimming, sliding or twitching motility) or fluid driven dispersal (clumping, rippling or rolling) (Hall-Stoodley & Stoodley 2005). For whatever reason bacteria detach from biofilms, or whatever the strategy bacteria use for dispersal, the dispersal phenomenon allows pathogenic organisms living in biofilms, to efficiently transfer and seed infective doses and cause persistent infections (Greenberg, 2003 & Costerton, *et al* 1999).

Biofilm physiology

Bacterial cells present within biofilms, capable of attaching to surfaces, differ profoundly in their phenotypes from their free-living planktonic counterparts (Donlan, 2002). The present favoured hypothesis that explains the phenotypic difference in planktonic and biofilm bacterial physiologies points to differential expression of genes in these two states (O'Toole, *et al* 2000).

Biofilm cells exhibit heterogeneous physiology. Bacteria produce exopolysaccharides, also known as glycocalyx, which assist the cell to firmly glue to the inert surfaces. Cell division inside the glycocalyx matrix results in the formation of microcolonies. Microcolonies coalesce to form layers of bacterial cells within the growing biofilm, and depending on the location of the particular bacterial cell within the biofilm, the physiological status changes (Caldwell & Lawrence 1986; Thien-Fah & O'Toole 2001). Cells located at the upper region of biofilms (surface biofilm cells) have better access to nutrients and oxygen compared to those found within. These

cells are metabolically active, likely to have envelopes permeable to nutrients and may also be capable of discharging their metabolic wastes more efficiently. Their characteristics may be very similar to those of planktonic bacterial cells (Anwar, *et al* 1992; Huang, *et al.* 1998; Xu, *et al* 2000). In contrast, cells within the thick glycocalyx (embedded biofilm cells) may not have as much access to nutrients and are more likely to be living in microaerobic conditions, increased osmotic pressure and pH variation (Beloin & Ghigo 2005). Core regions of the biofilm may also have a higher concentration of metabolic waste and the bacteria within this region are more likely to exhibit a physiological state similar to that of stationary phase bacteria. These cells may also be small, as they cannot divide efficiently and may largely lead a life of dormancy and low metabolic activity (Anwar, *et al* 1992; Xu, *et al* 2000).

Ample biochemical evidence supports the spatial complexity of the biofilm and the hypothesis that embedded biofilm bacteria face different conditions when compared to planktonic and surface biofilm bacterial growth. Recent gene expression studies have also come to similar conclusions (Beloin & Ghigo 2005). In *E. coli*, upto 38% of the bacterial genome may be affected by biofilm formation (Prigent-Combaret, *et al.* 1999). These process show that bacterial biofilm physiology is extremely complex and there is rich heterogeneity within different locations of the biofilm (Beloin & Ghigo 2005).

Antibiotic resistance in *Pseudomonas aeruginosa* biofilms

Antibiotic resistance in biofilms

Since the early 1950s, antimicrobial therapy has been a major tool in the treatment of infectious bacterial diseases. Almost all pathogenic microorganisms have developed some level of resistance to chemotherapeutic agents which is largely due to the failure to properly monitor antibiotic therapy (Madigan, *et al* 2003). Madigan *et*

al. (2003) reported some multi-drug resistant strains of *Pseudomonas aeruginosa* that are now untreatable with any known antimicrobial drugs.

Planktonic bacteria may become antibiotic resistant through either acquired or intrinsic mechanisms. Mutations within the bacterial genome resulting in resistance or lateral transfer of resistance genes (for example *via* the transfer of a resistance plasmid) are characteristic of acquired resistance. On the other hand Intrinsic resistance occurs through phenotypic changes resulting from differential expression of existing genetic material (Hogan & Kolter 2002; Schachter 2003).

Bacteria residing within biofilms are reportedly some 10 to 1000 times less susceptible to a wide variety of chemotherapeutic agents compared to their planktonic equivalents (Davies 2003; Gilbert, *et al.* 2002b). Besides a variety of physical factors characteristic of a biofilm (e.g. EPS acting as physical barrier to macrophages, chemotherapeutic agents, etc.), inherent cellular mechanisms, like differential expression of genes giving rise to altered phenotypes, may also play a role in the lower levels of antimicrobial susceptibility observed (Schachter 2003).

In addition to the effect of antimicrobials themselves, the structural and chemical heterogeneity of the biofilms are intimately related to the rise of resistant phenotypes (Schachter 2003; McBain, *et al*, 2003). Variable thickness of biofilms and non-uniform distribution of cells and polymers within the matrix constitutes the structural heterogeneity, while local variations in the concentrations of metabolites, products and microbial species within the biofilms, indicate the chemical heterogeneity found within biofilms (Murga, *et al* 1994, Stewart 2003). Chemical and physical heterogeneity within the biofilms may trigger differential expression of genes resulting in resistant bacterial phenotypes (McBain, *et al*, 2003 & Stewart 2003).

Reduced susceptibility of biofilms to antibiotics: Resistance mechanisms

Biofilm bacteria differ from planktonic bacteria, in their multicellularity. The formation of a biofilm involves a multicellular developmental process and depends on the ability of free-living bacteria to communicate and get together with each other (O'Toole, *et al* 2000). Within a biofilm, bacteria derive the same benefits as other multicellular organisms: strength in numbers, division of labour, functional specialization and adaptation to environmental stress. The resistance that biofilms employ against antibiotics may, also be a multicellular phenomenon. Hence, most of the hypotheses on the mechanisms of resistance exhibited by biofilm bacteria are inherently based on multicellular nature of the biofilm (Krasovec & Jerman 2003; Stewart & Costerton 2001).

There are currently four different hypothesised resistance mechanisms under study (Figure 5):

1. Slow penetration of the antimicrobial agent into the biofilm.
2. Altered chemical environment within biofilm, leading to zones having gradation in the growth rates of bacteria.
3. Adaptive stress responses.
4. Persister cells.

Figure 5: Four possible mechanisms of antimicrobial resistance. The yellow region on the top edge of the biofilm indicates the region of slow penetration of the antimicrobial. The region coloured red at the lower edge within the biofilm is the area where there exists high heterogeneity of cells due to altered microenvironments. Cells coloured green are those that exhibit a general stress response to the antimicrobials. The violet cells represent the persisters. Adapted from image library of Centre for Biofilm Engineering http://www.erc.montana.edu/Res-Lib99-SW/Image_Library/Biofilm_Control/ [Accessed on 02.03.2005]

Slow penetration of antibiotic into biofilms

Diffusion is the predominant transport process within biofilm cell aggregates (Stewart 2003). One of the hypothesese explaining biofilm resistance, is that the surface layers of a biofilm near the biofilm-bulk fluid interface is the only area exposed to a lethal dose of the antibiotic due to reaction-diffusion barrier and a short time of antibiotic exposure that limits the penetration potential of the antimicrobial (Stewart 2003; McBain, *et al.* 2003). If the hypothesis were true, in that, it was the only mechanism for biofilm resistance, then the antimicrobials would be lethal to the biofilm if it were exposed long enough for a total diffusion. However, this is not the case in most observations, as there seems to be some evidence of the antimicrobial being neutralised as it diffuses into the biofilm (McBain, *et al.* 2003). Some of the

antimicrobials that are reportedly affected by penetration limitation include chlorine (Stewart & Costerton 2001), hydrogen peroxide (Stewart, 2002) and β-lactam antibiotics (Anderl, *et al* 2000) and aminoglycoside antibiotics (Walters, *et al.* 2003).

Studies carried out with wild type strains carrying enzymes that deactivate the antimicrobial and mutant strains that lack these enzymes (but both strains are capable of forming normal biofilms), have shown that neutralization of the antibiotic as it diffuses into the biofilm plays a major role in its penetration potential (Anderl, *et al* 2000). For example, bioactive ampicillin fails to penetrate β-lactamase positive *Klebsiella pneumoniae* biofilms, as the enzyme cleaves the antibiotic faster than it diffuses (Anderl, *et al* 2000). Catalase positive strains of *Ps. aeruginosa* have also been reported to show resistance to hydrogen peroxide penetration (Stewart *et al*, 2000).

Recent studies have also indicated that many antimicrobials are capable of entering and diffusing within biofilms, without actually harming any cells. Antimicrobials of this nature, include ciprofloxacin (Anderl, *et al* 2000), chlorosulfamate (Stewart & Costerton 2001), rifampin (Zheng & Stewart 2002) and tetracycline (Stone, *et al.* 2002).

Altered microenvironments and slow growth

Microorganisms that have entered stationary phase are known to be generally resistant to antibiotics. Biofilms contain slowly growing or non-growing cells that exhibit similar characteristics to stationary phase planktonic cultures (Fux, *et al.* 2005). Such slow growth has been attributed to local depletion of nutrients within different layers of the biofilm. Alternatively, altered microenvironments within the biofilm, with respect to pH and oxygen levels may be antagonistic to the activity of the antimicrobial (McBain, *et al.* 2003).

The growth rate of microorganisms in biofilms is slow. Experimental comparisons have shown that average growth rate in biofilm cells can be as low as a few percent of their planktonic counterparts (Walters, *et al.* 2003). Biofilms in which the average specific growth rate is half the growth rate of planktonic cells may actually consist of a population of cells in which half are growing rapidly and the other half minimal. Experiments examining growth rate related effects under controlled growth conditions for planktonic cultures and biofilms of *Ps. aeruginosa, E. coli* and *Staphylococcus epidermidis* have shown that the sensitivities of both the planktonic and biofilm cells to either tobramycin or ciprofloxacin increased with increasing growth rates, thus supporting the suggestion that slow growth rates of biofilm cells protect the cells from antimicrobial action (Duguid, *et al.* 1992a & 1992b; Evans *et al.* 1991). Overall the evidence for growth rate being a critical factor in controlling antimicrobial resistance in biofilms is strong.

Stress responses

It is possible that the slow growth of microorganisms observed in biofilms is a direct consequence of a general stress response, rather than nutrient depletion (Brown & Barker 1999). Bacteria are capable of responding to numerous environmental stresses, like heat, cold, pH changes and chemical agents (Thien-Fah & O'Toole 2001). The molecular regulator of these responses is the stationary phase sigma factor, *RpoS*. Studies have indicated that *RpoS* is induced at high cell densities as judged by the production of trehalose (osmo-protectant) and catalase (effector of general stress response) (Liu, *et al* 2000). As biofilms are subject to benefits of high cell density, it is logical to propose that these cells would express *RpoS*. Accordingly, the presence of *RpoS* mRNA has been shown in sputum samples of cystic fibrosis patients suffering from chronic *Ps. aeruginosa* biofilm infections (Foley, *et al.* 1999). Finally,

stress responses within biofilms are implemented more effectively on antimicrobial challenge compared to planktonic cells (Maira-Litran *et al*, 2000a; De Kievit *et al*, 2001b). *Ps. aeruginosa* biofilm cells produce catalase production upon hydrogen peroxide treatment, while planktonic cells of the same reporter strain succumb to the effects of hydrogen peroxide before catalase can be produced (McBain, *et al.* 2003). This indicates that general stress response of bacteria within a biofilm are at a state of alert and be rapidly activated upon challenge.

Persisters

Regardless of the duration of treatment of biofilms with antibiotic a small fraction of the population remains recalcitrant (Keren, *et al.* 2004). This recalcitrant fraction of the population has been exhibited in kill versus time and kill versus concentration curves, as a significant tailing (residual number of cells that die slowly or do not die at all in presence of high concentration of antibiotics) (Keren, *et al.* 2004). The observed tailing could only be due to cells that are not affected by any antimicrobial challenge. The hypothetical state of these cells, where in they are protected from all types of antimicrobial insults, has been termed '*persisters*'. The cells in this state have been likened to dormant cells at stationary phase (Drenkard & Ausubel 2002; Lewis 2001; Spoering & Lewis 2001).

Presumably, antibiotics normally act against their targets in persisters as well, but unlike regular cells, they are not inhibited and do not die. The reason for this tolerance remains unknown. The suggestion that persisters are simply non-growing does not explain their phenotype. Indeed, fluoroquinolones kill non-growing cells, but leave persisters intact (Keren, *et al.* 2004). However, it is possible, that persisters are cells in a state of deep dormancy (Nystrom 2003).

In conclusion, it may be said that all these defense mechanisms are multicellular in nature. Penetration-limitation of antimicrobials within biofilms occurs due to neutralization of the activity of the antimicrobial. Such neutralization can only occur if there exists a quorum of cells, as lone cell cannot deplete the antimicrobial agent fast enough to ensure its own safety. Considering nutrient limitation and slow growth; an antibiotic that requires oxygen for effective killing may not be able to affect the depths of a biofilm due to microorganisms in the outer layers depleting the oxygen and effectively creating an anaerobic environment in the layers within. A single planktonic cell would not be able to sufficiently deplete local oxygen levels so as to inactivate the antimicrobial. Stress responses are also a multicellular phenomenon. The stationary phase sigma factor, *RpoS* is activated in the presence of a quorum of bacteria. The multicellular nature of the biofilm ensures a quick regulation of stress response and better adaptation to antimicrobial agent compared to the lone bacterial cell. Finally the persister hypothesis suggests that these cells are capable of withstanding major antimicrobial assaults. However, such a benefit comes at the cost of being dormant. No single cell may be a persister and at the same time have a rapid growth (as persisters are hypothesised to be dormant cells) (Lewis 2001). However, within the multicellular environment of biofilms, there exists the opportunity for persisters. Most of the cells within the biofilm are in relatively susceptible state, in which they can grow, synthesize glycocalyx, and replicate their genome. A few cells are transformed into the '*persister*' state. Although these cells may not be able to grow rapidly, they would survive and reseed the community in the event of a catastrophe (McBain, *et al.* 2003).

In summary, it has to be noted that no one of the mechanism outlined, offers an explanation for the persistent and tenacious resistance of a biofilm. It is more likely

that biofilms mount a multifaceted defense in which two or more of the mechanisms outlined operate in concert.

Aims and Objectives

The pathogenic effect of *Pseudomonas aeruginosa* is attributed to its virulence and survival factors (Fonesca *et al.* 2004). Common models of bacterial virulence analyze host-pathogen interactions under a defined set of conditions that usually do not include antibiotic therapy. Although the first stages of an infection usually occur without the presence of antibiotics, once a diagnosis is available most infectious diseases evolve under antibiotic treatment. Thus, besides being inhibitors of bacterial growth, antibiotics act as modulators of bacterial gene expression (Martinez & Baquero 2002).

A number of reports have indicated a general down-regulation of pathogenic determinants including biofilm formation of planktonic pathogenic organisms in the presence of sub-MICs of different antibiotics (Wilson, *et al.* 2002; Drago *et al.* 2001; Kim *et al.* 2001; Braga *et al.* 2000; Tateda *et al.* 1993) However, there exists the possibility that pathogenic organisms have already established biofilms and colonized the host before antibiotic therapy may commence. Hence, it is of prime importance to understand the effect of sub-MICs of antibiotics on bacteria within biofilms compared to their planktonic counterparts. A recent study (Fonseca *et al.* 2004) reported the inhibition of virulence factors of *Pseudomonas aeruginosa* in the presence of subinhibitory concentrations of antibiotics. However, this study was performed with planktonic cells, and not on biofilm bacteria as usually observed in clinical conditions.

This study adopts *Pseudomonas aeruginosa* ATCC 15692 (PAO1), which displays high levels of resistance to many antimicrobials, as the model organism to study bacterial virulence in the presence of sub-minimal inhibitory concentrations

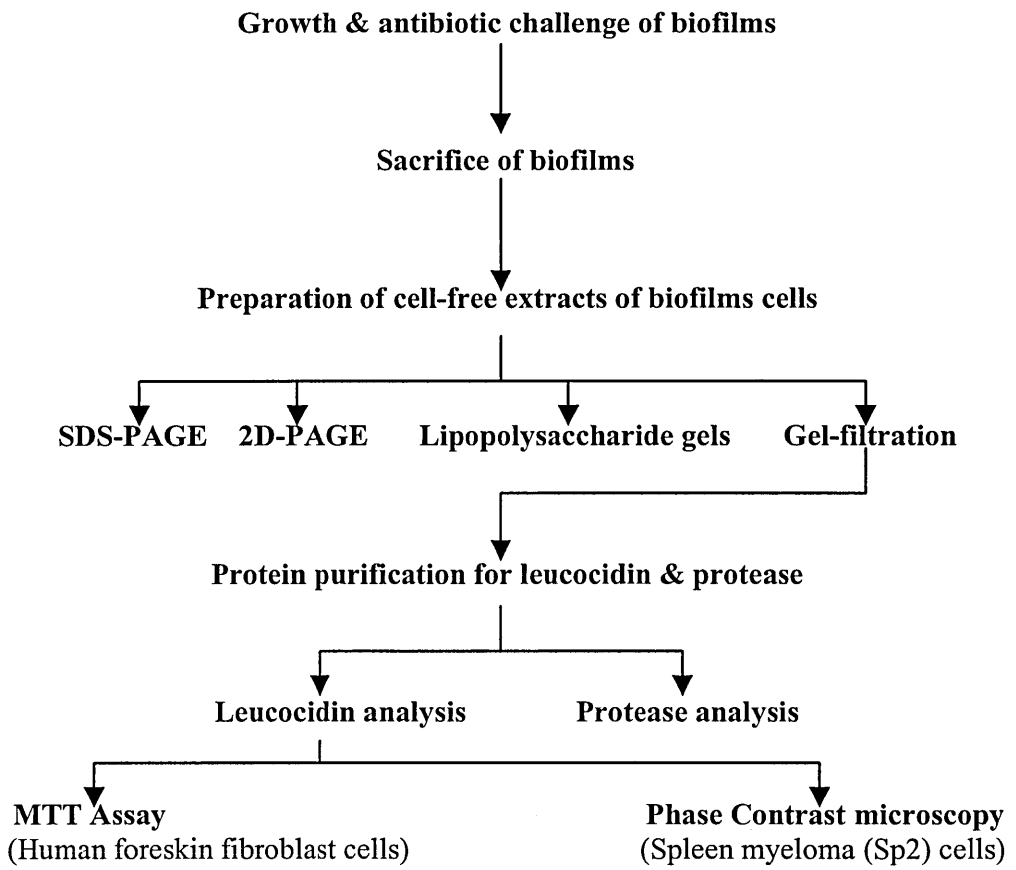
(sub-MICs) selected antibiotics. Previous studies have implicated alterations within the outer membrane proteins (OMPs), to be a possible reason for exclusion of harmful molecules within the bacterial cell (Abdel Malek *et al.* 2002; Al-Hmoud 2002; Winder *et al.* 2000; Brözel & Cloete 1994; Nikaido 1992). High affinities of antibiotics for certain OMPs may cause an import of antibiotics within the bacterial cell, resulting in inhibition of growth. It has been suggested that the bacteria may avoid such adverse growth effects due to antibiotics, by over-expression or loss of certain OMPs (Abdel Malek *et al.* 2002; Al-Hmoud 2002; Winder *et al.* 2000; Brözel & Cloete 1994; Nikaido 1992).

It is the aim of this study to investigate the effect of sub-minimal inhibitory concentrations (sub-MICs) of three antibiotics (ampicillin, nalidixic acid and streptomycin) on key pathogenic determinants (e.g. outer membrane proteins, leucocidin and other virulence factors) of monospecies and binary biofilms of *Pseudomonas aeruginosa* ATCC 15692 (PAO1) and *E. coli* ATCC 10000, respectively. It is hoped that sub-MICs of antibiotics will mimic subinhibitory chemotherapeutic doses and allow the bacteria to express pathogenic determinants comparable or similar to those in conditions of clinical infection.

Chapter Two: General Experimental Methods

Introduction

The aim of this chapter is to give the experimental detail of those techniques associated with the routine maintenance of cultures, testing protocols and analysis of bacterial physiology undertaken in the subsequent experimental chapters. Whilst the plan of the thesis changed over the period of the project, a flow chart (below) is given to indicate the relationship between certain tests / procedures and the sequence in which they were undertaken.



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. *Escherichia coli* cultures were grown in R2A media supplemented with 1% glycerol (Reasoner and Geldrich, 1985) and *Pseudomonas aeruginosa* cultures were grown in chemically defined media (CDM, replacing 0.5% succinic acid with glycerol; Dinning, 1995) for 24 h at 37°C in an orbital incubator (Gallenkamp, INA-305) at 190 rpm. Culture identification was determined using the API 20NE and API 20E tests (Biomerieux, France).

Reagents

Sigma, Poole, Dorset: Ammonium persulphate; ammonium sulphate; azocasein; casein; coomassie brilliant blue R-250; dithiothreitol; 5,5'-dithio-bis (2-nitrobenzoic acid); dimethyl sulfoxide (DMSO); ethylenediaminetetra-acetic acid; glutamine; glutathione; glycerol; lysostaphin; Thiazolyl blue (MTT); N-laurylsarcosine; 2-

mercaptoethanol; phenazine methosulphate (PMS); phenyl-methyl-sulphonyl-fluoride (PMSF); protease peptone number 3; proteinase K; polyethylene glycol 20000; Pyruvic acid; Rnase; sephadex G-100; sodium carbonate; sodium thiosulphate; soluble starch; Thiomersal; TEMED; N-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES); trichloroacetic acid (TCA) Triton X-100; 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; potassium cyanide (KCN); 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).

Ampicillin (AmP), Nalidixic acid (NaL), Streptomycin (ST) Antibiotic was purchased from Sigma (Pool, UK). Bio Gel P-100 and (2.5x100cm) column were purchased from Bio Rad (UK).

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℃, 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 $MgSO_47H_2O$ (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. 15.45 g L^{-1} technical agar (Oxoid number 3) was added as a gelling agent when required.

A modified CDM (MCDM) was also used in biofilm experiments, which was suitable for the optimal and non-advantageous growth of both microorganisms *Ps. aeruginosa* and *E. coli*. 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 1M 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 (Al-Hmoud, 2002).

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-coloured colonies and *E. coli*, which gives purple-coloured colonies (Figure 6). Aliquots (55.8g) of this medium were in 1L of distilled water. This medium was sterilized by autoclaving at 121 °C, 15 psi for 15 minutes.

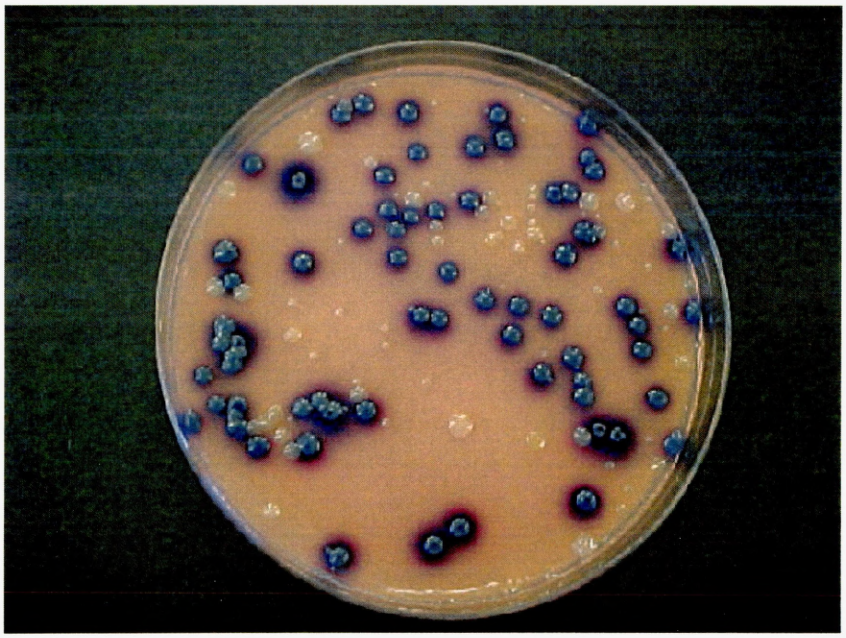


Figure 6: Chromogenic *E. coli* / Coliform plate showing two different coloured colonies; the purple ones represent *E. coli* ATCC 10000 and the straw ones represent *Ps. aeruginosa* ATCC 15692 (PAO1).

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 7). These data were used to determine which combination of tube and peristaltic pump speed were used in subsequent biofilm experiments.

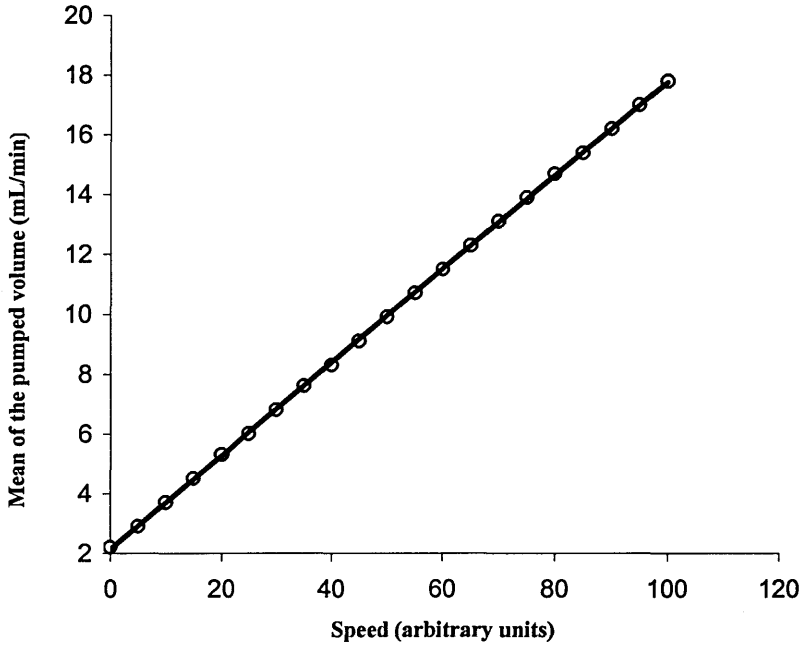


Figure 7: The standard curve for the peristaltic pump calibration. Error bars are calculated as the standard deviation of each data point. n = 5 replicates. (o) Mean of the pumped volume (mL min^{-1}). NB: SD = 0, hence error bars are inapparent.

The Sorbarod model: a simple *in vitro* model for growth control of bacterial biofilms (Hodgson *et al.*, 1995)

Sorbarod biofilm model is composed of silicone PVC tubing containing a single Sorbarod (Hodgson, *et al* 1995). 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 *Ps. aeruginosa* ATCC 15692 (PAO1) and *Escherichia coli* ATCC 10000 and binary biofilms (mixed) of *Ps. aeruginosa* ATCC 15692 (PAO1) and *Escherichia coli* ATCC 10000 were grown within Sorbarod filter plugs, which were perfused with modified Chemically Defined Medium (MCDM) (Al-Hmoud, 2002) at 37°C. An overnight culture of *Ps. aeruginosa* or *E. coli* grown in MCDM were used to inoculate (10 mL) from a syringe

dropwise onto a sterile pre-wetted Sorbarod (with the addition of 5 mL 0.9% (w/v) sterile normal saline), held within sterile PVC tubing. The rubber plunger seal was withdrawn from a sterile, disposable 2 mL syringe. The syringe was introduced into the PVC tubing containing the Sorbarod and a sterile, disposable needle (0.8 x 40 mm) inserted through the rubber seal (Figure 8). Media inlet tubing was attached *via* the needle and sterile MCDM was delivered into the unit at a rate of 2.2 mL min^{-1} (using peristaltic pump) at 37°C. 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. Pseudo-steady states were established at which the growth rate of the biofilm was reproducible, measurable and similar to likely *in vivo* growth rates (Brown & Williams, 1985). Steady state for *Ps. aeruginosa* was achieved at 102 h, for *E. coli* at 56 h and for binary biofilms (mixed) of *Ps. aeruginosa* and *E. coli* at 126 h. Sorbarods and eluate cells were collected and stored in the freezer at –20°C for later analysis.

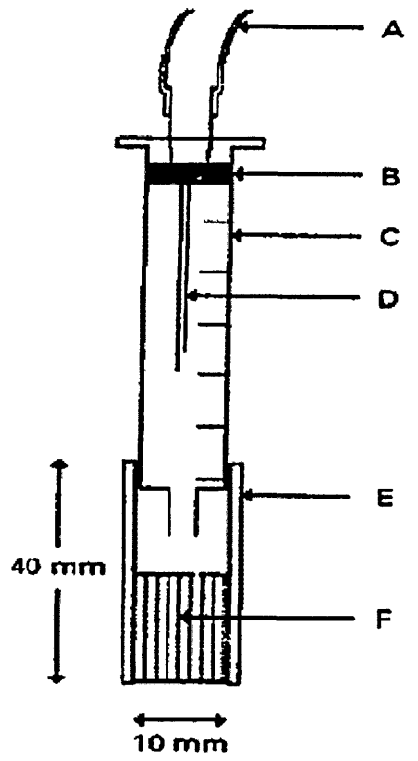


Figure 8: Diagram of the Sorbarod biofilm model showing silicone tubing to peristaltic pump and media vessel (A), rubber plunger seal (B), 2 mL disposable sterile syringe C), syringe needle (D), PVC tubing (E) and Sorbarod (F) (Hodgson *et al.*, 1995)

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.

Sacrifice of biolfim cells

The Sorbarod was aseptically removed from the PVC tubing with the use of a sterile scalpel blade. The Sorbarod was then placed into a sterile plastic centrifuge tube (20 mL) and 5 mL of sterile PBS buffer was added. The tube was then mixed using a vortexer for 30 minutes, until the Sorbarod was completely broken and

dispersed. The tube was then left to rest at room temperature for 20 minutes, until the components of the Sorbarod had settled out under gravity. The suspended cells were decanted into a sterile plastic centrifuge tube (20 mL) and centrifuged at 5000xg for 20 minutes. The cells were washed three times with sterile PBS (5 mL) by a process of resuspension and centrifugation. Cells were stored in a freezer at -20°C until required.

Preparation of cells

Sterile MCDM 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 (5mL) 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 labortechnic, 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 E_{260} nm, the minimum required time of homogenisation in order to yield the maximum absorbency was determined as 3 minutes for *Pseudomonas aeruginosa.*

The same procedure was carried out for determination of homogenization time (H_t) for *E. coli* 10000. By plotting homogenising time (H_t) against E_{260} nm, the

minimum required time of homogenisation in order to yield the maximum absorbency was determined as 3 minutes for *E. coli* 10000.

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 562nm. 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 BCA 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 calibration 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 absorbance was measured at 562nm (Novaspec II, LKB) and plotted against known protein concentrations to produce a standard curve (Figure 9).

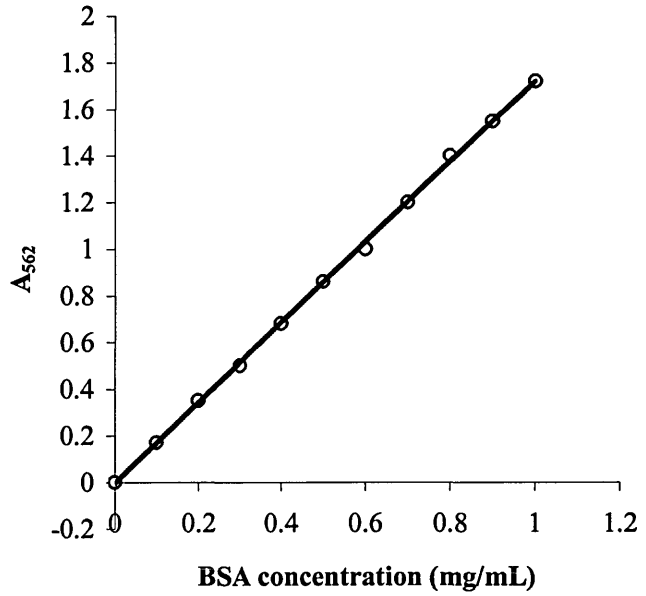


Figure 9: The standard curve for protein estimation. Error bars are calculated as the standard deviation of each data point. n = 3 replicates. (o) Mean absorbency at 562 nm. NB: SD = 0, hence error bars are inapparent.

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 (1mL) was added to those samples, which were incubated at 37°C for 30 minutes. Their absorbencies were measured at 562nm (versus a reagent blank) and compared against the standard curve (Figure 9) in order to estimate protein concentration.

Preparation of outer membrane proteins and sodium-dodecyl sulphate 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 applied 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 1mL of 0.9% (w/v) saline containing 100 μg of lysostaphin and incubated at 37°C for 2 hours 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 (MSE Microcentaur) 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 1 mmol $MgCl_2$. 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 membrane was sedimented out at 13000 rpm (MSE Microcentaur) for 1h at 4°C. The supernatants were removed and the pellets were washed with 1mL of distilled water. The pellets were resedimented out at 13000 rpm (MSE Microcentaur) 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-HC1 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-HC1; 0.5M; pH 6.8; made up to 10 mL with Tris-HC1; 0.5M; pH 6.8) 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 8.8 with HC1 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 persulphate 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 glass vessels at 4°C.

After nearly an hour, the separating gel was set and the butan-2-ol was removed from the polymerised gel. In order 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), 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 in the cracking buffer / protein mixture reached the bottom of the gel (between 1h to 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).

Protocol 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

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 using a syringe with a narrow gauge hypodermic needle. The tubes were filled approximately 5mm 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); 1M.
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-HC1 (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 8.8 with 1M HC1 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 3 h, 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 R-250 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 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 solution for as long as required (between 1 to 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 solution

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).

Developing solution: (12g of sodium carbonate (anhydrous); 37% formaldehyde, 100 µL and 0.0008g of sodium thiosulphate.$5H_2O$. This was of made up to 200 mL with distilled water). The above solutions only last for a maximum of two weeks.

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 / Storing *solution*: (50% methanol, 250 mL; made up to 500 mL with distilled water).

Reconstitution of Pharmacia low molecular weight (LMWt) markers

Low molecular weight calibration kit SDS-7 Dalton Mark VII-L (for SDS gel electrophoresis) is a lyophilised mixture of 7 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. 2 and 66 kDa.

Albumin, Bovine	: 66 kDa
Ovalbumin, chicken	: 45 kDa
Glyceraldehyde-3-phosphate Dehydrogenase, Rabbit	:36 kDa
Carbonic Anhydrase, Bovine	: 29 kDa
Trypsinogen, Bovin, PMSF treated	: 24 kDa
Trypsin Inhibitor, Soybean	: 20.1 kDa
α-Lactalbumin, Bovine	: 14.2 kDa

Reconstitution

(I) For Coomassie brilliant blue detection, 200 μL of 1x sample buffer (0.0625 M, "Tris"-HC1; 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.

Denaturation of proteins

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

Loading

Proteins markers were loaded in a range of 1 to 5 μL.

Two dimensional SDS-PAGE standards

Bio-Rad's 2D-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 (M_r). 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 12.3 to 78 kDa.

Ovotransferrin (hen egg)	: 76 to 78 kDa
Albumin (abovine serum)	: 66.25 kDa
Ovalbumin (hen egg)	: 42.7 kDa

Carbonicanhydrase (bovine erythrocyte) : 30 kDa

Myoglobin (equine) : 17.2 kDa

Cytochrome C (equine) : 12.3 kDa

Loading

Using silver staining 1 to 5 μL per mini gel.

Staining of LPS gels

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

Protocols for protein purification and separation on the basis of size

Preparation of phosphate buffered saline pH 7.2 (Dulbecco & Vogt 1954)

The PBS was made up from three separate solutions (A, B, and C).*Solution A*: Sodium Chloride (NaCl) 11.7g; Potassium Chloride 0.2g; Sodium phosphate dibasic

heptahydrate 1.15g; Potassium dihydrogen phosphate 0.2g, were added and dissolved in 800 mL distilled water.

Solution B: Calcium chloride 0.1g was added and dissolved in 100 mL distilled water.

Solution C: Magnesium chloride 0.1g was added and dissolved in 100 mL distilled water.

All solutions (A, B and C) were autoclaved separately, and reconstituted when cooled.

Preparation of gel columns

Each column was packed in a vertical borosilicate glass tube (2.5 cm, internal diameter across; Econo column, Bio-Rad, USA) having end caps fused to a porous polymer bed support (28 μm) that supported the gel. Approximately 20g Sephadex gel filtration media G-100 (Sigma, UK) or Bio Gel P-100 (Bio-Rad, UK) were suspended in 500 mL of 0.2M NaCl (prepared in PBS, pH 7.2) and allowed to swell for 72 hours. Suspensions of the gel were deaerated (5 to10 minutes with occasional swirling of suspension) *in vacuo*, before use.

Each column was prepared by pouring a thin slurry of appropriate (Sephadex G-100 or Bio Gel P-100) gel particles in buffer solution into a vertical tube (Econo column, Bio-Rad, UK) already partly filled with buffer, and at the same time allowing excess of liquid to percolate through the growing gel bed. The addition of gel was continued until a bed height of 95 to 97 cm. was obtained, and then a solvent reservoir was connected to the top of the column and the flow of the buffer maintained at a rate of approximately 4 mL h^{-1} for 6 days. Both Sephadex G-100 and Bio Gel P-100 columns were connected tandem to each other.

Procedure for column runs

All protein purification experiments were done with columns equilibrated with 0.2M NaCl (prepared in PBS, pH 7.2), and a flow rate through the columns of

approximately 4 mL h^{-1} was maintained by using a peristaltic pump (Watson Marlow Ltd., Cornwall, UK) attached to the solvent reservoir. All experiments were done at room temperature (20 ± 4°C).

Proteins were dissolved in the equilibration buffer (10 mL) and the solution was applied to the top of Sephadex G-100 column by layering under the buffer already present. The protein solution migrated from the Sephadex G-100 gel over to the Bio Gel P-100 gel until they reached the end of the column. Collection of the column effluent in fractions with a fraction collector (BROMMA 2070 ULTRORAC, Pharmacia LKB, Sweden) fitted with a 30 cm siphon. Collections were made as fractions of 2 mL per tube. Proteins were estimated spectrophotometrically by using 1 mL of each effluent fraction in a cuvette with a 1 cm light path. The wavelength was 280nm.

Microculture Tetrazolium (MTT) cell proliferation assay to determine activity of purified leucocidin

The reduction of tetrazolium salts is now widely accepted as a reliable way to examine cell proliferation (Goodwin, *et al*, 1995; Hynes, *et al*, 2003). The yellow tetrazolium MTT (3-(4, 5- dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH (Salter, *et al*, 1963; Mosmann, 1983). The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means. The MTT cell proliferation assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or oncosis, the reduction in cell viability. For human foreskin fibroblast cell type, the linear relationship between cell number and signal produced is established, thus allowing quantification of changes in the rate of cell proliferation.

Preparation of MTT assay solutions

The assay required preparation of four stock solutions.

Solution A: MTT (12mM in 0.2M NaCl (prepared in PBS) pH 7.2), 0.249g were dissolved in 50 mL buffer

Solution B: Phenazine methosulphate (PMS) 1mM, 0.0153g were dissolved in 50 mL of buffer.

Solution C: Potassium cyanide (KCN) 100 mM, 0.325g were dissolved in 50 mL of buffer.

Solution D: Sorensens glycerine (optional) (0.1M glycerine in 0.1 M NaCl at pH 10.5); 0.584g of sodium chloride were dissolved in 100 mL of distilled water; 0.37 mL of 0.1M glycerine were added to 50 mL of 0.1M NaCl.

Thiazolyl blue (MTT), Phenazine methosulphate (PMS) and Potassium cyanide (KCN) stock solutions were reconstituted in 5:2.5:1 ratios immediately prior to use to obtain the MTT assay solution.

MTT assay protocol (Goodwin, *et al*, 1995)

Human foreskin fibroblast cells were grown in a 25 cm^2 cell culture flask containing Dulbecco MEM (Minimum Essential Medium Eagle, Sigma, UK), 100 mL containing (10% FCS (foetal calf serum) 11 mL; 4mM glutamine, 2 mL; 1 mL antibiotic (penicillin / streptomycin; 50 units mL^{-1} penicillin and 50 $\mu g\ mL^{-1}$ streptomycin).

Culture cells from culture flask were trypsinized and were centrifuged (MSE MISTRAL 2000 UK) at 3500xg. The cells were washed twice in fresh buffer 0.2M NaCl (PBS, pH 7.2) and finally pellets were resuspended in 3 mL of Dulbecco MEM medium. 24-well microtiter plates containing approx. 450 μL of culture medium per well were seeded with the resuspended fibroblast cells at a density of 10^4 cells $well^{-1}$.

The plates were incubated at cell-line optimal (CLO) conditions at 37°C with 5% CO_2 for 72 h, until confluent or mat growth was observed.

In order to determine the cytotoxic activity of leucocidin, 50 µL of buffer 0.2M NaCl (PBS, pH 7.2) containing leucocidin (3.2 to 62.4 µg mL^{-1}) was added to each microtiter plate well containing fibroblast cells and incubated at 37°C and 5% CO_2 for either 30 min or 24 hours. The remaining viable cell number, after leucocidin treatment, was assayed by the addition of MTT assay solution (85 µL, 12% w/v). The plates were further incubated (37°C with 5% CO_2) for 2 hours, after which all supernatant from the wells were discarded.

Insoluble formazan resulting from the production of MTT within living cells was solubilised by the addition of 450 µL DMSO. An aliquot of 50 µL of Sorensen's glycerine was added at this stage to increase sensitivity and reduce variance caused by medium. Cells were spun down by centrifugation (MSE Microcentaur) at 10000 rpm for 2 min and the optical density for supernatant was measured at 540 nm using a spectrophotometer (Novaspec I LKB) against a media blank (Dulbecco MEM). The experiment was performed in triplicate.

Viable cell numbers corresponding to optical density readings were obtained from calibration curves constructed for the purpose (Figure 10).

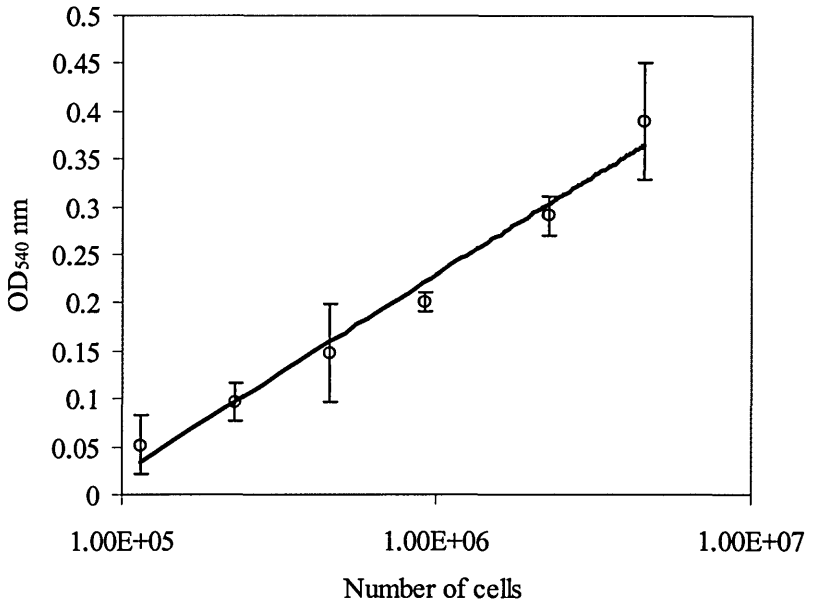


Figure 10: Standard calibration curve for Microculture Tetrazolium Assay (MTT). Error bars are calculated as the standard deviation of each data point. n = 3 replicates. (o) Mean absorbency at 540 nm.

Mouse Spleen Myeloma (Sp2) cell tissue culture

Sp2 cells (Sp2/0-Ag14 ECACC NO: 85072401) were cultured in RPMI-1640 (Sigma R0883) medium. 10 mL of a sterile medium (RPMI-1640; Sigma R0883) containing 10% FCS (Foetal Calf Serum) 11 mL; Glutamine 4mM 2 mL; 1 mL antibiotic (penicillin / streptomycin; Sigma P0906); 1mM sodium pyruvate 1 mL (Sigma S-8636) in a 25 cm^2 cell culture flask, was inoculated with mouse spleen myeloma Sp2 / 0-Ag14 (ECACC NO: 85072401) cells (1.4 x 10^5 cells mL^{-1}; 300 µL). The cell culture flask was incubated (Heraeus instrument, Germany B5060 EK-CO_2) for 72 h at 37°C with 5% CO_2 until mat growth of Sp2 cells was observed under microscope (Wilovert (Germany) Inverted microscope). Cytotoxic activity of leucocidin on Sp2 cells was observed under phase contrast microscope (LEICA DMR, UK) at 400X magnification (Scharmann, *et al*, 1976).

Chapter Three: The Effects of Selected Antibiotics on the Growth of *Pseudomonas aeruginosa* ATCC 15692 (PAO1) and *Escherichia coli* ATCC 10000 as both Planktonic and Biofilm Cultures

Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium belonging to the genus *Pseudomonas* that can be found in a variety of habitats including soil and fresh water streams as well as on plant and animal tissues (Hardalo & Edberg 1997; Boyd & Chakrabarty, 1995). Members of this genus are characterised by their ability to grow in water because of their simple nutritional requirements: they will even grow in distilled water and natural mineral water (Legnani *et al.,* 1999). The ecological versatility of this organism is partly credited to its large genome size (6.3 Mbp) (Bal, 2000). Selective expression of genes required for a particular habitat, will enable the organism to survive in adverse circumstances (Ewing & Green, 2000).*Ps. aeruginosa* is of particular interest as a human pathogen, which rarely causes infection in the healthy host. In all these environments, the microbes are found predominantly attached to solid surfaces forming biofilms (Pollack *et al.*, 1995).

The growth of these bacteria in natural environments is inhibited by periods of insufficient levels of energy and nutrients (Gilbert *et al.,* 1990). The bacterial cultures undergo a series of physiological or phenotypic changes, which enable the survival of some of the cells (Turner *et al.,* 2000). A series of rapid metabolic adjustments have also been reported during nutrient-limited conditions (Turner *et al.,* 2000). The metabolic changes activate the stringent response. A similar mechanism is also thought to take place in stressed cells present within the biofilm.

It was suggested that the stringent response might be at least partially responsible for the increased resistance to antibacterial agents at slow growth rates (Stewart, 2002; Gilbert *et al.,* 2002a & 2002b; Lewis, 2001; Greenway & 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 slows growth (Greenway & 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 estimate the rate of microbial reproduction, it is necessary to determine the numbers of microorganisms present. The growth of *Pseudomonas aeruginosa* can be estimated 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) (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 steps are taken to acclimate the microorganisms to laboratory culture conditions (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 & 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 470nm is less than 0.5, above this point the sample must be diluted (Lawrence & Maier, 1977).

Experimental approach

The overnight culture of Pseudomonas aeruginosa PAO1

25 mL of a sterile medium (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^{-1}.

The test culture of Pseudomonas aeruginosa PAO1

A fresh aliquot (25 mL) of medium (CDM) was inoculated with 250 μL of *Pseudomonas aeruginosa* PAO1 overnight culture. This was performed in duplicate (flasks A & B). The flasks were incubated at 37°C, 180 osc min^{-1}, 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 (E470) using a spectrophotometer (Novaspec I 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, as a compromise in the volume may cause a corresponding compromise in nutrients and space availability and cause the exponentially growing bacteria to undergo an early shift into the stationary phase. 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 & Maier, 1977). This diluted sample was discarded after reading the optical density. To calculate the optical density for this sample, E470 was recorded and multiplied by 10, thus giving the actual optical density of the sample.

Viable count

An aliquot (1 mL) of the identical test culture (flask B) was aseptically removed and serially diluted in the range of 10^{-2} to 10^{-8}. Aliquots (100 µL) of the dilutions were spread plated onto appropriate agar plates (CDM 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 1:

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

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 11 presents the growth curve of *Pseudomonas aeruginosa* PAO1 in CDM. CDM is a chemically defined medium, where the nutrient supply is generally lower than other medium. Therefore, it was expected that the growth would be considerably slower. The cells in this medium were in lag phase for 1 hour before they entered the exponential phase. The exponential phase was very long, lasting between 1 and 18 hours after inoculation. Mid exponential phase was at 9.5 hours after inoculation. The generation time in CDM was calculated to be 110 minutes (1.7 hours). The growth rate in CDM was slow because the nutrient sources in this medium were limited and it took the bacteria 1 hour to prepare for their proliferation. During the exponential phase bacterial growth was not rapid and increases in the bacterial biomass against time were slow. This result suggests 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, 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 each component such that one is restrictive and the remainder are present to a controlled excess.

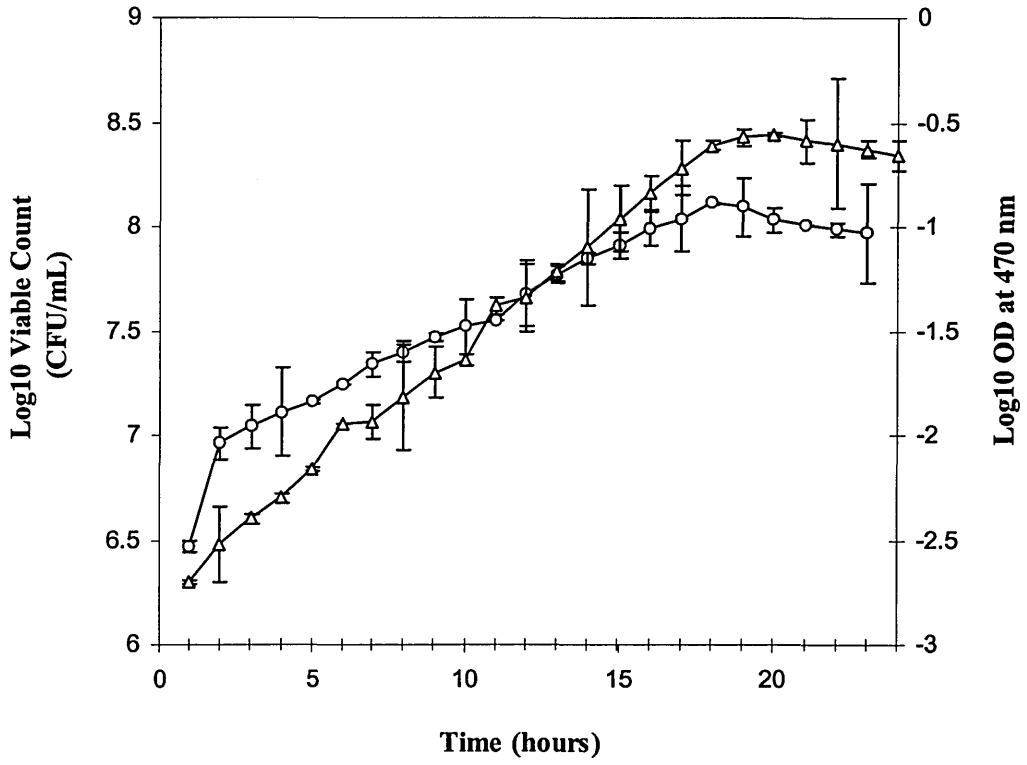


Figure 11: The observation of growth of *Pseudomonas aeruginosa* PAO1 in CDM. Error bars are calculated as the standard deviation of the individual data points. n = 3 replicates. (○) $\log_{10}$ viable count (CFU mL^{-1}); (Δ) $\log_{10}$ OD at 470 nm.

The control experiment for *Pseudomonas aeruginosa* PAO1 biofilm cultures

An overnight culture (OD 470nm = 1.00) of *Ps. aeruginosa* PAO1 grown in Modified Chemically Defined Medium (MCDM) (Dinning, 1995) was used to inoculate (10 mL) a pre-wetted (with the addition of 5 mL 0.9% (w/v) sterile normal saline) sterile Sorbarod filter, held within sterile PVC tubing (Figure 6) Sorbarod Model System Chapter 2). This was perfused with MCDM at 37°C, using a peristaltic pump and tubing calibrated to give a flow rate of 2.2 mL min^{-1}. The number 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 experiment 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 102 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 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).

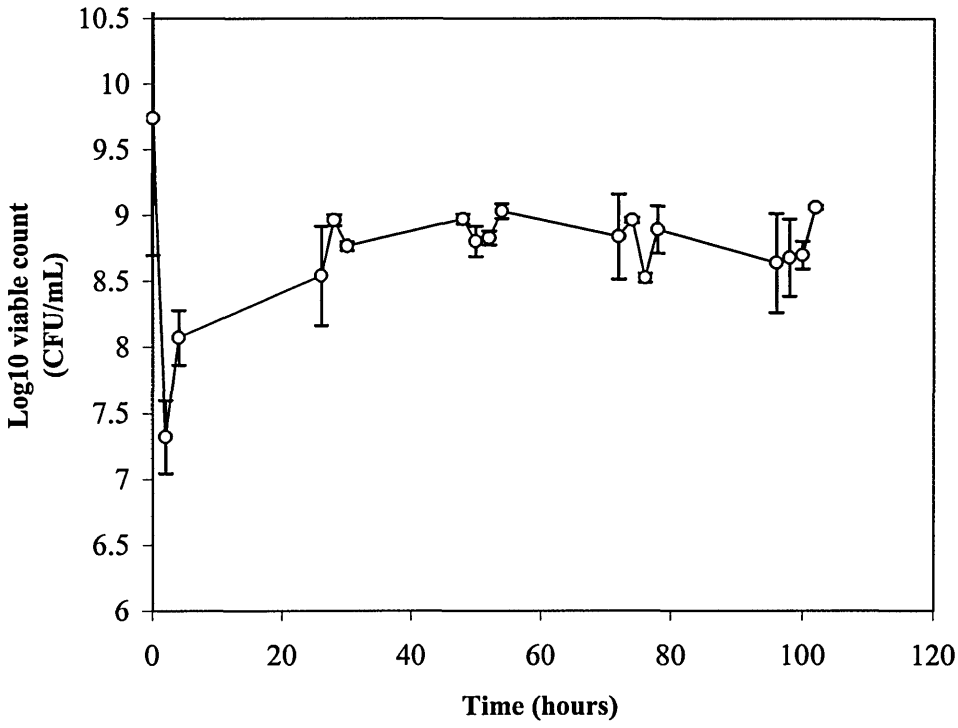


Figure 12: Graph of mean biofilm eluate counts (*Ps. aeruginosa* PAO1 perfused with MCDM) over Time. Error bars are calculated as the standard deviation of the individual data points. n = 3 replicates. (o) $\log_{10}$ viable count (CFU mL^{-1}).

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^{-9}. Aliquots (100 μL) were spread plated onto sterile CDM agar plates, in triplicate. The plates were incubated at 37°C for 48 hours and the subsequent colonies were counted.

The growth characteristics of *Escherichia coli* ATCC 10000

Escherichia coli is a Gram-negative bacterium that exhibits great tolerance towards many antimicrobial agents (Stone, *et al.,* 2002). 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 & 1983b).

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. 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 suggests 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).

The up-regulation of the *mar* operon results in multidrug- resistant phenotypes in *E. coli* planktonic cultures. However, Maria-Litran *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 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 phenotypes in *E. coli* planktonic cells) (Maira-Litran *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 (Maira-Litran *et al.*, 2000b).

Experimental approach

Test cultures

Sterile R2A medium, 25 mL in a 100 mL Erlenmeyer flasks (A, B) were 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 (25 mL) of medium (R2A) was inoculated with 250 µL of *E. coli* overnight culture; 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.

Viable count

1 mL of the identical test culture (flask B) was aseptically removed and serially diluted in the range of 10^{-2} to 10^{-8}. Aliquots (100 μL) of the dilutions were spread plated onto appropriate agar plates (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 1.

Results & Discussion

Figure 13 presents the growth curve of *Escherichia coli* ATCC 10000 in R2A medium, where the nutrient supply is considerably when compared to the CDM medium used with *Ps. aeruginosa.* Therefore, it was expected that the growth would be considerably higher. The lag phase in this medium was short, lasting 1 hour before they entered the exponential phase. The exponential phase lasted between 5 and 6 hours after inoculation. Mid-exponential phase was at 5.5 hours after inoculation. The generation time in R2A was calculated to be 18 minutes. This type of growth was expected in this kind of medium (R2A) is a nutrient-enriched medium and it is deficient in iron (Reasoner & Geldreich 1985).

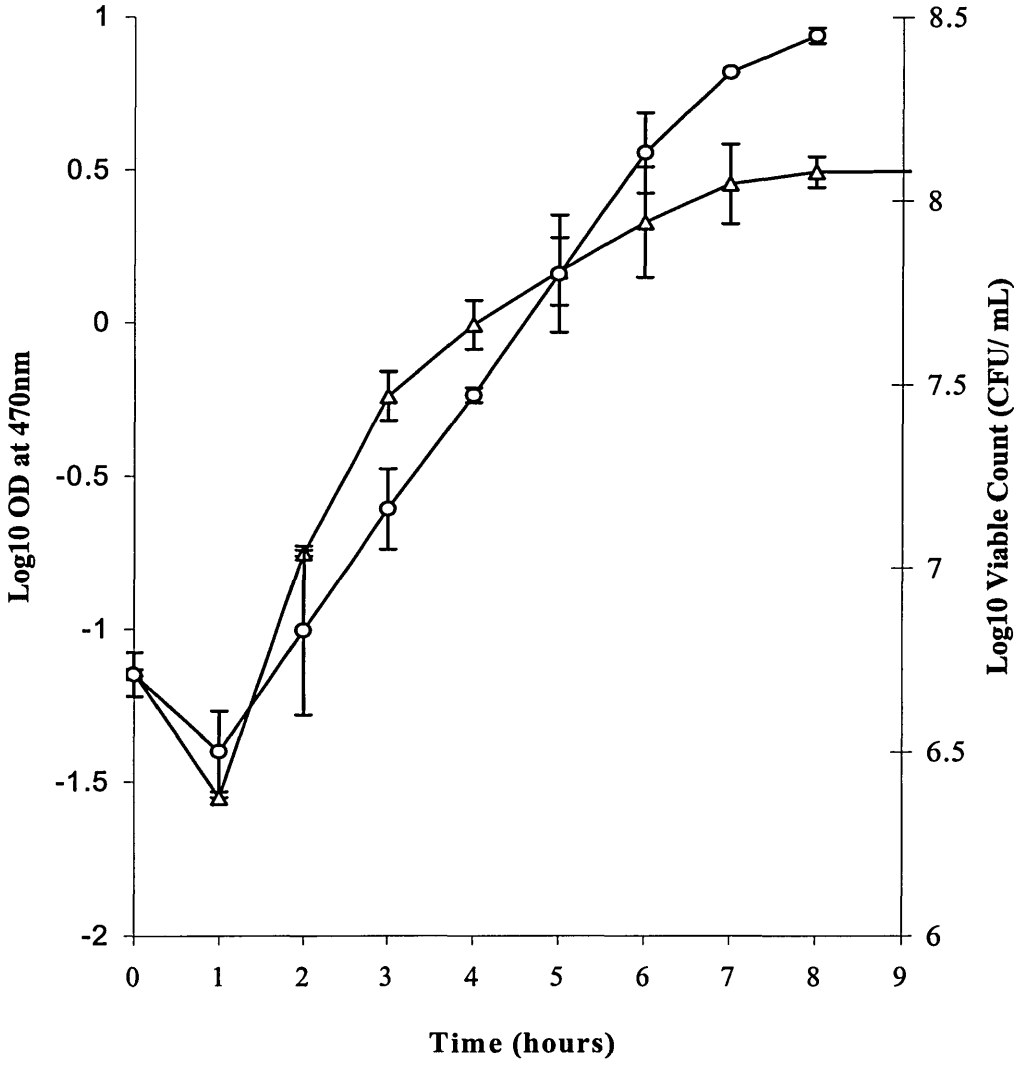


Figure 13: The observation of growth of *Escherichia coli* ATCC 10000 in R2A medium. Error bars are calculated as the standard deviation of the individual data points. n = 3 replicates. (o) $\log_{10}$ OD at 470 nm; (Δ) $\log_{10}$ viable count (CFU mL^{-1}).

The control experiment for *E. coli* ATCC 10000 biofilm cultures

Establishing *E. coli* biofilm on a Sorbarod filter was similar to that for *Ps. aeruginosa* PAO1 biofilm (Hodgson *et al.*, 1995; Chapter 2). 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 14).

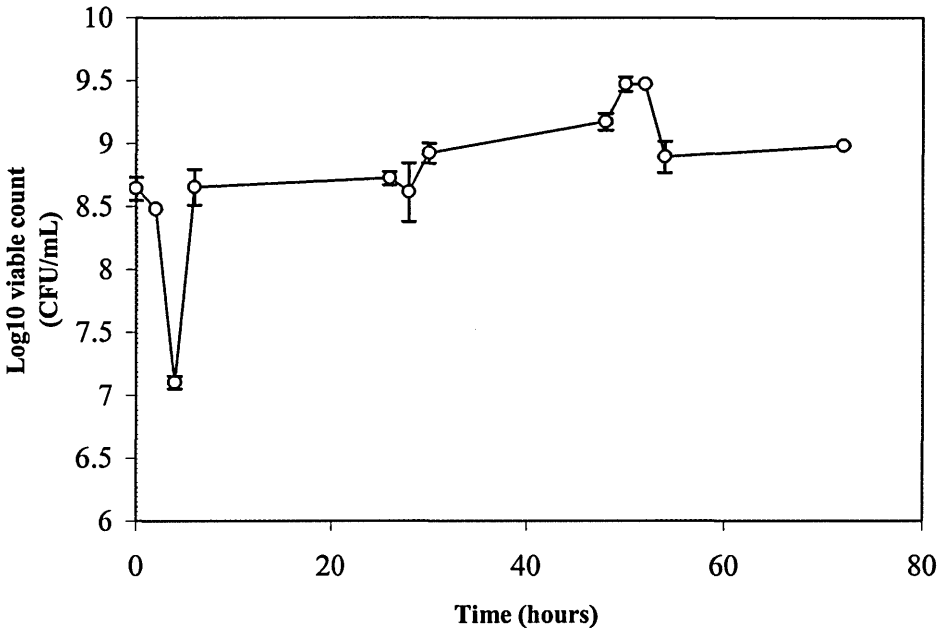


Figure 14: Graph of mean biofilm eluate counts (*Escherichia coli* ATCC 10000, perfused with modified (MCDM) over Time. Error bars are calculated as the standard deviation of the individual data points. n = 3 replicates. (o) $\log_{10}$ viable count (CFU mL^{-1}).

Studies on binary biofilms (*Pseudomonas aeruginosa* ATCC 15692 (PAO1) and *Escherichia coli* ATCC 10000)

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 multi-species 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.*, 2001b). The resultant biofilms may be thicker and more stable than monospecies biofilms and this could further influence their susceptibly to antimicrobials (Bourion & Cerf, 1996). 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 control experiment for binary biofilms (*Pseudomonas aeruginosa* ATCC 15692 (PAO1) and *Escherichia coli* ATCC 10000)

In order to establish a binary biofilm, the Sorbarod model was used (Hodgson *et al.*, 1995; Chapter 2). The Sorbarod filter was inoculated with mid-logarithmic phase of *E. coli* culture (10 mL). This was perfused with modified chemically defined

medium (MCDM) at 37°C. Modified CDM was suitable for the optimal and non-advantageous growth of both microorganisms *Ps. aeruginosa* and *E. coli.* 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 1M to 0.66 M) (Chapter 2). 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 (Al-Hmoud, 2002). 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 six-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 binary biofilm (Figure15).

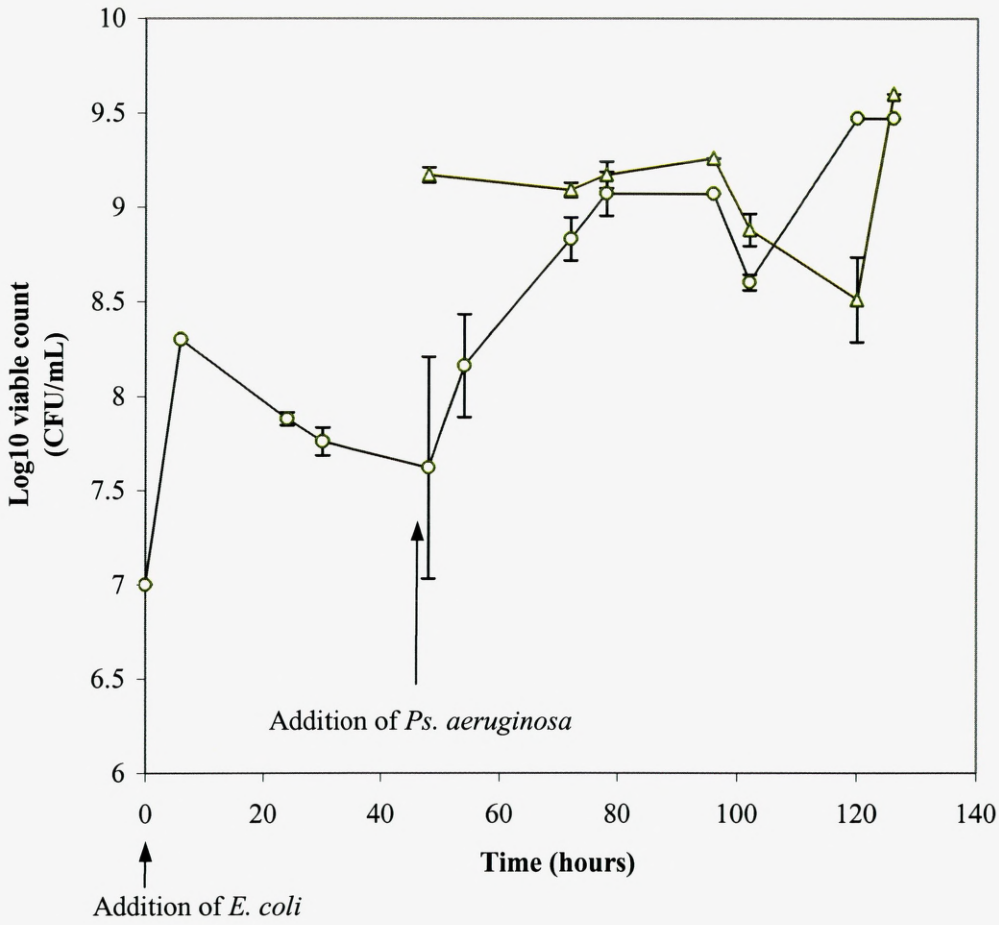


Figure 15: Graph of mean binary biofilm eluate counts for *Pseudomonas aeruginosa* ATCC 15692 (PAO1) and *Escherichia coli* ATCC 10000 over Time. Error bars are calculated as the standard deviation of the individual data points. n = 3 replicates. (o) $\log_{10}$ viable count (CFU mL^{-1}) for *E. coli*; (Δ) $\log_{10}$ viable count (CFU mL^{-1}) for *Pseudomonas aeruginosa*.

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^{-2} to 10^{-9}. Aliquots (100 μL) were spread plated onto sterile Chromogenic *E. coli* / Coliform agar plates (Oxide LTD., Basingstoke, Hampshire, England) in triplicate. The plates were in incubated at 37°C for 48 hours and the subsequent colonies were counted. Using the Chromogenic *E. coli* / Coliform agar was essential in this experiment to differentiate between both

types of bacteria. *Ps. aeruginosa* gives straw colonies while *E. coli* gives purple colonies on this agar (Figure 6 Chromogenic Medium, Chapter 2).

This experiment was performed by adding both bacteria (*Ps. aeruginosa* and *E. coli*) on the Sorbarod filter, simultaneously. The resultant binary biofilm was characterised by the dominance of *Ps. aeruginosa.* This may be partially explained by the extensive piliation of *Ps. aeruginosa.* Pratt & 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 has 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 has been suggested that type IV pili and type IV pili-mediated twitching motility in *Ps. aeruginosa* play a role in biofilm development (Pratt & Kolter, 1998). 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.

Results & Discussion

The relative concentrations of *Ps. aeruginosa* and *E. coli* in binary biofilms, as determined by observation of eluate cell numbers (Figure 15) could not be predicted from their relative abundance in monospecies communities (Figure 12 & 14). 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 (8.73 ± 0.1943 for *Ps. aeruginosa* and 8.44 ± 0.103 for *E. coli*) and those for monospecies biofilms (*Ps. aeruginosa* 9.1 ± 0.065 and *E. coli* 8.66 ± 0.325) indicate that binary biofilms are not simply a result of the addition of monoculture values. Therefore, the presence of *E. coli* cells has a negative effect on *Ps. aeruginosa* population numbers. 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 compositions may not accurately predict the biofilm population (Whiteley *et al.*, 2001b).

The growth inhibitory and antimicrobial activity of selected antibiotics on the growth of *Ps. aeruginosa* ATCC 15692 (PAO1) and *Escherichia coli* ATCC 10000 as both planktonic and biofilm Cultures.

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 (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 (McManus, 1997). Because resting stages are metabolically dormant and are not reproducing, they are unaffected by such antimicrobial agents (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 used in medical practice are aimed at eliminating infecting microorganisms or preventing the establishment of an infection (Chen & Stewart, 2000). In medicine, biofilms are responsible for numerous difficult-to-manage infections (Costerton *et al.,* 1999; Costerton, 2001). 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 (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.

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 (Russell, *et al* 1997).

Most of the common antibiotics used in medicine for treating bacterial infections are inhibitors of cell wall, protein synthesis or DNA replication (Neu, 1992; Goessens, 1993). For example, cephalosporins, aminglycosides, tetracyclines and quinolones are used to treat endocarditis, meningitis, tuberculosis and pneumonia (Foley & Gilbert, 1996). 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 (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 (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).

It has been proposed that intrinsic resistance in Gram-negative bacteria has the greatest significance (Russell, 1997). Resistance to antibiotics 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 barrier, thereby preventing the uptake of chemically unrelated molecules; Russell *et al.,* 1997; Russell *et al.,* 1998). Cell envelope changes have been 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 (CDR) efflux pumps and decreased OM permeability that confers this resistance (Masuda *et al.,* 1995).

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 up to 65% of bacterial infections (Potera, 1999). The mechanisms by which microorganisms in a biofilm evade killing by 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).

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 (Dodds *et al.,* 2000). 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 limitations occurs (Dodds *et al.,* 2000). Studies have shown that many antibiotics diffuse completely through the biofilm, but with a reduced rate of transfer (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 bound 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 (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). 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 (Stewart 2002; Gilbert, *et al.* 2002b).

The third mechanism of reduced biofilm susceptibility suggests 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). 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.

Antimicrobial susceptibility testing

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 used as an indicator of activity rather than something of real substantive value (Lambert & Pearson, 2002).

A useful definition of the MIC is "the *lowest concentration, which results 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 lowest 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 these experiments were to determine the Minimum Inhibitory Concentration (MIC) of selected antibiotics (ampicillin (AmP), nalidixic acid, (NaL) and streptomycin (ST)) against both monoculture and binary biofilms of *Ps. aeruginosa* ATCC 15692 (PAO1) and *Escherichia coli* ATCC 10000. 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 carried out. Besides yielding a set of MIC values for *Ps. aeruginosa* and *E. coli* grown as pure cultures in planktonic, biofilm and eluate forms, MIC determination also allowed selection of suitable sub-MICs of antibiotics. Additionally, mixed or binary biofilms of *Ps. aeruginosa* and *E. coli* were established and the MIC of these cultures and their eluate cells were also determined.

The antibiotics were selected from a broad range of classes, which include ß-lactams (ampicillin), quinolones (Nalidixic acid) and aminoglycosides (Streptomycin), respectively. The mode of action and targets of these different classes of antibiotics differ. While the ß-lactams, target the disruption of peptidoglycan synthesis due to competitive inhibition of the transpeptidation process; the quinolones (Nalidixic acid) and the aminoglycosides (streptomycin) disrupt internal processes of the bacterial cell. The quinolones bind the DNA gyrase and inhibit supercoiling of the

bacterial DNA, while the aminoglycosides inhibit protein synthesis by forming complexes with bacterial ribosomes. As indicated in Table 2 (Chapter 1), a number of antibiotics that fall within these general classes are currently being used for treatment against *Pseudomonas* infections.

Over the last 15 years, trends have indicated a decrease in susceptibility of*Ps. aeruginosa* towards these antibiotics (Hauser & Sriram 2005). Hence, there is reason to believe that this may be due to differential gene expression or adaptive mutations, leading to the expression of an unique set of outer membrane proteins responsible for the resistant phenotype. Hence, experiments were designed using sub-MICs of antibiotics to qualitatively visualise the changes in the outer membrane proteins (resulting from differential gene expression or adaptive mutations due to antibiotic influence) using SDS-PAGE and 2D-PAGE.

Monospecies and binary biofilms of*Ps. aeruginosa* ATCC 15692 (PAO1) and *Escherichia coli* ATCC 10000 were subjected to sub-minimum inhibitory concentrations (MIC/16) of ampicillin, nalidixic acid and streptomycin respectively. Although the use of sub-MICs had a negative effect on the growth rate of *Pseudomonas aeruginosa* and *E. coli*, biofilm formation was not hampered. Such biofilms were used for further investigation regarding the development of tolerance or resistance towards the selected antibiotics.

Minimum Inhibitory Concentrations (MICs) were determined for *Pseudomonas aeruginosa* and *E. coli* cultures in the planktonic, eluate and biofilm states. Separately MICs were also determined for binary biofilms. Determination of the MIC values would reflect the capacity of the bacteria to tolerate antibiotics. Changes in MIC values should indicate corresponding organismal changes in antibiotic susceptibility. For example, a positive increase in MIC values would suggest that the bacteria are more resistant to antibiotics, while a decrease would

indicate the opposite. In a different perspective, an increase in the MIC values may also indicate a corresponding increase in the pathogenic potential of the organism. This may be so as the organism is now capable of tolerating and causing disease in the presence of antibiotics. Although as indicated previously, a number of studies(Wilson *et al.* 2002; Drago *et al.* 2001; Kim *et al.* 2001; Braga *et al.* 2000) have reported a decrease in the pathogenic potential of the organism in the presence of antibiotics, this study aims to investigate this hypothesis in sub-MIC exposed biofilms.

Experimental approach

MIC determination for planktonic culture of Ps. aeruginosa PAO1

Aliquots (9 mL) of sterile Modified CDM medium (MCDM) were prepared and doubling increases of antibiotics (1μg mL^{-1} to 512 μg mL^{-1}) were added in 1 mL aliquots. Tubes were inoculated with 100 μL of a 16 h (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 after 48 h. The MIC was determined as the lowest concentration of the antibiotic showing no visible growth after 48 hours (Bloomfield, 1991; Figure 16).

MIC determination for biofilm and eluate culture of Ps. aeruginosa PAO1

A biofilm of *Ps. aeruginosa* PAO1 was established on a Sorbarod filter (Chapter 2; Hodgson *et al.,* 1995). This biofilm was perfused with MCDM at 37°C. This was only run for 102 hours (to reach steady-state). At 102 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 three selected antibiotics (ampicillin (AmP), nalidixic acid (NaL) and streptomycin (ST)) were determined according to the

tube dilution method (Bloomfield, 1991) using a standard inoculum (100 µL). This was performed for the eluate and attached cells (Figure 16).

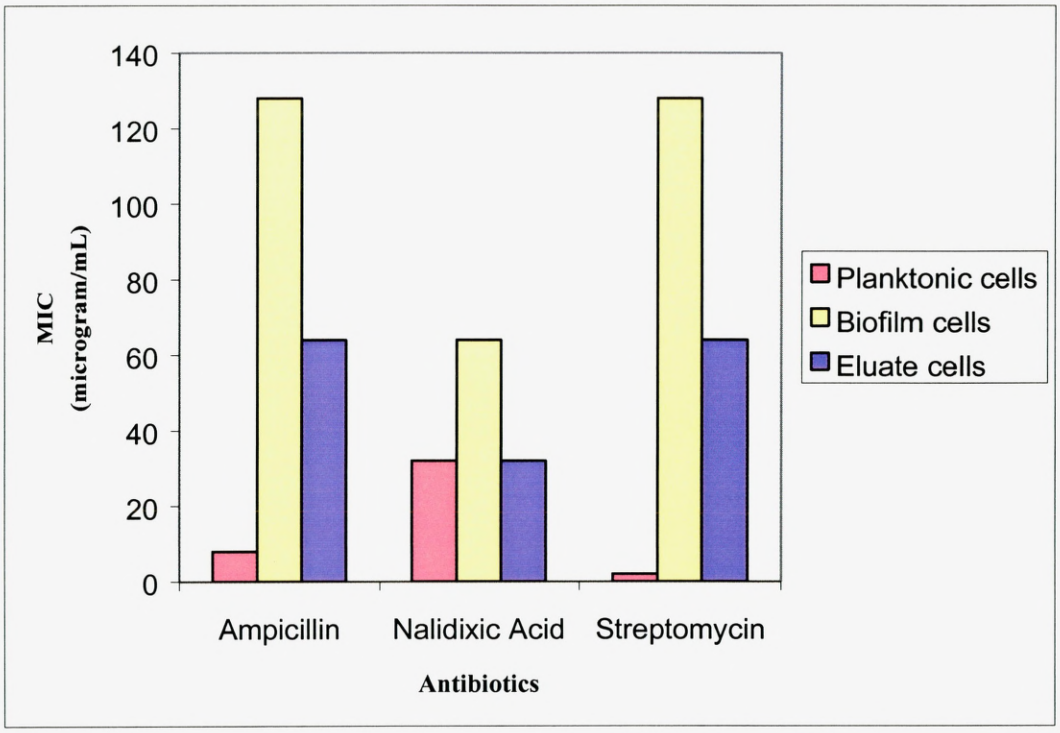


Figure 16: Summary of MIC results obtained against *Ps. aeruginosa* ATCC 15692 (PAO1) planktonic, biofilm and eluate cells cultured in modified CDM. Error bars are calculated as the standard deviation of the individual data points. n = 3 replicates. NB: SD = 0, hence error bars are inapparent.

MIC determination for planktonic culture of Escherichia coli ATCC10000

Aliquots (9 mL) of sterile MCDM prepared and doubling increases of antibiotics (1µg mL^{-1} to 512 µg mL^{-1}) were added in 1 mL aliquots (Figure 17). Tubes were inoculated with 100 µL of a 16 h (overnight) culture of *Escherichia coli* grown at 37°C, 180 osc min^{-1} and vortexed (Rotamixer, Hook and Tucker; UK). The tubes were incubated at 37°C and observed for growth after 48 h. The MIC was determined as the lowest concentration of the antibiotic showing no visible growth after 48 hours (Figure 17).

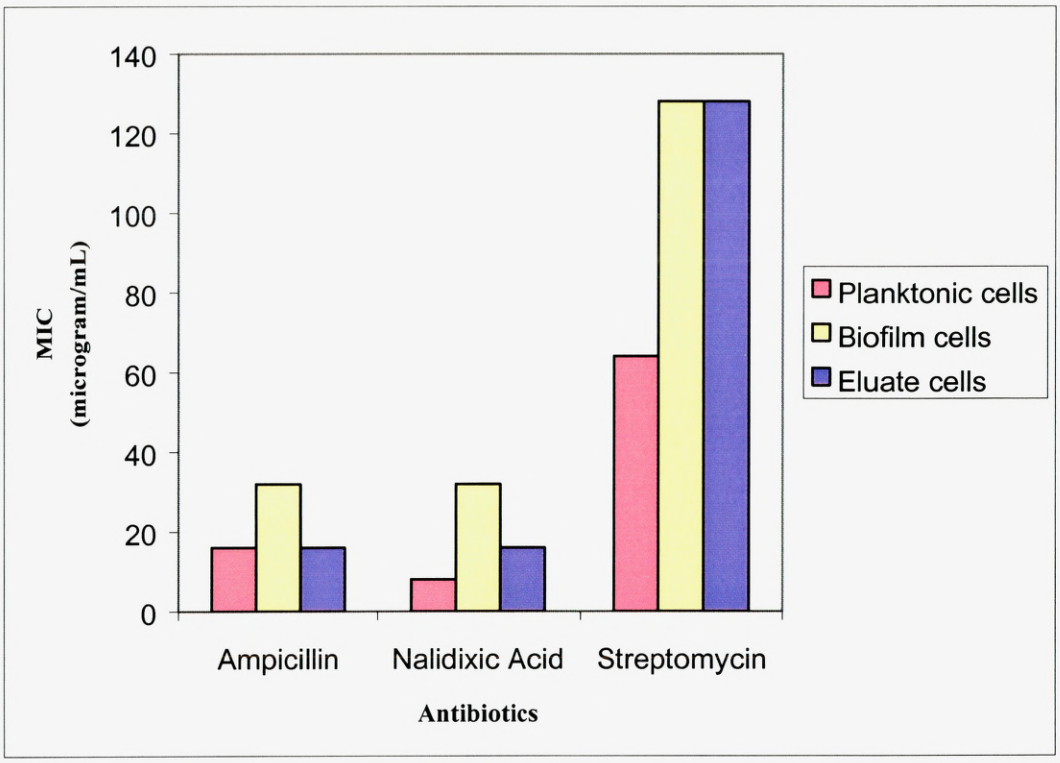


Figure 17: Summary of MIC results obtained against *E. coli* ATCC 10000 planktonic, biofilm and eluate cells cultured in modified CDM. Error bars are calculated as the standard deviation of the individual data points. n = 3 replicates. NB: SD = 0, hence error bars are inapparent.

MIC determination for biofilm and eluate culture of Escherichia coli ATCC 10000

A biofilm of *Escherichia coli* was established on a Sorbarod filter (Chapter 2; Hodgson *et al.,* 1995). This biofilm was perfused with MCDM at 37°C. This was only run for 56 hours (to reach steady-state). At 56 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 three selected antibiotics (ampicillin (AmP), nalidixic acid (NaL) and streptomycin (ST)) were determined according to the tube dilution method (Bloomfield, 1991) using a standard inoculum (100 μL). This was performed for the eluate and attached cells (Figure 17).

MIC determination for binary biofilm cells and eluate cells of Ps. aeruginosa ATCC 15692 (PAO1) and Escherichia coli ATCC 10000

In order to establish a binary biofilm, the Sorbarod model was used (Hodgson *et al.*, 1995; Al-Hmoud, 2002; Chapter 2). The Sorbarod filter was inoculated with mid-logarithmic phase of *E. coli* culture (10 mL). This was perfused with modified chemically defined medium (MCDM) at 37°C. Modified CDM was suitable for the optimal and non-advantageous growth of both microorganisms *Ps. aeruginosa* and *E. coli*. 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 0.028M, Al-Hmoud, 2002) and decreasing the concentration of carbon (solution C: the concentration was decreased from 1M to 0.66M, Al-Hmoud, 2002) (Chapter 2).

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 (Al-Hmoud, 2002). 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. At 126 hours, the Sorbarod filters were sacrificed and the MIC (Bloomfield, 1991) against these selected antibiotics AmP, NaL and ST were determined for both the attached and eluate cells as a mixed population (Figure 18).

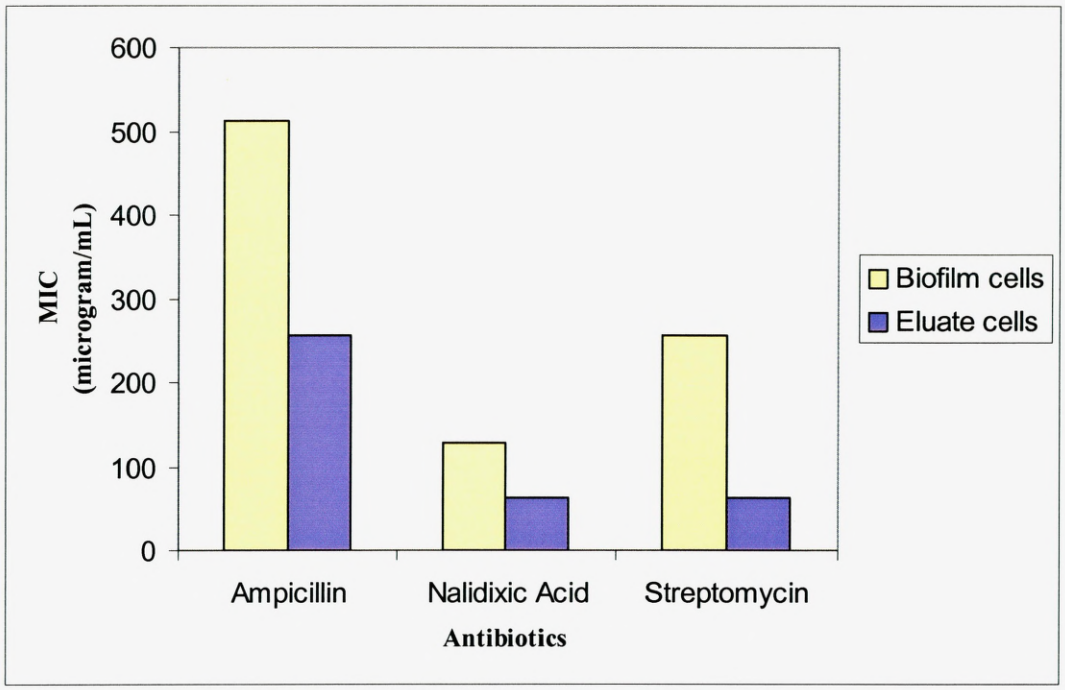


Figure 18: Summary of MIC results obtained against binary biofilm and eluate cells of *Ps. aeruginosa* ATCC 15692 (PAO1) and *E. coli* ATCC 10000 cultured in modified CDM. Error bars are calculated as the standard deviation of the individual data points. n = 3 replicates. NB: SD = 0, hence error bars are inapparent.

Results & Discussion

Minimal inhibitory concentration (MIC) analysis was carried out on *Ps. aeruginosa* PAO1 strains, in three states; planktonic, biofilm and eluate. The results show varying MIC values for antibiotics for each of these states, suggesting a change in the physiology of the organisms and their behaviour towards antibiotics. It can be seen that MIC values of biofilm and the eluate states of the bacteria were several times higher to that of the planktonic state (Table 6). Although it has been established elsewhere in this thesis that mechanisms involved with biofilm resistance to antibiotics are inherently due to the multicellular nature of the biofilm, a serious distinction has to be made at this stage regarding the nature of the resistance reported in this thesis. While measuring MICs using the tube dilution method, the biofilms were sacrificed and the cells were resuspended in media. This process eliminates some of the key proposed benefits of multicellular biofilm resistance and forces the

bacteria into a planktonic form. However, due to the abrupt nature of this experimental transition from biofilm to the planktonic form, it is highly unlikely that the biofilm cell has adopted a planktonic physiology. This is surmised from the fact that MIC values of biofilm cells are several times higher than their planktonic counterparts. Furthermore, as resistance due to the multicellular nature of biofilm is compromised in the experimental protocol, the resulting increase in the MIC can only be explained by an inherent cellular mechanism. It is proposed to investigate the possibility that the resistance seen is a result of outer membrane protein shifts and changes in the cell wall structure of the bacterial cell.

The eluate cells studied here are probably cells that detach from the biofilm and have undergone a physiological reversion back to the planktonic state. However, the increased tolerance exhibited towards selected antibiotics as concluded from the MIC values show that these cells haven't lost all their biofilm characteristics.

Table 6: Summary of MIC results obtained against binary biofilm, eluate and planktonic cells of (*Ps. aeruginosa* ATCC 10000 (PAO1) and *Escherichia coli* (ATCC 10000) and monospecies biofilms cultured in modified CDM.

Antibiotic	**Type of cells**	***Ps. aeruginosa* (ATCC 15692) (PAO1)**	***E. coli* (ATCC 10000)**	**Binary (mixed) cells**
		MIC ($\mu g\ mL^{-1}$)	**MIC ($\mu g\ mL^{-1}$)**	**MIC ($\mu g\ mL^{-1}$)**
Ampicillin	Planktonic Cells	8	16	nd
	Biofilm Cells	128	32	512
	Eluate Cells	64	16	256
Nalidixic acid	Planktonic Cells	32	8	nd
	Biofilm Cells	64	32	128
	Eluate Cells	32	16	64
Streptomycin	Planktonic Cells	2	64	nd
	Biofilm Cells	128	128	256
	Eluate Cells	64	128	64

nd = Not determined

What is indeed interesting from the results indicated in Figure 18 is that binary biofilms have an increased antibiotic tolerance to that observed with monospecies

biofilms (in binary biofilm cells the MIC value with ampicillin was 512 μg mL^{-1}, with nalidixic acid 128 μg mL^{-1} and with streptomycin 256 μg mL^{-1}, while in the monospecies biofilms (in *Ps. aeruginosa* PAO1 the MIC value with ampicillin was 128 μg mL^{-1}, with nalidixic acid was 64 μg mL^{-1} and with streptomycin was 128 μg mL^{-1} while (in *E. coli* ATCC 10000 the MIC value with ampicillin was 32 μg mL^{-1}, with nalidixic acid was 32 μg mL^{-1} and with streptomycin the MIC was 128 μg mL^{-1}). The practical significance of this observation is heightened, when considering the fact that in medical and environmental contexts, biofilms exist as mixed communities and that they may offer a synergistic resistance to antimicrobials. The results in these analyses reveal that bacteria living in biofilms can be up to 7-fold more resistant to antibiotics than the corresponding planktonic population of the same single or mixed species.

The increase in the MIC values provides ample ground to investigate the hypothesis that post-biofilm formation, the virulence and resistance of the organism increases. Although it is known that antibiotic resistance of the organism increases after biofilm formation (Davies 2003; Gilbert, *et al.* 2002a), there is no current evidence of virulence of the organism increasing post-biofilm formation. Based on the initial MIC results, studies to determine the activity of the virulence factors before and after biofilm formation were designed. In order to investigate the inherent cellular resistance exhibited by the biofilm cells against antibiotics, the cell envelope characteristics were probed for the presence or absence of OMPs that may shed a clue to their increased resistance.

Chapter Four: Studies of the Outer Membrane Proteins of Planktonic and Biofilm Cultures of *Pseudomonas aeruginosa* ATCC 15692 (PAO1) and *Escherichia coli* ATCC 10000 Challenged with sub-MICs of Selected Antibiotics

Introduction

Pseudomonas aeruginosa is one of the most antibiotic resistant of the non-spore-forming bacteria. Intrinsic resistance exhibited by this organism is higher than any other Gram-negative bacteria (Hancock, 1997). Although three main mechanisms of intrinsic resistance has been attributed to *Pseudomonas aeruginosa* (outer membrane impermeability, drug efflux mechanisms and antibiotic degrading enzymes; McDonnell & Russell 1999, Jo *et al.*, 2003), low outer membrane permeability is the property that distinguishes *Ps. aeruginosa* from other Gram-negative bacteria (McDonnell & Russell 1999). Hence, antibiotic resistance has been primarily proposed to be due to cell envelope characteristics (Yoshihara & Nakae, 1989).

The outer cell envelope of *Pseudomonas aeruginosa* is composed mainly of proteins (outer membrane proteins, OMPs) and lipopolysaccharide (LPS) (Backhed *et al.*, 2003). Alterations in the outer membrane proteins (OMPs) and the integrity of the LPS influence the permeability of the outer membrane to antimicrobial agents (Koebnik *et al* 2000). The presence of LPS in the outer membrane confers very low permeability for hydrophobic antibiotics (Koebnik *et al* 2000; Hogan and Kolter, 2002). However, the overall permeability of the outer membrane depends upon the number and properties of pore-forming proteins, generally called porins (Trias & Nikaido, 1990). Porins are proteins that form pores in the outer membrane by the clustering of three protein subunit molecules (trimeric) (Koebnik *et al.*, 2000), by

folding into β-pleated sheets to form a closed barrel. *Ps. aeruginosa* also exhibits resistance to small hydrophilic antibiotics, like tetracyclines and β-lactams. *Ps. aeruginosa* has 12- to 100-fold lower outer membrane permeability than *Escherichia coli* (Russell & Chopra 1996; Hancock 1998). Recently, the *Ps. aeruginosa* genome was published and three large paralogous families of outer membrane proteins have been identified (Stover, *et al* 2000). The OprD family of specific porins (19 genes), the TonB-family of gated porins and the OprM family of outer membrane proteins involved in efflux or secretion (18 genes) (Stover, *et al* 2000). Most porins of *Ps. aeruginosa* are specific for certain substrates (e.g. for basic amino acids (OprD), or for phosphate (OprP); Hancock *et al.*, 1990). These porins permit entry of structural analogues (eg: OprD, which is the protein by which the antibiotic imipenem enters through the outer membrane of *Ps. aeruginosa* (Nikaido, 1992)). On the other hand OprF (38 kDa), which is the major non-specific OMP, has poor efficiency intransport (Hancock 1998), although it forms both large and small porins. Despite these variations in membrane permeability in the planktonic forms of *Pseudomonas aeruginosa*, the periplasmic concentrations of many antibiotics can 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. It is now well understood that outer membrane impermeability must be coupled with a secondary antibiotic resistance mechanism, like active drug efflux, for effective resistance against antibiotics (Jo *et al.*, 2003).

Although it may be said that multi drug efflux systems act as a major resistance mechanism in planktonic *Ps. aeruginosa*, evidence indicates the contrary within biofilms. Recently, De Kievit *et al.* (2001b) revealed that the expression of the four well-characterised multidrug resistant (MDR) pumps (MexAB-OprM, MexCD-OprJ, MexFE-OprN, and MexXY) is not increased in *Ps. aeruginosa* biofilms. This

suggests that the other factors, such as decreased membrane permeability resulting from specific molecular organisation of lipopolysaccharides and porins may be responsible for the innate resistance to antibiotics by this population of bacteria (Tattawasarat, *et al.* 2000).

Pseudomonas aeruginosa grows as biofilms in nature and is also associated with chronic lung infections in patients with cystic fibrosis (CF) (Hoiby, 2002). Chronicity of infections has been recently attributed to antibiotic resistance of biofilms (Costerton, 2001, Hall-Stoodley & Stoodley, 2005, Fux *et al.*, 2005). This chapter aims to investigate alteration of outer membrane proteins (OMPs) as a possible source for structural envelope modifications responsible for antibiotic resistance. Accordingly, monospecies and binary biofilms of *Pseudomonas aeruginosa* were tested for alterations of outer membrane proteins in the presence of sub-minimal inhibitory concentrations of selected antibiotics (ampicillin, nalidixic acid and streptomycin).

Experimental approach

In this study, outer membrane proteins of monospecies and binary biofilms, eluate and planktonic cells of *Ps. aeruginosa* and *Escherichia coli* in the presence of the MIC/16 of antibiotics AmP-, NaL- and ST-sensitive and resistant cells were analyzed using SDS-PAGE, in order to examine any alterations in protein profiles. Two different methods for preparation of OMPs were used (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; Chapter 2). Eluate OMPs were prepared for the same analyses by the method of Pugsley *et al.*, (1986; Chapter 2). However, before OMPs could be isolated from bacterial cell walls, biofilms had to be grown on Sorbarod filters as described below.

Growth of monospecies and binary biofilms of *Ps. aeruginosa* ATCC 15692 (PAO1) and *Escherichia coli* ATCC 10000

Monospecies biofilms of *Ps. aeruginosa* ATCC 15692 (PAO1) and *Escherichia coli* ATCC 10000 were established on Sorbarod filters (Chapter 2; Hodgson *et al.,* 1995). The Sorbarod adherent cells (*Ps. aeruginosa* and *E. coli*) were initially perfused with MCDM (modified chemically defined medium) in the presence of the MIC/16 of selected antibiotics (ampicillin (AmP); nalidixic acid (NaL) and streptomycin (ST)) at 37°C for 102 hours and 56 hours respectively, until biofilm steady state was achieved for both organisms. Subsequent to the formation of mature biofilms (i.e. after steady state was achieved) the Sorbarod filters were sacrificed and the adherent cells were removed and resuspended in sterile normal saline (0.9% NaCl in distilled water (w/v)). Control and antibiotic treated biofilm and eluate cells were stored separately at –20°C for later analysis.

In order to establish a binary biofilm, the modified Sorbarod model was used (Al-Hmoud, 2002; Chapter 2). The Sorbarod filter was inoculated with mid-logarithmic phase of *E. coli* culture (10 mL), and supplemented with modified chemically defined medium (MCDM) in the presence of the MIC/16 of selected antibiotics (ampicillin (AmP), nalidixic acid (NaL) and streptomycin (ST)) at 37°C. The *E. coli* biofilm was run for 48 hours (approaching biofilm 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 cells before introducing *Ps. aeruginosa* to the community. The system was run for 126 hours until a combined biofilm steady state resulting from the presence of both bacteria was achieved. The Sorbarod filters were sacrificed (the adherent cells were removed and resuspended in sterile normal saline) and both biofilm and eluate cells were collected and centrifuged

(IEC Centra-4B) at 5000 g for 20 minutes. Cell pellets were collected and stored at–20°C for all subsequent analysis.

Modified CDM was suitable for the optimal and non-advantageous growth of both microorganisms, *Ps. aeruginosa* and *E. coli*. The concentration of nitrogen (NH_4Cl) in CDM (solution A) was increased from 0.0187M to 0.028M and the concentration of carbon (solution C) was decreased from 1M to 0.66M (Al-Hmoud, 2002). These modifications in the concentrations of carbon and nitrogen allowed the doubling times for both bacteria to approximate each other.

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 treated with the anionic detergent, sodium dodecylsulphate (SDS) or sodium lauryl sulphate (SLS) to form negatively charged complexes. The amount of SDS bound by a protein, and so the charge on the complex, is roughly proportional to its size. Roughly, about 1.4g SDS is bound per gram of protein, although there are exceptions to this rule (Deyl, 1979). The proteins are generally denatured and solubilized when bound to SDS, and the complex adopts the form of a negatively charged prolate ellipsoid or rod of length roughly proportional to the protein's molecular weight. Thus, proteins of acidic or basic pI (isoelectric point) form negatively charged complexes that can be separated on the basis of differences in charge and size by electrophoresis through a sieve-like matrix of polyacrylamide gel (Smith, 1984). Sample preparation for SDS-PAGE and 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 formallows simultaneous electrophoresis of more than one sample and thus, is ideal for comparative purposes.

Preparation of separating and stacking gel mixtures, were undertaken as described in Chapter 2. Outer membrane protein profiles were obtained using a Power Pack 300 (constant current of 35 mA for 1.5 h) and then stained with Coomassie brilliant blue R250 or Silver staining (described in detail in Chapter 2).

The analysis of OMP profiles

The first stages in analysing the gels (image capture) were performed using Phoretix imaging analysis software (non-Linear Dynamics Ltd, Newcastle upon Tyne, England). However, this software was faulty and hence, the molecular weights and R_f (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. Lane 1 in each gel demonstrates the standard molecular weights (M_r) markers for that gel. These (M_r) markers are given below in Table 7.

By plotting molecular weights of markers against their R_f values, a standard calibration curve was constructed (Figure 19). However, R_f values for these standard molecular weights may vary slightly from one gel to another. This standard calibration curve was used to determine the molecular weights of all the proteins on the gel. The R_f for each band was estimated according to Equation 2:

R_f = distance migrated by protein/distance migrated by dye (Equation 2)

Table 7: Standard molecular weight ladder for SDS Polyacrylamide Gel Electrophoresis (14.2 to 66 kDa)		
Protein	**M_r (kDa)**	**R_f**
Albumin, Bovine	66	0.34
Ovalbumin, chicken	45	0.49
Glyceraldehyde-3- phosphate Dehydrogenase, Rabbit	36	0.52
Carbonic Anhydrase, Bovine	29	0.62
Trypsinogen, Bovin, PMSF treated	24	0.77
Trypsin Inhibitor, Soybean	20.1	0.86
α-Lactalbumin, Bovine	14.2	0.98

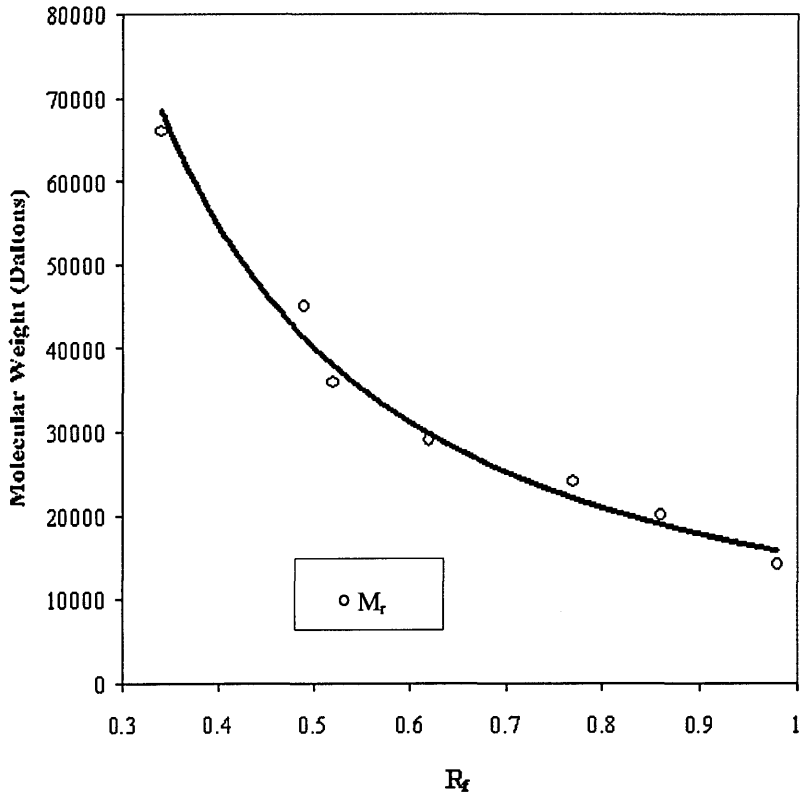


Figure 19: The standard calibration curve generated by plotting the molecular weight (M_r) of the low range molecular weight standards vs. the R_f.

Results & Discussion

Upon exposure of *Ps. aeruginosa* PAO1 biofilm and eluate cells to sub-MICs of antibiotics (AmP, NaL and ST) some distinct variations in the number of outer membrane proteins (OMPs) between the planktonic, biofilm and eluate cells were observed. In particular, it was possible to observe the presence of 'novel' proteins within sub-MIC antibiotic-exposed treatments (NB: for the purposes of this research, a novel protein is defined as one which has a greater than 1 kDa shift from a protein in a similar position on a control gel, but which is no longer present in the treated gel).

OMP shifts in *Ps. aeruginosa* PAO1 biofilm and eluate cells exposed to sub-MICs of ampicillin (AmP), nalidixic acid (NaL) and streptomycin (ST)

A 44.7 kDa outer membrane protein present in control (cells unexposed to antibiotics) (lane 3, Figure 20), was not apparent after cells were exposed to sub-MICs of antibiotics of AmP, NaL and ST (lanes 4, 5 & 6 respectively, Figure 20). Similarly a 37 kDa OMP present in control (lane 3, Figure 20) was not present in (lanes 4 and 5), after cells were exposed to sub-MICs of antibiotics AmP and NaL. However, this OMP appeared in cells exposed to sub-MICs of streptomycin (lane 6, Figure 20; Table 8). 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 relatively similar molecular weight has been assumed to indicate a small apparent molecular weight change in a single protein rather than the coupled disappearance/ appearance of two proteins.

A 71 kDa outer membrane protein was absent in both sub-MIC controls of planktonic and eluate cells (lanes 2 and 4, Figure 21), but appeared after cells were exposed to sub-MICs of antibiotics AmP, NaL and ST (Lane 5, 6 & 7, Figure 21). Two outer membrane proteins of 43 kDa and 40.2 kDa were absent from

control samples of both planktonic and eluate cells (lanes 2 and 4, Figure 21), but appeared after cells were exposed to sub-MIC of antibiotics with nalidixic acid (lane 6, Figure 21; Table 9).

All subsequent analysis of results from biofilm and eluate samples of *E. coli* and binary cultures were carried out in a similar fashion as indicated below (Table 8, parts 1 and 2; Table 9, parts 1, 2 and 3). These analyses (gels were analysed manually) are not given here for the purposes of brevity.

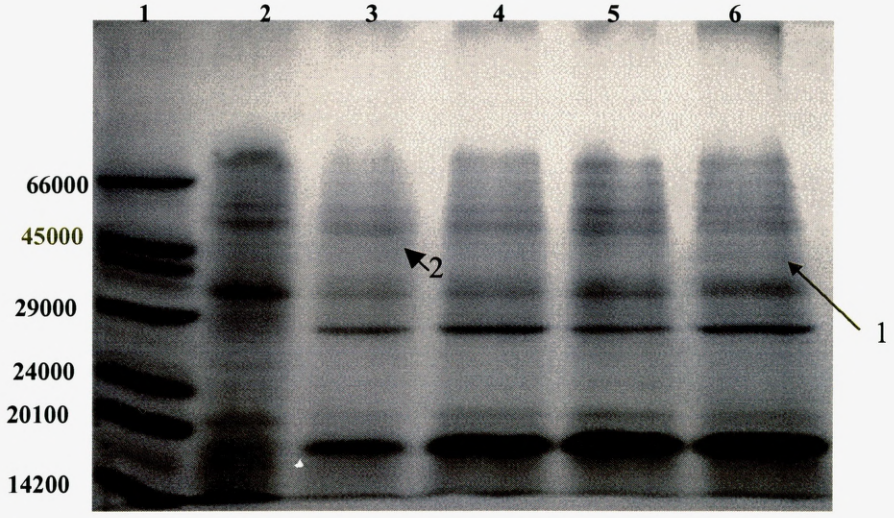


Figure 20: Outer membrane protein profile of *Ps. aeruginosa* ATCC 15692 (PAO1) biofilm cells cultured in modified CDM and exposed to different treatment of antibiotics. Lane 1: molecular weights markers; Lane 2: *Ps. aeruginosa* planktonic cells control (776 μg protein well^{-1}); Lane 3: *Ps. aeruginosa* biofilm cells control (729 μg protein well^{-1}); Lane 4: *Ps. aeruginosa* biofilm cells (739 μg protein well^{-1}) with treatment of ampicillin at sub-MIC (8 μg mL^{-1}); Lane 5: *Ps. aeruginosa* biofilm cells (732 μg protein well^{-1}) with treatment of nalidixic acid at sub-MIC (4 μg mL^{-1}); Lane 6: *Ps. aeruginosa* biofilm cells (736 μg protein well^{-1}) with treatment of streptomycin at sub-MIC (8 μg mL^{-1}). Arrow (1) indicates the 37 kDa OMP. Arrow (2) indicates the 44.7 kDa OMP.

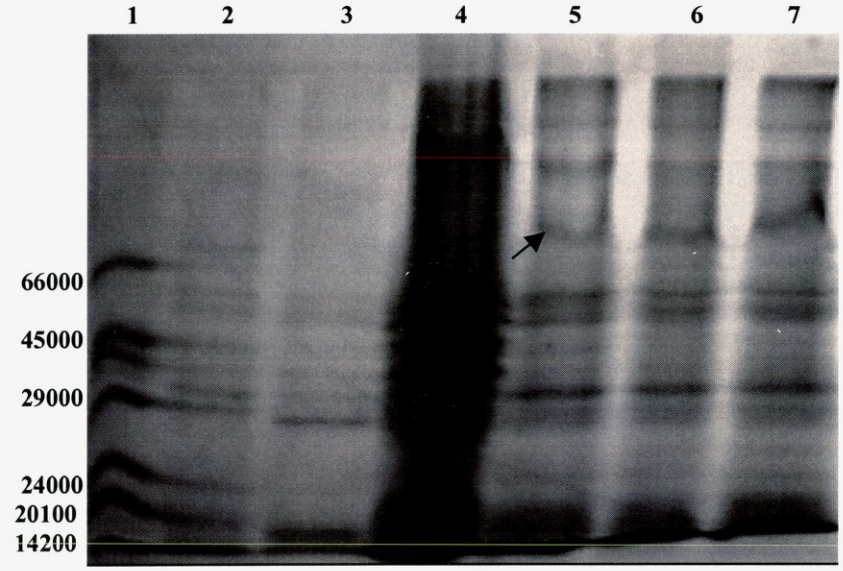


Figure 21: Outer membrane protein profile of *Ps. aeruginosa* PAO1 eluate cells cultured in modified CDM and exposed to different treatment of antibiotics. Lane 1: molecular weights markers; Lane 2: *Ps. aeruginosa* planktonic cells control (776 μg protein well^{-1}); Lane 3: *Ps. aeruginosa* biofilm cells control (729 μg protein well^{-1}); Lane 4: *Ps. aeruginosa* eluate cells control (752 μg protein well^{-1}); Lane 5: *Ps. aeruginosa* eluate cells (731 μg protein well^{-1}) with treatment of ampicillin at sub-MIC (8 μg mL^{-1}); Lane 6: *Ps. aeruginosa* eluate cells (728 μg protein well^{-1}) with treatment of nalidixic acid at sub-MIC (4 μg mL^{-1}); Lane 7: *Ps. aeruginosa* eluate cells (736 μg protein well^{-1}) with treatment of streptomycin at sub-MIC (8 μg mL^{-1}). Arrow indicates the 71 kDa OMP.

Table 8 (part 1): Measurement data indicating Band number, Molecular weight (M_r) and Retardation factor (R_f) for Outer Membrane Proteins of *Ps. aeruginosa* PAO1 biofilm cells exposed to different treatment of antibiotics and cultured in modified CDM (Band no: band number; Co: corresponding protein to Lane 3 (NB: only the test lanes 4, 5 & 6 are compared to lane 3); N: novel protein).

Lane 1 (molecular weight markers)				Lane 2 (*Ps. aeruginosa* planktonic cells control)				Lane 3 (*Ps. aeruginosa* biofilm cells control)			
Band no.	M_r (kDa)	R_f	Co	Band no.	M_r (kDa)	R_f	Co	Band no.	M_r (kDa)	R_f	Co
1	66	0.34	-	1	(72.2)	(0.11)	-	1	(73.7)	(0.09)	-
2	45	0.49	-	2	(71)	(0.13)	-	2	(68.7)	(0.16)	-
3	36	0.52	-	3	(68.2)	(0.17)	-	3	(66)	(0.20)	-
4	29	0.62	-	4	(66)	(0.20)	-	4	(63.2)	(0.24)	-
5	24	0.77	-	5	(64)	(0.23)	-	5	(61.2)	(0.27)	-
6	20.1	0.86	-	6	(62.5)	(0.25)	-	6	(59.7)	(0.29)	-
7	14.2	0.98	-	7	(60.5)	(0.28)	-	7	57.7	0.32	-
-	-	-	-	8	(57.7)	(0.32)	-	8	53	0.35	-
-	-	-	-	9	51	0.36	-	9	48	0.38	-
-	-	-	-	10	48	0.38	-	10	44.7	0.41	-
-	-	-	-	11	44.7	0.41	-	11	41.7	0.44	-
-	-	-	-	12	41.7	0.34	-	12	38.7	0.47	-
-	-	-	-	13	37	0.50	-	13	37	0.50	-
-	-	-	-	14	33.5	0.55	-	14	36	0.52	-
-	-	-	-	15	30.5	0.60	-	15	33	0.56	-
-	-	-	-	16	28.2	0.64	-	16	29.5	0.63	-
-	-	-	-	17	26.7	0.67	-	17	26.7	0.67	-
-	-	-	-	18	23.7	0.74	-	18	22.7	0.78	-
-	-	-	-	19	21.7	0.79	-	19	19.5	0.85	-
-	-	-	-	20	19.2	0.86	-	20	17.5	0.90	-
-	-	-	-	21	17	0.91	-	-	-	-	-
-	-	-	-	22	15.7	0.95	-	-	-	-	-

KEY:

( ): Molecular weights estimated from linear equation derived from standard calibration curve (Fig 19)

- : No data for that cell

Table 8 (part 2): Measurement data indicating Band number, Molecular weight (M_r) and Retardation factor (R_f) for Outer Membrane Proteins of *Ps. aeruginosa* biofilm cells exposed to different treatment of antibiotics and cultured in modified CDM (Band no: band number; Co: corresponding protein to Lane 3 (NB: only the test lanes 4, 5 & 6 are compared to lane 3); N: novel protein).

Lane 4 (*Ps. aeruginosa* biofilm cells with treatment of ampicillin at sub-MIC (8 μg mL^{-1}).				Lane 5 (*Ps. aeruginosa* biofilm cells with treatment of nalidixic acid at sub-MIC (4 μg mL^{-1}).				Lane 6 (*Ps. aeruginosa* biofilm cells with treatment of streptomycin at sub-MIC (8 μg mL^{-1}).			
Band no.	M_r (kDa)	R_f	Co	Band no.	M_r (kDa)	R_f	Co	Band no.	M_r (kDa)	R_f	Co
1	(59.7)	(0.29)	6	1	(59.2)	(0.30)	6	1	(59.2)	(0.30)	6
2	49.5	0.37	N	2	49.5	0.37	N	2	46.7	0.39	N
3	45.7	0.40	N	3	45.7	0.40	N	3	44.7	0.41	10
4	41.7	0.44	11	4	43.7	0.42	10	4	41.7	0.44	11
5	41	0.45	11	5	41.7	0.44	11	5	41	0.45	11
6	40.2	0.46	N	6	41	0.45	11	6	37	0.50	13
7	33	0.56	15	7	33	0.56	15	7	32.2	0.57	15
8	32.2	0.57	15	8	32.2	0.57	15	8	30.5	0.60	16
9	30.5	0.60	16	9	30.5	0.60	16	9	29	0.62	16
10	29.5	0.63	16	10	29.5	0.63	16	10	27.7	0.65	17
11	27.7	0.65	17	11	27.7	0.65	17	11	25	0.71	N
12	26.2	0.68	17	12	26.2	0.68	17	12	23.7	0.75	18
13	24.5	0.72	N	13	25	0.71	N	13	20.2	0.83	19
14	22.5	0.77	18	14	23	0.76	18	14	17.5	0.90	20
15	19.7	0.84	19	15	20.2	0.83	19	-	-	-	-
16	17.5	0.90	20	16	17.5	0.90	20	-	-	-	-

KEY:

( ): Molecular weights estimated from linear equation derived from standard calibration curve (Fig 19)

- : No data for that cell

Table 9 (part 1): Measurement data indicating Band number, Molecular weight (M_r) and Retardation factor (R_f) for Outer Membrane Proteins of *Ps. aeruginosa* PAO1 eluate cells exposed to different treatment of antibiotics and cultured in modified CDM (Band no: band number; Co: corresponding protein to Lane 4 (NB: only the test lanes 5, 6 & 7 are compared to lane 4); N: novel protein).

Lane 1 (molecular weight markers)				Lane 2 (*Ps. aeruginosa* planktonic cells control)				Lane 3 (*Ps. aeruginosa* biofilm cells control)			
Band no.	M_r (kDa)	R_f	Co	Band no.	M_r (kDa)	R_f	Co	Band no.	M_r (kDa)	R_f	Co
1	66	0.34	-	1	(72.2)	(0.11)	-	1	(73.7)	(0.09)	-
2	45	0.49	-	2	(68.2)	(0.17)	-	2	(68.7)	(0.16)	-
3	36	0.52	-	3	(66)	(0.20)	-	3	(66)	(0.20)	-
4	29	0.62	-	4	(64)	(0.23)	-	4	(63.2)	(0.24)	-
5	24	0.77	-	5	(62.5)	(0.25)	-	5	(61.2)	(0.27)	-
6	20.1	0.86	-	6	(60,5)	(0.28)	-	6	(59.7)	(0.29)	-
7	14.2	0.98	-	7	(58.5)	(0.31)	-	7	57,7	0.32	-
-	-	-	-	8	51	0.36	-	8	53	0.35	-
-	-	-	-	9	48	0.38	-	9	48	0.38	-
-	-	-	-	10	44.7	0.41	-	10	44.7	0.41	-
-	-	-	-	11	41.7	0.34	-	11	41.7	0.44	-
-	-	-	-	12	37	0.50	-	12	38.7	0.47	-
-	-	-	-	13	33.5	0.55	-	13	37	0.50	-
-	-	-	-	14	30.5	0.60	-	14	36	0.52	-
-	-	-	-	15	28.2	0.64	-	15	33	0.56	-
-	-	-	-	16	26.7	0.67	-	16	29.5	0.63	-
-	-	-	-	17	23.7	0.74	-	17	26.7	0.67	-
-	-	-	-	18	21.7	0.79	-	18	22.7	0.78	-
-	-	-	-	19	19.2	0.86	-	19	19.5	0.85	-
-	-	-	-	20	17	0.91	-	20	17.5	0.90	
-	-	-	-	21	15.7	0.95	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-

KEY:

( ): Molecular weights estimated from linear equation derived from standard calibration curve (Fig 19)

- : No data for that cell

Table 9 (part 2): Measurement data indicating Band number, Molecular weight (M_r) and Retardation factor (R_f) for Outer Membrane Proteins of *Ps. aeruginosa* eluate cells exposed to different treatment of antibiotics and cultured in modified CDM (Band no: band number; Co: corresponding protein to Lane 4 (NB: only the test lanes 5, 6 & 7 are compared to lane 4); N: novel protein).

Lane 4 (*Ps. aeruginosa* eluate cells control).				Lane 5 (*Ps. aeruginosa* eluate cells with treatment of ampicillin at sub-MIC (8 μg mL^{-1}).				Lane 6 (*Ps. aeruginosa* eluate cells with treatment of nalidixic acid at sub-MIC (4 μg mL^{-1}).			
Band no.	M_r (kDa)	R_f	Co	Band no.	M_r (kDa)	R_f	Co	Band no.	M_r (kDa)	R_f	Co
1	(73.7)	(0.09)	-	1	(73)	(0.10)	1	1	(73.7)	(0.09)	1
2	(66.7)	(0.19)	-	2	(71)	(0.13)	N	2	(71)	(0.13)	N
3	38.7	0.47	-	3	(67.5)	(0.18)	2	3	(66.7)	(0.19)	2
4	37.7	0.49	-	4	(59.7)	(0.29)	N	4	(64.7)	(0.22)	N
5	36	0.52	-	5	57	0.33	N	5	(61.7)	(0.26)	N
6	32.2	0.57	-	6	48	0.38	N	6	(61.2)	(0.27)	N
7	29.5	0.63	-	7	45.7	0.41	N	7	(59.7)	(0.29)	N
8	26.2	0.68	-	8	41.7	0.44	N	8	57	0.33	N
9	24.5	0.72	-	9	38.7	0.47	3	9	44.7	0.41	N
10	23.5	0.75	-	10	37.7	0.49	4	10	43	0.43	N
11	21.7	0.79	-	11	36	0.52	5	11	40.2	0.46	N
12	20.5	0.82	-	12	33.5	0.55	N	12	36	0.52	4
13	19.5	0.85	-	13	32.2	0.57	6	13	36	0.52	5
14	18	0.89	-	14	30.5	0.60	7	14	33	0.56	6
15	15.7	0.95	-	15	26.7	0.67	8	15	30.5	0.60	7
-	-	-	-	16	24	0.73	10	16	29.5	0.63	7
-	-	-	-	17	23.5	0.75	10	17	26.7	0.67	8
-	-	-	-	18	21	0.81	11	18	25	0.71	9
-	-	-	-	19	19.5	0.85	13	19	23.5	0.75	10
-	-	-	-	20	18.2	0.88	14	20	21.5	0.80	11
-	-	-	-	-	-	-	-	21	19.7	0.84	13
-	-	-	-	-	-	-	-	22	18.2	0.88	14

KEY:

( ): Molecular weights estimated from linear equation derived from standard calibration curve (Fig 19)

- : No data for that cell

Table 9 (part 3): Measurement data indicating Band number, Molecular weight (M_r) and Retardation factor (R_f) for Outer Membrane Proteins of *Ps. aeruginosa* eluate cells exposed to different treatment of antibiotics and cultured in modified CDM (Band no: band number; Co: corresponding protein to Lane 4 (NB: only the test lanes 5, 6 & 7 are compared to lane 4); N: novel protein).

Lane 7 *(Ps. aeruginosa* eluate cells with treatment of streptomycin at sub-MIC (8 μg mL^{-1})			
Band no.	M_r (kDa)	R_f	Co
1	(73.7)	(0.09)	1
2	(71)	(0.13)	N
3	(67.5)	(0.18)	N
4	(66.7)	(0.19)	2
5	(65.2)	(0.21)	N
6	(63.2)	(0.24)	N
7	57	0.33	N
8	45.7	0.40	N
9	43.7	0.42	N
10	41.7	0.44	N
11	38.7	0.47	3
12	37.7	0.49	4
13	36	0.52	5
14	33	0.56	6
15	30.5	0.60	7
16	29	0.62	7
17	27.2	0.66	8
18	25.7	0.69	8
19	24	0.73	10
20	23.7	0.74	10
21	21.7	0.79	11
22	19.7	0.84	13
23	18.2	0.88	14

KEY:

( ): Molecular weights estimated from linear equation derived from standard calibration curve (Fig 19)

- : No data for that cell

OMP shifts in *Escherichia coli* ATCC 10000 biofilm and eluate cells exposed to sub-MICs of ampicillin (AmP), nalidixic acid (NaL) and streptomycin (ST)

In *E. coli* biofilm cells two outer membrane proteins with molecular weights of 37.7 kDa and 36 kDa were present in control (lane 3, Figure 22), but were undetectable in cells exposed to sub-MICs of NaL and ST (lanes 5 and 6, Figure 22). After treatment with sub-MICs of AmP and nalidixic acid (lanes 4 and 5, Figure 22), a 23.5 kDa OMP was no longer apparent.

In *E. coli* eluate cells, a 21.5 kDa outer membrane protein absent from control samples (lane 4, Figure 23), appeared in cells exposed to sub-MICs of nalidixic acid (lane 6, Figure 23). Two proteins with molecular weights of 22.7 kDa and 23.5 kDa were absent from control antibiotic unexposed cells (lane 4, Figure 23), but both OMPs were present in cells exposed to sub-MICs of AmP, NaL and ST respectively (lanes 5, 6, & 7, Figure 23). A 36.5 kDa OMP was only present in control samples, but inapparent in cells exposed to sub-MICs of AmP and ST (lanes 5 and 7, Figure 23).

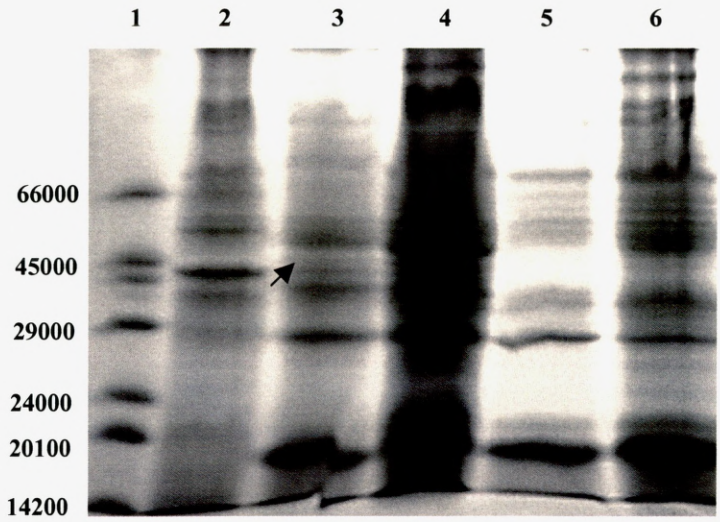


Figure 22: Outer membrane protein profile of *Escherichia coli* biofilm cells cultured in modified CDM and exposed to different treatment of antibiotics. Lane 1: molecular weights markers; Lane 2: *E. coli* planktonic cells control (745 μg protein well^{-1}); Lane 3: *E. coli* biofilm cells control (700 μg protein well^{-1}); Lane 4: *E. coli* biofilm cells (784 μg protein well^{-1}) with treatment of ampicillin at sub-MIC (2 μg mL^{-1}); Lane 5: *E. coli* biofilm cells (794 μg protein well^{-1}) with treatment of nalidixic acid at sub-MIC (2 μg mL^{-1}); Lane 6: *E. coli* biofilm cells (673 μg protein well^{-1}) with treatment of streptomycin at sub-MIC (8 μg mL^{-1}). Arrow indicates the 37.7 kDa OMP.

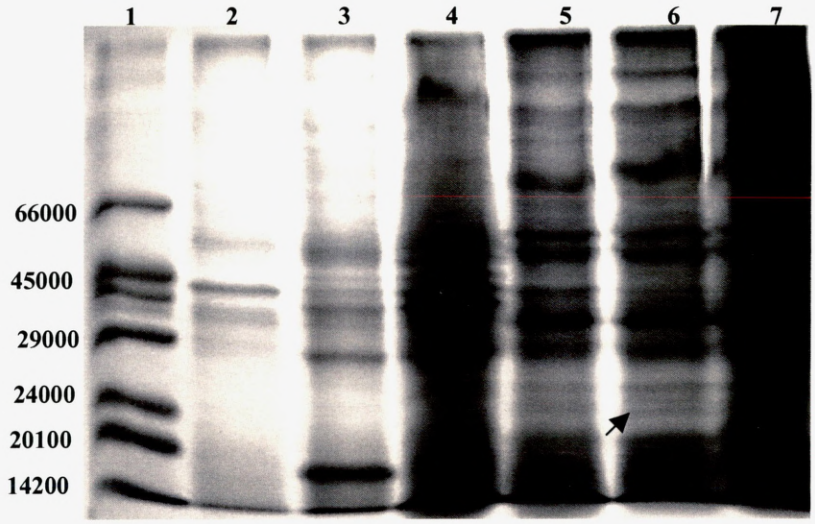


Figure 23: Outer membrane protein profile of *Escherichia coli* eluate cells cultured in modified CDM and exposed to different treatment of antibiotics. Lane 1: molecular weight markers; Lane 2: *E. coli* planktonic cells control (745 μg protein well^{-1}); Lane 3: *E. coli* biofilm cells control (700 μg protein well^{-1}); Lane 4: *E. coli* eluate cells control (723 μg protein well^{-1}); Lane 5: *E. coli* eluate cells (707 μg protein well^{-1}) with treatment of ampicillin at sub-MIC (1μg mL^{-1}); Lane 6: *E. coli* eluate cells (697 μg protein well^{-1}) with treatment of nalidixic acid at sub-MIC (1 μg mL^{-1}); Lane 7: *E. coli* eluate cells (702 μg protein well^{-1}) with treatment of streptomycin at sub-MIC (8 μg mL^{-1}). Arrow indicates the 21.5 kDa OMP.

OMP shifts in sub-MIC of AmP, NaL and ST exposed cells of binary biofilm cells and eluate cells of *Ps. aeruginosa* PAO1 and *E. coli* ATCC 10000.

In binary biofilms (mixed) cells a 44.7 kDa OMP observed in control cells was inapparent in cells exposed to sub-MIC of antibiotics of AmP and ST (compare lane 4 to lanes 5 and 7, Figure 24). However, this OMP was apparent in cells treated with sub-MICs of nalidixic acid (lane 6, Figure 24). Two other OMPs with molecular weights of 66 kDa and 23.7 kDa were present in control cells (lane 4, Figure 24),but were inapparent in cells exposed to sub-MICs of AmP, NaL and ST (lane 5, 6, & 7, Figure 24).

A 48 kDa was apparent in cells exposed to sub-MICs of antibiotics of AmP and NaL (compare lane 4 to lanes 5 and 6, Figure 24). Another OMP with a molecular weight of 38.7 kDa was present in control samples (lane 4, Figure 24), but inapparent in cells exposed to sub-MICs of antibiotics of AmP and NaL (lanes 5 and 6, Figure 24). Similarly a 35 kDa OMP was present in controls, and inapparent in cells treated with sub-MICs of antibiotics of AmP, NaL and ST (lane 5, 6 & 7, Figure 24).

Figure 25 illustrates SDS-PAGE OMP profiles of binary eluate cells. An outer membrane protein with a molecular weight of 22.7 kDa was absent in controls (cells that have not been treated with antibiotics), but was present after cells were exposed to sub-MICs of ampicillin (compare lane 7 to lane 8, Figure 25). Another 66.7 kDa only appeared in cells exposed to sub-MICs of AmP and ST (compare lane 7 to lanes 8 and 10). Two OMPs with molecular weights, 61.7 kDa and 24 kDa were induced only after cells were exposed to sub-MICs of AmP, NaL and ST (compare lane 7 to lanes 8, 9 and 10, Figure 25). A 70.2 kDa OMP was only present in control samples (lane 7, Figure 25) and another 51 kDa OMP was inapparent in the presence of sub-MICs of antibiotics of AmP and nalidixic acid (lanes 8 and 9, Figure 25).

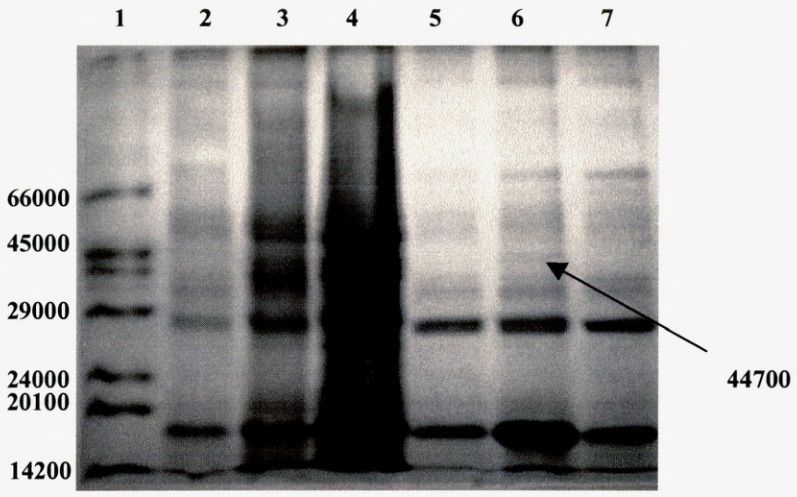


Figure 24: Outer membrane protein profile of binary biofilm cells cultured in modified CDM and exposed to different treatment of antibiotics. Lane 1: molecular weights markers; Lane 2: *Ps. aeruginosa* PAO1 biofilm cells control (729 μg protein well^{-1}); Lane 3: *Escherichia coli* ATCC 10000 biofilm cells control (700 μg protein well^{-1}); Lane 4: Binary biofilm cells control (787 μg protein well^{-1}); Lane 5: Binary biofilm cells (715 μg protein well^{-1}) with treatment of ampicillin at sub-MIC concentration (32 μg mL^{-1}); Lane 6: Binary biofilm cells (705 μg protein well^{-1}) with treatment of nalidixic acid at sub-MIC concentration (8 μg mL^{-1}); Lane 7: Binary biofilm cells (723 μg protein well^{-1}) with treatment of streptomycin at sub-MIC concentration (16 μg mL^{-1}). Arrow indicates the 44.7 kDa OMP.

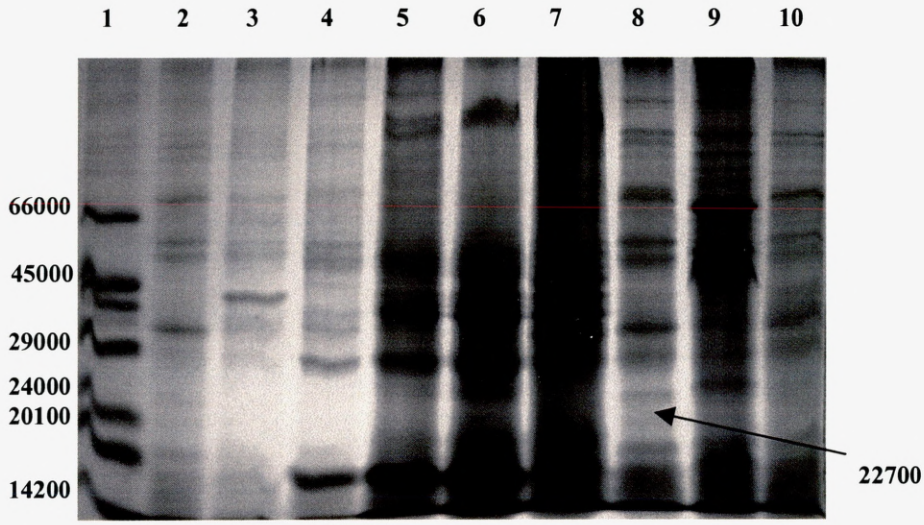


Figure 25: Outer membrane protein profile of binary eluate cells cultured in modified CDM and exposed to different treatment of antibiotics. Lane 1: molecular weights markers; Lane 2: *Ps. aeruginosa* PAO1 planktonic cells control (776 μg protein well^{-1}); Lane 3: *Escherichia coli* 10000 planktonic cells control (745 μg protein well^{-1}); Lane 4: *Ps. aeruginosa* biofilm cells control (729 μg protein well^{-1}), Lane 5: *Escherichia coli* biofilm cells control (784 μg protein well^{-1}), Lane 6: Binary biofilm cells control (787 μg protein well^{-1}); Lane 7: Binary biofilm eluate cells control (744 μg protein well^{-1}); Lane 8: Binary eluate cells (721 μg protein well^{-1}) with treatment of ampicillin at sub-MIC concentration (16 μg mL^{-1}); Lane 9: Binary eluate cells (724 μg protein well^{-1}) with treatment of nalidixic acid at sub-MIC concentration (4 μg mL^{-1}); Lane 10: Binary eluate cells (716 μg protein well^{-1}) with treatment of streptomycin at sub-MIC concentration (4 μg mL^{-1}). Arrow indicates the 22.7 kDa OMP.

Variations in outer membrane profiles between *Ps. aeruginosa* and *E. coli* monospecies and binary biofilm and eluate cells following exposure to sub-MICs of antibiotics, ampicillin, nalidixic acid and streptomycin are apparent. Some outer membrane proteins appeared in both sensitive and treated cells, while others appeared with slight alterations in their molecular weights. It is also evident that some OMPs were lost from the cells following antibiotic treatments. Others appeared as novel proteins in the treated cells. These results indicate a complex series of internal metabolic and external outer membrane associated changes induced by the presence of sub-MICs of antibiotics. Such changes are indicative of antibiotic acting at a diversity of sites internal to the bacterial cell.

The mode of growth (biofilm, eluate or planktonic) affects the sensitivity of the bacterial species towards antibiotics. It may be noted that variations in the OMP profiles may also be characteristic to the mode of growth. Significant variations in the OMP profiles following exposure to different antibiotics may lead to epitopic changes in the bacterial membrane. Outer membrane proteins (OMPs) implanted within the bacterial membrane are capable of selective intake of solutes (including antimicrobials). Over-expression or loss of certain OMPs may lead to epitopic changes in the bacterial membrane, leading to subsequent resistance or loss of sensitivity (Abdel Malek, 2002).

One-dimensional SDS-PAGE, while capable of providing valuable information that could be used for initial analysis of OMPs, is not considered a definitive approach to their proteomic analysis. In order to identify these OMPs, mass spectroscopic analysis has to be carried out and 2D-PAGE analysis of these proteins would naturally precede it. Hence, 2D-PAGE analysis of these proteins (OMPs) was undertaken.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of outer membrane proteins isolated from monospecies and binary biofilms of *Ps. aeruginosa* ATCC 15692 (PAO1) and *Escherichia coli* ATCC 10000

The results obtained from SDS-PAGE analysis illustrated significant differences in the outer membrane proteins. Although SDS-PAGE analysis of antibiotic treated and control samples indicated shifts in protein patterns, further analysis using 2D-PAGE was undertaken. Such analysis, besides guaranteeing separation of all outer membrane proteins being analysed, would also ensure correct interpretation of results. For instance, a thick protein band visualised on SDS-PAGE may be interpreted as over-expression of a single protein. However, theoretically this may also be due to two proteins having similar molecular weights co-localizing at the same position on the gel. In order to discount the latter possibility 2D-PAGE analysis was performed on all test samples. Likewise, novel protein bands present within antibiotic treated samples (37 kDa protein band in streptomycin treated *Ps. aeruginosa* biofilm cells, 44.7 kDa protein band visualised in nalidixic acid treated binary biofilm sample, 22.7 kDa protein band in the ampicillin treated eluate cell samples, etc.) were investigated using 2D-PAGE so as to ascertain the actual number of proteins that make up the band.

Experimental approach

Since O'Farrell (1975) introduced the improved technique for high-resolution two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). It has become one of the most powerful tools for the separation and characterisation of proteins from complex mixtures (O'Farrell, 1975). This form of separation depends on the molecular weights of polypeptides and 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 according to their isoelectric points and subsequently according to molecular weight in the second dimension. Consequently, it has sufficient resolution to separate individual proteins as discrete spots on the gel (O'Farrell, 1975). There are two different methods for performing the first-dimensional stage (isoelectric focusing; IEF). The first method is by pre-running the first dimensional gel (tube gel) without the sample to establish the pH gradient. In the second method, 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, *et al.*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) can resolve over 1000 proteins (Celis & Bravo 1983).

Preparation of tube-gels (first dimension protein separation), isoelectric focusing of protein samples and preparation of slab gels (second dimension protein separation) were performed as described in Chapter 2.

For 2D-PAGE analysis, biofilms were grown on Sorbarod filters as described earlier in this chapter (Page 97). Biofilm OMPs were extracted according to the method described by Hodgson *et al.* (1995) and eluate OMPs were extracted according to Pugsley *et al.* (1986) samples were stored at -20°C for later analysis. Outer membrane protein (OMP) samples used for 2D-PAGE analyses were those of *Pseudomonas aeruginosa* monospecies biofilms treated with sub-MICs of streptomycin and binary biofilms (*Pseudomonas aeruginosa* and *Escherichia coli*) treated with all three antibiotics (AmP, NaL and ST).

Analysis of OMP profiles

Figure 26 illustrates the two-dimensional electrophoretic protein pattern of 2D-PAGE markers separated according to their molecular weights and pI's (isoelectric points; points at which the proteins have a net neutral charge). The IEF range is between pH 3.5 and pH 10; the pI for each protein was estimated manually and compared to the standard pI (Table 10) in order to determine the molecular weight for each spot. The R_f value for each standard protein was calculated according to Equation 2 (Page 99). By plotting the molecular weights (M_r; Daltons) against R_f values, a calibration curve was constructed (Figure 27). For the sample gels (Figures 28, 29 & 30) the R_f value of proteins were estimated from Equation 2 and then extrapolated onto the calibration curve, in order to determine the molecular weight. The individual numbered proteins (on each sample gel) and their corresponding estimated molecular weights, R_f and pI (analysed manually) are shown in Table 10.

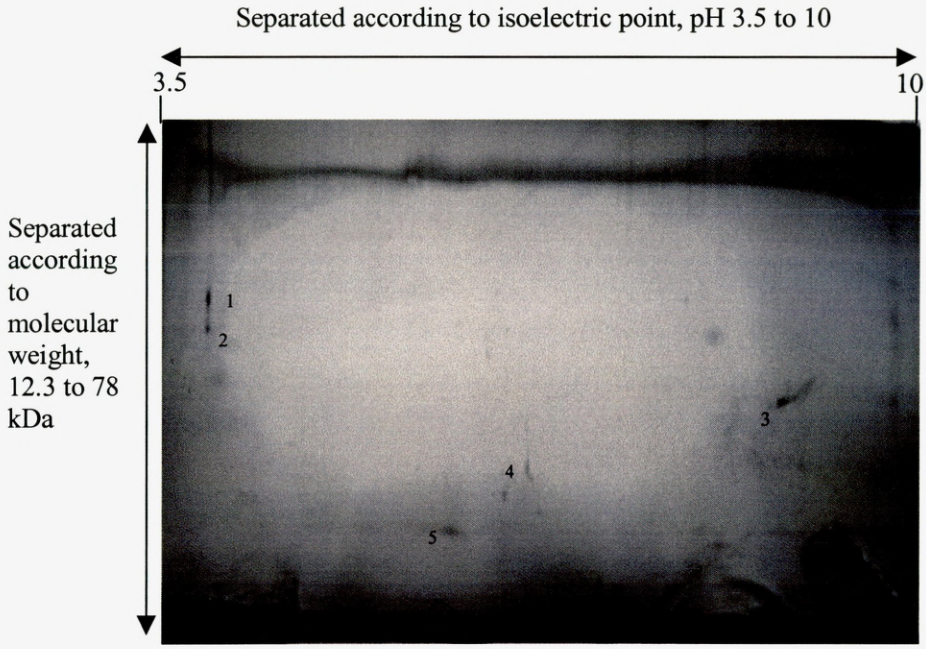


Figure 26: Two-dimensional electrophoretic protein pattern of 2D-PAGE standards separated according to their molecular weight and pI. Protein concentration: 792 μg protein gel^{-1}. Numbers may be to the left or right of protein, but not above or below.

Table 10: Standard Molecular Weights and Isoelectric points (pI).

Protein	**Molecular Weight (kDa)**	**pI (pH)**
Ovotransferrin (hen egg)	76- 78	3.9
Albumin (a bovine serum)	66.25	3.9
Ovalbumin (hen egg)	42.7	8.7
Carbonicanhydrase (bovine erythrocyte)	30	6.6
Myoglobin (equine)	17.2	N
Cytochrome C (equine)	12.3	6.0
N: Not detected due to manufacturers error.		

Table 11: Measurement data indicating Protein number, Molecular weight (M_r), Retardation factor (R_f) and Isoelectric point (pI) of the proteins identified in the 2D-PAGE standard proteins gel.			
Protein no.	**M_r (kDa)**	**R_f**	**pI (pH)**
1	76	0.35	3.9
2	66.2	0.42	3.9
3	42.7	0.56	8.7
4	30	0.68	6.6
5	12.3	0.82	6

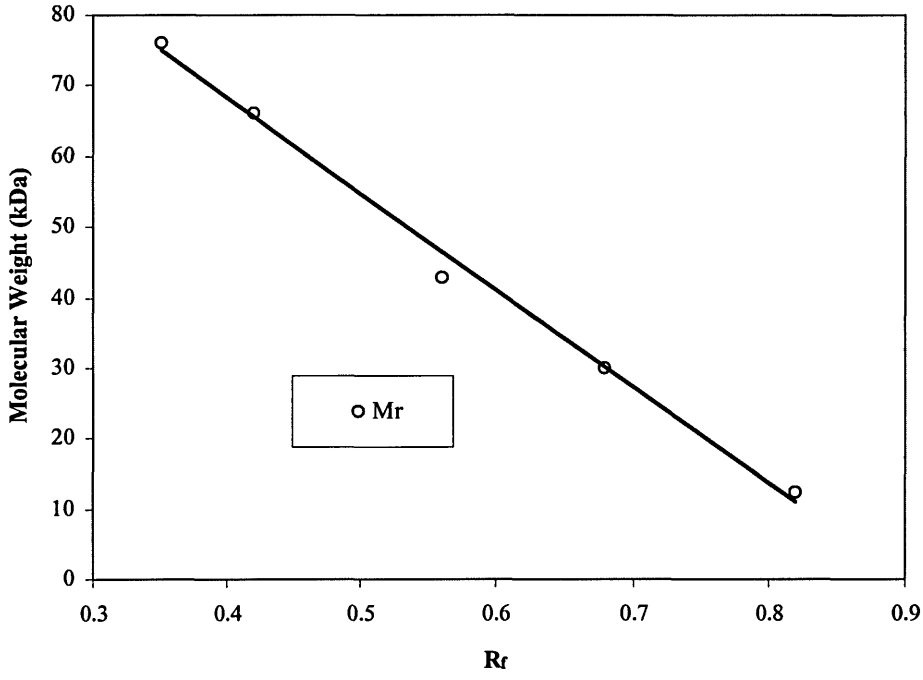


Figure 27: Calibration curve generated by plotting the molecular weights of 2D-PAGE standards in Dalton's vs R_f values.

Results & Discussion

One of the primary aims of carrying out SDS-PAGE and 2D-PAGE analyses on outer membrane proteins of *Pseudomonas aeruginosa* biofilms was to isolate those potential proteins that may be responsible for a common mechanism of antibiotic resistance. Hence, three different antibiotics were chosen (AmP, NaL and ST) and

SDS-PAGE enabled the identification of common novel proteins that became apparent and those that became inapparent, for all different antibiotic treatments. In order to further investigate this subset of proteins derived from comparison of all three antibiotic treatments (data not given here), 2D-PAGE of only one antibiotic treated sample was necessary, and so streptomycin was chosen as the representative compound.

The outer membrane proteins of *Ps. aeruginosa* biofilm cells exposed to the antibiotic streptomycin at sub-MIC (8 $\mu g\ mL^{-1}$) were analysed and compared against proteins of biofilm culture control (Table 12). Key proteins, that were visible in the biofilm control and with treated biofilm cells, were identified and compared, such that the molecular weight and isoelectric points of proteins from treated biofilm cells could be estimated against those of the control cells. The most prominent difference between the gels containing proteins from planktonic control cells (Figure 28) and biofilm control cells (Figure 29) compared to those treated with streptomycin at sub-MIC (8 $\mu g\ mL^{-1}$) (Figure 30), is the disappearance of various high molecular weight proteins (80 kDa to 98 kDa) and the presence of OMPs with basic isoelectric points after exposure to sub-MIC of streptomycin. An outer membrane protein with a molecular weight of 59.2 kDa (pI 8.1) appeared following exposure of *Ps. aeruginosa* biofilm cells to sub-MIC of streptomycin (8 $\mu g\ mL^{-1}$), but was not found in biofilm control cells. This protein appears to be the same OMP (59.2 kDa), which appeared upon the exposure of biofilm cells to sub-MIC of streptomycin (8 $\mu g\ mL^{-1}$) in SDS-PAGE gels (lane 6, Figure 20). Similarly another 36.7 kDa (pI 4.1) OMP appeared following the exposure of the biofilm cells to sub-MIC of streptomycin (8 $\mu g\ mL^{-1}$), which could be the same protein found in similar conditions on SDS-PAGE gels (Lane 6, Figure 20). Finally a 98 kDa (pI 4.5) OMP was present in biofilm control samples, but disappeared after exposure to the sub-MIC of streptomycin (8 $\mu g\ mL^{-1}$).

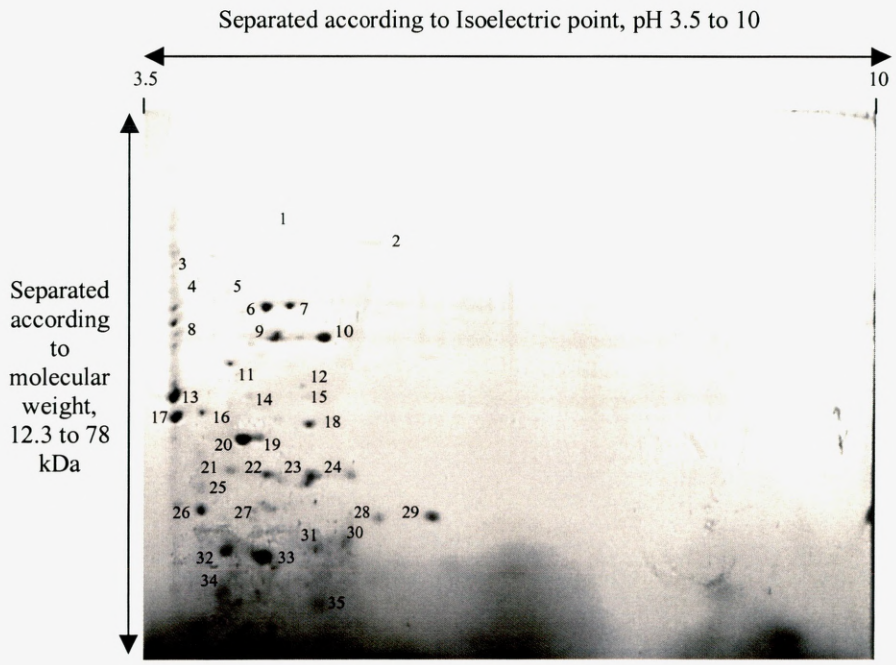


Figure 28: Two-dimensional gel electrophoresis for outer membrane protein (OMP) profiles of *Pseudomonas aeruginosa* PAO1 planktonic cells control cultured in MCDM. The OMPs are separated by the isoelectric point (reading left to right) and molecular weight (top to bottom). Protein concentration: 793 μg protein gel^{-1}. Numbers may be to the left or right of protein.

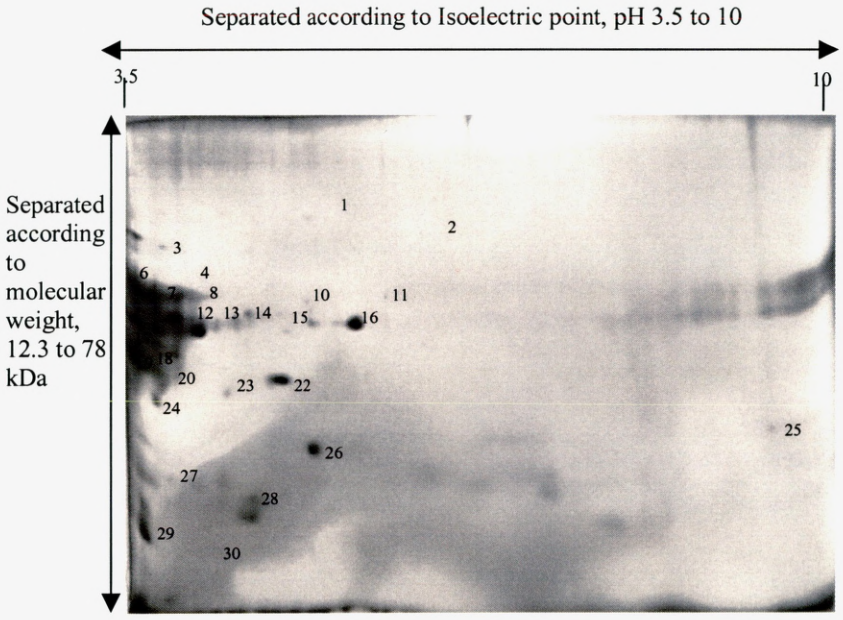


Figure 29: Two-dimensional gel electrophoresis for outer membrane protein (OMP) profiles of *Pseudomonas aeruginosa* PAO1 biofilm cells control cultured in MCDM. The OMPs are separated by the isoelectric point (reading left to right) and molecular weight (top to bottom). Protein concentration: 747 μg protein gel^{-1}. Numbers may be to the left or right of protein.

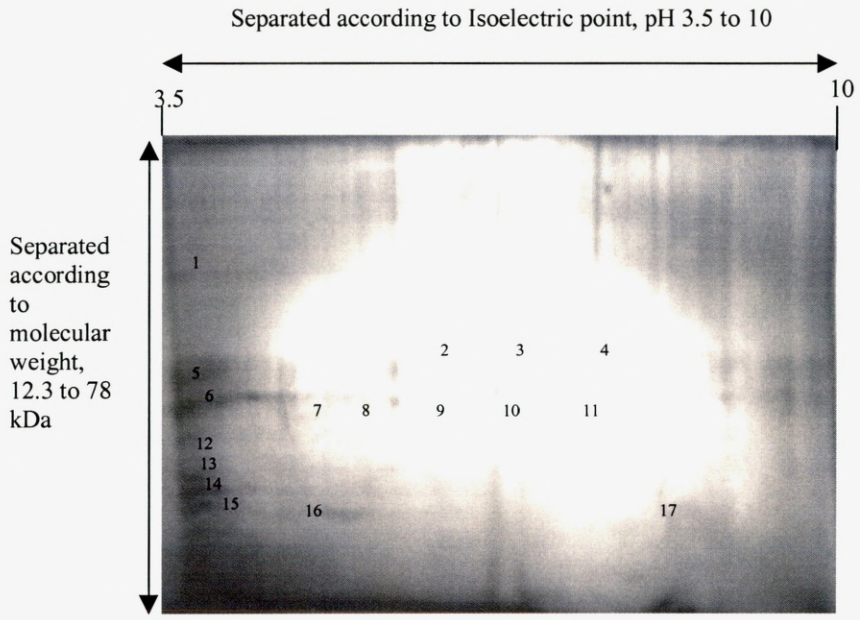


Figure 30: Two-dimensional gel electrophoresis for outer membrane protein (OMP) profiles of *Pseudomonas aeruginosa* biofilm cells exposed to streptomycin at sub-MIC (8 μg mL^{-1}) and cultured in MCDM. The OMPs are separated by the isoelectric point (reading left to right) and molecular weight (top to bottom). Protein concentration: 753 μg protein gel^{-1}. Numbers may be to the left or right of protein.

Table 12 (part 1): Measurement data indicating Protein number, Molecular weight (M_r) and Retardation factor (R_f) and Isoelectric point (pI) of the proteins identified from the Two-Dimensional gel analysis of *Ps. aeruginosa* biofilm cells exposed to streptomycin at sub-MIC (8 μg mL^{-1}) and compared against *Ps. aeruginosa* biofilm cells (control), cultured in modified CDM, (Co: corresponding protein to Figure 29 (NB: only Figure 30 is compared to Figure 29); N: novel protein).

Figure 26 Standard molecular weight					**Figure 28** *Ps. aeruginosa* planktonic cells control				
Protein no.	M_r (kDa)	R_f	pI	Co.	Protein no.	M_r (kDa)	R_f	pI	Co.
1	76	0.35	3.9	-	1	96	0.19	4.5	-
2	66.2	0.42	3.9	-	2	92	0.22	5.6	-
3	42.7	0.56	8.7	-	3	88	0.25	3.9	-
4	17.2	0.81	6.6	-	4	85	0.27	4	-
5	12.3	0.83	6.0	-	5	81	0.30	4.5	-
-	-	-	-	-	6	77	0.33	4.6	-
-	-	-	-	-	7	76	0.35	4.9	-
-	-	-	-	-	8	70.2	0.38	3.9	-
-	-	-	-	-	9	70.2	0.38	4.8	-
-	-	-	-	-	10	64	0.43	5.3	-
-	-	-	-	-	11	59.2	0.46	4.3	-
-	-	-	-	-	12	55.2	0.49	5.1	-
-	-	-	-	-	13	55.2	0.49	3.9	-
-	-	-	-	-	14	55.2	0.49	4.5	-
-	-	-	-	-	15	51.2	0.52	5.1	-
-	-	-	-	-	16	51.2	0.52	3.9	-
-	-	-	-	-	17	48.5	0.54	4.2	-
-	-	-	-	-	18	42	0.56	5.1	-
-	-	-	-	-	19	42	0.56	4.5	-

Table 12 (part 2): Measurement data indicating Protein number, Molecular weight (M_r) and Retardation factor (R_f) and Isoelectric point (pI) of the proteins identified from the Two-Dimensional gel analysis of *Ps. aeruginosa* biofilm cells exposed to streptomycin at sub-MIC (8 μg mL^{-1}) and compared against *Ps. aeruginosa* biofilm cells (control), cultured in modified CDM, (Co: corresponding protein to Figure 29 (NB: only Figure 30 is compared to Figure 29); N: novel protein).

Figure 26 Standard molecular weight					**Figure 28** *Ps. aeruginosa* planktonic cells control				
Protein no.	M_r (kDa)	R_f	pI	Co.	Protein no.	M_r (kDa)	R_f	pI	Co.
-	-	-	-	-	20	39.5	0.61	4.8	-
-	-	-	-	-	21	39.5	0.61	4.3	-
-	-	-	-	-	22	39.5	0.61	4.6	-
-	-	-	-	-	23	39.5	0.61	5.1	-
-	-	-	-	-	24	34.2	0.65	5.4	-
-	-	-	-	-	25	30	0.68	4.2	-
-	-	-	-	-	26	30	0.68	4.2	-
-	-	-	-	-	27	30	0.68	4.6	-
-	-	-	-	-	28	27.5	0.70	5.8	-
-	-	-	-	-	29	27.5	0.70	6.2	-
-	-	-	-	-	30	20.5	0.75	4.2	-
-	-	-	-	-	31	20.5	0.75	5.3	-
-	-	-	-	-	32	20.5	0.75	5.4	-
-	-	-	-	-	33	19	0.76	4.6	-
-	-	-	-	-	34	12.3	0.82	4.2	-
-	-	-	-	-	35	7.2	0.85	5.3	-
-	-	-	-	-	36	3	0.88	4.2	-

- : No data for that cell

Table 12 (part 3): Measurement data indicating Protein number, Molecular weight (M_r) and Retardation factor (R_f) and Isoelectric point (pI) of the proteins identified from the Two-Dimensional gel analysis of *Ps. aeruginosa* biofilm cells exposed to streptomycin at sub-MIC (8 μg mL^{-1}) and compared against *Ps. aeruginosa* biofilm cells (control), cultured in modified CDM, (Co: corresponding protein to Figure 29 (NB: only Figure 30 is compared to Figure 29); N: novel protein).

Figure 29 *Ps. aeruginosa* biofilm cells (control)					**Figure 30** *Ps. aeruginosa* biofilm cells treated with sub-MIC of streptomycin at (8 μg mL^{-1})				
Protein no.	M_r (kDa)	R_f	pI	Co.	Protein no.	M_r (kDa)	R_f	pI	Co.
1	98.7	0.17	4.5	-	1	81	0.30	4.1	N
2	96	0.19	5.4	-	2	59.2	0.46	6.2	N
3	92	0.22	3.6	-	3	59.2	0.46	7.2	N
4	89.2	0.24	3.9	-	4	59.2	0.46	8.1	N
5	88	0.25	3.6	-	5	55.2	0.49	4.1	20
6	77	0.34	3.6	-	6	48.5	0.54	4.1	23
7	77	0.34	3.7	-	7	48.5	0.54	4.8	23
8	77	0,34	4.1	-	8	48.5	0.54	5.2	23
9	73	0.36	4.7	-	9	48.5	0.54	6.2	23
10	73	0.36	5.4	-	10	48.5	0.54	7.2	23
11	73	0.36	6	-	11	48.5	0.54	8.1	23
12	68.7	0.39	3.9	-	12	37	0.63	4.1	N
13	66.2	0.42	4.3	-	13	27.5	0.70	4.1	N
14	66.2	0.42	5	-	14	21.7	0.74	4.1	N
15	66.2	0.42	5.4	-	15	16.5	0.78	4.1	N
16	66.2	0.42	5.8	-	16	16.5	0.78	5.7	N
17	62	0.44	3.7	-	17	1	0.91	8.8	N

- : No data for that cell

Table 12 (part 4): Measurement data indicating Protein number, Molecular weight (M_r) and Retardation factor (R_f) and Isoelectric point (pI) of the proteins identified from the Two-Dimensional gel analysis of *Ps. aeruginosa* biofilm cells exposed to streptomycin at sub-MIC (8 μg mL^{-1}) and compared against *Ps. aeruginosa* biofilm cells (control), cultured in modified CDM, (Co: corresponding protein to Figure 29 (NB: only Figure 30 is compared to Figure 29); N: novel protein).

Figure 29 *Ps. aeruginosa* biofilm cells (control).					**Figure 30** *Ps. aeruginosa* biofilm cells treated with sub-MIC of streptomycin at (8 μg mL^{-1}).				
Protein no.	M_r (kDa)	R_f	pI	Co.	Protein no.	M_r (kDa)	R_f	pI	Co.
18	58	0.47	3.9	-	-	-	-	-	-
19	58	0.47	4.3	-	-	-	-	-	-
20	55.2	0.49	3.6	-	-	-	-	-	-
21	52.5	0.51	3.9	-	-	-	-	-	-
22	49.7	0.53	5	-	-	-	-	-	-
23	48.5	0.54	4.5	-	-	-	-	-	-
24	42	0.56	3.7	-	-	-	-	-	-
25	35	0.64	9.5	-	-	-	-	-	-
26	31	0.67	5.4	-	-	-	-	-	-
27	26.2	0.71	3.7	-	-	-	-	-	-
28	15	0.79	4.7	-	-	-	-	-	-
29	13	0.81	3.6	-	-	-	-	-	-
30	5.7	0.86	4.5	-	-	-	-	-	-

- : No data for that cell

Figures 31, 32, 33 & 34 demonstrate the outer membrane protein profiles of binary biofilm cells (control and those treated with the sub-MIC of antibiotics- ampicillin, nalidixic acid and streptomycin, respectively), separated by two-dimensional gel electrophoresis. A comparison of the outer membrane profiles of biofilm cells (control and antibiotic treated) is shown in Table 13.

Figure 32 demonstrates the outer membrane proteins of binary biofilm cells exposure to the sub-MIC of ampicillin at (32 μg mL^{-1}). The significant difference between biofilm control gel (Figure 31) and biofilm treated gel (Figure 32) is the appearance of various proteins with high molecular weights (100 kDa to 111 kDa) and also the observation of OMPs with alkaline isoelectric points. An outer membrane protein with a molecular weight 31 kDa (pI 7.6) was apparent in antibiotic treated biofilm cells, but this OMP was absent in the biofilm control cells. This protein could be the same OMP (30 kDa), which appeared upon the exposure of biofilm cells to sub-MIC of ampicillin (32 μg mL^{-1}) in SDS-PAGE gels (Lane 4, Figure 24).

Comparison of the 2D-PAGE OMP profiles of binary biofilms, control (Figure 31) and treated cells (Figure 33), indicate marked differences between the two gels. A 48.5 kDa (pI 5.2) protein, absent from control samples, was apparent in cells exposed to sub-MIC of nalidixic acid (8 μg mL^{-1}). This protein appears to be the same OMP (48 kDa) that appeared in SDS-PAGE samples treated with sub-MIC of nalidixic acid (8 μg mL^{-1}) (lane 6, Figure 24). In binary biofilms exposed to sub-MICs of streptomycin (16 μg mL^{-1}) (Figure 34), a 73 kDa (pI 5.0) and 92 kDa (pI 3.5) protein were apparent. Again, OMPs with molecular weights of 41 kDa (pI 5.0) and 35 kDa (pI 4.7) were present in binary biofilm control cells, but absent in cell samples exposed to the sub-MIC of streptomycin (16 μg mL^{-1}).

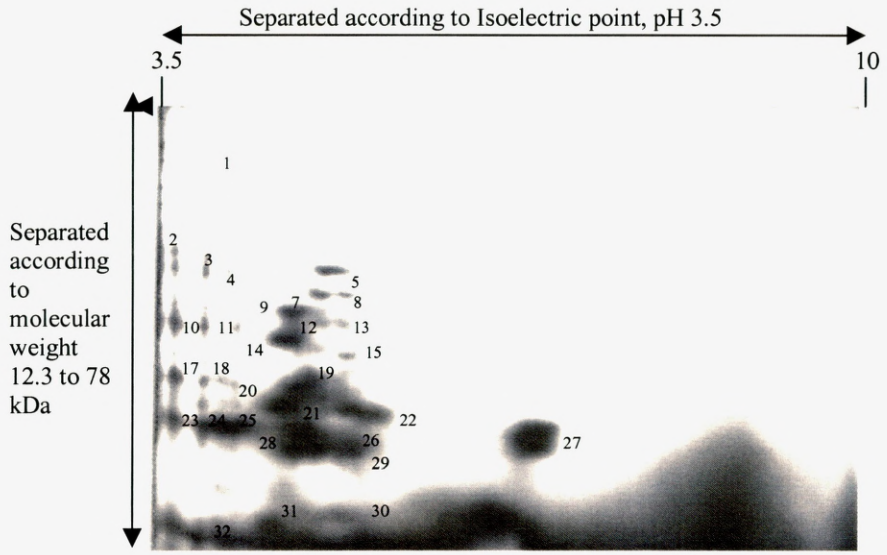


Figure 31: Two-dimensional gel electrophoresis for outer membrane protein (OMP) profiles of binary biofilm cells control cultured in MCDM. The OMPs are separated by the isoelectric point (reading left to right) and molecular weight (top to bottom). Protein concentration: 796 μg protein gel^{-1}. Numbers may be to the left or right of protein.

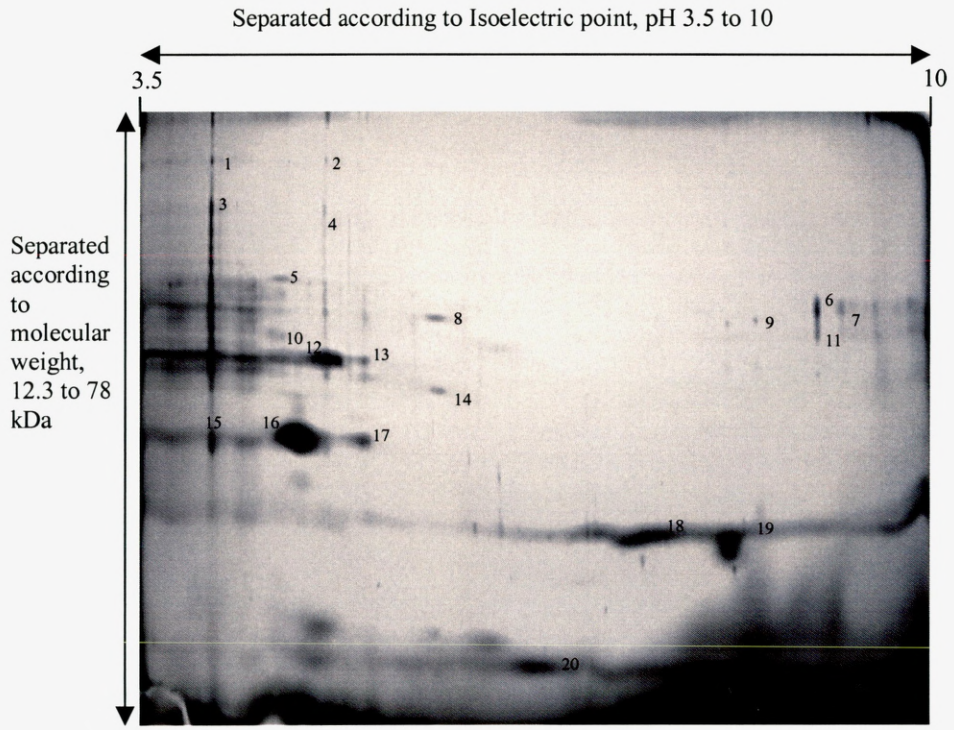


Figure 32: Two-dimensional gel electrophoresis for outer membrane protein (OMP) profiles of binary biofilm cells exposed to ampicillin at sub-MIC concentration (32 μg mL^{-1}) and cultured in modified CDM. The OMPs are separated by isoelectric point (reading left to right) and molecular weight (top to bottom). Protein concentration: 731 μg protein gel^{-1}. Numbers may be to the left or right of protein.

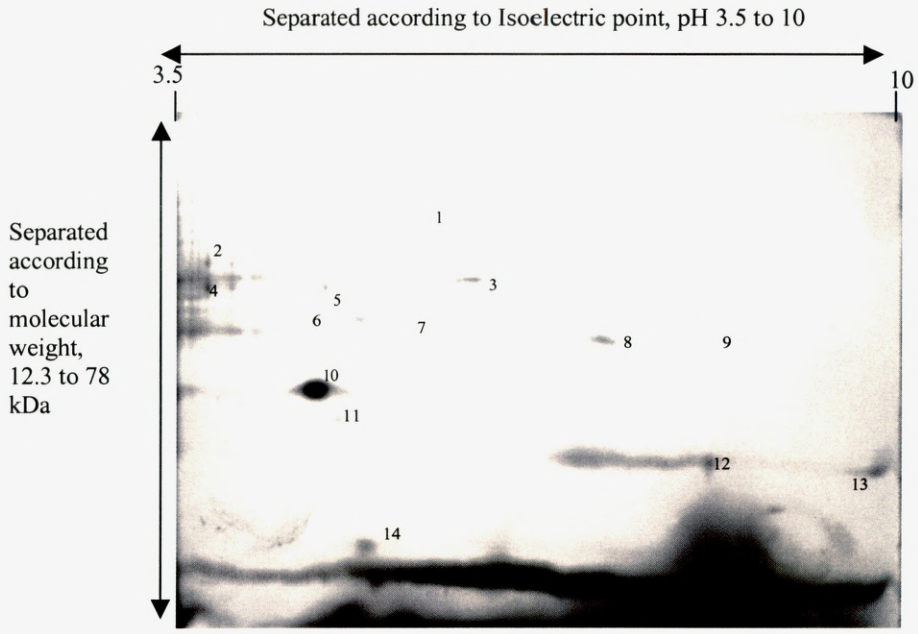


Figure 33: Two-dimensional gel electrophoresis for outer membrane protein (OMP) profiles of binary biofilm cells exposed to nalidixic acid at sub-MIC (8 μg mL^{-1}) and cultured in modified CDM. The OMPs are separated by isoelectric point (reading left to right) and molecular weight (top to bottom). Protein concentration: 712 μg protein gel^{-1}. Numbers may be to the left or right of protein.

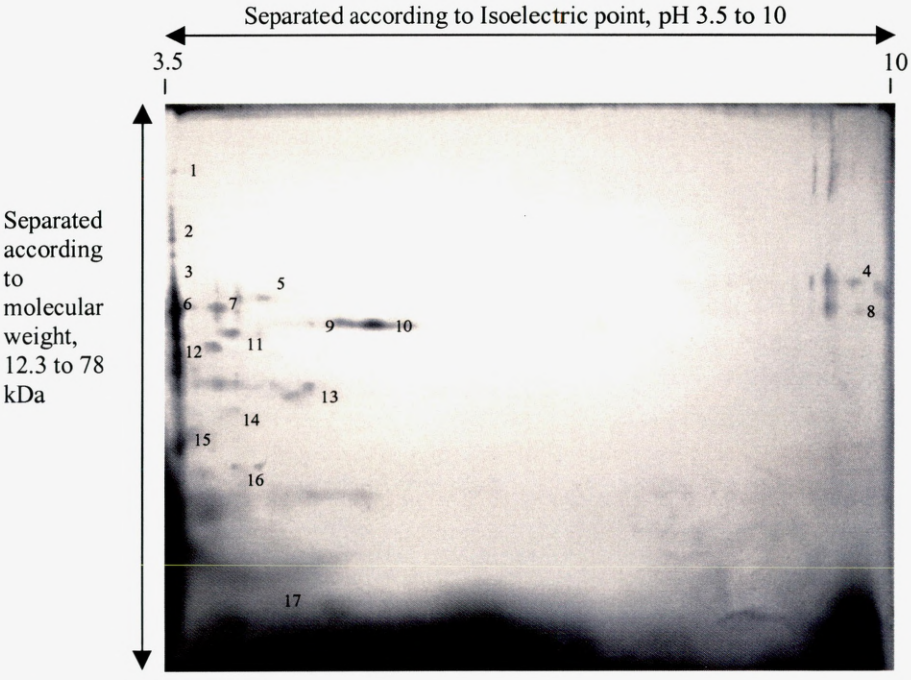


Figure 34: Two-dimensional gel electrophoresis for outer membrane protein (OMP) profiles of binary biofilm cells exposed to streptomycin at sub-MIC (16 μg mL^{-1}) and cultured in modified CDM. The OMPs are separated by isoelectric point (reading left to right) and molecular weight (top to bottom). Protein concentration: 729 μg protein gel^{-1}. Numbers may be to the left or right of protein.

Table 13 (part 1): Measurement data indicating Protein number, Molecular weight (M_r) and Retardation factor (R_f) and Isoelectric point (pI) of the proteins identified from the Two-Dimensional gel analysis of binary biofilm cells exposed to different treatment of antibiotics and compared against binary biofilm cells (control), cultured in modified CDM, (Co: corresponding protein to Figure 31; N: novel protein).

Figure 31 Binary biofilm cells control.					**Figure 32** Binary biofilm cells with the treatment of ampicillin at sub-MIC (32 μg mL^{-1}).				
Protein no.	M_r (kDa)	R_f	pI	Co.	Protein no.	M_r (kDa)	R_f	pI	Co.
1	92	0.20	4.3	-	1	86	0.25	4.1	3
2	91	0.21	3.6	-	2	82.5	0.28	5.1	4
3	87	0.24	4.0	-	3	81	0.29	4.1	5
4	82	0.28	4.0	-	4	81	0.29	5.1	5
5	82	0.28	5.3	-	5	77.5	0.32	4.7	7
6	80	0.30	4.7	-	6	72.5	0.36	9.0	9
7	77.5	0.32	4.9	-	7	72.5	0.36	9.4	9
8	76	0.33	5.3	-	8	66.5	0.41	6.0	11
9	73.5	0.35	4.6	-	9	62	0.44	8.6	13
10	70	0.38	3.6	-	10	57	0.48	4.7	16
11	67	0.40	3.9	-	11	57	0.48	9.0	16
12	65	0.42	4.9	-	12	52	0.52	5.1	19
13	62	0.44	5.3	-	13	52	0.52	5.5	19
14	61.5	0.45	4.7	-	14	48	0.57	6.0	N
15	61.5	0.45	5.5	-	15	37.5	0.63	4.1	22
16	57	0.48	4.2	-	16	37.5	0.63	4.9	22
17	57	0.48	3.6	-	17	37.5	0.63	5.5	22
18	56	0.49	4.0	-	18	31	0.68	7.6	28

Table 13 (part 2): Measurement data indicating Protein number, Molecular weight (M_r) and Retardation factor (R_f) and Isoelectric point (pI) of the proteins identified from the Two-Dimensional gel analysis of binary biofilm cells exposed to different treatment of antibiotics and compared against binary biofilm cells (control), cultured in modified CDM, (Co: corresponding protein to Figure 31; N: novel protein).

Figure 31 Binary biofilm cells control.					**Figure 32** Binary biofilm cells with the treatment of ampicillin at sub-MIC (32 $\mu g\ mL^{-1}$).				
Protein no.	M_r (kDa)	R_f	pI	Co.	Protein no.	M_r (kDa)	R_f	pI	Co.
19	52.5	0.51	5.2	-	19	29	0.69	8.4	28
20	52.5	0.51	4.5	-	20	7.2	0.85	6.8	31
21	41.5	0.61	5.2	-	-	-	-	-	-
22	36.5	0.65	5.9	-	-	-	-	-	-
23	35	0.66	3.6	-	-	-	-	-	-
24	35	0.66	4.0	-	-	-	-	-	-
25	35	0.66	4.5	-	-	-	-	-	-
26	33	0.67	5.7	-	-	-	-	-	-
27	33	0.67	7.0	-	-	-	-	-	-
28	30	0.68	4.9	-	-	-	-	-	-
29	27.5	0.70	5.8	-	-	-	-	-	-
30	13	0.81	5.7	-	-	-	-	-	-
31	8.2	0.84	4.0	-	-	-	-	-	-
32	5.7	0.86	4.0	-	-	-	-	-	-

- : No data for that cell

Table 13 (part 3): Measurement data indicating Protein number, Molecular weight (M_r) and Retardation factor (R_f) and Isoelectric point (pI) of the proteins identified from the Two-Dimensional gel analysis of binary biofilm cells exposed to different treatment of antibiotics and compared against binary biofilm cells (control), cultured in modified CDM, (Co: corresponding protein to Figure 31; N: novel protein).

Figure 33 Binary biofilm cells with the treatment of nalidixic acid at sub-MIC (8 μg mL^{-1}).					**Figure 34** Binary biofilm cells with the treatment of streptomycin at sub-MIC (16 μg mL^{-1}).				
Protein no.	M_r (kDa)	R_f	pI	Co.	Protein no.	M_r (kDa)	R_f	pI	Co.
1	90	0.22	5.6	2	1	107	0.11	3.5	N
2	81	0.29	6.4	4	2	92	0.20	3.5	1
3	81	0.29	7.2	4	3	83	0.28	3.5	4
4	78	0.31	5.1	7	4	81	0.30	9.4	5
5	74	0.34	5.6	9	5	79.7	0.31	4.4	6
6	71	0.37	5.4	10	6	77	0.33	3.5	7
7	67	0.40	5.6	11	7	77	0.33	3.9	7
8	67	0.40	7.5	11	8	77	0.33	9.4	7
9	67	0.40	8.5	11	9	73	0.36	5	9
10	56	0.49	4.9	18	10	73	0.36	5.4	9
11	49	0.55	5.2	N	11	68.7	0.39	4.1	N
12	36.5	0.65	8.5	22	12	67	0.40	3.9	11
13	32.5	0.68	9.8	26	13	55.2	0.49	4.6	18
14	20.5	0.78	5.4	N	14	51.2	0.52	4.1	N
15	13	0.81	5.6	30	15	47.5	0.58	3.5	N
-	-	-	-	-	16	29	0.69	3.8	28
-	-	-	-	-	17	3	0.88	4.1	N

- : No data for that cell

The results obtained from SDS-PAGE and 2D-PAGE analysis showed significant differences between biofilm and eluate cells, on one hand and control and cells treated with sub-MICs of antibiotics on the other hand. While many outer membrane proteins were common in both control and treated cell samples, others appeared to be novel (proteins that are not present in the control, but present in the treated samples). Furthermore, some outer membrane proteins disappeared from cells following exposure to sub-MICs of antibiotics. These significant alterations in outer membrane profiles after exposure to antibiotics may lead to a bacterial surface phenotype that minimises antibiotic entry into cells or may even result in an over expression of surface proteins that pump-out accumulating concentrations of antibiotics into the external environment.

Although it is outside the scope of this work to identify the proteins that are responsible for the observed resistance, some clues may be derived from primary proteomic analysis carried out in this study by a process of protein elimination. An SDS-PAGE and 2D-PAGE analysis of control and antibiotic treated cells in the biofilm state has indicated a subset of unique proteins (novel proteins and proteins that have disappeared) to the selected antibiotic treatment. A similar analysis carried out with eluate cells also resulted in unique protein subsets (data not given here). However, a comparison of these two subsets of proteins (derived from biofilm and eluate samples) should yield a few common proteins that may be responsible for the resistance offered by the organism against the antibiotic. The proteins that are not similar may have no relation (may be outer membrane proteins that are produced as a direct consequence of addition of the antibiotic, but have no role in resistance) or only a secondary relation to antibiotic resistance. For example the disappearance of a 38.7 kDa protein from biofilm and eluate cells treated with NaL (Table 8, Parts 1 & 2; Table 9, Part 2) may be responsible for the nalidixic acid-resistant phenotype. Such a

comparison between outer membrane protein profiles of different states (biofilm state and eluate cell state) is attempted on the assumption that the physiologies of these two states are very similar, as concluded from relatively similar MIC results (Chapter 3, Table 6). If the cell physiologies are grossly different as in biofilm and planktonic states, there exist the possibility that the bacteria may exhibit different resistant phenotypes for the same antibiotic, and the above comparison would not have been possible. A similar logic applied to the protein subsets derived from other antibiotic treatments (ampicillin and streptomycin; data not given here) did not yield any similar proteins. However, this may be due to manual error in estimation of molecular weights of proteins. For instance, a 45.7 kDa protein observed in NaL-treated biofilm cells by SDS-PAGE (Table 8, Parts 1 & 2), might actually be the 44.7 kDa protein observed in the corresponding eluate samples (Table 9, Part 2). However, this cannot be confirmed unless further analysis (e.g. MALDI-TOF mass spectrometric analysis) of these proteins is done.

It is interesting to note that the 45.7 kDa protein (SDS-PAGE) appears to be present in all antibiotic (ampicillin, streptomycin and nalidixic acid) treated samples (Table 8, Part 2; Table 9, Parts 2 & 3). This observation may indicate a common mode of resistance for all three different antibiotics. The presence of common protein profiles observed in comparisons of the biofilm and eluate states of *Pseudomonas aeruginosa* further strengthens the hypothesis, that the resistance against antibiotics is an inherent cellular based mechanism.

There is no definitive evidence as to how shifts in outer membrane proteins in response to antibiotic treatments affect the overall pathogenicity of the organism. However, some logical conclusions may be made. OMP shifts may result in epitopic changes of the bacterial membrane. Besides an increase in resistance to antibiotics, epitopic fluctuations by bacteria (resulting from OMP) shifts may lead to antigenic

shifts and subsequent evasion of the immune response mounted against it. Such increases in the resistance and bacterial evasion tactics will positively influence the survival of these pathogens *in vivo*, and indirectly increase the pathogenic potential of these organisms, provided virulence of these organisms is not hampered by the presence of the antibiotic. Hence, when considered as a determinant of pathogenicity, increases in bacterial antibiotic resistance may equate to a concurrent increase in pathogenicity of *Pseudomonas aeruginosa.*

Chapter Five: Studies of the Effects of sub-MICs of Selected Antibiotics on Pathogenic Factors of *Pseudomonas aeruginosa* ATCC 15692 (PAO1) and *Escherichia coli* ATCC 10000 Grown as Biofilms

Introduction

The term 'virulence' is described as the relative capacity of a microbe to cause disease or a reduction in host fitness (Poulin & Combes, 1999; Cassadeval & Pirofski, 2002). At present, the concept of virulence has also been used as a characteristic that distinguishes pathogenic bacteria from non-pathogenic ones (Cassadeval & Pirofski, 2001). Despite the traditional concept of virulence, there is a degree of uncertainty regarding the exact definition of the term. The problem results from the variability present in the disease causing ability of a microbial species in different hosts. For example, virulence can only be totally expressed in a susceptible host and not in a healthy host. Again, the degree of host susceptibility causes a variation in the degree of virulence. In short, phenotypic expression of virulence is dependent on host-microbe interactions, despite being an inherent microbial character (Cassadeval & Pirofski, 2001).

If disease is the phenotypic result of virulence expression, then the factors that organisms possess that can cause disease are termed *virulence factors*. Thus, virulence factors are determinants of pathogenicity and they have been defined as "*components that impair virulence when deleted, but not viability*" (Cassadeval & Pirofski, 1999; 2001). Virulence factors may have different functional roles, either directly or indirectly related to reducing host fitness. They may include factors that help bacteria attach to cell surfaces (adhesins, pili, etc.), escape and evade host immune detection (flagella, capsule), destroy host cells (cytotoxins, LPS) and survive in the host (e.g., leucocidin) (Wilson *et al.*, 2002).

The success of a bacterial pathogen with regard to colonization, survival and growth within the host depends on its ability to sense and respond to different host environments during the infection process (Cassadeval & Pirofski, 2003). Virulence factors play a major role in this context and in the response strategy employed by bacteria during the process of host infection. Hence, regulation of these virulence factors *in vivo* is critical for successful infection. Bacterial pathogens regulate virulence using common regulatory mechanisms that include alternate sigma factors and two-component signal transduction systems (Wilson *et al.*, 2002). While alternate sigma factors have been shown to regulate gene expression in stressful conditions (stationary phase, nutrient limitation, oxidative stress, osmotic stress, etc.) two-component signal transduction systems often depend on cell density (e.g. quorum sensing) (Wilson *et al.*, 2002). Both these factors: (1) stress conditions and (2) high cell densities are present within bacterial biofilms, and thus, may be ideal for virulence gene expression. Although it is evident that bacterial biofilms are responsible for chronic infections and as such, may have a basal level of virulence (Cryer *et al.*, 2004), this section presents the effects of selected antibiotic stress (ampicillin, nalidixic acid and streptomycin) on *Pseudomonas* monospecies and binary biofilm virulence.

The lipopolysaccharide (LPS) in Gram-negative organisms (e.g., *Ps. aeruginosa* and *E. coli*) is located within their outer membranes. They are often referred to as endotoxins, as their release from bacterial surfaces catalyses pathogenesis and other manifestations of Gram-negative infections (e.g. septic shock) (Gutsmann *et al.*, 2000). The biological effects of LPS-related pathogenesis are often a result of activation of a signalling cascade that leads to a disease phenotype following transport-protein-mediated or hydrophobic interactions with a specific signal protein on host cell membranes (Gutsmann *et al.*, 2000).

Many classes of antibiotics are known to induce release of various amounts of LPS *in vitro*, and thus, are thought to contribute to increases in virulence potential of Gram-negative pathogens during antibiotic treatment (Tsuji *et al.*, 2003). However, clinical investigations of the phenomenon of antibiotic-induced endotoxin release in infections have raised conflicting reports. Simpson *et al.* (2000) have shown that patients treated with the antibiotic ceftazmide had significantly higher systemic endotoxin concentrations after the first dose. Other investigators have reported no significant differences in release of endotoxin (Tsuji *et al.*, 2003).

As no consensus could be deduced on LPS-related pathogenesis or virulence potential of pathogens in the presence of antibiotics, the present study was designed and aims to circumvent this problem by investigating virulence based on relative production of the individual components of LPS. The rationale behind this is the understanding that, changes observed in LPS patterns of biofilm pathogens in presence of antibiotics are linked to increases in antibiotic resistant phenotypes and hence, the overall virulence potential of the microorganism.

Leucocidin and proteases produced by *Pseudomonas aeruginosa* contribute directly to its virulence potential. Leucocidins have cytotoxic activity (Hirayama *et al.*, 1984; Ohnishi *et al.*, 1994). They are capable of attacking and destroying host cells, especially those of the immune system (e.g., leukocytes or white blood cells (WBCs) (Ohnishi *et al.*, 1994). This will enable the bacteria (in this case, *Pseudomonas*) to survive and may even thrive in hostile host environments. While a number of *Pseudomonas* cytotoxins have been described in recent times, this thesis will focus on virulence studies carried out on the earliest cytotoxin (leucocidin) characterised. Furthermore, as cytotoxins produced by *Pseudomonas* exhibit their activity by oncosis (death of host cell characterised by swelling), fluctuations in virulence (after monospecies and binary biofilms of *Pseudomonas* were challenged

with antibiotics) were directly assayed as a measure of leucocidin activity (oncosis) (Dacheux *et al.*, 2000) on Spleen-myeloma cell line (Sp2 cells), using phase contrast microscopy.

Proteases produced by *Pseudomonas aeruginosa* break down specific host proteins leading to some of the characteristic clinical manifestations of disease (Wilson *et al.*, 2002). Examples of *Pseudomonas* proteases include elastase (*LasB*), alkaline protease, *LasA* protease and protease IV (Matsumoto, 2004). While elastase is able to cleave type I, III and IV collagen, alkaline protease of*Pseudomonas* is more specific to type I collagen (Heck *et al.*, 1986; Matsumoto, 2004). Protease IV has also been recently attributed to degrade cellular matrix proteins (Caballero *et al.*, 2004). Besides breakdown of cellular matrix proteins a number of other host immune response factors (plasma proteins, immunoglobins, coagulation and complement factors) are reportedly affected by proteases (Caballero *et al.*, 2004). The activity of proteases is known to aid in the pathogenesis of tissue invasion and hemorrhagic tissue necrosis often observed in *Ps. aeruginosa* infections (e.g., keratitis) (Matsumoto, 2004). Azocasein protease assays were used to measure changes in the virulence of *Pseudomonas aeruginosa* (after antibiotic challenge of biofilms), as a result of protease activity.

Pseudomonas aeruginosa biofilms (monospecies and binary biofilms of *Ps. aeruginosa* ATCC 15692 (PAO1) and *E. coli* ATCC 10000) were subjected to sub-MICs of the above-mentioned antibiotics and fluctuations in virulence levels were assayed for three major virulence factors: (1) lipopolysaccharide (LPS), (2) leucocidin and (3) proteases (described also in Chapter 1).

Experimental approach

Monospecies and binary biofilms of *Ps. aeruginosa* ATCC 15692 (PAO1) and *Escherichia coli* ATCC 10000 were established on a Sorbarod filters (Chapter 2; Al-Hmoud, 2002; Hodgson *et al.,* 1995). The biofilms were perfused with MCDM containing MIC/16 of selected antibiotics (ampicillin (AmP), nalidixic acid (NaL) and streptomycin (ST) at 37°C. The control and sub-MIC antibiotic treated biofilms were sacrificed after specific time intervals (corresponding to development of monospecies and binary biofilm steady states; 102 hours for *Ps. aeruginosa* ATCC 15692 (PAO1), 56 hours for *Escherichia coli* ATCC 10000 and 126 hours for binary biofilms). LPS was extracted and proteases and leucocidin were purified from sacrificed biofilm cells, eluate cells and planktonic cells after lysis.

Lipopolysaccharide (LPS) extractions

Lipopolysaccharide was extracted from biofilm, eluate and planktonic cells according to Preston and Penner (1987). Biofilm cells (developed on Sorbarod filters), eluate cells and planktonic cells were harvested in 5 mL of cold phosphate buffered saline (PBS). Cultures were collected in tubes and cell density was measured at E_{600}. Cell suspensions were diluted in PBS to an E_{600} of 0.6. An aliquot (1.5 mL) of the diluted cell suspension was centrifuged for 1.5 minutes at 5000xg (IEC Centra-4B). The supernatant was discarded. The pellets were solubilised in 0.2 mL of lysis buffer (Glycerol, 20%; 2-β-mercaptoethanol, 5%; SDS, 4.6%; 0.125M Tris HCl (pH 6.8), bromophenol blue, 0.004%) and heated at 100°C for 10 min. The samples were cooled to room temperature. An aliquot (0.04 mL) of lysis buffer containing 2.5 mg mL^{-1} proteinase K was added to the cool lysates. The samples were incubated at 60°C for one hour, followed by incubation in a boiling water bath for 5 minutes. Samples were cooled and used for LPS-PAGE.

Determination of lipopolysaccharide profiles

The LPS profiles were prepared using a 12% separator gel and a 5% stacker gel as described in (Chapter 2) without the addition of SDS. An aliquot (10 to 20 μL) of the samples were added to the wells and the gels were run at 35 mA for approximately 1.5 hours or until the leading samples had reached the end of the gel. The gels were fixed and stained according to the method of Preston & Penner (1987; Chapter 2).

LPS shifts in *Ps. aeruginosa* PAO1 biofilm and eluate cells exposed to sub-MICs of ampicillin, nalidixic acid and streptomycin

Figure 35 illustrates the LPS profile of *Ps. aeruginosa* biofilm cells (control and treated with the sub-MIC of antibiotics (AmP, NaL and ST)). LPS bands are known to migrate according to their molecular weight. However, unlike negatively charged protein molecules (under the influence of SDS), there may be molecular weight anomalies in LPS migration patterns, due to charge and to mass differences of individual LPS molecules. The gel shows general differences in banding patterns. The range of LPS bands corresponding to the high molecular weight protein markers (27 kDa to 74 kDa; lane 1) show marked differences with respect to spacing and intensity of bands. While control (without antibiotic treatment) samples of the biofilm cells showed a number of bands in this region (36 kDa to 72 kDa; lane 3; Figure 35) and planktonic cells showed bands in two different regions (64 kDa to 74 kDa and 32 kDa to 45 kDa; lane 2), after cells were exposed to sub-MICs of antibiotics (AmP, NaL and ST) treated biofilm samples (lanes 4, 5 and 6 respectively) exhibited few bands ranging between (44 kDa to 65 kDa). Furthermore, the few bands seen also showed diminished staining (indicative of lower concentrations of LPS). The LPS molecules that band at high molecular weight regions are the O antigens. Hence, a decrease in

number of bands in antibiotic treated samples within this region would mean there are either short O chains (lower M_r) or there are none at all.

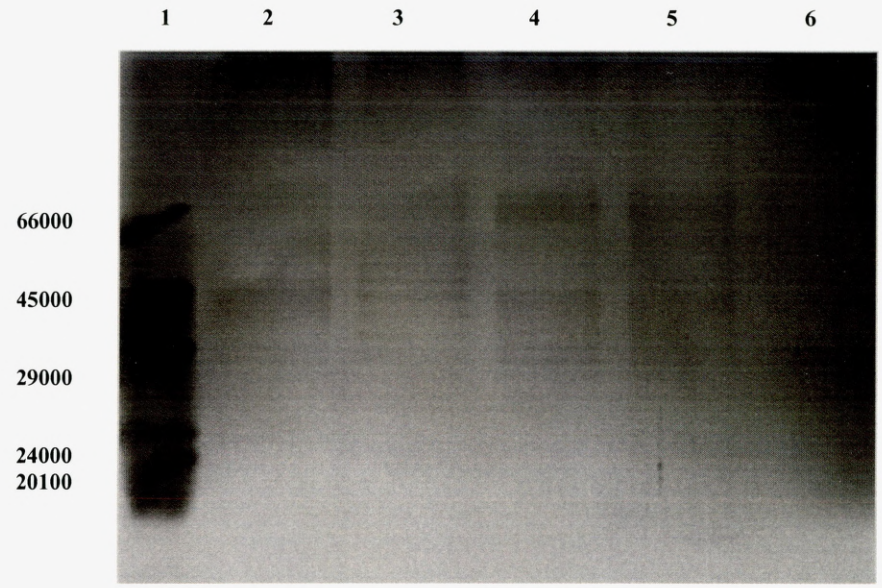


Figure 35: Silver-stained LPS-PAGE for LPS of *Ps. aeruginosa* PAO1 biofilm cells cultured in modified CDM and exposed to different treatment of antibiotics. Lane 1: molecular weight markers; Lane 2: *Ps. aeruginosa* planktonic cells control; Lane 3: *Ps. aeruginosa* biofilm cells control; Lane 4: *Ps. aeruginosa* biofilm cells with treatment of ampicillin at sub-MIC (8 μg mL^{-1}); Lane 5: *Ps. aeruginosa* biofilm cells with treatment of nalidixic acid at sub-MIC (4 μg mL^{-1}); Lane 6: *Ps. aeruginosa* biofilm cells with treatment of streptomycin at sub-MIC (8 μg mL^{-1}).

Figure 36 demonstrates the LPS of *Ps. aeruginosa* eluate cells (control and treated with sub-MIC of antibiotics (AmP, NaL and ST)). As observed in the previous gel (Figure 35), general differences in banding patterns between control and treated cells could be seen in the range of LPS bands corresponding to the O antigen region. High molecular weight LPS bands observed in biofilm control samples (32 kDa to 71 kDa; Lane 4; Figure 35), some bands (35 kDa to 66 kDa) were inapparent after cells were exposed to sub-MICs of antibiotics (AmP, NaL and ST) (lane 5, 6 and 7 respectively). A thick band was apparent in eluate control with a molecular weight about (32 kDa; lane 4; Figure 36), but inapparent after cells were exposed to sub-MICs of NaL and ST (lanes 6 and 7 respectively). The antibiotic response of eluate cells (as judged by their LPS profiles) is very similar to that of biofilm cells. This

gives further credence to previous observations made, of relatively high MIC values for both biofilm and eluate cells towards selected antibiotics.

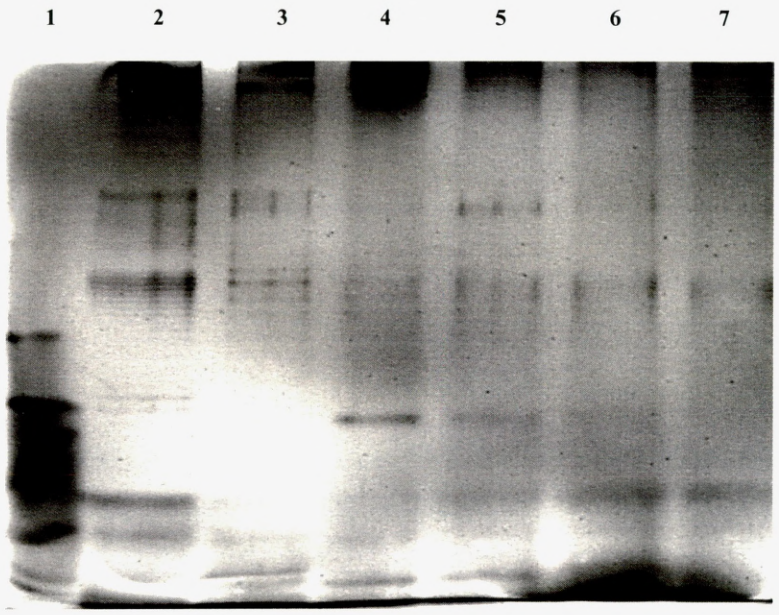


Figure 36: Silver-stained LPS-PAGE for LPS of *Ps. aeruginosa* PAO1 eluate cells cultured in modified CDM and exposed to different treatment of antibiotics. Lane 1: molecular weight markers; Lane 2: *Ps. aeruginosa* planktonic cells control; Lane 3: *Ps. aeruginosa* biofilm cells control; Lane 4: *Ps. aeruginosa* eluate cells control; Lane 5: *Ps. aeruginosa* eluate cells with treatment of ampicillin at sub-MIC (8 μg mL^{-1}); Lane 6: *Ps. aeruginosa* eluate cells with treatment of nalidixic acid at sub-MIC (4 μg mL^{-1}); Lane 7: *Ps. aeruginosa* eluate cells with treatment of streptomycin at sub-MIC (8 μg mL^{-1}).

E. coli biofilm cells (control; lane3; Figure 37) and cells treated with sub-MIC of antibiotics, AmP (lane 4), NaL (lane 5) and ST (lane 6) showed few bands (if any) corresponding to O antigen (high molecular weight bands; see Figure 37). However, significant differences in high molecular weight bands (O antigen) were observed when compared to planktonic cells (28 kDa to 68 kDa; lane 2; Figure 37). This indicates that *E. coli* cells down-regulate O antigen production as they shift to the biofilm state from the planktonic state. 26 kDa to 29 kDa bands were present in biofilm control cells (lane 3; Figure 37), but inapparent after cells were exposed to sub-MICs of AmP, NaL and ST (lanes 4, 5 and 6 respectively). These low molecular weight bands correspond to the Lipid A region of LPS. Banding pattern changes in

this region would indicate changes in the epitopic nature of the endotoxin. The reflection of such changes on endotoxin (Lipid A) towards virulence of the organism is not clear at present.

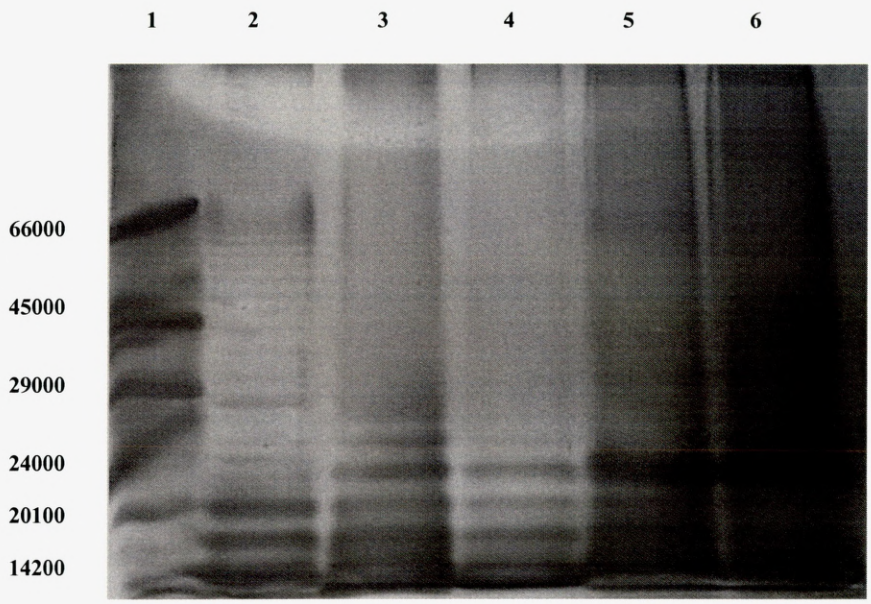


Figure 37: Silver-stained LPS-PAGE for LPS of *Escherichia coli* biofilm cells cultured in modified CDM and exposed to different treatment of antibiotics. Lane 1: molecular weight markers; Lane 2: *E. coli* planktonic cells control; Lane 3: *E. coli* biofilm cells control; Lane 4: *E. coli* biofilm cells with treatment of ampicillin at sub-MIC (2 μg mL^{-1}); Lane 5: *E. coli* biofilm cells with treatment of nalidixic acid at sub-MIC (2 μg mL^{-1}); Lane 6: *E. coli* biofilm cells with treatment of streptomycin at sub-MIC (8 μg mL^{-1}).

LPS profiles of *E. coli* eluate cells show marked differences from their biofilm counterparts. While *E. coli* biofilm cells down regulate O antigens, the eluate cells (control, lane 3, Figure 38) express O antigens. 26 to 74 kDa bands were present in control cells (lane 3; Figure 38), but some bands were inapparent in the range of 42 kDa to 62 kDa after cells were exposed to sub-MIC of antibiotics Amp, NaL and ST (lanes 4, 5 and 6 respectively). The presence of sub-MICs of antibiotics seems to cause suppression of O antigens (decrease in the number and intensity of high molecular weight bands within antibiotic treated samples). Two thick bands were present in control cells (lane 3; Figure 38) with low molecular weights (14 kDa to 20 kDa). These bands were inapparent after cells were exposed to sub-MIC of antibiotic

(AmP, NaL and ST; lanes 4, 5 and 6 respectively). The presence of antibiotics also caused changes in the banding patterns of the lipid A (lower M_r) region of LPS.

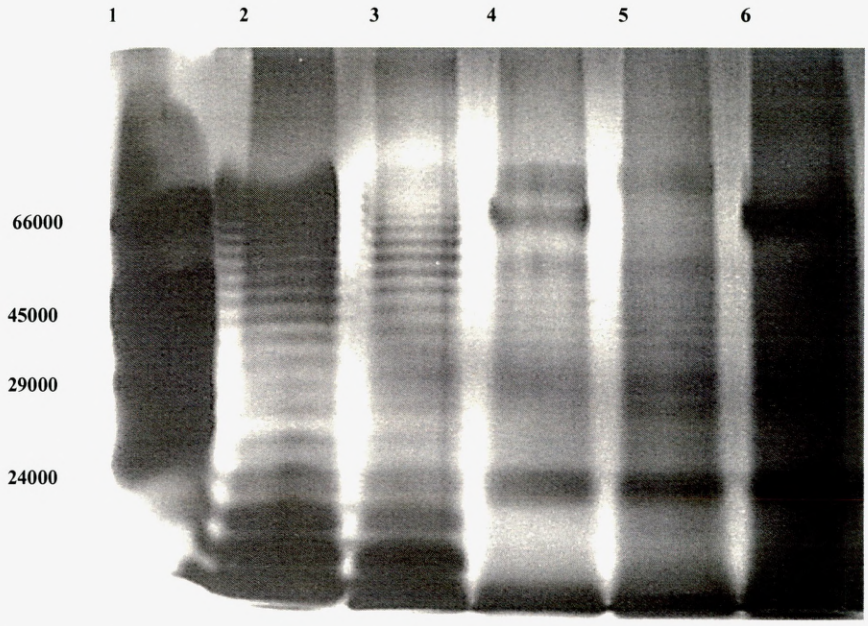


Figure 38: Silver-stained LPS-PAGE for LPS of *Escherichia coli* eluate cells cultured in modified CDM and exposed to different treatment of antibiotics. Lane 1: molecular weight markers; Lane 2: *E. coli* planktonic cells control; Lane 3: *E. coli* eluate cells control; Lane 4: *E. coli* eluate cells with treatment of ampicillin at sub-MIC (1μg mL^{-1}); Lane 5: *E. coli* eluate cells with treatment of nalidixic acid at sub-MIC (1 μg mL^{-1}); Lane 6: *E. coli* eluate cells with treatment of streptomycin at sub-MIC (8 μg mL^{-1}).

Biofilms formed by *Pseudomonas aeruginosa* and *E. coli* in the presence of each other (binary biofilms, lane 4, Figure 39) indicated LPS profile pattern similarities to those obtained from individual species at a time (monospecies biofilms, lanes 2 and 3 respectively). 26 to 68 kDa bands patterns were present in control binary biofilm cells (lane 4; Figure 39), but some bands in the range of 52 kDa to 64 kDa were inapparent after cells were exposed to sub-MIC of antibiotics (AmP, NaL and ST; lanes 5, 6 and 7 respectively). Two thick bands with lower molecular weights (14 kDa to 18 kDa) were present in control cells (lane 4; Figure 39), but inapparent after cells were exposed to sub-MIC of antibiotics (AmP, NaL and ST; lanes 5, 6 and 7 respectively). A 26 kDa band was absent in control cells (lane 4; Figure 39), but apparent after treatment with sub-MIC of ST (lane 6; Figure 39). However, it is

observed that the LPS profile patterns of binary biofilms were significantly different from the monospecies biofilms when treated with antibiotics. A number of LPS bands corresponding to O antigen (52 kDa to 64 kDa) disappeared in sub-MIC antibiotic treated cells (AmP (lane 5), NaL (lane 6) and ST (lane 7; Figure 39) and two thick bands within lipid A regions (14 kDa to 18 kDa) were present in control cells (lane 4; Figure 39), were inapparent after cells were exposed to sub-MIC of antibiotics treated samples (AmP (lane 5), NaL (lane 6) and ST (lane 7; Figure 39).

Figure 40 illustrates the LPS of binary eluate cells (control; lane 7) and cells treated with sub-MIC of antibiotics (AmP, NaL and ST; lanes 8, 9 and 10 respectively). Interestingly, sub-MIC antibiotic treated samples of binary biofilm eluate cells exhibit an increase in the production of lipid A, as can be seen from the strong low molecular weight bands in the region between 14.2 kDa to 27 kDa (Figure 40; lanes 8, 9 and 10 respectively) and maintain a decrease of O antigen production when compared to their binary biofilm counterparts (lanes 5, 6 and 7, Figure 39).

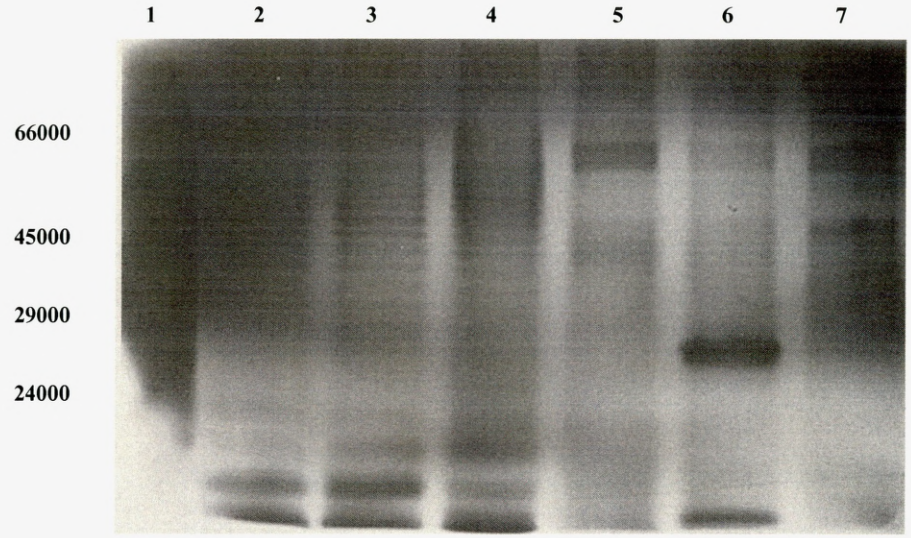


Figure 39: Silver-stained LPS-PAGE for LPS of binary biofilm cells cultured in modified CDM and exposed to different treatment of antibiotics. Lane 1: molecular weights markers; Lane 2: *Ps. aeruginosa* PAO1 biofilm cells control; Lane 3: *Escherichia coli* ATCC 10000 biofilm cells control; Lane 4: Binary biofilm cells control; Lane 5: Binary biofilm cells with treatment of ampicillin at sub-MIC concentration (32 μg mL^{-1}); Lane 6: Binary biofilm cells with treatment of nalidixic acid at sub-MIC concentration (8 μg mL^{-1}); Lane 7: Binary biofilm cells with treatment of streptomycin at sub-MIC concentration (16 μg mL^{-1}).

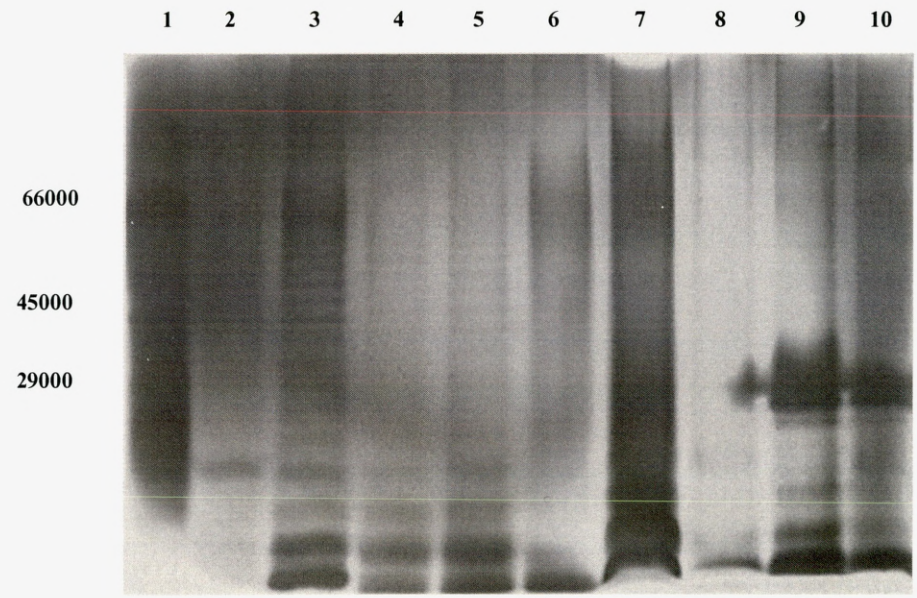


Figure 40: Silver-stained LPS-PAGE for LPS of binary eluate cells cultured in modified CDM and exposed to different treatment of antibiotics. Lane 1: molecular weights markers; Lane 2: *Ps. aeruginosa* PAO1 planktonic cells control; Lane 3: *Escherichia coli* 10000 planktonic cells control; Lane 4: *Ps. aeruginosa* biofilm cells control, Lane 5: *Escherichia coli* biofilm cells control, Lane 6: Binary biofilm cells control; Lane 7: Binary biofilm eluate cells control; Lane 8: Binary eluate cells with treatment of ampicillin at sub-MIC concentration (16 μg mL^{-1}); Lane 9: Binary eluate cells with treatment of nalidixic acid at sub-MIC concentration (4 μg mL^{-1}); Lane 10: Binary eluate cells with treatment of streptomycin at sub-MIC concentration (4 μg mL^{-1}).

Results & Discussions

The expression of LPS components (particularly O antigen) appears to be environmentally regulated (Makin & Beveridge, 1996a & 1996b; Rocchetta *et al.*, 1999; Norman *et al.*, 2002). Accordingly, the pattern of LPS molecules within LPS-PAGE may vary. Comparisons of LPS from the planktonic and biofilm cells exhibit a general decrease in O antigen production in the latter phenotype and hence, a transition from the smooth to the rough phenotype (Giwercman *et al.*, 1992). Although this is the case for *E. coli* (Figure 37, lanes 2 and 3), the results obtained for *Pseudomonas aeruginosa* (Figure 35, lanes 2 and 3) indicate no change in LPS patterns. Nevertheless, the presence of antibiotics caused a decrease in the production of O antigens in both *E. coli* and *Pseudomonas aeruginosa* samples. A shift from the planktonic stage to the biofilm stage corresponds to a similar transition from acute to chronic infection (Goldberg and Pier, 1996, Rocchetta *et al.*, 1999, Sabra *et al.*, 2003). Loss of O antigens in the biofilm phase will thus, help the cells evade host immune response and adapt to the chronic infection lifestyle. The loss of O antigens may result from internal biofilm stress (Sabra *et al.*, 2003). The presence of antibiotics in the immediate biofilm environment might constitute further stress and may possibly cause further down regulation of O antigens.

Perhaps development of resistance to antibiotics by reduced permeability may be another reason for the observed decrease in the production of O antigens in the presence of antibiotics. Although the exact mechanism of resistance to different classes of antibiotics at the present time is outside the scope of this study, generalisations may be made. A decrease of O antigen in ampicillin treated samples (lane 4, Figure 35; lane 5, Figure 36 and lane 4, Figure 37) will increase the relative proportion of hydrophobic lipid A portions of LPS. This will undoubtedly increase the hydrophobicity of the outer membrane. Hydrophilic antibiotics like β-lactams

(ampicillin) and aminoglycosides (streptomycin) will find the membrane more hydrophobic and an effective barrier to cross (Lambert, 2002). Furthermore, an increase in cell membrane hydrophobicity may also attract moderately hydrophobic enzymes, like β-lactamases, towards it and release them into the external environment, causing a neutralisation of β-lactams outside the cell (Giwercman *et al.*, 1992).

Pseudomonas aeruginosa PAO1 possesses two distinct types of LPS O polysaccharides (A band and B band LPS). Previous studies have shown that aminoglycosides bind maximally to the LPS containing both A and B bands (A^+B^+), moderately to LPS containing either one of these bands (A^+B^-/ A^-B^+) and least to LPS containing no O antigen (A^-B^-) (Kadurugamuwa, *et al.*, 1993; Makin and Beveridge, 1996b). The near absence of any bands either corresponding to the A band and B band in *Pseudomonas aeruginosa* streptomycin-treated biofilm and eluate samples (Figure 35, lane 6 and Figure 36, lane 7 respectively) indicates that the LPS phenotype is responsible for the reduction in attachment of streptomycin to the membrane.

Quinolones (esp. nalidixic acid) are hydrophobic antibiotics (Jain and Rajeshwari, 2002; Hirai *et al.*, 1986). Generally, a complete LPS (presence of both A and B bands or complete O antigen) is responsible for creating a permeability barrier for these antibiotics and also prevents them from accumulating inside cells (Hirai *et al.*, 1986). An absence of hydrophobic B bands may also result in a resistant phenotype as the relatively hydrophilic A bands may repel hydrophobic antibiotics. Indeed, the observations (Figure 35, lane 5; Figure 36, lane 6 and Figure 37, lane 5) seem to indicate a general down-regulation of B bands.

In clinical conditions, multispecies biofilms may be the norm rather than monospecies biofilms (Donlan, 2001). Analysis of LPS patterns in binary biofilms show results similar to those of monospecies biofilms. In general, the presence of sub-

MICs of antibiotics, higher molecular weight regions of O antigens are down regulated. Down regulation is further accentuated in the case of eluate cells from binary biofilms treated with sub-MICs of antibiotics. These changes in the surface membrane may result in epitopic changes of cells resulting in effective host-immune evasion mechanisms (Goldberg and Pier, 1996).

A cursory glance of the different LPS profiles generated for eluate and biofilm cell physiologies indicates a decrease in the number and intensity of bands in the presence of antibiotics with respect to control samples. However, interpreting this observation to mean a decrease in virulence in the presence of sub–MICs of antibiotics may be misleading. In the present study detailed analysis shows a pattern of evidence suggesting adaptation of cells to a new environment (namely, in the presence of antibiotics) rather than a decrease in virulence. Indeed, the evidence may point to increases in the overall virulence potential of the biofilm pathogen (*Pseudomonas aeruginosa*) in the presence of antibiotics. However, the earlier supposition that antibiotic resistance resulting from LPS pattern changes may lead to increases in virulence has to be tested with other virulence factors. Hence, the study of cytotoxins (leucocidin) and proteases of *Pseudomonas aeruginosa* was subsequently attempted.

Purification of Leucocidin and Protease from monospecies and binary biofilms of *Ps. aeruginosa* ATCC 15692 (PAO1) and *E. coli* ATCC 10000 as control and challenged with sub-MIC of selected antibiotics (ampicillin, nalidixic acid and streptomycin).

Previously, Scharmann and co-workers (1976) have described purification of leucocidin from *Pseudomonas aeruginosa* strain I 58 using ammonium sulphate precipitation (20% saturation) and gel filtration techniques. Similar protocols were

employed in this study to purify and isolate leucocidin from *Ps. aeruginosa* ATCC 15692 (PAO1). In order to test fluctuations in virulence potential of leucocidin produced by *Ps. aeruginosa* biofilm cells, when challenged with antibiotics and in the presence of other organisms like *E. coli* (binary biofilms are a good simulation of multispecies biofilms present in nature and certain infectious conditions), it is critical to check for functionally similar cytotoxic molecules produced by *E. coli* that may co-precipitate and be purified along with fractions of pseudomonal-leucocidin. Undetected co-purification of functionally similar molecules would certainly indicate erroneous fluctuations of virulence potential that may be attributed to the target molecule. Hence, fractions similar to those containing pseudomonal-leucocidins were isolated from *E. coli* and tested for activity. A similar line of reasoning was applied to pseudomonal-proteases isolated from fractions that eluted later than those of *Pseudomonas*-leucocidin in the purification process. Proteins present within these fractions were detected and confirmed using SDS-PAGE and were stored at –20°C for further analysis.

Purification of leucocidin and protease from planktonic cells of *Ps. aeruginosa* ATCC 15692 (PAO1) (Control).

Preparation of autolysate

250 mL of a sterile medium (CDM), in a 1000 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^{-1}. After 22 h cultivation *Ps. aeruginosa* cells were washed once with Phosphate buffered saline (pH 7.2; PBS; Dulbecco & Vogt, 1954), then cells were resuspended in PBS to one-tenth of the original culture volume and shaken for 73 h at 37°C. The supernatant of the autolysate was collected

by centrifugation (MSE Superspeed 50, UK; 37000xg, 40 min; Scharmann, 1976a). All purification steps were carried out at 4°C.

Ammonium sulphate fractionation

The supernatant fluid from autolysed *Ps. aeruginosa* cultures (150 mL) was cooled to 4°C and 37.5 mL of a saturated ammonium sulphate solution (20% saturation, pH 7.2) was added dropwise with stirring. Stirring was continued over-night and the precipitate collected by centrifugation (MSE Superspeed 50, UK) at 22000xg for 15 min. The precipitate was dissolved in (10 mL) PBS containing 0.2M NaCl (pH 7.2), stirred overnight, and the soluble fraction obtained by centrifugation (MSE Superspeed 50, UK; 22000xg, 15 min; Scharmann, 1976).

Gel filtration

The supernatant fluid from step 1 (10 mL) was applied to a column (2.5 x 100 cm) of Sephadex G-100 superfine previously equilibrated with PBS containing 0.2 M NaCl (pH 7.2), and eluted with the same buffer. The flow rate was 4 mL h^{-1} (2 mL per tube). Fractions were collected (first 97 fractions from the Sephadex G-100 column alone) and the optical density for all fractions was measured at 280nm. Shortly before the leucocidin activity was expected to appear in the eluate, the effluent was transferred to a second column (2.5 x 100 cm) of Bio Gel P-100 equilibrated with PBS containing 0.2M NaCl (pH 7.2). This transfer was made by connecting the draining tube of the Sephadex column to the adapter of the Bio Gel column (fractions from 98 to 270 were collected from both columns (Figure 41; Fractions 1 to 40 were not considered for analysis). This *tandem* gel filtration (Scharmann, 1976a) was used to separate leucocidin and protease from monospecies and binary biofilms of *Ps. aeruginosa* ATCC 15692 (PAO1) and *E. coli* ATCC 10000.

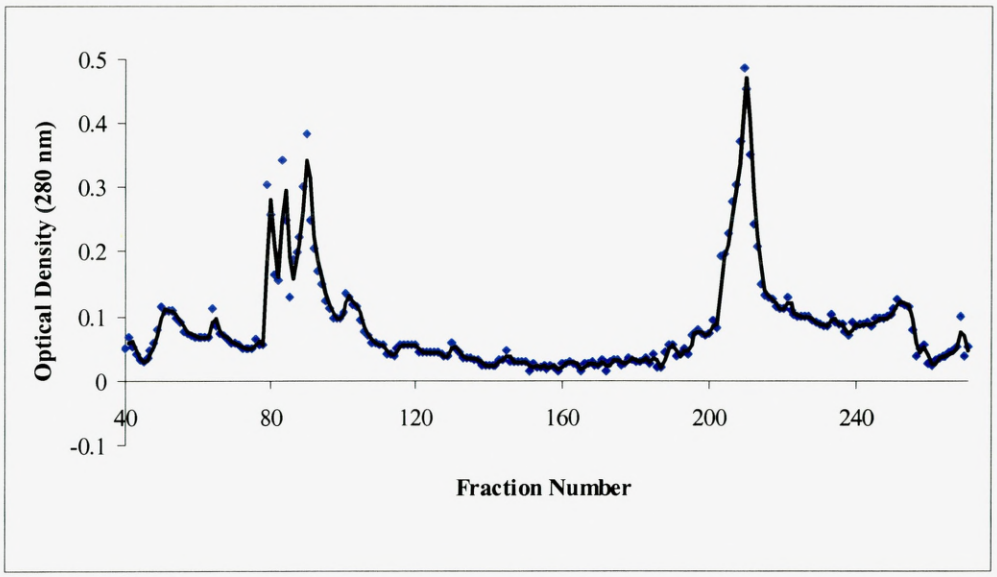


Figure 41: Elution profile of protein fractions of *Ps. aeruginosa* PAO1 planktonic cells control. *'Tandem'* gel filtration of leucocidin on Sephadex G-100 superfine and Bio Gel P-100. 10 mL of sample was applied to Sephadex G-100 column (2.5 x 100 cm) equilibrated with phosphate buffered saline. The flow rate was 4 mL h^{-1} (2 mL tubes). Effluent was analyzed for protein at (E280nm). Fractions 209 to 213 were combined and dialysed before further analysis.

SDS-PAGE profile of leucocidin from Ps. aeruginosa ATCC 15692 (PAO1) planktonic cells

Elution profiles from previous studies carried out by Scharmann (1976b), showed the 27.5 kDa protein, leucocidin to fractionate between fractions 120 to 140 (flow rate used 5.4 mL h^{-1}). In this study, peaks corresponding to proteins in and around fractions 200 to 220 on elution profiles (flow rate used 4 mL h^{-1}; Figure 41) were analysed using SDS-PAGE, to confirm the presence of leucocidin.

SDS-PAGE profiles of leucocidin fractions were prepared using a 10% separator and 5% stacker gel SDS-PAGE as described in Chapter 2. Aliquots (10 to 20 μL) of the samples were added to the wells and the gels were run at 35 mA for approximately 1.5 hours or until the leading samples had reached the end of the gel. The gels were stained in Silver stain as described in Chapter 2.

Figure (42A and 42B) illustrate leucocidin fractions of *Ps. aeruginosa* PAO1 planktonic cells between fractions 200 to 219. Leucocidin protein appeared as a single band on the SDS-PAGE gel with a molecular weight of 29 kDa (lanes 10 to 14).

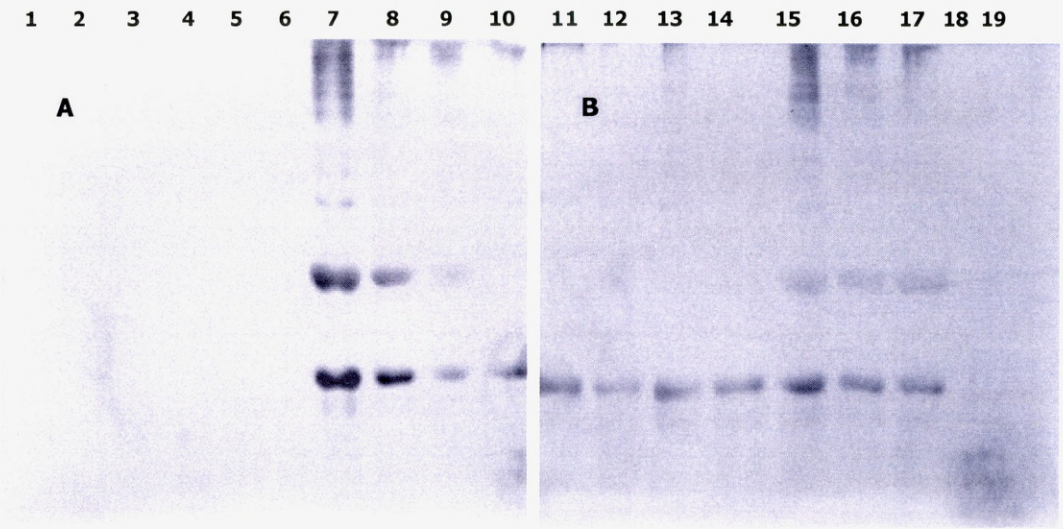


Figure 42: SDS-PAGE profile of leucocidin fractions of *Ps. aeruginosa* PAO1 planktonic cells. (A) Fractions 200 to 209 (lanes 1 to 10; Figure 41). (B) Fractions 210 to 219 (lanes 11 to 20; Figure 41).

Purification of leucocidin and protease from biofilm cells of *Ps. aeruginosa* ATCC 15692 (PAO1)

SDS-PAGE analysis of 20% ammonium sulphate protein precipitated fractions of planktonic *Pseudomonas* cell lysates, indicated the presence of leucocidin in fractions 200 to 219. However, the aim of the current investigation was to analyse changes in the virulence potential of leucocidin produced by biofilms of *Ps. aeruginosa*. Investigations to identify the leucocidin fractions in biofilm *Pseudomonas* cells were therefore undertaken.

Biofilms of *Ps. aeruginosa* ATCC 15692 (PAO1) were established on Sorbarod filters (Chapter 2; Hodgson *et al.,* 1995). These biofilms were perfused with MCDM at 37°C. Biofilm steady state was achieved within 102 hours. The Sorbarod filters were sacrificed (the adherent cells were removed and washed once with phosphate buffered saline, pH 7.2, PBS; Dulbecco & Vogt, 1954) and resuspended in

PBS to one-tenth of the original culture volume. Autolysis of cells was carried out in a shaker set at 37°C for 73 hours. The supernatant of the autolysate was collected by centrifugation (MSE Superspeed 50, UK; 37000xg, 40 min; Scharmann, 1976a). Ammonium sulphate precipitation and gel filtration of the biofilm derived autolysate was carried-out in a fashion similar to that for planktonic cells. The elution profile is exhibited below (Figure 43). A similar purification strategy was used to collect target fractions (containing leucocidin and proteases) from all samples (with different variables). The leucocidin fraction profiles were analysed by SDS-PAGE, using a 10% separating and 5% stacking gel as described in Chapter 2. An aliquot (10 to 20 μL) of the samples were added to the wells and the gel was run at 35 mA for approximately 1.5 hours or until the sample reached the end of the gel. The gels were stained in Silver stain as described in Chapter 2.

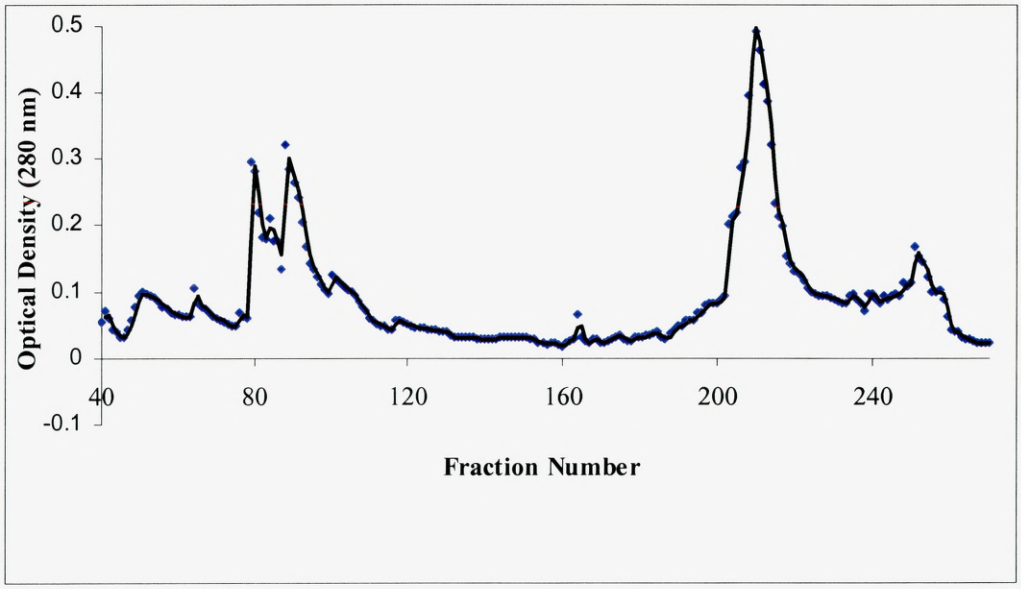


Figure 43: Elution profile of protein fractions of *Ps. aeruginosa* PAO1 biofilm cells control. *'Tandem'* gel filtration of leucocidin on Sephadex G-100 superfine and Bio Gel P-100. 10 mL of sample was applied to Sephadex G-100 column (2.5 x 100 cm) equilibrated with phosphate buffered saline. The flow rate was 4 mL h^{-1} (2 mL tubes). Effluent was analyzed for protein at (E_{280}nm). Fractions 209 to 213 were combined and dialysed.

Figure 44 illustrates SDS-PAGE analysis of leucocidin fractions of *Ps. aeruginosa* PAO1 biofilm cells. Lanes 1 and 7 show standard molecular weight

protein markers (14.2 to 66 kDa, Dalton mark VII–L, Sigma-Aldrich Company Ltd., Gillingham, UK). Leucocidin protein fractions (column fractions 209 to 213) appeared as a single band on the gel with a molecular weight of 29 kDa (lanes 2, 3, 4, 5 and 6). The molecular weight of leucocidin determined at present (29 kDa) appears to be slightly at variance to previous results published by Scharmann (1976b), 27.5 kDa and Lutz (1979), 25.1 kDa.

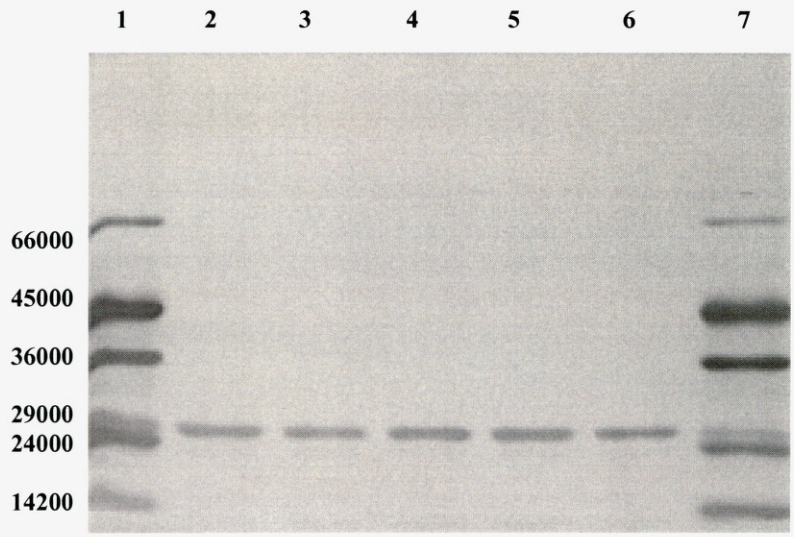


Figure 44: SDS-PAGE profile of leucocidin fractions of *Ps. aeruginosa* PAO1 biofilm cells (control). The gel shows fractions 209 to 213 (lanes 2 to 6; Figure 43). Lane 1 and 7 contain standard molecular weight marker (14.2 to 66 kDa).

The elution profile and initial SDS-PAGE analysis of leucocidin proteins of *Pseudomonas aeruginosa* PAO1 may be compared to those samples treated with the antibiotic ampicillin (Figure 45 and 46). Ampicillin was chosen as the representative antibiotic used to challenge *Pseudomonas* biofilms and to study virulence, due to its functional significance as a broad-spectrum antibiotic.

The elution profile (Figure 45) indicated peaks corresponding to leucocidin as analysed by SDS-PAGE (Figure 46) within similar fractions to those obtained in the control samples (*Pseudomonas aeruginosa* biofilm samples without sub-MIC ampicillin treatment). Both control and antibiotic challenged samples showed the

29kDa leucocidin protein. This indicates no significant modifications of leucocidin protein occurred in the presence of ampicillin.

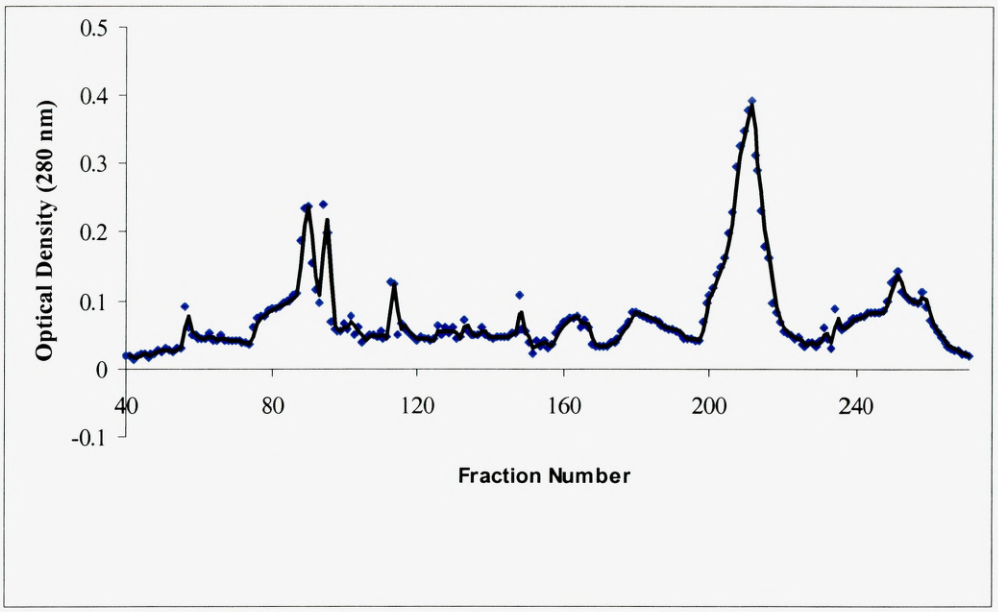


Figure 45: Elution profile of protein fractions of *Ps. aeruginosa* PAO1 biofilm cells with sub-MIC of ampicillin (8 μg mL^{-1}). *'Tandem'* gel filtration of leucocidin on Sephadex G-100 superfine and Bio Gel P-100. 10 mL of sample was applied to Sephadex G-100 column (2.5 x 100 cm) equilibrated with phosphate buffered saline. The flow rate was 4 mL h^{-1} (2 mL tubes). Effluent was analyzed for protein at (E_{280}nm). Fractions 209 to 213 were combined and dialysed.

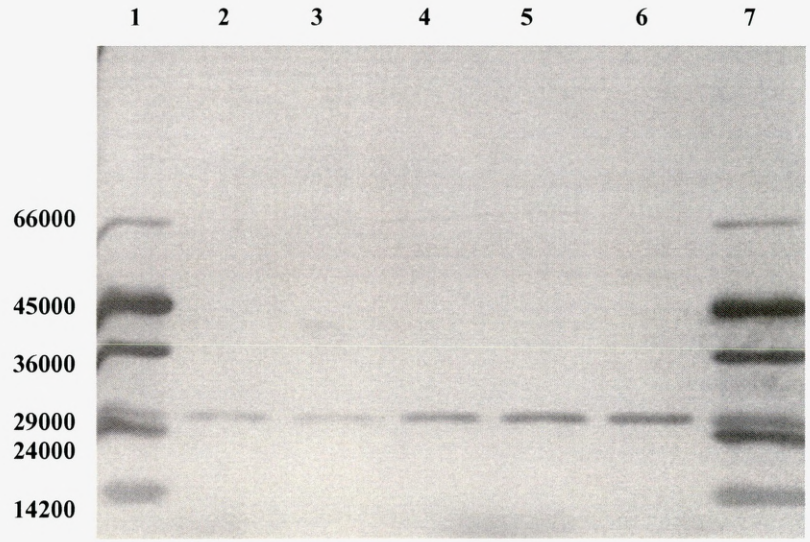


Figure 46: SDS-PAGE profile of leucocidin fractions of *Ps. aeruginosa* PAO1 biofilm cells in the presence of sub-MIC of ampicillin (8 μg mL^{-1}). The gel represents fractions 209 to 213 (lanes 2 to 6; Figure 45). Lane 1 and 7 represent standard molecular weight marker (14.2 to 66 kDa).

Elution profiles for all remaining samples (monospecies and binary biofilms of *Ps. aeruginosa* ATCC 15692 (PAO1) and *E. coli* ATCC 10000; control and antibiotic treated) are represented below (Figures 47, 48, 49 and 50). While the process of purification was similar to that outlined above, the steady state of biofilm development for *E. coli* biofilms varied to those of *Pseudomonas aeruginosa*. *E. coli* biofilms reached a steady state within 56 hours as opposed to 102 hours of *Pseudomonas* biofilms. Again, binary biofilms reached steady state only after 126 hours. *Pseudomonas aeruginosa* biofilms were grown in chemically defined medium (CDM). However, CDM had to be modified (MCDM) with respect to their C:N ratios for better growth of *E. coli* monospecies and binary biofilms. 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 (Al-Hmoud, 2002).

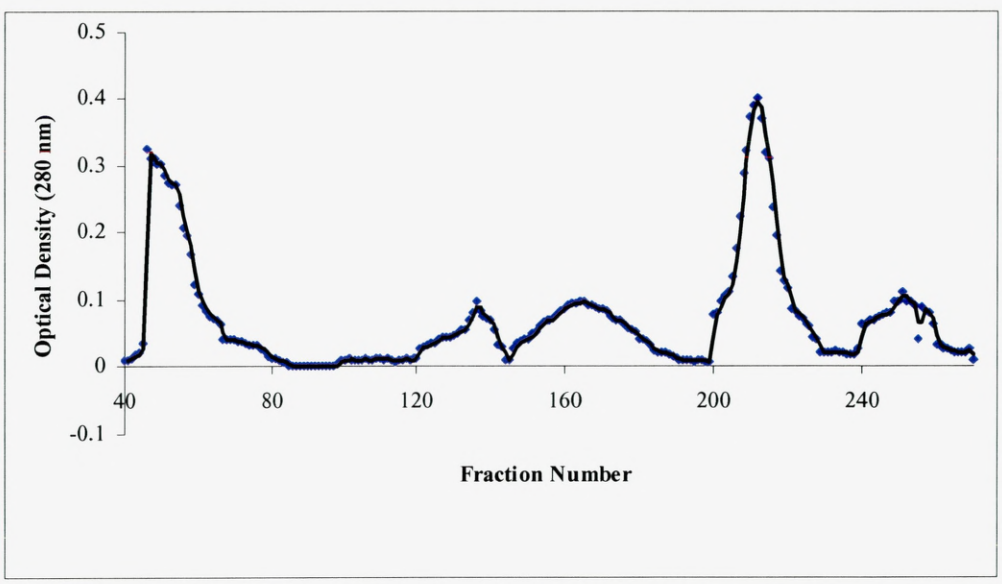


Figure 47: Elution profile of protein fractions of *Ps. aeruginosa* PAO1 biofilm cells with sub-MIC of nalidixic acid (4 μg mL^{-1}). *'Tandem'* gel filtration of leucocidin on Sephadex G-100 superfine and Bio Gel P-100. 10 mL of sample was applied to Sephadex G-100 column (2.5 x 100 cm) equilibrated with phosphate buffered saline. The flow rate was 4 mL h^{-1} (2 mL tubes). Effluent was analyzed for protein at (E_{280}nm). Fractions 210 to 213 were combined dialysed.

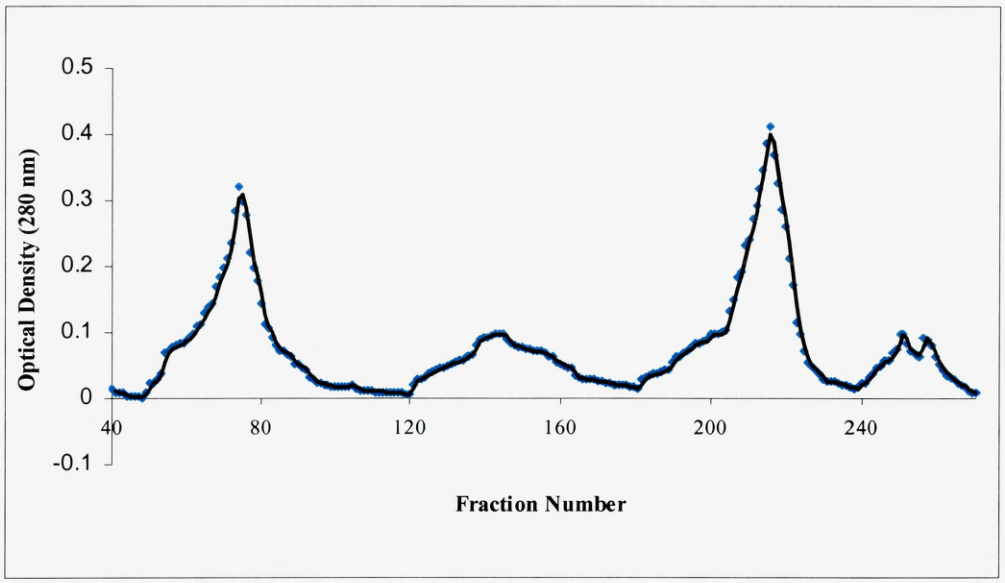


Figure 48: Elution profile of protein fractions of *Ps. aeruginosa* PAO1 biofilm cells with sub-MIC of streptomycin (8 μg mL^{-1}). *'Tandem'* gel filtration of leucocidin on Sephadex G-100 superfine and Bio Gel P-100. 10 mL of sample was applied to Sephadex G-100 column (2.5 x 100 cm) equilibrated with phosphate buffered saline. The flow rate was 4 mL h^{-1} (2 mL tubes). Effluent was analyzed for protein at (E_{280}nm). Fractions 214 to 218 were combined dialysed.

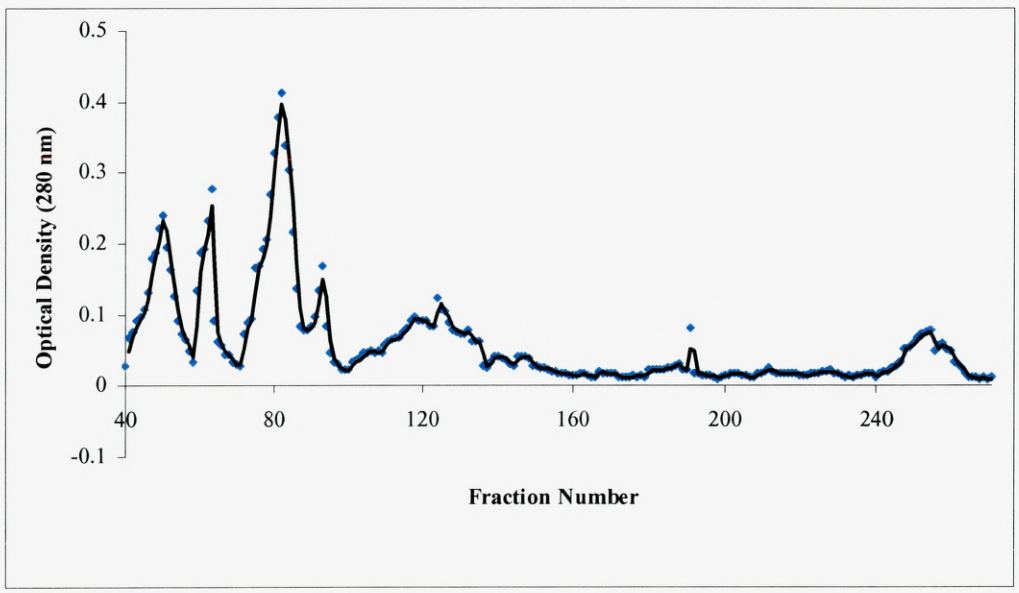


Figure 49: Elution profile of protein fractions of *E. coli* ATCC 10000 biofilm cells with sub-MIC of ampicillin (2 μg mL^{-1}). *'Tandem'* gel filtration of leucocidin on Sephadex G-100 superfine and Bio Gel P-100. 10 mL of sample was applied to Sephadex G-100 column (2.5 x 100 cm) equilibrated with phosphate buffered saline. The flow rate was 4 mL h^{-1} (2 mL tubes). Effluent was analyzed for protein at (E_{280}nm). No leucocidin fractions obtained.

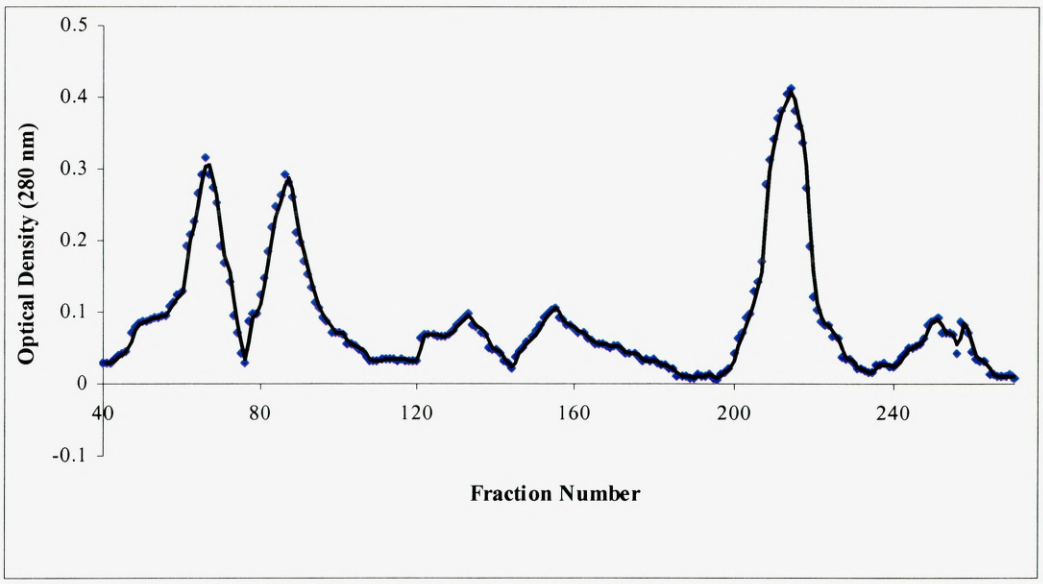


Figure 50: Elution profile of protein fractions of binary biofilms of *Ps. aeruginosa* PAO1 and *E. coli* ATCC 10000 with sub-MIC of ampicillin (32 μg mL^{-1}). *'Tandem'* gel filtration of leucocidin on Sephadex G-100 superfine and Bio Gel P-100. 10 mL of sample was applied to Sephadex G-100 column (2.5 x 100 cm) equilibrated with phosphate buffered saline. The flow rate was 4 mL h^{-1} (2 mL tubes). Effluent was analyzed for protein at (E_{280}nm). Fractions 212 to 216 were combined dialysed.

Twin peaks of leucocidin and protease were visible in all elution profiles, except those of *E. coli* biofilms wherein only a single protease peak appeared. Leucocidin generally appeared between fractions 210 to 220, while protease peaks were found between fractions 250 to 260 in all samples.

Interestingly, protein peaks obtained corresponding to fractions preceding leucocidin (40 to 100), shifted significantly when challenged with antibiotics. The shift in peaks was also observed when monospecies biofilms were converted to binary biofilms. Although it is not intended at present to determine the nature of these proteins, a preliminary analysis using SDS-PAGE was performed. The results are indicated in Figures 51 to 56.

Investigation of proteins in column fractions preceding leucocidin

The fractions visualised in the elution profiles (40 to 100; all samples), preceeding those of leucocidin contained unrecognised proteins. The principle applied during gel filtration of protein mixtures is one of size. The gel beads act as a sieve

through which smaller molecular weight protein molecules are trapped (due to high diffusibility), while the high molecular weight proteins are pulled down by gravitational forces more easily and thus, are collected as fractions earlier in the purification process. Accordingly, the fractions collected before those of leucocidin were expected to have molecular weights greater than that of leucocidin (29 kDa). Initial characterisation of the proteins present in the highly variable region was attempted and the results are shown below (Figures 51 to 56).

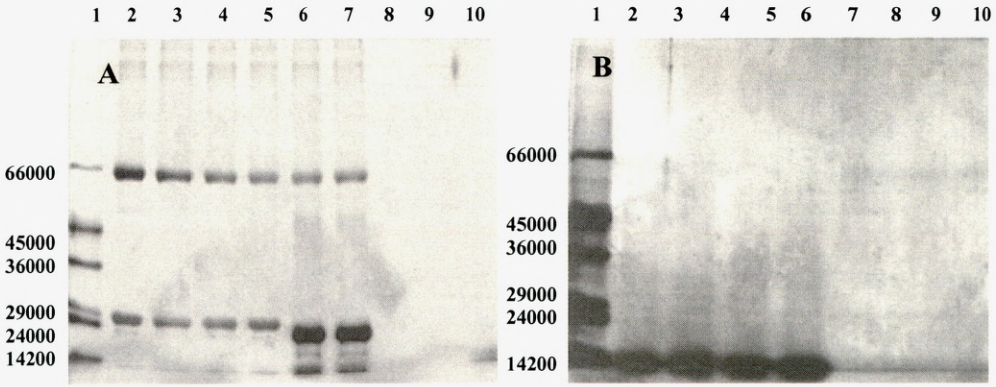


Figure 51: SDS-PAGE profile of unknown proteins of *Ps. aeruginosa* PAO1 biofilm cells (control). A) Fractions 80 to 88 are represented in lanes 2 to 10 (Figure 43). B) Fractions 89 to 95 are represented in lanes 2 to 10. Lane 1 represents standard molecular weight marker (14.2 to 66 kDa).

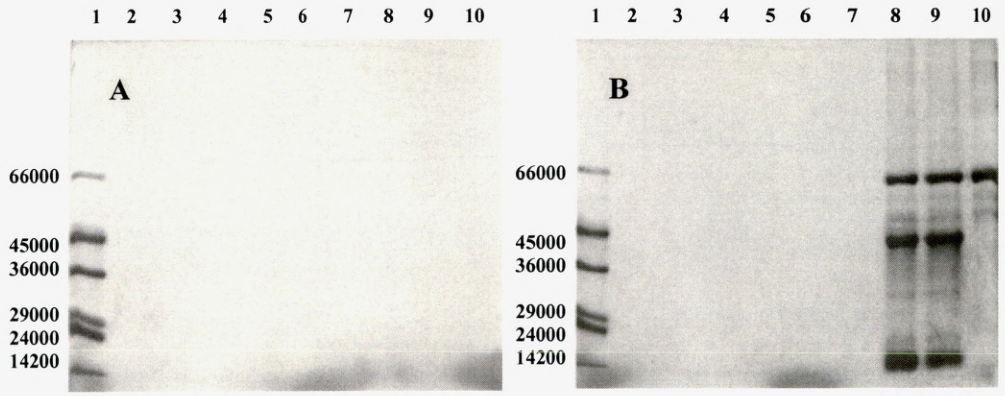


Figure 52: SDS-PAGE profile of unknown proteins of *Ps. aeruginosa* PAO1 biofilm cells in the presence of sub-MIC of ampicillin. A) Fractions 55 to 60 (lanes 1 to 7; Figure 45) and 80 to 82 (lanes 8 to 10; Figure 45). B) Fractions 83 to 91 (lanes 2 to 10; Figure 45) Lane 1 represents standard molecular weight marker (14.2 to 66 kDa).

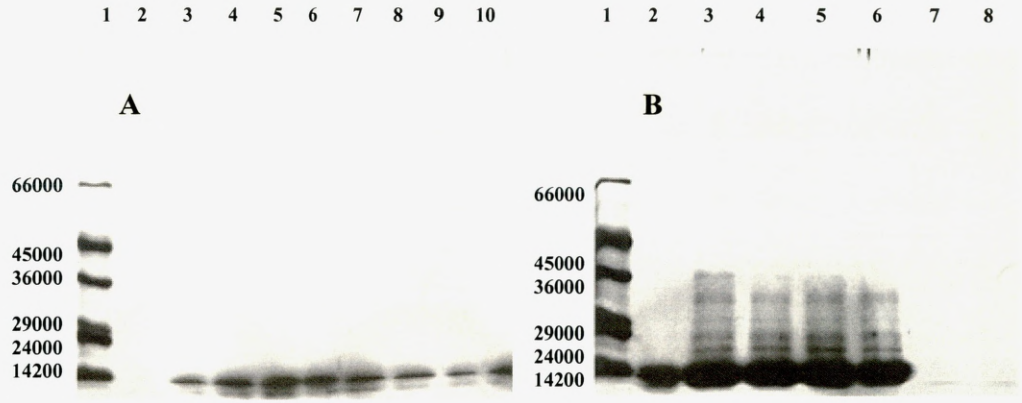


Figure 53: SDS-PAGE profile of unknown proteins of *Ps. aeruginosa* PAO1 biofilm cells in the presence of sub-MIC of nalidixic acid. A) Fractions 45 to 53 (lanes 2 to 10; Figure 47). B) Fractions 54 to 60 (lanes 2 to 8; Figure 47). Lane 1 represents standard molecular weight marker (14.2 to 66 kDa).

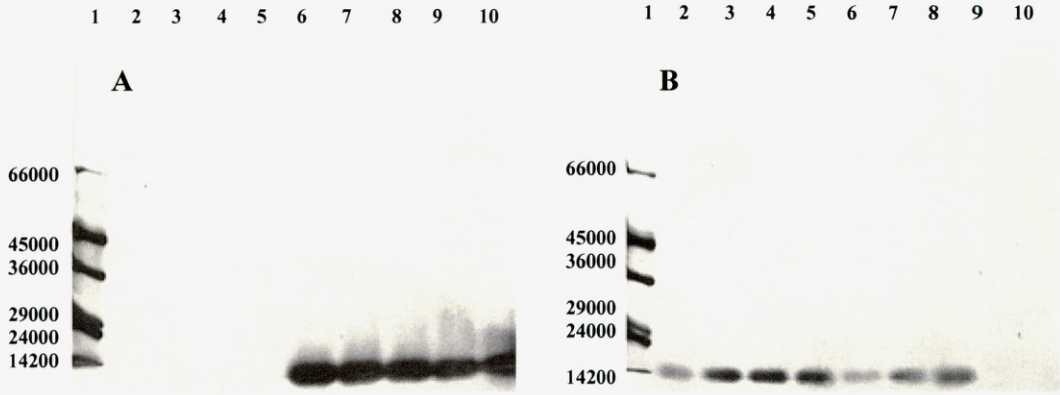


Figure 54: SDS-PAGE profile of unknown proteins of *Ps. aeruginosa* PAO1 biofilm cells in presence of sub-MIC of streptomycin. A) Fractions 64 to 72 (lanes 2 to 10; Figure 48) and B) Fractions 73 to 81 (lanes 2 to 10; Figure 48) Lane 1 represents standard molecular weight marker (14.2 to 66 kDa).

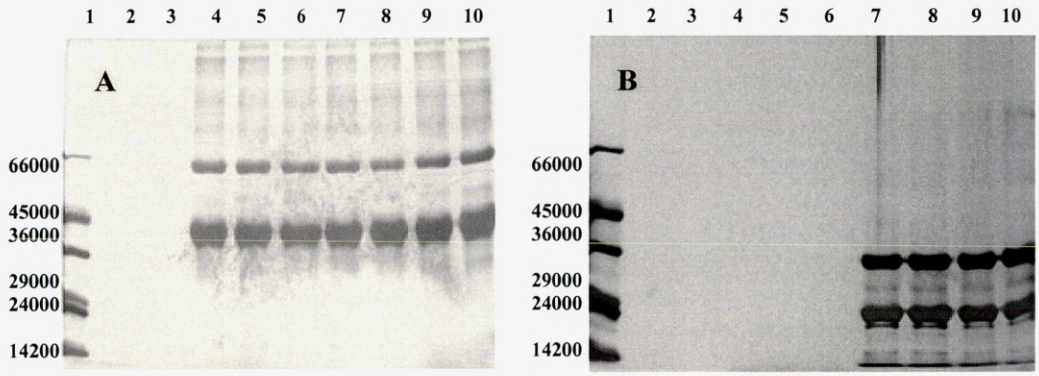


Figure 55: SDS-PAGE profile of unknown proteins of binary biofilms of *Ps. aeruginosa* PAO1 and *E. coli* ATCC 10000 with sub-MIC of ampicillin. A) Fractions 60 to 68 (lanes 2 to 10; Figure 50) and B) Fractions 78 to 86 (lanes 2 to 10; Figure 50). Lane 1 represents standard molecular weight marker (14.2 to 66 kDa).

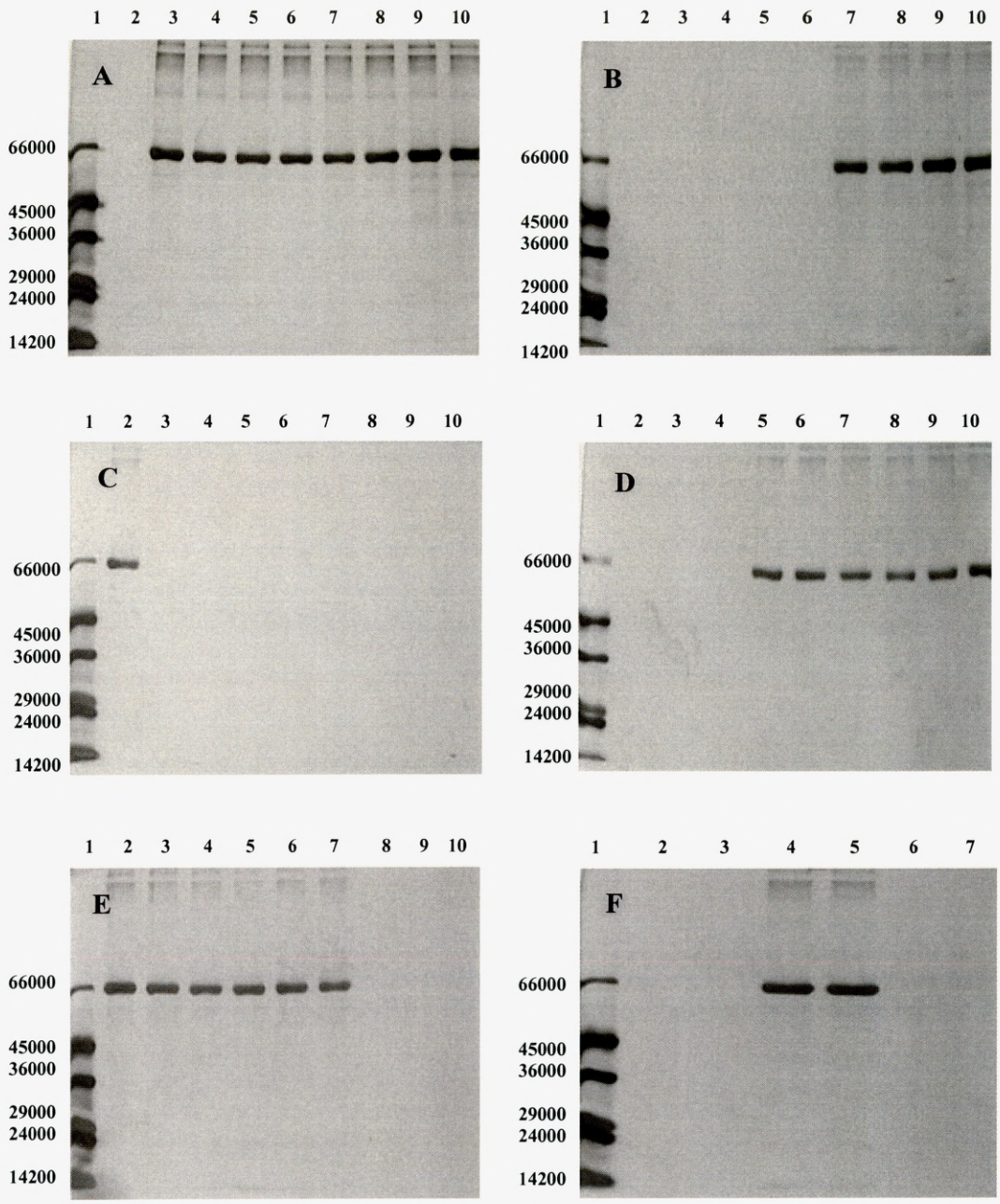


Figure 56: SDS-PAGE profiles of unknown proteins of *E. coli* ATCC 10000 biofilm cells in presence of sub-MIC of ampicillin. A) Fractions 45 to 53 (lane 2 to 10; Figure 49), B) Fractions 54 to 62 (lane 2 to10; Figure 49), C) Fractions 63 to 71 (lane 2 to 10; Figure 49), D) Fractions 72 to 80 (lanes 2 to 10; Figure 49), E) Fractions 81 to 89 (lane 2 to 10; Figure 49), and F) Fractions 90 to 95 (lane 2 to 7; Figure 49). Lane 1 represents standard molecular weight marker (14.2 to 66 kDa).

Surprisingly, some of the fractions (Figure 54A; lanes 6, 7, 8, 9 and 10 and Figure 54B; lanes 2, 3, 4, 5, 6, 7, 8 and 9) indicated proteins having lower molecular weights than that of leucocidin (29 kDa). The molecular weights of the proteins from

the highly variable region (fractions 40 to 100) were analysed using SDS-PAGE and the results are tabulated below (Table 14).

Table 14: Proteins appearing in elution fractions that correspond to the highly variable region (fraction 40 to 100) preceding leucocidin fraction (fractions 209-213). Molecular weights of proteins were calculated manually and expressed as kilodaltons (kDa)

Fractions No.	*Ps.aeruginosa* biofilm cells (control)	*Ps.aeruginosa* biofilm cells with sub-MIC (AmP)	*Ps.aeruginosa* biofilm cells with sub-MIC (NaL)	*Ps.aeruginosa* biofilm cells with sub-MIC (ST)	*E. coli* biofilm cells with sub-MIC (AmP)	Binary biofilm cells with sub-MIC (AmP)
45-50	-	-	12	-	90.2, 88.5, 84, 77.7, 66, 55	-
50-55	-	-	12	-	90.2, 88.5, 84, 77.7, 66, 55, 50	-
55-60	-	9	12, 38, 35.5, 33, 31.5, 28.7, 25.2, 23.2, 20, 12	14.2	88.7, 86.7, 77.7,66, 43, 18.2	-
60-65	-	-	-	14.2	88.7, 86.7,77.7, 66, 54, 51, 43	90.7, 88.5, 76.7, 69.2, 66, 38
65-70	-	-	-	14.2	-	90.7, 88.5, 76.7, 69.2, 66, 38, 53, 32.5
70-75	-	-	-	14.2	-	-
75-80	-	-	-	14.2	90.2, 88.2, 66	-
80-85	65, 27, 12, 48, 20	9	-	-	90.2, 88.2, 66	74.2,63.7 ,56, 46,33.5, 31.5, 24, 21, 15.5, 13.2
85-90	33, 29,28, 14.2	9, 62, 52.7, 48.7, 42, 29.7, 18, 14	-	-	90.2, 88.2, 66	74.2,63.7 ,56, 46,33.5, 31.5, 24, 21, 15.5, 13.2
90-95	33, 29, 28, 14.2	62, 52.7, 44, 14.2	-	-	90.2, 88.7, 79, 64	-

Key:
- : No data in the cell

It is not entirely clear as to why low molecular weight proteins co-fractionated with high molecular weight proteins. However, there may be two possible explanations for this phenomenon

1. Some low molecular weight proteins may be very loosely packed and hence, have an effective large 3-D size. Since gel filtration sieves proteins based on size, these loosely packed lower molecular weight proteins may be less diffusible and slip through along with proteins of high molecular weight.
2. It may also be that a few low molecular weight proteins may have a relatively low diffusion rate or solubility rate, hence they may be able to compete with their higher molecular weight counterparts and fractionate simultaneously.

It is probable that both these factors play a role in the column purification of proteins, resulting in low molecular weight proteins co-fractionating with high molecular weight proteins.

Although it may be observed that a number of proteins with varying sizes are present within this highly variable region, SDS-PAGE analysis proves that the most abundant proteins are of low molecular weights. They may be any protein from the total protein profile of *Pseudomonas aeruginosa* that are capable of precipitating at 20% saturation of ammonium sulphate. In all probability the low molecular weights may indicate that they are siderophores or siderophore-like proteins. It may also be noted that for a subset of the low molecular weight proteins, the fractionating pattern changes when challenged with the antibiotics. For instance, a 14.2 kDa protein present within fractions 85 to 95 (Table 14) of *Ps. aeruginosa* biofilm control cells, fractionates in samples 55 to 75, when these cells are treated with streptomycin at sub-MIC (8 $\mu g\ mL^{-1}$). This indicates that solubility of that particular protein has decreased when the cells are treated with streptomycin or in other words the protein may have mis-folded resulting in an inactive protein. However, further investigation

(e.g., MALDI spectrophotometric analysis to determine protein identity) of such proteins is needed to provide any definitive conclusions.

Determination of leucocidin activity on fibroblast cells by Microculture Tetrazolium Assays (MTT assay)

Microculture tetrazolium assays (MTT) are widely used to investigate the mechanisms of both cell activation and cell damage (Goodwin, *et al*, 1995). They are colourimetric assays, which are based upon the bioreduction of a tetrazolium salt to an intensely coloured formazan. Bioreduction occurs when activated cells cause tetrazolium salt to change into soluble formazan (Goodwin, *et al*, 1995). Ideal colorimetric assays should utilize colourless substrates that may be modified to coloured products by any living cell and not dead cells or tissue culture medium. The activity of bacterial dehydrogenase enzymes, present only within active mitochondria, causes cleavage of the tetrazolium ring by a reduction process, giving rise to a coloured product. As active mitochondria may only be seen in viable cells, MTT assays are highly specific and sensitive assays for measuring live cells (Mosmann, 1983).

Leucocidin produced by *Pseudomonas aeruginosa* exhibits cytotoxic action against eukaryotic cells. Cytotoxicity is characterised by abnormal cell swelling followed by membrane-rupture and cell death. As the bio-reduction of MTT by live eukaryotic cells is directly proportional to their cell concentration and exhibits a linear relationship (Figure 10; Chapter 2), any change in the number of viable cells is immediately reflected upon the reduction of MTT and its coloured end-product. Hence, a decrease in the concentration of viable eukaryotic cells on addition of active pseudomonal-leucocidin may be accurately quantified as a function of the live cells remaining after treatment. The number of cells killed by the action of leucocidin is

directly proportional to its concentration. Activity of leucocidin was determined as a measure of its efficiency and ability to kill eukaryotic tissue-culture cells.

Experimental approach

10 mL of sterile medium (Dulbecco Minimum Essential Medium Eagle-Dulbecco MEM; Sigma M2279; also described in Chapter 2), containing 10% FCS (Foetal Calf Serum) 11 mL; Glutamine (4mM) 2 mL; 1 mL antibiotic (penicillin / streptomycin; Sigma P0906; described in Chapter 2) in a 25 cm^2 cell culture flask, was inoculated with 300 µL human foreskin fibroblast cells (1.4 x 10^5 cells mL^{-1}). The cell culture flask was incubated in an incubator (Heraeus Instruments, Germany B5060, EK-CO_2) for 72 h at 37°C with 5% CO_2 until confluent growth of fibroblast cells was observed under microscope (Wilovert (Germany) Inverted microscope). The activity of leucocidin (3.2 to 62.4 µg mL^{-1}) on tissue-cultured fibroblast cells was analysed using MTT assay (described in Chapter 2)

Colourimetric MTT (tetrazolium) assay (Mosmann, 1983; Goodwin,*et al*, 1995).

1 mL of the MTT assay supernatant was removed (from 24 well plates) and the optical density was measured at 540 nm using a spectrophotometer (Novaspec I LKB) against a media blank (Dulbecco MEM). This was performed at two time points (30 minutes and 24 hours). If the absorbency was greater than 0.5, a dilution of the assay supernatant was made (1:10; Lawrence and Maier, 1977). In order to calculate the actual optical density for the sample, E_{540} was multiplied by a factor 10. Cell numbers corresponding to observed optical densities were obtained from a standard calibration curve constructed for the purpose (presented in Chapter 2).

Results & Discussion

Ps. aeruginosa leucocidin, sometimes referred to as cytotoxin (CTX), is a 29-kDa protein endowed with cytolytic activity towards leucocytes and other types of cells (Ohnishi *et al.*, 1994). It is initially synthesized as a 31 kDa precursor protein (pro-CTX) and converted into active toxin only on the proteolytic cleavage of 20 amino acid region from its C-terminus (Hayashi *et al.*, 1989; Ohnishi *et al.*, 1994). The present study isolated and purified the 29 kDa active cytotoxin from monospecies and binary biofilms of *Ps. aeruginosa* PAO1 and probed its virulence potential when challenged with sub-MIC of ampicillin (8 $\mu g\ mL^{-1}$).

The activity of leucocidin was determined by MTT assay. As the assay was specific for viable cells, cells killed as a result of cytotoxic activity of leucocidin could be accurately estimated. Figure 57 illustrates the measurement of leucocidin activity by MTT assay after 30 minutes and 24 hours at an optical density of 540nm. It may be noted that leucocidin activity could be observed within 30 minutes of addition into fibroblast cultures. Analysis of the data (Figure 57) illustrating the number of viable cells after the addition of purified active leucocidin shows a distinct pattern of events also reflected when analysing virulence potential of monospecies and binary Pseudomonal-proteases challenged with sub-MIC of ampicillin.

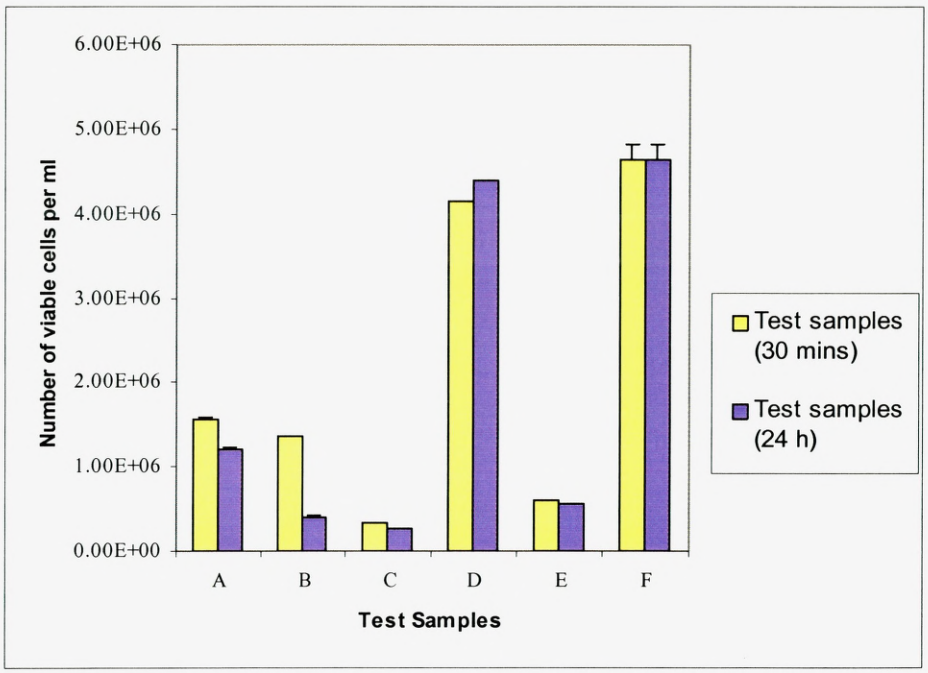


Figure 57: Measurement of leucocidin activity by MTT assay at 30 minutes and 24 hours at an optical density of 540nm; Y axis units 1.00E+06 to 6.00E+6 corresponds to 1.0 x 10^6 to 6.0 x 10^6 viable cells per mL, respectively. (A) leucocidin of *Ps. aeruginosa* PAO1 planktonic cells; (B) leucocidin of *Ps. aeruginosa* PAO1 biofilm cells; (C) leucocidin of *Ps. aeruginosa* PAO1 biofilm cells with sub-MIC of antibiotic (ampicillin 8 μg mL^{-1}); (D) leucocidin of *E. coli* biofilm cells with sub-MIC of (ampicillin 2 μg mL^{-1}); (E) leucocidin of binary biofilm cells with sub-MIC of (ampicillin 32 μg mL^{-1}); (F) is control untreated cells only MTT was added on the fibroblast cells (4.4 x 10^6 mL^{-1}). Error bars are calculated as the standard deviation of the individual data points. n = 3 replicates.

The most active leucocidin was purified from *Pseudomonas aeruginosa* biofilm cells treated with sub-MIC of ampicillin (8 μg mL^{-1}). Parallel *E. coli* biofilm fractions, treated with sub-MIC of ampicillin (2 μg mL^{-1}) showed minimal cytotoxicity. This indicates there is no active cytotoxin within *E. coli* fractions similar to those of *Ps. aeruginosa* leucocidin. This could also be confirmed from elution profiles of *E. coli* (challenged with ampicillin) lacking any peaks within the target fractions (fractions 200 to 219, Figure 49).

It is also noted that planktonic and biofilm cells of *Pseudomonas* devoid of treatment of ampicillin showed significant cytotoxic activity. While it may be usual for planktonic cells to exhibit cytotoxic activity, it is surprising to find an increase in leucocidin activity in biofilm cells due to the relatively dormant nature of bacterial cells within biofilms and also because of the chronic nature of biofilm infection.

Finally, activity of leucocidin isolated from binary biofilms challenged with ampicillin, was second only to corresponding monospecies biofilm of *Pseudomonas aeruginosa*. This indicates that even in clinical settings (where the probability of finding multispecies or binary biofilm infections is high) treatment with antibiotics may actually worsen prognosis rather than improve it.

It is also noted that the above analysis of the activities of leucocidin from different samples (control and antibiotic treated) were carried out at constant volumes, but not constant leucocidin protein concentrations. Thus, a high activity in one sample relative to a lower activity in another may be due to higher leucocidin concentrations in the former and may not constitute a significant increase in virulence potential due to leucocidin. In order to normalise the data with respect to leucocidin protein concentration, quantitative estimates of leucocidin present within target fractions were determined spectroscopically and the virulence potential reanalysed. Although slight variations in protein concentrations were observed within constant fraction volumes, the pattern of activity of the different samples described above remained the same. The results are shown in Figure 58 as percent cells killed due to leucocidin activity. As noted previously, increased cytotoxicity of fibroblast was observed for monospecies and binary biofilms challenged with ampicillin. These results show that presence of sub MICs of antibiotics cause an increase in virulence potential of *Pseudomonas aeruginosa* biofilms.

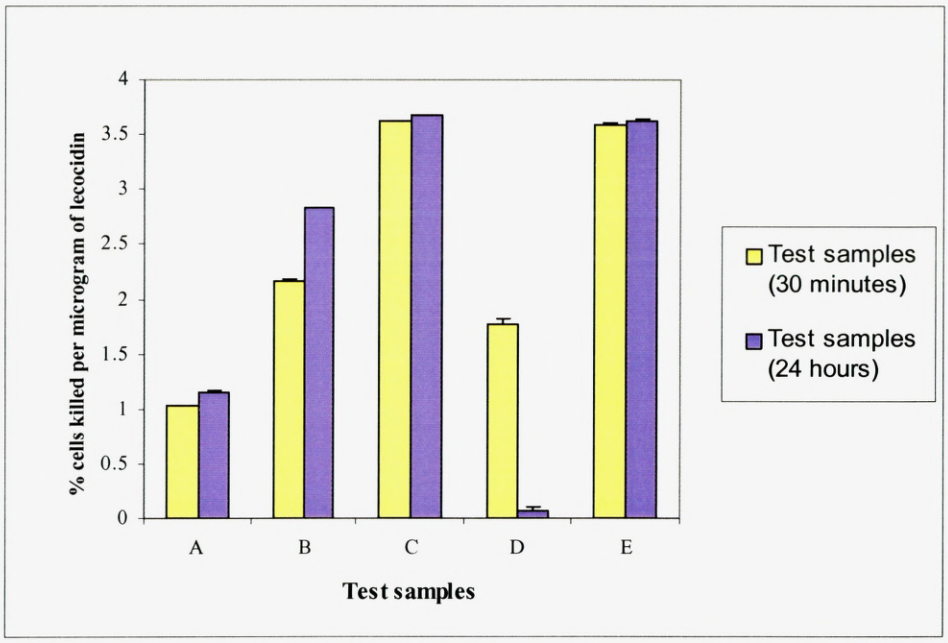


Figure 58: Percent cells killed after treatment of leucocidin from different samples at 30 minutes and 24 hours; (A) leucocidin of *Ps. aeruginosa* PAO1 planktonic cells; (B) leucocidin of *Ps. aeruginosa* PAO1 biofilm cells; (C) leucocidin of *Ps. aeruginosa* PAO1 biofilm cells with sub-MIC of antibiotic (ampicillin 8 μg mL^{-1}); (D) leucocidin of *E. coli* biofilm cells with sub-MIC of (ampicillin 2 μg mL^{-1}); (E) leucocidin of binary biofilm cells with sub-MIC of (ampicillin 32 μg mL^{-1}). Error bars are calculated as the standard deviation of the individual data points. n = 3 replicates.

Phase contrast microscopic analysis of the cytotoxic action of leucocidin of *Pseudomonas aeruginosa* PAO1 on Spleen-myeloma cell line (Sp2) cells (Sp2/0-Ag14 EC ACC NO: 85072401)

Pseudomonal-leucocidins are cytotoxic to different human cells. Scharmann and colleagues (1976) have shown these proteins to be active against human leucocytes of various cell types (hence the name, leucocidins). Cells killed due to the action of leucocidin, showed signs characteristic to accidental death or oncosis (death characterised by swelling of the target cell). Subsequently, they were found to be active against different host cells. In an earlier experiment, cytotoxic activity of leucocidin against human foreskin fibroblast cells was demonstrated using MTT assay. In order to discount the possibility of natural death of cells (apoptosis) in presence of leucocidin, phase contrast microscopy was performed on tissue cultured

Spleen myeloma cells. Phase contrast microscopy would help determine the morphological characteristics of the population of cells targeted by leucocidin, and thus help determine the cause of death, either by apoptosis or oncosis. Furthermore, the degree of oncosis (swelling) may also be a reliable measure for quantifying the activity of the toxin (leucocidin).

Experimental approach

10 mL of a sterile medium (RPMI-1640; Sigma R0883; described in Chapter 2), containing (10% FCS (Foetal Calf Serum) 11 mL; Glutamine (4mM) 2 mL; 1 mL antibiotic (penicillin / streptomycin; Sigma P0906); 1mM sodium pyruvate in a 25 cm^2 cell culture flask, was inoculated with Spleen-myeloma cell line Sp2 / 0-Ag14 (ECACC NO: 85072401 cells (1.4 x 10^5 cells mL^{-1}; 300 µL). The cell culture flask was incubated in an incubator (Heraeus instrument, Germany B5060 EK-CO_2) for 72 h at 37°C with 5% CO_2 until mat growth of (Sp2) cells was observed under microscope (Wilovert (Germany) Inverted microscope). Morphological alterations of the Sp2 cells after leucocidin treatment was observed by using phase contrast microscopy (LEICA DMR) at 400x on a glass slide with a cover glass.

Preparation of Sp2 cells on glass slide

An aliquot (10 µL) of Sp2 cells was taken and mixed with 5 µL of leucocidin solution or with 5 µL of PBS containing 0.2M NaCl (pH 7.2) as control on a glass slide. This was performed at two time points (30 minutes and 24 hours). A cover glass was added and the observations on the phase contrast microscope (LEICA DMR) at 400x were made at room temperature.

Results & Discussion

Eukaryotic cell death may be classified into two distinct types based on morphological and biochemical changes. Suicidal cell death or programmed cell death (also called apoptosis) is characterised morphologically by *'half moon'* like-(condensed chromatin) structures within shrunken nuclei. Apoptosis is also often characterised by degradation of chromatin leading to the release of 200bp DNA oligomers. The second type of cell death characterised by cellular and nuclear swelling, vacuolization and cell membrane disintegration is described as *'accidental'* cell death or oncosis (Dacheux *et al.*, 2000).

Microscopic analysis of morphology of SP2 cells after leucocidin treatment (purified from different samples) reveals cell death by oncosis (Figure 59). These results also indicate that death of fibroblast cells observed in previous MTT assays after treatment with leucocidin fractions were not due to natural causes (apoptosis), but were accidental or induced by the presence of the leucocidin.

Although all fractions containing leucocidin were able to cause oncosis, the degree varied. Leucocidins from antibiotic-exposed cells were more active and were able to cause much more swelling of Sp2 cells than their less active non-antibiotic-exposed controls counterparts. The degree of swelling matched the virulence potential of leucocidin. Accordingly Figure 59 shows maximum degree of oncosis (death of cell characterised by swelling) to *Pseudomonas aeruginosa* biofilm cells exposed to ampicillin (sample C, Figure 60). Leucocidin purified from binary biofilms of *Pseudomonas* showed at least 10% decrease in activity when compared to their monospecies counterparts, yet remained significantly higher than control samples (A, planktonic *Pseudomonas* cells; B, biofilm *Pseudomonas* cells) that were

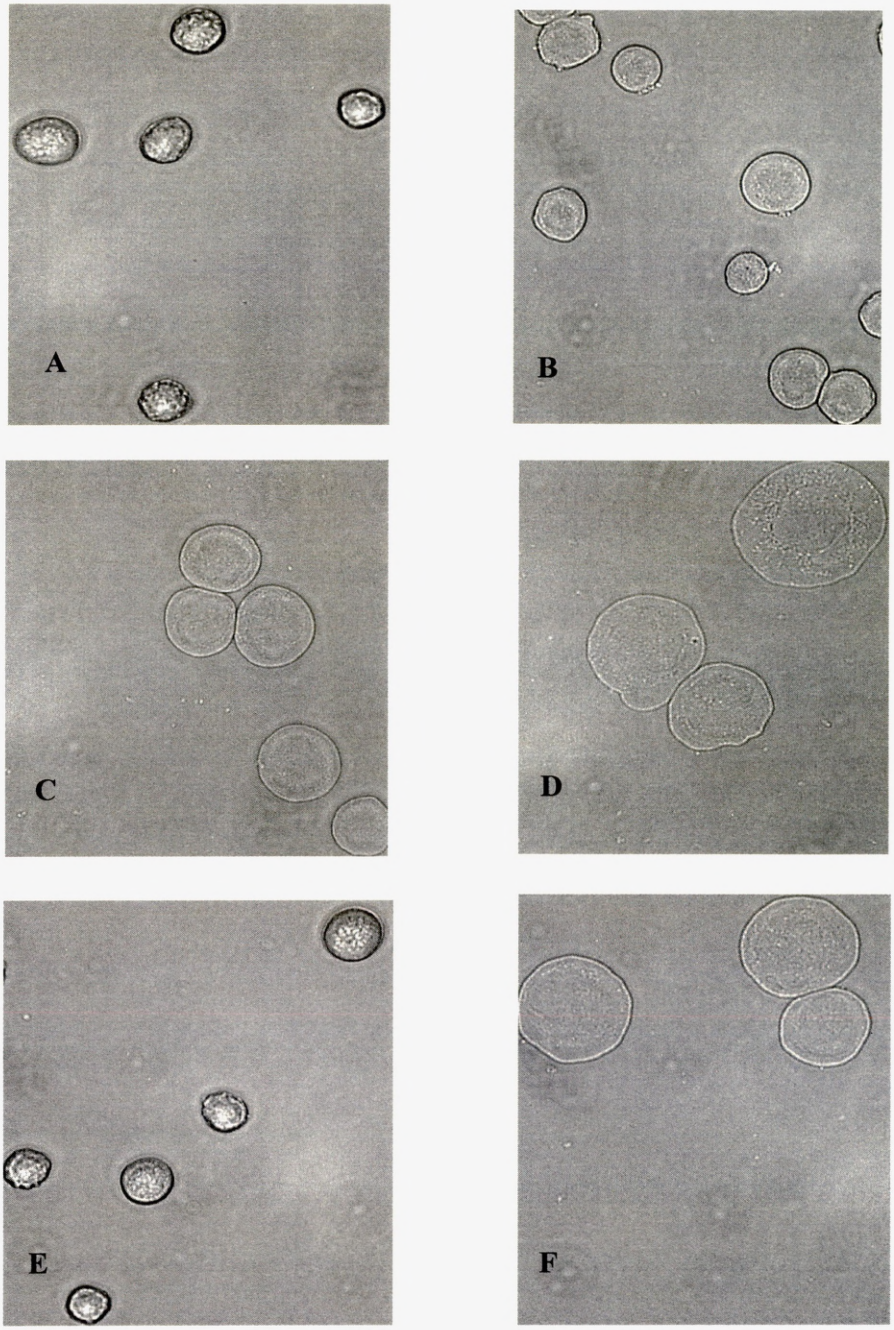


Figure 59: Activity of leucocidin against Sp2 cells after 30 minute exposure (Magnification: 400 x). (A) Sp2 cells treated with 0.2M NaCl, (pH 7.2; PBS; control). (B) Sp2 cells after exposure to leucocidin produced by planktonic *Ps. aeruginosa* PAO1 cells, (C) Sp2 cells treated with leucocidin produced by *Ps. aeruginosa* PAO1 biofilm cells; (D) Sp2 cells exposed to leucocidin produced by *Ps. aeruginosa* PAO1 biofilm cells treated with sub-MIC of antibiotic (ampicillin 8 μg mL^{-1}); (E) Sp2 cells treated with parallel fractions to leucocidin obtained from *E. coli* ATCC 10000 biofilm cells treated with sub-MIC of antibiotic (ampicillin 2 μg mL^{-1}); (F) Sp2 cells after being exposed to leucocidin produced by binary biofilm cells of *Ps. aeruginosa* PAO1 and *E. coli* ATCC 10000 in the presence of sub-MIC of antibiotic (ampicillin 32 μg mL^{-1}).

not exposed to ampicillin. Although parallel fractions collected from *E. coli* biofilms exposed to ampicillin, did not show any significant cytotoxic activity, this does not

discount the possibility of *E. coli* producing some molecules that may quench the activity of Pseudomonal-leucocidin, thus explaining the decrease of leucocidin activity of binary biofilms. Alternatively, this observed decrease in leucocidin activity may be the result of a simple dilution of leucocidin production within the binary biofilm structure. The morphological results obtained (Figure 59 and 60) yielded leucocidin activities that were in agreement with previous results from MTT assay

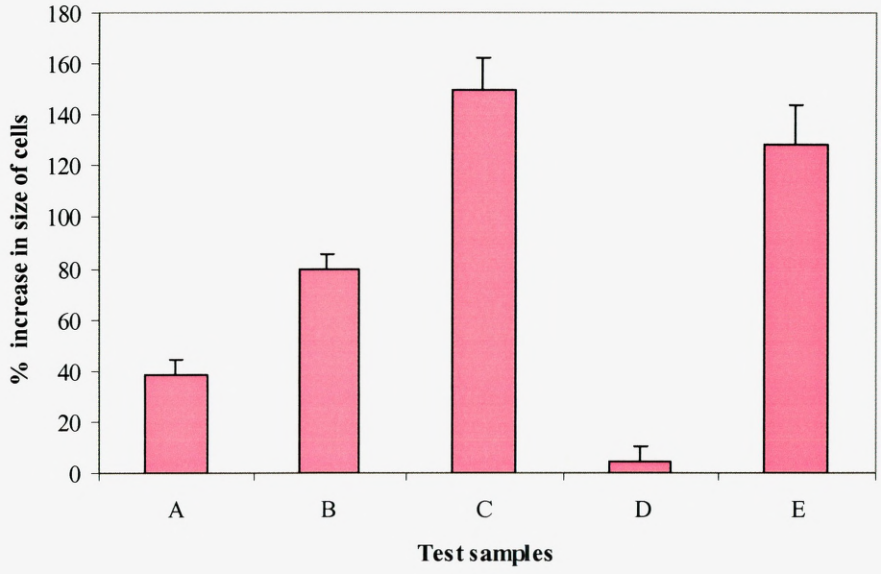


Figure 60: Percent increase in diameter of Sp2 cells after treatment with leucocidin for 30 minutes; (A) percent increase in size of Sp2 cells after treated with leucocidin of *Ps. aeruginosa* ATCC 15692 (PAO1) planktonic cells control; (B) percent increase in size of Sp2 cells treated with leucocidin of *Ps. aeruginosa* ATCC 15692 (PAO1) biofilm cells control; (C) percent increase in size of Sp2 cells treated with leucocidin of *Ps. aeruginosa* ATCC 15692 (PAO1) biofilm cells with sub-MIC of antibiotic (ampicillin 8 μg mL^{-1}) ; (D) percent increase in size of Sp2 cells treated with leucocidin of *E. coli* ATCC 10000 biofilm cells with sub-MIC of antibiotic (ampicillin 2 μg mL^{-1}) ; (E) percent increase in size of Sp2 cells treated with leucocidin of binary biofilm cells of *Ps. aeruginosa* ATCC 15692 (PAO1) and *E. coli* ATCC 10000 with sub-MIC of antibiotic (ampicillin 32 μg mL^{-1}). Error bars are calculated as the standard deviation of the individual data points. n = 3 replicates.

In summary, it may be noted that activities of leucocidin (virulence factor) produced by *Pseudomonas aeruginosa* PAO1 biofilm cells, increase in the presence of sub-MICs of antibiotics. This has been elegantly demonstrated using MTT assay and phase contrast microscopy. The results of these experiments are tabulated below

(Table 15). The table shows percent reduction (killing) of cells per mL per microgram of leucocidin and percent increase in the mean diameter of Sp2 cells. From these results, it may be concluded that the increase in virulence of monospecies and binary biofilms of *Pseudomonas aeruginosa* PAO1 in the presence of sub-MIC antibiotics would increase the obvious effects of pathogenicity.

Table 15: Measures of the cytotoxic effect of leucocidin on human fibroblast cells and Sp2 cells

Sample	Time of exposure (hours)	% Reduction in cells (killing)	% Cells remaining	Total concentr-ation of leucocidin ($\mu g\ mL^{-1}$)	% Cells killed per μg of protein per mL	% increase in diameter of SP2 cells
A	0.5	64.5	35.5	62.4	1.03	55
	24	72.7	27.3		1.16	
B	0.5	69.3	30.7	32	2.16	81
	24	90.6	9.4		2.83	
C	0.5	92.5	7.5	25.6	3.61	150
	24	93.9	6.1		3.66	
D	0.5	5.7	94.3	3.2	1.78	3.44
	24	0.15	99.85		0.048	
E	0.5	86.3	13.7	24	3.59	132
	24	87.2	12.8		3.63	

KEY:

A- purified leucocidin of *Ps. aeruginosa* planktonic cells (control)

B- purified leucocidin of *Ps. aeruginosa* biofilm cells (control)

C- purified leucocidin of *Ps. aeruginosa* biofilm cells with sub-MIC of ampicillin (8 $\mu g\ mL^{-1}$)

D- purified leucocidin of *E.coli* 10000 biofilm cells with sub-MIC of ampicillin (2 $\mu g\ mL^{-1}$)

E- purified leucocidin of binary biofilm cells of *Ps. aeruginosa* and *E. coli* with sub-MIC of ampicillin (32 $\mu g\ mL^{-1}$)

F- Untreated cells (Fibroblast cells with MTT). Final concentration of cells was 4.4×10^6 cells per mL

Isolation, identification and assay of protease activity from monospecies and binary biofilms of *Ps. aeruginosa* ATCC 15692 (PAO1) and *E. coli* ATCC 10000

Investigations of *Ps. aeruginosa* biofilms indicate an increase in virulence potential of leucocidin (virulence determinant) in the presence of sub-MICs of antibiotics. It was decided to investigate whether this observation was true for other virulence factors (proteases) produced by *Ps. aeruginosa* biofilms.

Proteases are assumed to play a major role during acute infections. Pulmonary haemorrhages, which occur during invasive infection of *Pseudomonas aeruginosa*, have been traditionally attributed to hyperactivity of its proteases (Van Delden and Iglewski, 1998). The human lung tissue is composed of elastin that helps it expand and contract. Elastin is also responsible for the resilience of blood vessels. The synergistic elastolytic activity of two *Pseudomonas* proteases, Las A and Las B, degrades elastin and is responsible for the gradual destruction of lung epithelia in cystic fibrosis patients (Galloway, 1991). Las B elastase also degrades fibrin and collagen (Heck *et al.*, 1986). The involvement of alkaline protease produced by *Pseudomonas* in corneal infections is said to be substantial (Van Delden and Iglewski, 1998).

Experimental approach

Previously it has been observed that proteases interfere with the purification of leucocidins as they co-precipitate in 20% ammonium sulphate and have similar molecular weights (Scharmann, 1976b). Accordingly, fractions obtained near to those of leucocidin were probed for proteases using zymography. Zymography is an electrophoretic method for measuring proteolytic activity. It is based on sodium dodecyl sulphate gel impregnated with a protein substrate (usually casein), which is degraded by the proteases during the incubation period (Leber & Balkwill, 1997). The

protease activity on the gel is visualised as a zone of clearance. Background Coomassie blue staining reveals sites of proteolysis as white bands on a dark blue background. The band intensity can be linearly related to the amount of protease loaded (Thangam & Rajkumar, 2002).

Once samples containing proteases were identified, their activities were determined by azocasein assay. Azocasein is a chemically modified protein composed of casein (a milk protein) to which an orange sulphanilamide group has been attached. Proteolysis releases the sulphanilamide group resulting in an orange-red colour (Chantawannakul, *et al.* 2002; Tomarelli, *et al.* 1949). The intensity of the colour is directly proportional to the activity of proteases (Chantawannakul, *et al.* 2002).

Zymography assay (Leber & Balkwill, 1997)

The protease profiles were visualised on non-denaturing 10% separating gels containing 0.5 mg mL^{-1} casein and 4% stacking gel (described in Chapter 2). Sample aliquots (10 to 20 µL) were loaded to the wells and the gel was run at 35 mA for approximately 1.5 hours or until the leading samples had reached the end of the gel. Gels were soaked for 1 h in 2.5% Triton X-100 on a shaker followed by two brief washes in collagenase buffer (50mM Tris-HCl (pH 7.6), 0.2M NaCl, 5mM $CaCl_2$, 0.25 (v/v) Triton X-100 (King *et al*, 1996) and finally incubated for 18 h at 37°C within collagenase buffer. Prior to staining, gels were briefly rinsed in distilled water. The gels were fixed and stained in Coomassie brilliant blue R-250 for 3 h and then destained in destaining solution as (described in Chapter 2) until clear regions of substrate degradation were visualized.

Azocasein assay (Chantawannakul, *et al.* 2002)

An aliquot (20 µL) of the proteases were incubated at 37°C in a mixture (400 µL) containing 2% azocasein (230 µL), and 0.2 M N-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES) buffer pH 7.0 (150 µL) for 2 h. In order to terminate the reaction, 1.2 mL of 10% trichloroacetic acid (TCA) was added. All samples were allowed to stand for 15 min and the supernatant (1.4 mL) was collected after centrifugation (10,000xg; 5 min). An equivalent volume of 1 M NaOH was then added and the solution was mixed thoroughly prior to measuring the absorbance at 440nm (Brock *et al*, 1982).

Protease activity was measured in terms of enzyme units. One unit protease activity corresponds to a change in absorbance (440nm) of 0.001 per minute. The amount of azocasein capable of being hydrolysed per minute per unit activity of protease was previously determined to be 1.3 µg (Wolz and Bond, 1995).

Results & Discussion

Elution profiles of all samples indicated a protein peak immediately after leucocidin. Fractions corresponding to the observed peak (240 to 259) were analysed using zymography for the presence of proteases. Figure 61A and 61B show zymograms produced from protease fractions of planktonic *Ps. aeruginosa* PAO1 cells. Although fractions 240 to 249 did not contain any protease (as analysed from zymograms, Figure 61A), zymograms containing fractions 250 to 259 (Figure 61B) showed very good clearance bands indicative of strong proteases. As similar peaks were visualised in the elution profiles of all samples corresponding to fractions 240 to 260, it was assumed that the peaks corresponded to proteases.

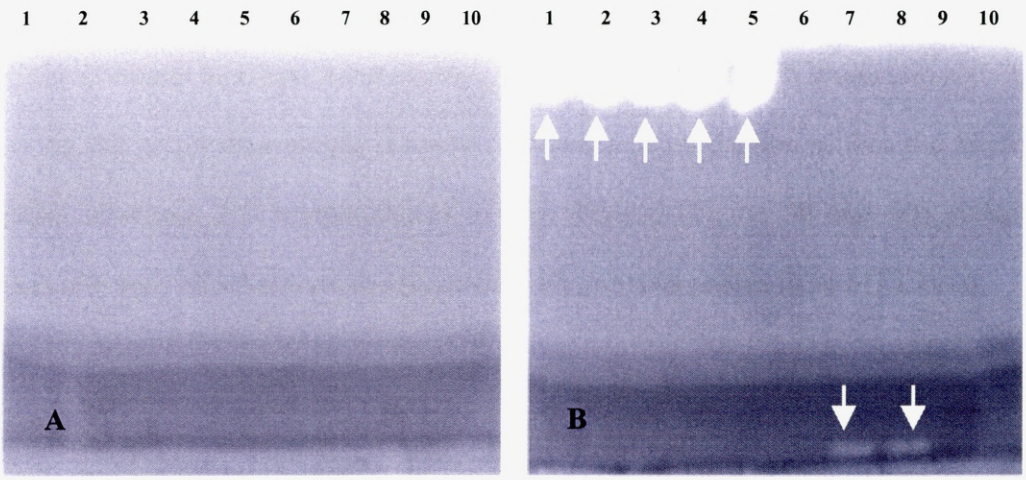


Figure 61: Zymogram analysis of protease fractions of *Ps. aeruginosa* PAO1 planktonic cells. (A) Lanes 1 to 10 contain fractions 240 to 249 (Figure 41). (B) Lane 1 to 10 contains fractions 250 to 259 (Figure 41). Arrows indicate clearance bands corresponding to proteases.

Fraction samples containing proteases were used for azocasein activity assays. The rationale behind conducting protease activity assays was to check for activity fluctuations similar to those observed for leucocidin. The results of the assay are tabulated below (Table 16).

Table 16: Protease activities of monospecies and binary biofilms of *Ps. aeruginosa* ATCC 15692 (PAO1) and *E. coli* ATCC 10000, were tested with azocasein.

Test samples protease	Absorbency (440nm)	Protease activity (EU)	Units μg^{-1} of protease	Azocasein $\mu g\ mL^{-1}\ min^{-1}$
A	0.682	14.22	0.070	0.091
B	0.775	16.14	0.060	0.078
C	1.424	29.66	0.130	0.169
D	0.355	7.40	0.045	0.058
E	0.913	19.02	0.130	0.169

KEY:

A- Proteases of *Ps. aeruginosa* planktonic cells (control)

B- Proteases of *Ps. aeruginosa* biofilm cells

C- Proteases of *Ps. aeruginosa* biofilm cells in presence of sub-MIC of ampicillin (8 $\mu g\ mL^{-1}$)

D- Proteases of *E. coli* 10000 biofilm cells in presence of sub-MIC of ampicillin (2 $\mu g\ mL^{-1}$)

E- Proteases of binary biofilm cells of *Ps. aeruginosa* and *E. coli* in presence of sub-MIC of ampicillin (32 $\mu g\ mL^{-1}$)

It may be noted that activity of proteases were analysed from all samples (both control and antibiotic- (ampicillin) challenged samples of *Ps. aeruginosa* and *E. coli* biofilms). The most active proteases were found from monospecies and binary biofilms of *Pseudomonas aeruginosa* treated with sub-MICs of ampicillin. *E. coli* biofilms treated with sub-MICs of ampicillin (2 $\mu g\ mL^{-1}$) showed the least activity. Interestingly, no synergistic protease activity effects were observed in binary biofilms treated with ampicillin. Planktonic cells of *Pseudomonas aeruginosa* produced proteases that were more active than biofilm cells. This may be expected as cells within biofilms are said to be in a dormant stage and hence, may not produce active proteases. However, from the results, we may conclude that the activity of proteases significantly increases in the presence of sub-MICs of ampicillin.

Conclusion

The concept that virulence factors are microbial characteristics that determine the capacity for causing disease (pathogenesis) has led to the investigation and identification of microbial traits that effect and mediate virulence (Casadevall & Pirofski, 2001). Accordingly, bacterial LPS pattern changes may be classified as *'mediators of virulence'* (changes in the LPS present within the outer bacterial envelope may indirectly result in evasion of bacteria from host immune system), and proteases and cytotoxins (leucocidins) may be denoted *'effectors of virulence'* (proteases and cytotoxins are capable of directly destroying or damaging host cell targets).

The results presented in this chapter indicate significant rearrangement of LPS structure of biofilm cells of *Pseudomonas aeruginosa* in the presence of antibiotics (ampicillin). This was accompanied by an increase in antibiotic resistance (Table 6; Chapter 3). Such increases in resistance will enable the bacteria to survive in an antibiotic containing environment and may be an indicator of increase in overall virulence potential of the organism. Similar increases in virulence were observed for leucocidin and proteases produced by the biofilm cells. While the activity of leucocidin was observed to increase by at least 50%, protease activity increased 100%, in the presence of sub-MIC antibiotics (ampicillin). The increase in virulence activity of biofilm phenotypes reported herein has not been previously predicted or even expected, as biofilm cells are said to have a chronic infection phenotype, indicating long-term infection characterised by low levels of pathogenicity (Fux *et al.*, 2005).

Moderately virulent microorganisms producing clinical symptoms are more frequently treated with antibiotics than non-virulent ones (Martinez and Baquero, 2002). Selective antibiotic pressure against these intermediately virulent forms may

result in either decrease in pathogenicity (sub-virulent forms) over several bacterial cell divisions, or increase in pathogenicity (hyper-virulent forms), in the first instance because they are not always detected and thus not always treated, and in the second, because the host dies without giving them enough time to be in contact with inhibitory concentrations of antibiotics (Martinez and Baquero, 2002). The results obtained in the present work suggest the latter possibility, wherein biofilms present in sub-MIC antibiotic environments increase their virulence, possibly to escape the selective pressure and inhibitory effects of antibiotics.

Chapter Six: General Discussion

Individuals whose natural defenses are severely impaired are at a risk of contracting opportunistic infections (Marie *et al.*, 2005). While it was the normal microflora associated with the human body that was responsible for opportunistic infections in the preantibiotic era, more recently, environmental pathogens have been observed to play a major role in causing infections (Martinez and Baquero, 2002). The reason for such a transition has been hypothesised to be due to the excessive use of antibiotics in clinical settings (Dancer, 2004).

Antibiotics eliminate susceptible human microflora, prevent infections and thus, act as a 'second line of defense' when the body's natural immune response is low. However, environmental microorganisms with an inherently high level of resistance are capable of efficiently tackling antibiotics (the second line of defense) and may convert into human pathogens, especially in the case of an immunocompromised patient (Quinn, 1998). Perhaps it is this ability of environmental pathogens (having no pathogenic determinants to tackle human immune responses, save antibiotic tolerance) that allows them to infect the immunocompromised host (Martinez and Baquero, 2002). For instance, the opportunistic human pathogen *Pseudomonas aeruginosa* is ubiquitous in nature and is not able to cause infection in a human host unless the immune system is impaired. It is this inherent resistance towards antibiotics offered by *Ps. aeruginosa* (by virtue of its large genome and repertoire of antibiotic resistant genes accumulated in evolutionary time) that enables the bacterium to persist in the immounocompromised host during antibiotic treatment (Martinez and Baquero, 2002).

In recent years, the replacement of traditional opportunistic pathogens with more resistant ones has been observed. Susceptibility rates have steadily been shown to decrease over time, and this trend has been attributed to selective antibiotic

pressures resulting in bacterial adaptation within the human body (Van Eldere, 2003; Henwood *et al.*, 2001). Indeed from a bacterial standpoint, the acquisition of resistance to antibiotics must be a fairly simple process when considering the tremendous antibiotic stress bacterial pathogens undergo (bacteria associated with humans undertaking antibiotic chemotherapy have encountered at least 10^{11} molecules of antibiotic), the incredible biochemical versatility they possess (they are capable of living in a wide range of pH and temperatures) and the kind of life style they lead, wherein they have frequent exchanges of genetic material including antibiotic resistance genes (Hamilton-Miller, 2004).

Is the process of acquired resistance to antibiotics reversible?

While it is true that acquired resistance to antibiotics results from their excessive use, the question remains as to the possibility of these resistant strains reverting to their susceptible forms. Several studies have indicated a biological fitness cost endured by the bacterium that accepts resistance plasmids or mutations resulting in antibiotic resistance (Bjorkman & Andersson, 2000; Andersson & Levin, 1999). Thus, theoretically if the use of the antibiotic is withdrawn, the susceptible forms of the bacteria should outgrow the resistant forms and the process may be reversible (Andersson, 2003). However, this is not the case in reality as mutations in the resistant strain compensate their lack of biological fitness, making the acquisition of resistance in bacteria an irreversible process (Kugelberg *et al.*, 2005). The practical implications of antibiotic resistance, as noted above, form the basis of the present investigation conducted and the interpretations presented within this section.

The primary aim of this thesis is to understand the effect of sub-minimal inhibitory concentrations (sub-MICs) of selected antibiotics (ampicillin, nalidixic acid and streptomycin) on *Pseudomonas aeruginosa* biofilms (both monospecies and

binary biofilms). The effects were analysed with respect to protein profile pattern changes and activity differences in outer membrane proteins and virulence factors; lipopolysaccharide (LPS), cytotoxins (leucocidin) and proteases respectively.

Clinical importance of sub-minimal inhibitory concentrations (sub-MICs) of antibiotics

Sub-minimal inhibitory concentrations (sub-MICs) of antibiotics have much practical implication in a clinical setting. Antibiotics create concentration gradients within the human body during chemotherapy (Baquero, 2001). Such concentration gradients arise largely due to pharmacokinetic factors such as different diffusion rates into various tissues, metabolism, local inactivation or differences in the elimination rates from various body sites. Hence, inhibitory concentrations of antibiotics may not reach target bacterial pathogens in infection sites. Rather, most bacteria are in contact with sub-MICs of antibiotics (Baquero, 2001). Although it is now known that bacteria in their planktonic state are often vulnerable to changes elicited by sub-MICs of antibiotics, there are no current studies indicating the effects of sub-MICs of antibiotics on the virulence of bacteria in the biofilm state. The present study of *Ps. aeruginosa* biofilms in presence of sub-MICs of antibiotics, assumes critical importance in the light of the fact that *Pseudomonas* infections are often found in the form of biofilms.

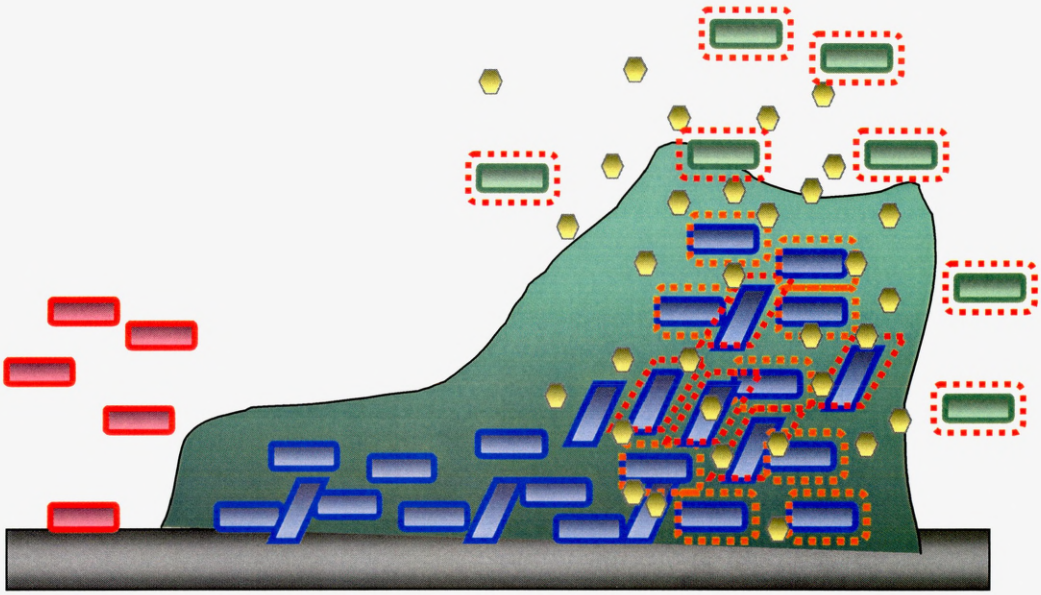
Bacterial outer membrane protein shifts may be involved in antibiotic resistance

Determination of MICs of selected antibiotics (ampicillin, nalidixic acid and streptomycin) in the planktonic state and biofilm states of *Pseudomonas aeruginosa* showed significant differences (Figure 16; Table 6). MIC levels exhibited were 7-fold higher in biofilm cells treated with antibiotics compared to their planktonic

counterparts (Table 6). Cells that detach from the biofilms (eluate cells) had an overall lesser MIC when compared to biofilm MICs (Table 6). However, the eluate MICs were much greater than those of the corresponding planktonic forms. This may be because the detached cells are undergoing physiological reversion to the planktonic state. Although some literature ascribes multicellularity as the root cause of biofilm resistance (Krasovec & Jerman, 2003), the evidence suggested in this thesis indicates some form of inherent cellular mechanism, thus, making resistance more individualistic. It is hypothesised that the cellular resistance observed is due to changes in the outer membrane proteins (Figure 62).

In Figure 62, a general understanding of the hypothesis (proposed above) is illustrated. Planktonic cells approaching a surface adhere to it and finally form a biofilm (Davey and O'Toole, 2000; Stoodley *et al.*, 2002). Biofilm cells differ significantly in their physiologies to planktonic cells (Beloin and Ghigo, 2005). Many of their outer membrane proteins may be significantly different from those present in their planktonic state (lanes 2 and 3, Figure 20; Chapter 4). However, when these biofilm cells encounter antibiotics, they further alter the expression of their outer membrane proteins so as to ultimately prevent intracellular accumulation of lethal levels of antibiotics (compare lane 3 to lanes 4, 5 and 6, Figure 20; Chapter 4). Biofilm cell clusters that detach from biofilms (eluate cells) may revert to their planktonic forms (Hoiby, 2001). However, they may retain outer-membrane proteins that may be related to the antibiotic resistant phenotype (compare Table 8 (part 2) to Table 9 (part 2 and 3); Chapter 4) (significantly higher MIC values of eluate cells to those of planktonic forms, also support this suggestion). The hypothesis (Figure 62) above assumes critical importance in light of the fact that outer membrane proteins (OMPs) present within the outer envelope of bacteria play important roles in excluding harmful molecules from the cell (Abdel Malek *et al.*, 2002). Many

antimicrobial agents enter the cell through OMPs. Over-expression or loss of certain OMPs may lead to an increase in resistance towards antimicrobials (Winder *et al.*, 2000).



Key:

( ) – Planktonic cell phenotype

( ) – Eluate cell phenotype with altered outer membrane proteins (in response to antibiotics)

( ) – Biofilm cell phenotype

( ) – Biofilm cell phenotype with altered outer membrane proteins (in response to antibiotics)

( ) – Antibiotic molecules

( ) – Biofilm matrix

Figure 62: Alteration of biofilm and eluate cell outer membrane proteins in the presence of antibiotics (ampicillin, nalidixic acid and streptomycin). The presence of antibiotic molecules in and around biofilms alters bacterial surface membrane proteins. Alteration happens in a subset of the outer membrane proteins in the biofilm and eluate cell phenotypes. While most of the proteins altered (appearance of novel proteins or disappearance of proteins) may not have a direct relationship to antibiotic resistance (i.e. some outer membrane proteins may result from presence of antibiotics, but may not be responsible for the resistance phenotype exhibited by the cell), there may be a subset of altered proteins that may be responsible for antibiotic resistance. This subset of resistance-related proteins might be similar for biofilm and eluate cell phenotypes due to strong similarities in their cell physiologies.

In order to identify proteins that may be involved in resistance, SDS-PAGE and 2D-PAGE analysis of outer membrane proteins was performed. The approach

involved investigating outer membrane protein profiles of *Ps. aeruginosa* in two states, biofilm and eluate. As the resistance shown by the eluate forms (adjudged by MICs) were much closer to that of the biofilm state cells, it was suggested that the physiology and the mechanism of resistance in these two states might also be similar. Thus, a comparison of antibiotic treated and control samples in each of these states (biofilm and eluate) would reveal candidate outer membrane proteins responsible for resistance towards a selected antibiotic and a comparison between the candidate proteins derived from these two states would further select common proteins that may be responsible for antibiotic resistance to selected antibiotics in these two states. Accordingly a 38.7 kDa protein, apparent in control cells was inapparent in the presence of nalidixic acid treatment of biofilms and eluate cells. Furthermore, the presence of this protein in control samples of both biofilm and eluate cells suggests that it may play a major role in the resistance exhibited by *Pseudomonas* biofilm infections. It is to be noted that the antibiotic treatment was performed MIC/16 and thus, the disappearance of the 38.7 kDa protein in response to the presence of nalidixic acid may actually have clinical significance.

Changes in LPS structure contribute to antibiotic resistance in *Ps. aeruginosa*

Most antibiotics used for treatment of *Pseudomonas* infections have to first penetrate the outer membrane, cell wall and inner membrane, in order to reach their targets. The innate resistance exhibited by *Pseudomonas aeruginosa* against antibiotics has been attributed to the low permeability of the cell envelope (Hauser and Sriram, 2005). LPS is a major constituent of the Gram-negative outer membrane and hence, changes observed in its structure may result in heightened antibiotic permeability barriers.

In the present study, high molecular weight LPS banding patterns were seen to differ in binary and monospecies *Pseudomonas aeruginosa* biofilms (Figure 35 and Figure 39). High molecular weight LPS bands have generally been attributed to the O chain of the LPS structure. Disappearance of these bands in the sub-MIC antibiotic treated samples indicates a phenotypic switch from the more virulent S-form (responsible for acute infections) to the less virulent R-form (involved in chronic infections). Thus, it may be that the biofilm bacteria are gearing up for a chronic infection lifestyle when exposed to sub-MICs of antibiotics.

It has been previously reported that outer membrane phospholipid content increases in parallel with the switch from the S to the R form (Giwercman *et al.*, 1992). A down regulation of the high molecular weight fraction of the LPS (O chain) will thus result in an increase in the ratio of core Lipid A content to O antigen. Thus, the bacterial outer membrane becomes more hydrophobic. Hydrophilic antibiotic molecules will as a result find it more difficult to penetrate the hydrophobic outer membrane. Besides this general outer membrane-repelling action, the increase in the hydrophobicity of the outer membrane may also cause moderately hydrophobic proteins, like β-lactamases, to escape into the outer milieu (Giwercman *et al.*, 1992). Such a leakage may result in neutralisation of antibiotics before they have a chance to interact with the bacterial cell.

Virulence has been defined as the relative capacity of a microbe to cause damage in a host (Casadevall & Pirofski, 2000). While mechanisms of antibiotic resistance help bacteria to adapt to an antibiotic-containing environment, virulence mechanisms help the bacteria to evade or resist the host immune system. Thus, it may be assumed that both antibiotic resistance and virulence are similar adaptive mechanisms induced in order to survive stress (either host invasion or antibiotic treatment) (Martinez & Baquero, 2002).

There exist many examples where bacterial virulence and antibiotic resistance determinants contribute to *in vivo* infective versatility of the bacterium. For instance, virulence determinants of the pathogen *Legionella pneumophilla* allow it to travel intracellularly from one cell to another without much potential antibiotic contact. The poor permeability offered by mammalian cells against various families of antibiotics, allows this pathogen to escape detrimental effects of antibiotics (Barker *et al.*, 1995). Again multidrug resistance (MDR) efflux pumps primarily used by bacteria to exclude different antibiotics from building high concentrations within the cell, has also been reported to extrude bile salts, allowing the pathogen to colonize intestinal tracts (Thanassi *et al.*, 1997). Thus, virulence and antibiotic resistance determinants contribute to each other's effects. As suggested above, evolutionary and ecological links may be established between bacterial virulence and antibiotic resistance. However, little is known about the nature of bacterial virulence when challenged with sub-MICs of antibiotics, especially virulence responses of biofilm bacteria.

In a recent investigation, Fonseca and co-workers (2004) showed that sub-MIC concentrations of piperacillin / tazobactam combination had profound negative impact on virulence parameters of *Pseudomonas aeruginosa.* Their results indicated significant decrease in adhesion values, biofilm formation, flagellum mediated swimming and type IV mediated twitching motility, and led to the conclusion that sub-MICs of piperacillin / tazobactam could interfere with pathogenic potential of clinical isolates of *Pseudomonas aeruginosa.* However, it may be noted that their investigation was performed on planktonic forms of *Pseudomonas*, rather than their more common clinical manifestations as biofilms. The present work outlined in this thesis assumes critical importance, in that the studies were carried out on monospecies and binary biofilms of *Pseudomonas aeruginosa* and *E. coli.*

Are resistant organisms more or less virulent?

Current evidence shows that planktonic bacteria may be inhibited by sub-MICs of antibiotics (Fonseca, *et al* 2004). Such growth limitation causes a decrease in virulence. However, when bacteria become resistant to antibiotics (as in the case of biofilm bacteria when exposed to sub-MICs of antibiotics), will there be any fluctuation in virulence properties?

In a clinical setting, patients suffering from *Pseudomonas* bacterial infections may be administered antibiotics. However, if the pathogenic bacteria are resistant to the antibiotics applied, competing flora may be eradicated resulting in the rapid proliferation of antibiotic resistant phenotypes (Dancer, 2004). Although antibiotic aided colonization of mammalian tissues by resistant bacteria may be classified as an indirect increase in virulence, resistant bacteria may also exhibit a more direct increase. In the present study three virulence determinants, lipopolysaccharide (LPS), proteases and cytotoxins (leucocidin), were analysed in biofilm bacteria in the absence and presence of sub-MICs of selected antibiotics (ampicillin, nalidixic acid and streptomycin). While the concentration of virulence factors (leucocidin and proteases) did not significantly alter, their activities increased in the presence of sub-MICs of selected antibiotics (Table 15 and Table 16; Chapter 5). Hence, qualitative and quantitative measurements of virulence parameters (lipopolysaccharide, leucocidin and proteases) determined that the virulence of biofilm bacteria (*Pseudomonas aeruginosa*) increases when treated with sub-MICs of selected antibiotics.

Lipopolysaccharide (LPS)

LPS was analysed for its contribution in implementing the antibiotic resistance phenotype in *Pseudomonas aeruginosa* (Figure 35). It was noted that the

disappearance of high molecular weight O antigens in the presence of sub-MICs of antibiotics might result in resistance by creating a permeability barrier to hydrophilic antibiotic molecules. However, down regulation of O antigens, may also contribute to the virulence of this organism *in vivo*, provided there is some basal level of immunity in the infected patient.

The endotoxic effect of LPS is due to its lipid A component (Gutsmann, *et al* 2000). However, it is the O chain attached to the lipid A and core polysaccharide that elicits a strong immune response (Trautmann, 1998). A strong immune response mounted against O antigen of *Pseudomonas aeruginosa* in acute infections will mediate opsonophagocytic killing of bacteria (Goldberg & Pier, 1996; Preciado *et al.*, 2005). Results from the present study indicate a possible shift in phenotype of *Pseudomonas* from the S to the R form (Figure 35) and thus, a corresponding shift from acute to chronic lifestyle. This observation may have clinical significance, as in the absence of antibiotics the pathogen has only to deal with the immune response against it. However, it is suggested that when antibiotics, in addition to already existing immune response bring about a synergistic effect, the pathogen can no longer afford to keep the O side chain and thus, down regulates its production. This may help the pathogen to confuse the immune system and escape its detrimental effects (Goldberg & Pier, 1996).

Leucocidin

Investigations carried out on leucocidin (29 kDa cytotoxin) produced by *Pseudomonas aeruginosa* biofilms in presence of ampicillin, have shown an unmistakable increase in its virulence potential (Figure 58). *Pseudomonas aeruginosa* biofilms treated with sub-MIC of ampicillin (8 $\mu g\ mL^{-1}$) showed at least 50% increase in its leucocidin cytotoxic activity when compared to controls (biofilm samples

unexposed to any antibiotics) and more than 200% increase in activity, when compared to cytotoxic activity of leucocidin from *Pseudomonas aeruginosa* planktonic cells (Figure 58). This dramatic increase in virulence potential was reconfirmed when measures of oncosis were determined from phase contrast images of Sp2 cells treated with leucocidin isolated from different samples (Figure 59). It was assumed that the more virulent the leucocidin, the greater would be the degree of oncosis (as determined by increase in diameter of cells). Accordingly, *Pseudomonas aeruginosa* biofilm samples treated with sub-MIC of ampicillin exhibited the maximum amount of cytotoxic activity towards Sp2 cells. Sp2 cell diameter increased more than 50% when treated with leucocidin isolated from *Pseudomonas aeruginosa* biofilms treated with sub-MIC of ampicillin (8 $\mu g\ mL^{-1}$), compared to control samples (biofilm cells unexposed to antibiotics), and more than 250% when compared to *Pseudomonas aeruginosa* planktonic cells (Figure 59; Figure 60 and Table 15).

An increase in the virulence potential of leucocidin from *Pseudomonas aeruginosa* biofilms in the presence of sub-MIC of ampicillin (8 $\mu g\ mL^{-1}$) might actually translate to a similar observation in clinical conditions. Accordingly, antibiotic treatment of patients suffering from chronic biofilm infections may cause more harm than good. Both LPS pattern changes and increased activity of leucocidin of *Ps. aeruginosa* treated with sub-MIC of ampicillin (8 $\mu g\ mL^{-1}$) indicate an increase in virulence of the organism. However, further investigations were carried out to determine if a similar increase in virulence might also occur in proteases produced by *Ps. aeruginosa.*

Proteases

Proteases produced by *Pseudomonas aeruginosa* were identified by zymography (Figure 61). Activity of these proteases was assayed using azocasein as a

substrate. Proteolytic release of sulphanilamide group attached to azocasein was estimated at a wavelength of 440nm, and was found to be proportional to protease activity. *Pseudomonas aeruginosa* monospecies biofilms exposed to sub-MIC of ampicillin (8 μg mL^{-1}) showed maximum protease activity (Table 16). More than 100% increase in protease unit activity was observed for protease produced by *Pseudomonas aeruginosa* biofilm samples treated with ampicillin and about 85% increase in activity was observed when compared with proteases produced by planktonic *Pseudomonas aeruginosa*. These results are indicative of an increase in virulence in the presence of sub-MIC of ampicillin (8 μg mL^{-1}), and thus may have clinical significance.

Antibiotic exposure and virulence in binary biofilms

In most natural and industrial environments biofilms consist of complex communities consisting of multiple microbial species acting in consortium (Elvers *et al.*, 2001). Complex inter-species and intra-species interactions are said to take place within such biofilms that may influence the overall dynamics of biofilm formation and antimicrobial susceptibilities (Elvers *et al.*, 2002). Multispecies biofilms may be more stable and have characteristics pertaining to enhanced antimicrobial resistance. Although there exist many examples for complex mixed species natural and industrial biofilms (Elvers *et al.*, 2002), published reports on multiple species medical biofilms are still a rarity (however, complex mixed species biofilms have been intensively studied in the case of dental plaques (Elvers *et al.*, 2001). However, the existence of a binary or multiple species medical biofilm is a strong possibility, when viewed in light of the common major biofilm pathogens isolated from infection sites in the human body. For instance, *E. coli* and other Gram-negative organisms are common biofilm pathogens isolated from urinary catheter infections and bacterial prostitis (Fux

et al., 2003). There exists the possibility that these different Gram-negative organisms exist as binary or multispecies biofilms.

Previous passages in this discussion have related to the effect of antibiotics on the resistance and virulence of monospecies biofilms of *Pseudomonas aeruginosa.* It was seen that changes in outer-membrane protein expression and LPS patterns of monospecies biofilms could be linked to the increased resistance characteristics of monospecies *Ps. aeruginosa* biofilms. Also the virulence of monospecies biofilms with respect to key virulence factors, leucocidin and proteases increased at least two-fold in the presence of antibiotics (Figure 58; Table 16). Analysis of results (pertaining to antibiotic resistance and virulence) obtained from antibiotic-challenged binary biofilms also indicates results broadly similar to monospecies biofilms (Figure 58; Figure 59; Table 6; Table 16).

2D-PAGE analysis indicated appearance of several novel outer membrane proteins, while others were inapparent in the presence of sub-MICs of ampicillin (8 $\mu g\ mL^{-1}$). Interestingly a comparison of the novel proteins that were apparent in the different antibiotic (AmP, NaL and ST) treated samples yielded no common proteins, indicating that expression of these proteins may be related to unique antibiotic resistance mechanisms specific to a particular antibiotic. Alternatively, these may be proteins that are produced by the organism to adapt to the environment (modified with antibiotics) and may not be directly involved in resistance. A comparison of OMPs that were inapparent after antibiotic treatment of binary biofilms exhibited proteins that may be related to resistance. Among these were 88 kDa, 58 kDa, 41 kDa, 35 kDa and a 27.5 kDa proteins (Table 13 parts 1, 2 and 3; Chapter 4). Down regulation of key OMPs may prevent antibiotics from accumulating within cells, and thus form a possible source for antibacterial resistance. However, further proteomic analysis (e.g.,

mass spectrometric analysis) of the above mentioned proteins are required before a more complete picture of their role in antibiotic resistance emerges.

LPS banding patterns of binary biofilms were similar to those of monospecies biofilms. Generally, a number of high molecular weight bands corresponding to O antigenic regions, previously apparent in control cells, became inapparent on addition of sub-MIC of antibiotics. The disappearance of high molecular weight bands was further accentuated in the case of eluate samples treated with sub-MICs antibiotics. While lipid A regions (low molecular weight bands) were minimal for biofilm cells treated with sub-MIC antibiotics, eluate cells exposed to sub-MIC of nalidixic acid (4 $\mu g\ mL^{-1}$) showed more bands corresponding to these regions (compare Figures 39 and 40, Chapter 5). This could only mean that the endotoxin production increased in eluate samples treated with antibiotics when compared to biofilm cells. However, it still remains to be seen if such an increase translates to an increase in virulence associated with endotoxin of *Ps. aeruginosa.*

Cytotoxins (leucocidin) and proteases isolated from binary biofilms exhibited marginal decreases in activity when compared to monospecies biofilms of *Pseudomonas aeruginosa* treated with sub-MIC of antibiotics (see Tables 15 and 16). This observation may not be surprising, as gel column fractions corresponding to *Ps. aeruginosa* proteases and leucocidins need not contain the same proteins as those from *E. coli.* Hence, a synergistic effect resulting from similar enzymes from these two species co-fractionating, leading to an increase in activity of either of these enzymes may be discounted. However, the results still indicate that the binary biofilms of *Pseudomonas aeruginosa* are stable entities and may exhibit enhanced virulence comparable to monospecies biofilms of the same species. The results obtained in this section, indicating increases in virulence of binary biofilms of *Pseudomonas aeruginosa* in presence of sub-MICs of selected antibiotics, is in sharp

contrast to those reported by Fonseca *at al.* (2004). Although Fonseca and colleagues reported a decrease in virulence and hence, pathogenic potential of *Pseudomonas aeruginosa* in presence of sub-MIC antibiotics, it may be noted that their work was carried out on planktonic forms and not biofilms. This observation gives credence to the suggestion that physiological differences between these two states (planktonic and biofilm) may play major roles in determining virulence potential of *Pseudomonas* in presence of sub-MICs of selected antibiotics (ampicillin, nalidixic acid and streptomycin).

Conclusions

Immunocompromised patients infected with *Pseudomonas* infections (as in the case of secondary *Pseudomonas* infections in cystic fibrosis patients) are treated with a range of antibiotics spanning different classes (Hauser and Sriram, 2005). The results discussed here arise from experiments carried out with three such classes of antibiotics, viz. β-lactams (ampicillin), quinolones (nalidixic acid) and aminoglycosides (streptomycin).

In response to sub-MICs of antibiotics, biofilm bacteria have been shown to behave in diverse ways. As a result of adapting to a new environment (the presence of antibiotics), biofilm bacteria change their outer membrane protein and LPS patterns. Changes in the LPS structure also suggest the possible leak of antibiotic inactivating proteins (β-lactamases). Novel proteins that appear and those that become reduced or undetectable (possibly porin proteins that have selective affinity for some antibiotics) might indicate phenotypic changes towards resistance, as well as epitopic changes. Such changes will help the bacteria resist antibiotics that target them and will help them survive in antibiotic-rich environments.

On the virulence front, the LPS changes (disappearance of the O antigen) may suggest mechanisms that help the pathogen evade host immune response. While no significant quantitative changes in the production of proteases and leucocidins were observed, qualitative changes were seen. Activities of proteases and leucocidins significantly increased with respect to control samples in the presence of sub-MIC of selected antibiotics.

These results suggest a possible model (Figure 63) for *in vivo* biofilm pathogenesis and virulence in the presence of sub-MICs of selected antibiotics (ampicillin, nalidixic acid and streptomycin). Figure 63 illustrates the probable behaviour of an *in vivo* biofilm model towards sub-MIC of antibiotics. These biofilms may have characteristics similar to those found in patients suffering from cystic fibrosis or other biofilm-related diseases. Biofilms of *Pseudomonas aeruginosa* are major complications that result in secondary infections of cystic fibrosis patients (Hoiby *et al.*, 2001). However, as opposed to planktonic forms, their pathogenic potential is significantly lower, and as such they are more important in chronic infections (Hoiby *et al.*, 2001). Hence, these pathogens may express only that level of virulence (basal) needed to develop chronic infections. Current medical treatment of medical biofilms involves antibiotics, either singly or in combination (Aaron *et al.*, 2002). The rationale behind using antibiotics is their ability to decrease viability and virulence of planktonic forms and hence, a corresponding decrease in their pathogenic potential (Fonseca *et al.*, 2004). However, results presented within this thesis show that biofilms and planktonic states of *Pseudomonas* are physiologically different and thus, behave differently in the presence of sub-MICs of antibiotics. Although the concentration of virulence proteins (leucocidins and proteases) do not alter significantly, their cytotoxic and proteolytic activities are enhanced in the presence of sub-MICs of selected antibiotics (ampicillin, nalidixic acid and streptomycin) and

may cause much more damage to host factors than can be expected for chronic infections unexposed to antibiotics (Table 15 and Table 16; Chapter 5). This would result in an increase in the pathogenic potential of the organism and hence, its disease causing ability. Hence, the use of antibiotics to treat *in vivo* biofilm infections may actually worsen prognosis rather than improve it, if the patient is allowed to develop sub-MIC levels of antibiotic at the infected site.

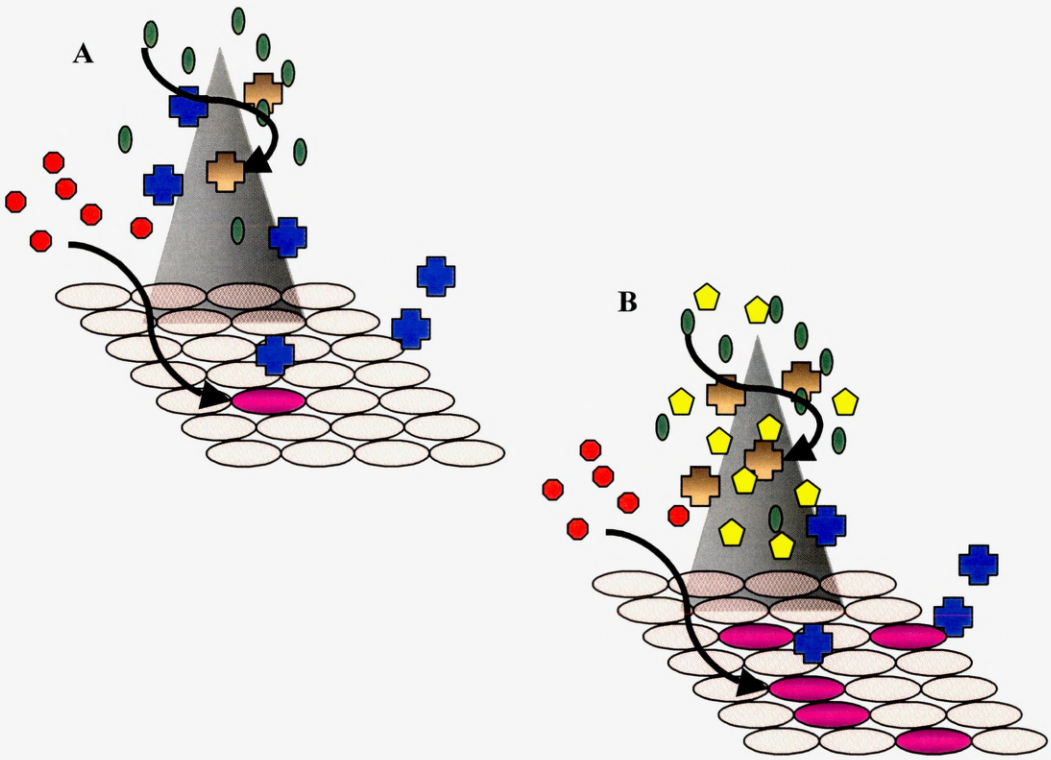


Figure 63: Schematic representation illustrating a possible biofilm model of pathogenesis in presence of sub minimal inhibitory concentrations of antibiotics (ampicillin, nalidixic acid and streptomycin). In the absence of antibiotics (Figure 63A), biofilm bacteria ( ) secrete proteases ( ) and leucocidins ( ) having basal levels of virulence, and may be partly responsible for virulence. The ratio of damaged epithelial cells ( ) to healthy epithelia ( ) is low. Similarly the ratio of damaged leucocytes or white blood cells ( ) to healthy ones ( ) is also basal. However, in the presence of antibiotics ( ) (Figure 63B), although there is no significant increase in the levels of production of virulence factors (proteases and leucocidins), the activity of these determinants significantly increase causing damage to more cells.

Suggestions for future work

Although the results presented within this thesis attribute antibiotic resistance to inherent bacterial cellular mechanisms, further work has to be carried out to confirm this hypothesis.

(1) Manual determination of molecular weights of proteins from SDS-PAGE and 2D-PAGE may not result in reproducibly accurate results. Hence, advanced proteomic techniques like MALDI-TOF spectrometry have to be employed to identify all novel outer membrane proteins and those proteins that have disappeared in the presence of sub-MICs of antibiotics. Identification will greatly enhance the process of protein elimination and comparison that has been adopted in this thesis to identify outer membrane proteins that may potentially play a role in antibiotic resistance.

(2) Significant quantitative changes in protein fractions which emerged before leucocidin and protease fractions for sub-MIC antibiotic treated samples when compared to controls. Although SDS-PAGE analysis was carried out for these proteins, further proteomic analysis has to be carried out to determine any possible relation to antibiotic resistance.

(3) Quantitative and qualitative analysis of fluctuations of virulence in presence of sub-MICs of antibiotics were carried out only for LPS, and secreted virulence factors (leucocidins and proteases) of biofilms. Other major virulence parameters like EPS in biofilms or flagella mediated swimming, type IV pili mediated twitching, etc. of bacterial cells detached from biofilms (eluate cells) may also be tested.

(4) The spread of biofilm-related infections within the host is related to detachment of infective doses of cells from biofilms. Circulation of LPS released from planktonic forms into the blood is responsible for their systemic endotoxic effects. Monitoring detachment of biofilm cells and release of biofilm LPS in the

presence of sub-MICs of antibiotics may help give a more accurate prediction of virulence fluctuations.

(5) Based on the results obtained from *in vitro* virulence assays, the *in vivo* model of biofilm virulence (illustrated in Figure 63) was proposed. It now remains to test this hypothesis in a suitable chronic infection model.

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