CHAPTER ONE

LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous gram-negative bacterium and highly successful in colonising a diversity of environments (Palleroni, 1992a; 1992b). Not only is it an opportunistic pathogen of humans, causing infections in immunocompromised patients such as those with cancer or AIDS as well as those suffering from cystic fibrosis and burns (Van Delden and Iglewski, 1998; Ramsey and Wozniak, 2005), but it has also been shown to infect plants and insects (Schroth *et al.*, 1992; Rahme *et al.*, 1995). The success of *P. aeruginosa* in colonising these diverse environments is attributed to its ability to synthesise a large number of different virulence factors such as alginate, pili and lipopolysaccharides, and secreted virulence factors, including toxins, proteases and haemolysins (Liu, 1974; Doring *et al.*, 1987; Lazdunski *et al.*, 1990; Van Delden and Iglewski, 1998; Ramsey and Wozniak, 2005).

In the vast majority of ecological niches, *P. aeruginosa* can grow in association with surfaces, which leads to the formation of biofilms (Zobell, 1943; Characklis, 1973; Costerton *et al.*, 1995). Biofilms have been defined as structured communities of bacterial cells that are enclosed in a self-produced polymeric matrix and adhere to biotic and abiotic surfaces, an interface or each other (Costerton *et al.*, 1995). Biofilm formation occurs in response to a variety of environmental signals (Davey and O'Toole, 2000; Stanley and Lazazzera, 2004) that leads to a number of changes in gene regulation that cause the adhering cells to become phenotypically (Costerton *et al.*, 1995; Davies and Geesey, 1995; Sauer *et al.*, 2002) and metabolically (Costerton *et al.*, 1999; Davey and O'Toole, 2000) distinct from their planktonic counterparts. The complex biofilm architecture also provides an opportunity for metabolic cooperation, and niches are formed within the spatially well-organised systems. Consequently, the bacteria are exposed to an array of distinct physicochemical conditions within a biofilm that can result in differential gene expression (Davey and O'Toole, 2000; O'Toole *et al.*, 2000a; Whiteley *et al.*, 2001; Sauer and Camper, 2001; Sauer *et al.*, 2002).

Recent studies have suggested that biofilm formation occurs as a sequential, developmental process (O'Toole *et al.*, 2000a; Sauer *et al.*, 2002; Stoodley *et al.*, 2002). Current models, based largely on *P. aeruginosa*, depict biofilm formation commencing when planktonic bacterial cells attach irreversibly to a surface. This attachment is followed by growth into a mature, structurally complex biofilm and culminates in the dispersion of detached bacterial cells into the bulk fluid (Sauer *et al.*, 2002). Notably, the bacteria within each of the stages of biofilm development are believed to be physiologically distinct from cells in the other stages. Using two-dimensional polyacrylamide gel electrophoresis, Sauer *et al.* (2002) reported that the average difference in protein expression between each developmental stage was 35% of detectable proteins, and the most profound differences were observed when planktonic cells were compared to mature biofilm cells. The phenotypic heterogeneity within the biofilm has been interpreted as a specialisation or division of labour, similar to cellular differentiation seen in multicellular organisms (O'Toole *et al.*, 2000a; Webb *et al.*, 2003a).

Not surprisingly, several reports have therefore indicated that biofilms should be regarded as multicellular organisms and that biofilm bacteria exhibit cooperative unselfish behaviour (O'Toole *et al.*, 2000a; Klausen *et al.*, 2003; Webb *et al.*, 2003a). Biofilm bacteria do indeed display at least some similarities with multicellular organisms, *e.g.* sensing of their surroundings (O'Toole *et al.*, 2000b; Otto and Silhavy, 2002), a means to communicate with each other via secretion of autoinducer molecules (Davies *et al.*, 1998; Lazdunski *et al.*, 2004) and they can undergo a process similar to programmed cell death (Webb *et al.*, 2003b). However, it may be erroneous to refer to biofilm bacteria as multicellular organisms since they do not permanently differentiate. Rather they are more likely to be interactive communities in that they respond to their environmental surroundings by adapting their gene expression to suit their own needs for survival (Jefferson, 2004). Nevertheless, biofilm formation provides its members with a number of benefits. In addition to the advantage of resistance to environmental changes (Donlan and Costerton, 2002; Jefferson, 2004), the biofilm bacteria may benefit from a number of properties of a communal existence, including division of metabolic burden (Geesey, 2001; Yarwood *et al.*, 2004), gene transfer (Clark and Warren, 1979; Roberts *et al.*, 1999; Ghigo, 2001; Molin and Tolker-Nielsen, 2003) and altruistic behaviour (Rice and Bayles, 2003).

1.2 BIOFILM FORMATION BY *P. aeruginosa*

Over the past few years, much progress has been made towards understanding the development of bacterial biofilms. This progress has been largely due to the recent focus of analysing biofilms using genetic (O'Toole and Kolter, 1998a; 1998b; Whiteley *et al.*, 2001; Finelli *et al.*, 2003), proteomic (Sauer and Camper, 2001; Sauer *et al.*, 2002) and molecular biological (Tolker-Nielsen *et al.*, 2000; De Kievit *et al.*, 2001a; Klausen *et al.*, 2003) approaches. In addition, extensive biophysical, structural and chemical analysis of bacterial biofilms has led to a basic model for biofilm structure (Costerton *et al.*, 1995; Tolker-Nielsen *et al.*, 2000). In this model, bacteria form microcolonies surrounded by copious amounts of exopolysaccharide and between the microcolonies are water-filled channels (Costerton *et al.*, 1995). It has been suggested that these channels serve to promote the influx of oxygen, organic substrates and nutrients, and the efflux of carbon dioxide and metabolic by-products (DeBeer *et al.*, 1994; Costerton *et al.*, 1995; 1999).

1.2.1 Steps in biofilm development

It has been proposed that microbial biofilm formation may be a further example of a bacterial developmental process (Davey and O'Toole, 2000; O'Toole *et al.*, 2000a; Stoodley *et al.*, 2002), not unlike that observed in cell cycle-controlled swarmer-to-stalk cell transition in *Caulobacter crescentus* (Dworkin, 1999), sporulation in *Bacillus subtilis* (Branda *et al.*, 2001) and fruiting body formation in *Myxococcus xanthus* (Shimkets, 1999). Similar to these developmental systems, building a biofilm requires a series of discreet and well-regulated steps. While the exact molecular mechanisms may differ from organism to organism, the stages of biofilm development appear to be conserved among a wide range of microbes (Fig. 1.1). These stages include attachment of free-floating bacterial cells to a surface, the growth and aggregation of cells into microcolonies followed by growth into mature, structurally complex biofilm (maturation), and the dispersal of detached bacterial cells into the bulk fluid (O'Toole *et al.*, 2000a; Sauer *et al.*, 2002).

1.2.1.1 Reversible attachment

Prior to surface colonisation, a preconditioning film, composed of proteins, glycoproteins and organic nutrients, is believed to form on the attachment surface, thus resulting in a nutritionally rich zone that is metabolically favourable for bacterial cells (Marshall *et al.*, 1971; 1985; Beveridge *et al.*, 1997). Once a surface has been conditioned, its properties are

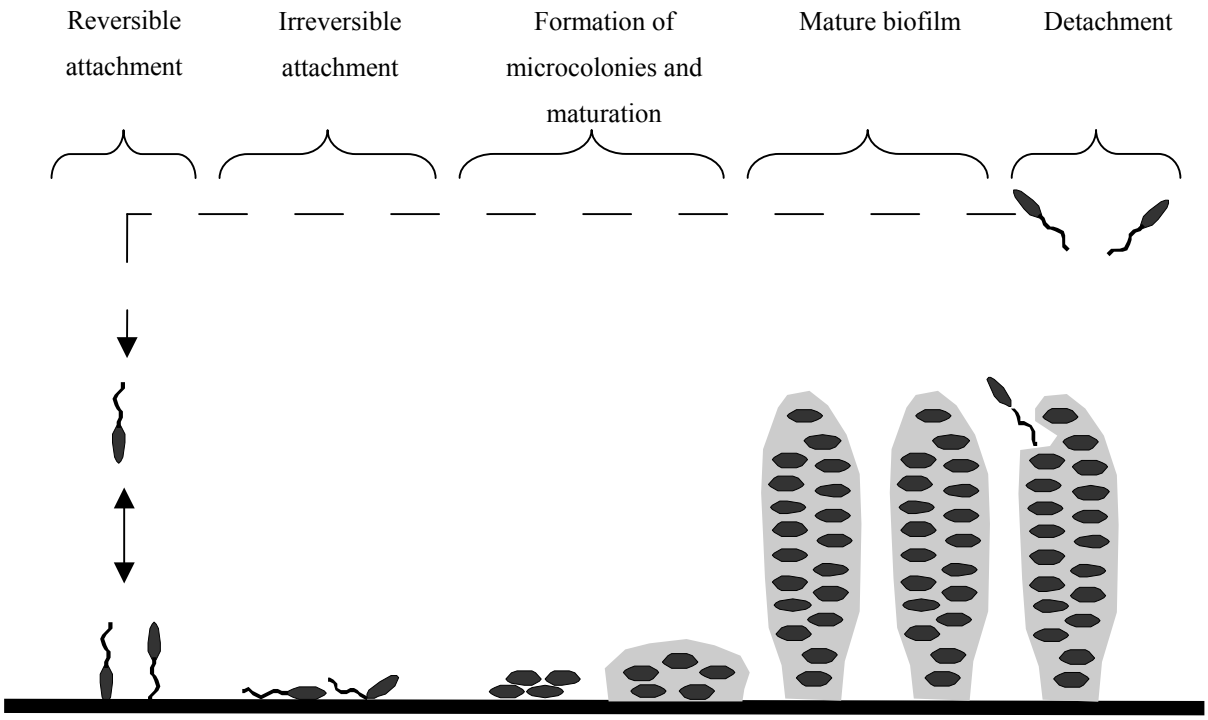

Fig. 1.1 **Model of biofilm development. In response to environmental cues, planktonic cells initiate cell-to-surface and cell-to-cell contacts resulting in the formation of microcolonies. In response to developmental signals, microcolonies undergo differentiation to form a well-developed biofilm characterised by pillar- or mushroom-like structures surrounded by bacterial exopolysaccharides (EPS) and interspersed with fluid-filled channels. Once the biofilm has reached critical mass, some of the biofilm cells may detach to colonise other surfaces (Modified from O'Toole *et al.*, 2000a and Stoodley *et al.*, 2002).**

permanently altered so that the affinity of an organism for a native or a conditioned surface can be quite different (Hermansson and Marshall, 1985; Boland *et al.*, 2000). Planktonic bacteria may be brought into close approximation of the conditioned surface by either a random (*e.g.* sedimentation and liquid flow) or in a directed fashion (*e.g.* chemotaxis and active motility) (Quirynen *et al.*, 2000). Initial attachment of the bacteria to the conditioned surface is then facilitated by van der Waals forces, electrostatic and hydrophilic interactions and specific interactions, or by a combination of these, depending on the proximity of the organism to the attachment surface (Van Loosdrecht *et al.*, 1990; Carpentier and Cerf, 1993; Zottola and Sasahara, 1994; An *et al.*, 2000). The individual adherent cells that initiate biofilm formation on a surface are surrounded by only small amounts of exopolymeric material and many are capable of independent movement by means of pilus-mediated twitching or gliding (O'Toole and Kolter, 1998a). These cells are, however, not yet committed to the process of biofilm formation and many may leave the surface to resume the planktonic lifestyle (Sauer *et al.*, 2002).

1.2.1.2 Irreversible attachment

Following the initial reversible attachment to a surface, the bacteria must not only maintain contact with the substratum but also grow in order to develop a mature biofilm. Thus, the stage of reversible attachment is followed by a phase during which production of bacterial exopolysaccharides (EPS) results in more stable attachment by forming organic bridges between the cells and substratum (Notermans *et al.*, 1991). Reporter gene studies have established that expression of the *P. aeruginosa* alginate biosynthetic genes *algC* (Davies *et al.*, 1993; Davies and Geesey, 1995) and *algD* (Hoyle *et al.*, 1993; Rice *et al.*, 1995) are up-regulated within 15 min following initial attachment to a surface, with a concomitant increase in alginate production. Although the production of alginate has been considered to form the structural and mechanical framework required for biofilm formation (Davies and Geesey, 1995; Stoodley *et al.*, 2002), recent reports, however, have indicated that EPS other than alginate is essential for *P. aeruginosa* biofilm formation (Friedman and Kolter, 2004; Jackson *et al.*, 2004; Matsukawa and Greenberg, 2004).

Transition from reversible to irreversible attachment is also mediated by pili, fimbriae and fibrillae (Jacob-Dubuisson *et al.*, 1993; Jones *et al.*, 1995; Rudel *et al.*, 1995; Pratt and Kolter, 1998). Whereas flagellar-mediated motility is important in establishing initial cell-surface

contacts, twitching motility has been shown to be required for maturation of *P. aeruginosa* biofilms under quiescent conditions (O'Toole and Kolter, 1998a). Twitching motility refers to a mode of surface translocation mediated by type IV pili (Wall and Kaiser, 1999) in which the pili are believed to extend and retract, thus propelling the cells along the surface (Palmer, 1999). Specifically, twitching motility is required for the formation of microcolonies within the biofilm by facilitating interactions of bacteria with one another at the surface, forming groups of cells, thereby helping to strengthen the degree of attachment to a surface (O'Toole and Kolter, 1998a).

Microscopy observations have shown that initial surface attachment in *P. aeruginosa* proceeds from transient cell pole-mediated interactions (reversible attachment) to stable surface interactions that occur via the long axis of the cell body (irreversible attachment) (Sauer *et al.*, 2002). Recently, a new class of *P. aeruginosa* biofilm mutant was described that was able to initiate surface attachments but failed to form microcolonies in flow cell-grown biofilms, despite being proficient in twitching and swimming motility (Caiazza and O'Toole, 2004). The transposon insertion was subsequently mapped to open reading frame PA5346, which encodes a protein of unknown function, and was designated *sadB*. Since the mutant cells were arrested at reversible attachment, it was proposed that *sadB* may be required for the transition from reversible to irreversible attachment, but the exact mechanism by which SadB promotes this transition is not yet known.

1.2.1.3 Biofilm maturation

Once the bacteria have irreversibly attached to a surface, the process of biofilm maturation begins. During this process, binary division of irreversibly attached cells causes the daughter cells to spread outward and upward from the attachment point to form microcolonies or cell clusters (Heydorn *et al.*, 2000; Tolker-Nielsen *et al.*, 2000). Alternatively, the attached cells can be redistributed by surface motility (O'Toole and Kolter, 1998a; 1998b; Klausen *et al.*, 2003) and/or single cells may be recruited from the bulk fluid to the developing biofilm (Tolker-Nielsen *et al.*, 2000). The nature of the surface that is being colonised and the physical and chemical conditions of the environment will determine which of the mechanisms of biofilm formation will dominate (Hall-Stoodley and Stoodley, 2002; Stoodley *et al.*, 2002). Maturation of the biofilm results in the generation of mushroom- or pillar-like structures interspersed with fluid-filled channels (Costerton *et al.*, 1995; Tolker-Nielsen *et al.*, 2000),

and once fully developed, a biofilm generates altered patterns of bacterial growth, physiological cooperation and metabolic efficiency (Costerton *et al.*, 1995; 1999; Rice *et al.*, 2000; Geesey, 2001; Werner *et al.*, 2004).

Notably, the biofilm cells display altered behaviour in gene expression. In a recent study, mature biofilms of *P. aeruginosa* were shown to have a radically different protein profile from planktonic bacteria grown in chemostats (Sauer *et al.*, 2002). As much as 50% of the detectable proteome (over 800 proteins) was shown to have a six-fold or greater difference in expression. Of these, more than 300 proteins were detectable in mature biofilm samples that were undetectable in planktonic bacteria. The identified proteins fell into five major classes, *i.e.* metabolism, phospholipid and lipopolysaccharide (LPS)-biosynthesis, membrane transport and secretion, as well as adaptation and protective mechanisms (Sauer *et al.*, 2002). By making use of DNA microarrays to compare gene expression of biofilm and planktonic *P. aeruginosa* PAO1 grown either in chemostats or in once-flow through tubing, Whiteley *et al.* (2001) reported that 73 genes displayed alterations in expression. The genes identified to be up-expressed in mature biofilms were genes encoding proteins involved in translation, metabolism, gene regulation and membrane transport and/or secretion, whilst flagella and pilin genes were down-regulated, as was the gene encoding RpoS, which regulates the general stress response (Venturi, 2003).

1.2.1.4 Detachment

The growth potential of the bacterial biofilm is ultimately limited by the availability of nutrients in the immediate environment, the expression of quorum-sensing molecules released in response to nutrient limitation, accumulation of toxic by-products and other factors, including pH, oxygen perfusion, carbon source availability and osmolarity (Puskas *et al.*, 1997; Allison *et al.*, 1998; Davies *et al.*, 1998; O'Toole and Kolter, 1998b; O'Toole *et al.*, 2000b; Prigent-Combaret *et al.*, 2001; Sauer *et al.*, 2004). At some point, the biofilm reaches critical mass and a dynamic equilibrium is reached at which the cells may detach and together with progeny of other biofilm cells may colonise other surfaces (Korber *et al.*, 1989).

Although it has generally been believed that bacterial cells leave the biofilm after division of cells in the outer layers of the biofilm matrix, recent studies have suggested that the detachment process may be more complex than originally thought. Several recent studies

have reported pronounced activity and cellular differentiation localised to the center of mature biofilm structures, which led to the dispersal of cells from inside the structure, leaving behind large transparent cavities, or hollow "shells" made up of non-motile cells (Tolker-Nielsen *et al.*, 2000; Sauer *et al.*, 2002). Several mechanisms for biofilm dissolution and consequently, cell dispersal have been proposed. Enzymes such as polysaccharide lyases that degrade the extracellular polysaccharide matrix have been reported to play a role in biofilm dissolution in several organisms (Sutherland, 1999; Kaplan *et al.*, 2003). Boyd and Chakrabarty (1994) reported that induction of alginate lyase expression in *P. aeruginosa* substantially decreased the amount alginate produced, which corresponded with a significant increase in the number detached cells. It was thus suggested that the role of alginate lyase in wild-type *P. aeruginosa* might be to cause a release of cells from solid surfaces or biofilms, thereby aiding in the dispersal of these organisms. Recently, death of a subpopulation of cells has also been observed as a normal feature of biofilm development in *P. aeruginosa* (Webb *et al.*, 2003b). Cell death occurred inside microcolony structures, and killed only a subpopulation of cells within the biofilm. *P. aeruginosa* cell death was linked to the expression of a Pf1-like filamentous prophage of *P. aeruginosa* (Webb *et al.*, 2003b). It was proposed that prophage-mediated cell death might be an important mechanism of differentiation inside *P. aeruginosa* microcolonies, which facilitates subsequent dispersal of a subpopulation of surviving cells (Webb *et al.*, 2003b).

1.3 STRUCTURAL COMPONENTS AND CELL-TO-CELL SIGNALLING MOLECULES REQUIRED FOR BIOFILM FORMATION

Of the processes leading to the formation of biofilms, bacterial structural components required for initial attachment have been best characterised, primarily through mutation analysis. The rate and extent of attachment of bacterial cells to a surface is influenced by cell surface hydrophobicity, presence of flagella, pili and adhesins, outer membrane proteins and production of extracellular polymeric substances (EPS) (O'Toole and Kolter, 1998a; 1998b; DeFlaun *et al.*, 1999; Genevaux *et al.*, 1999; Espinosa-Urgel *et al.*, 2000; Yoon *et al.*, 2002; Finelli *et al.*, 2003; Jackson *et al.*, 2004). In addition, evidence suggests that the primary development of a biofilm might be regulated at the level of population density-dependent gene expression controlled by cell-to-cell signaling molecules such as acylated homoserine lactones (McLean *et al.*, 1997; Allison *et al.*, 1998; Davies *et al.*, 1998).

1.3.1 Importance of flagella, pili and adhesins

Although earlier studies have suggested that simple chemical models could account for the bacterial behaviour during the initial stages of attachment (Marshall *et al.*, 1971; McEldowney and Fletcher, 1986), subsequent studies, mainly through transposon mutagenesis, have shown that structural components such as flagella, pili and adhesins play an important role in bacterial interaction with the surface. The primary function of flagella in biofilm formation is believed to be in transport and in initial cell-to-surface interactions. This has been based on observations that the absence of flagella impaired the ability of *P. fluorescens* and *P. putida* to colonise potato and wheat roots (De Weger *et al.*, 1987; DeFlaun *et al.*, 1994), and reduced cellular adhesion of *P. aeruginosa* and *P. fluorescens* to a polystyrene surface (O'Toole and Kolter, 1998a; 1998b). Similarly, the absence of flagella in *Vibrio cholerae* (Watnick *et al.*, 1999) and in *Escherichia coli* (Genevaux *et al.*, 1996; Pratt and Kolter, 1998) prevented the mutant strains in forming biofilms resembling those formed by the wild-type bacteria on polyvinylchloride (PVC). More recent evidence obtained though proteomic analysis has indicated that gene expression of flagellar components (*fleN* and *flgG*) was down-regulated in 6-h biofilms of *P. putida* (Sauer and Camper, 2001). In addition, expression of the *flgD*, *fliD* and *flgE* genes was reported to be repressed in 24-h biofilms of *P. aeruginosa* (Whiteley *et al.*, 2001). These observations seem to indicate that after initial cell-to-surface contact, the flagella become dispensable for further biofilm development. However, several studies have subsequently shown that the initial down-regulation of flagella is transitory and motile bacteria are present in biofilms at later stages of biofilm development, suggesting a role for flagella in the detachment of cells from the biofilm (Sauer and Camper, 2001; Sauer *et al.*, 2002).

Pili and pilus-associated adhesins have also been shown to be important for the adherence to and colonisation of surfaces. Expression of *sfaA*, the gene encoding S-fimbrial adhesins, of a pathogenic strain of *E. coli* has been reported to be up-regulated upon attachment (Schmoll *et al.*, 1990). In *E. coli*, attachment is reduced by mutations in the curlin biosynthetic gene *csgA* (Vidal *et al.*, 1998; Dorel *et al.*, 1999), and in the type I pili biosynthetic gene *fimH*, which encodes the mannose-specific adhesin (Pratt and Kolter, 1998). Similarly, mutations in the mannose-sensitive hemagglutinin pilus of *V. cholerae* also result in a reduction of adhesion to surfaces (Watnick *et al.*, 1999). In *P. aeruginosa*, type IV pili mutants have been shown to be impaired in their ability to form microcolonies (O'Toole and Kolter, 1998a), whilst Vallet *et*

al. (2001) reported that a *cupA* mutant was defective in biofilm formation in *P. aeruginosa*. The *cupA* gene cluster encodes the components of a chaperone/usher pathway that is involved in assembly of fimbrial subunits such as P pili and type I pili, but not type IV pili (Soto and Hultgren, 1999), suggesting that pili other than type IV pili may be involved in initial attachment of *P. aeruginosa* to surfaces. The importance of pili in the initial stages of biofilm formation has been supported by proteomic analysis indicating the up-regulation of gene expression of type IV pili components (*pilR*, *pilC* and *pilK*) in 6-h old biofilms of *P. putida* (Sauer and Camper, 2001). In mature biofilms of *P. aeruginosa*, expression of the *pilA* gene, which encodes the type IV pilin subunit, was repressed. This may indicate that although type IV pili are involved in the initial steps of biofilm formation, they may not be required for maintenance of the mature biofilm (Whiteley *et al.*, 2001). In addition to a role for type IV pili in the initial phase of biofilm development (O'Toole and Kolter, 1998a), a model for *P. aeruginosa* biofilm development has recently been proposed in which type IV pili-driven bacterial migration plays a key role in structural formation in the late stage of biofilm development (Klausen *et al.*, 2003). According to the model, the formation of mushroom-shaped structures in *P. aeruginosa* biofilms occurs through stalk formation by proliferation of bacteria that have down-regulated twitching motility and cap formation by bacteria that climb the microcolony stalks by the use of type IV pili and aggregate on top.

1.3.2 Importance of membrane proteins

Membrane proteins have been reported to have a substantial influence on attachment and may also play a role in early biofilm development. Attachment of *E. coli* to abiotic surfaces leads to alterations in the composition of outer membrane proteins, which suggests that physical interactions with the surface lead to an alteration of the surface characteristics of the cell envelope (Otto *et al.*, 2001). Mutations in surface and membrane proteins, including a calcium-binding protein, a hemolysin, a peptide transporter, and a potential glutathione-regulated K^+ efflux pump caused defects in attachment of *P. putida* to corn seeds (Espinosa-Urgel *et al.*, 2000). The genome sequence of *P. aeruginosa* PAO1 (Stover *et al.*, 2001) encodes a 163 known or predicted outer membrane proteins of which 64 are found as part of three families of porins, the OprD family of specific porins (19 members), the OprM family of efflux porins (18 members), and the Ton B-interacting family of global porins (35 members). The remainder is grouped together as general/nonspecific porins (Hancock and Brinkman, 2002). Although the function of only a few is known, it can be expected that some of these

may play a role in *P. aeruginosa* biofilm formation. The first outer membrane protein reported to play a role in *P. aeruginosa* biofilm development was OprF, and appears to be required for biofilm development under anaerobic conditions (Yoon *et al.*, 2002). Using *in vivo* expression technology (IVET) to study gene expression in mature biofilms followed by analysis of mutant strains, Finelli *et al.* (2003) reported that loss of the putative porin OpdF has detrimental effects on *P. aeruginosa* biofilm formation. However, the exact function of these porins in biofilm formation is not yet known, nor is it known during which stage of biofilm development they may be required.

The expression of genes encoding several different membrane proteins has also been shown to be up-regulated in *P. putida* biofilm cells grown on silicone tubing. These included *nlpD*, which encodes an outer membrane lipoprotein, *potB*, which encodes a component of the polyamine ABC transporter, *mexA*, the gene for a resistance/nodulation/cell division/multidrug efflux pump and *ybaL*, which encodes a probable K^+ efflux transporter. In *P. aeruginosa*, the expression of *tatA* and *tatB*, both of which encode translocation proteins, *tolA*, which encodes a product affecting lipopolysaccharide structure, and *omlA*, which encodes an outer membrane protein, was up-regulated in mature biofilm cells compared to their planktonic counterparts (Whiteley *et al.*, 2001). In a subsequent study, Sauer *et al.* (2002) reported that expression of porin protein E1 and two probable protein components of an ABC transporter was also up-regulated in *P. aeruginosa* biofilm cells.

1.3.3 Importance of extracellular polysaccharides

Bacterial extracellular polysaccharides (EPS) may also influence attachment and initial biofilm development, since these factors contribute to cell surface charge, which affects electrostatic interactions between bacteria and the substratum (Van Loosdrecht *et al.*, 1989). Adhesiveness of *Pseudomonas* species has been reported to be related to the presence and composition of polysaccharides (Williams and Fletcher, 1996). Substantially reduced attachment to biotic and abiotic surfaces was observed in O-polysaccharide-deficient *Pseudomonas* spp. (Dekkers *et al.*, 1998; DeFlaun *et al.*, 1999), whilst changes in *P. aeruginosa* lipopolysaccharide (LPS) resulted in an altered attachment behaviour (Makin and Beveridge, 1996). For example, a *P. aeruginosa* strain containing a mutant B-band LPS showed reduced attachment to hydrophilic surfaces and increased attachment to hydrophobic

surfaces. *E. coli* strains with mutations in the LPS core biosynthetic genes *rfaG*, *rfaP* and *galU* also displayed reduced attachment to surfaces (Genevaux *et al.*, 1999).

Following attachment of bacteria to a surface, numerous changes in gene expression are initiated, which may enable the bacteria to adapt to the changing environment. In *P. aeruginosa*, expression of the *algC* and *algD* genes has been reported to be up-regulated following bacterial adhesion (Davies *et al.*, 1993; Davies and Geesey, 1995; Hoyle *et al.*, 1993). Both these genes form part of the alginate biosynthetic operon (*algD-algA* PA3540-PA3551), which controls alginate synthesis, while the *algC* gene is also required for LPS core biosynthesis (Gacesa, 1998; Ramsay and Wozniak, 2005). The expression of *algC* was shown to be activated as early as 15 min after the bacterial cell attaches to either a Teflon or glass substratum and cells that did not undergo *algC* up-regulation were less able to remain attached to the surface relative to cells in which expression is activated (Davies and Geesey, 1995). Thus, *algC* appears to not only be required for initial cell-to-surface attachment, but it may also be important to maintain attachment. Garrett *et al.* (1999) have extended upon these studies by reporting the existence of a link between the regulation of flagellar biosynthesis and alginate production. Induction of the alternative sigma factor *algT* (also known as σ^{22}/AlgU), which controls alginate biosynthesis, resulted in decreased expression of the *fliC* gene, which encodes flagellin, the structural subunit of flagella. Thus, induction of *algT* results in increased alginate synthesis and a coordinate decrease in flagellum synthesis. Garrett *et al.* (1999) proposed that *algT* activates a negative effector of flagellum synthesis, although the precise mechanism by which *algT* modulates *fliC* expression is not known. Interestingly, expression of *mucC*, a negative regulator of alginate synthesis, was found to be up-regulated in biofilm cells of *P. putida* (Boucher *et al.*, 2000; Nunez *et al.*, 2000; Sauer and Camper, 2001), indicating that alginate expression, in contrast to *P. aeruginosa*, is down-regulated in biofilm cells of *P. putida* following attachment.

In addition to its role in facilitating irreversible attachment, the production of EPS also appears to play a role in determining the biofilm structure (Stoodley *et al.*, 2002). Microbial EPS are biosynthetic polymers that can be highly diverse in chemical composition and include polysaccharides, which can be highly branched with a wide variety of linkages and side groups, as well as nucleic acids, proteins and phospholipids (Flemming *et al.*, 2000; Leriche *et al.*, 2000; Sutherland, 2001). Recent studies have suggested that alginate expression is not required for *in vitro* biofilm formation by non-mucoid *P. aeruginosa* strains

(Hentzer *et al.*, 2001; Nivens *et al.*, 2001; Wozniak *et al.*, 2003). Moreover, several independent groups have reported the involvement of alternative polysaccharide-encoding genes in the initiation of biofilm formation by non-mucoid *P. aeruginosa* strains PAO1 and PA14. These gene clusters, designated *psl* (polysaccharide synthesis locus) and *pel* (pellicle formation), are required for biofilm development in *P. aeruginosa* and encode either a mannose- or a glucose-rich exopolysaccharide, respectively (Friedman and Kolter, 2004; Jackson *et al.*, 2004; Matsukawa and Greenberg, 2004). Despite alginate not being required for biofilm formation by non-mucoid *P. aeruginosa* strains *in vitro*, it does appear to play an important role in determining biofilm structure. Not only does *P. aeruginosa* FRD1, a mucoid strain, form thick structurally differentiated biofilms (Nivens *et al.*, 2001), but structural complexity could be induced in the flat undifferentiated biofilm of a non-mucoid strain of wild-type *P. aeruginosa* PAO1 by overexpression of alginate (Hentzer *et al.*, 2001).

In addition to EPS, the biofilm matrix also contains a significant amount of nucleic acids (Whitchurch *et al.*, 2002; Matsukawa and Greenberg, 2004). It is yet to be established if the nucleic acids found in EPS have a structural role or are merely remnants of lysed cellular debris. However, it has been suggested that extracellular DNA may play a structural role in the early events of biofilm formation since microcolonies could be dissipated in 60-h biofilms when exposed to DNase I (Whitchurch *et al.*, 2002). Interestingly, Ghigo (2001) reported that the presence of conjugative plasmids induced biofilm formation and hypothesised that the high cell densities in biofilms also favour higher rates of horizontal transfer of plasmid DNA.

1.3.4 Importance of quorum sensing

Although quorum sensing is normally associated with the regulation of *P. aeruginosa* virulence factors (Van Delden and Iglewski, 1998; Lazdunski *et al.*, 2004), several studies have linked quorum sensing and biofilm formation (Davies *et al.*, 1998; De Kievit *et al.*, 2001a). Acylated homoserine lactones (AHLs), which are quorum sensing signal molecules, have been shown to be present both in aquatic biofilms grown on submerged stones (McLean *et al.*, 1997) and in biofilms formed on urethral catheters (Stickler *et al.*, 1998). *P. aeruginosa* possesses two complete, semi-independent quorum sensing systems, designated *las* and *rhl*. These two quorum-sensing systems are inter-related in that LasR activates the expression of the *rhlR* and *rhlI* genes (Latifi *et al.*, 1996; Pesci *et al.*, 1997; Whiteley *et al.*, 1999; Parsek and Greenberg, 2000). Each quorum sensing system consists of a

transcriptional activator, LasR (Gambello *et al.*, 1993) and RhlR (Brint and Ohman, 1995), and a cognate autoinducer synthetase, LasI and RhlI respectively. LasI directs the synthesis of the autoinducer *N*-(3-oxododecanoyl)-L-homoserine lactone ($3OC_{12}$-HSL) (Pearson *et al.*, 1994), whilst RhlI directs the synthesis of the autoinducer *N*-butyryl-L-homoserine lactone (C_4-HSL) (Pearson *et al.*, 1995).

The formation of a mature differentiated biofilm by *P. aeruginosa* has been reported to be dependent on the synthesis of $3OC_{12}$-HSL (Davies *et al.*, 1998). Whereas a *P. aeruginosa lasI* mutant produced biofilm that was much thinner and lacked the three-dimensional architecture of the wild-type biofilm, a *rhlI* mutant biofilm closely resembled the wild-type biofilm, suggesting that the *las* quorum system, but not the *rhl* quorum system, is important for *P. aeruginosa* biofilm development into three-dimensional structures. In addition, when the autoinducer $3OC_{12}$-HSL was added to the growth medium of *lasI* mutant bacteria, the cells developed biofilms that were indistinguishable from the wild-type organism (Davies *et al.*, 1998). Furthermore, reporter gene studies by De Kievit *et al.* (2001a) indicated that up-regulation of the *lasI* gene corresponded with the metamorphosis of *P. aeruginosa* microcolonies to the three-dimensional architecture characteristic of mature biofilms. In contrast, *rhlI* expression fluctuated very little during biofilm development. However, only approximately 5 to 15% of the cells expressed *rhlI*, and these cells were concentrated around the base of the biofilm.

In contrast to the above studies, which clearly indicate a role for the *las* quorum sensing system in *P. aeruginosa* biofilm development, Heydorn *et al.* (2002) reported no differences in the biofilm structure and density between *P. aeruginosa lasI* mutant and wild-type PAO1 strains. These results suggest that twitching motility is not required for microcolony formation under conditions of flow and that cell-to-cell signaling via *lasI-lasR* quorum sensing is not required for development of mature biofilms. These discordant results may be due to differences in flow, experimental duration, nutritional source and method of analysis when compared to the conditions used in the above-mentioned investigations.

Analysis of the *P. aeruginosa* transcriptome (Schuster *et al.*, 2003; Wagner *et al.*, 2003) has led to the identification of 315 quorum-induced and 38 quorum-repressed genes, representing *ca.* 6% of the *P. aeruginosa* genome (Schuster *et al.*, 2003). Although quorum sensing may therefore play an important role in regulating gene expression, the importance of cell-to-cell

signaling molecules in biofilm formation specifically has been addressed in a study by Whiteley *et al.* (1999). In a global screen, nearly 40 widely different genes were isolated whose expression was found to be activated by AHLs. However, the role of these genes in *P. aeruginosa* biofilm development still awaits further analysis. Sauer *et al.* (2002) have subsequently reported that quorum sensing does not account for all biofilm-specific protein production in *P. aeruginosa*. The results obtained imply that quorum sensing accounts for only a portion of the total number of genes whose regulation is altered during the irreversible stage of biofilm development and that the physiological change in attached bacteria is not due solely to induction by the $3OC_{12}$-HSL autoinducer. These results furthermore imply that undiscovered biofilm regulons probably exist and suggests that the quorum sensing systems are under intercellular, as well as extracellular control (Sauer *et al.*, 2002; Stoodley *et al.*, 2002).

1.4 REGULATION OF BIOFILM FORMATION

The transition from planktonic growth to surface-attached growth follows a complex pathway and appears to require a regulatory cascade that controls the temporal and spatial expression of biofilm-specific genes (O'Toole *et al.*, 2000a; Stoodley *et al.*, 2002). As evidenced from the preceding sections, a plethora of genes associated with biofilm development has been identified. However, sorting out the roles of these and identifying the underlying regulating processes and factors may be a much more complex task. This is mainly due to the cyclical and dynamic nature of biofilm formation, *i.e.* external signals trigger alterations in the expression of a subset of genes required for biofilm formation and the formation of a biofilm then alters the microenvironment of its own inhabitants, which leads to additional alterations in gene expression. Complicating the study of gene expression further is that biofilms are heterogeneous with respect to gene expression due to the local variations in pH, nutrient and oxygen availability, and concentrations of bacterial metabolites (DeBeer *et al.*, 1994; Xu *et al.*, 1998; Sternberg *et al.*, 1999). Nevertheless, genetic and molecular techniques used in combination with advanced microscopy techniques have contributed greatly towards unravelling some regulatory processes and factors underlying the biofilm formation process.

1.4.1 Two-component signal transduction pathways

Attachment of bacterial cells to a surface is dependent both on chance (*i.e.* whether or not the bacterium comes in direct contact with the surface) and on favourable cell-surface interactions to overcome the repulsive forces generated between the two surfaces (Van Loosdrecht *et al.*, 1990; Geesey, 2001). Although the initial contact of a bacterial cell with a surface is not necessarily regulated, evidence has been presented that indicates that the formation of stable cell-surface interactions is regulated by two-component signalling systems. Such systems comprise two proteins: a sensor kinase that perceives an environmental signal or cue, autophosphorylates, and then activates its partner, a protein known as a response regulator. The activated response regulator subsequently functions as the transcriptional activator of one or more genes whose production allows the bacterium to rapidly adjust to environmental conditions (Lengeler *et al.*, 1999).

Two different two-component signalling systems have been identified in *E. coli* that indicate that these bacteria can sense contact with a surface and, in response, alter gene expression to promote stable cell-surface interactions. The first of these is the CpxA/CpxR signalling system, which is composed of CpxA, a sensor kinase and phosphatase, and CpxR, a response regulator (Raivio and Silhavy, 1997). When *E. coli* cells interact with a hydrophobic surface, the Cpx pathway is activated and the transduction of the signals through this pathway is dependent on the outer membrane protein NlpE, which may be the direct sensor of contact with a surface (Otto and Silhavy, 2002). Consistent with stable cell-surface interactions being required for biofilm formation by *E. coli*, a *cpxR* mutant strain forms less stable cell-surface interactions in comparison with the wild-type strain (Otto and Silhavy, 2002). Although the genes regulated by the Cpx pathway that are required to enable stable cell-surface interactions are as yet undefined, it is known that the Cpx signalling pathway positively regulates P-pili (Hung *et al.*, 2001), which may play a role in surface adhesion.

The second *E. coli* two-component signalling system, the EnvZ/OmpR signalling system, appears to have a role in promoting stable cell-surface interactions in response to increased osmolarity, since this system is activated to generate phosphorylated OmpR under conditions of increasing osmolarity (Pratt and Silhavy, 1995). Phosphorylated OmpR may contribute to biofilm formation by binding to and activating the promoter of *csgD*, which is a positive regulator of transcription of the *csgAB* operon that encodes the structural subunits required for

curli biosynthesis (Romling *et al.*, 1998). Curli have been reported previously to enhance biofilm formation (Vidal *et al.*, 1998; Prigent-Combaret *et al.*, 2000). It has been proposed that the EnvZ/OmpR signalling system may represent a mechanism by which *E. coli* can respond to surfaces under nutrient-limiting conditions (Stanley and Lazazzera, 2004). Under conditions when the medium has a low concentration of nutrients, the nutritionally rich zone on the attachment substratum represents a zone of higher osmolarity compared to the surrounding medium. Consequently, the EnvZ/OmpR system may provide the cells with a mechanism for responding to such an osmolarity gradient by promoting adhesion and biofilm formation. Conversely, under conditions of high osmolarity in the surrounding medium, which have been reported to inhibit biofilm formation by *E. coli* and *P. fluorescens* (O'Toole and Kolter, 1998b; Prigent-Combaret *et al.*, 2001), the cells would remain in the planktonic phase and thus be able to relocate to more favourable environments.

A small number of other regulatory factors involved in *P. aeruginosa* biofilm development have also been described. Amongst these is a two-component-type regulatory gene, *pvrR*, which controls the phenotypic switch between wild-type morphology and a rough small colony variant. The variant has been reported to posses increased biofilm forming capacity and antibiotic tolerance (Drenkard and Ausubel, 2002). It has also been reported that GacA, the global virulence response regulator of the GacS/GacA two-component regulatory system (Reimmann *et al.*, 1997; Rahme *et al.*, 1997), is involved in *P. aeruginosa* biofilm formation in that cells lacking GacA adhered to surfaces but failed to develop into mature biofilm structures (Parkins *et al.*, 2001). The mutant strain failed to form microcolonies and displayed a 10-fold reduction in biofilm formation capacity relative to the wild-type strain. These defects in biofilm development were found to be independent of the *las* and *rhl* quorum sensing systems, alginate production and twitching motility (Parkins *et al.*, 2001). However, both the signal to which GacS responds, as well as the GacA-regulated genes required for biofilm formation has not yet been identified.

Finelli *et al.* (2003) recently identified a new regulator involved in *P. aeruginosa* biofilm formation. The new regulator is encoded by an open reading frame designated PA3782, which appears to code for a transcriptional regulator of the AraC-XylS family. This family of transcriptional regulators is involved in regulation of carbon metabolism, production of virulence factors, including fimbriae and adhesins, and responds to environmental stresses (Gallegos *et al.*, 1997). Although *P. aeruginosa* lacking PA3782 was significantly impaired

in its ability to form normal biofilms, further research is needed to identify genes whose expression is under PA3782 control in order to understand its role in biofilm formation.

More recently, Kuchma *et al.* (2005) described a three-component regulatory system that appears to be required for biofilm maturation by *P. aeruginosa*. The system, designated SadARS, is comprised of genes encoding for a putative sensor histidine kinase (*sadS*, PA3946) and two response regulators (*sadR*, PA3947 and *sadA*, PA3948). Although nonpolar mutations in any of the *sadARS* genes did not confer defects in growth or early biofilm formation, swimming, or twitching motility, it did, however, result in biofilms with an altered mature structure in flowing systems. The mutant biofilms appeared to be more homogeneous than the wild-type strain in that they failed to form large and distinct macrocolonies and showed a drastic reduction in water channels. DNA microarray studies were subsequently used to identify downstream targets of the SadARS system and among the genes regulated by the SadARS system were those required for type III secretion. Since mutations in the type III secretion genes were shown to result in strains with enhanced biofilm formation, is has been proposed that the SadARS regulatory system may function to promote biofilm formation, possibly in part by repressing the type III secretion system genes. However, the mechanism by which SadARS regulates expression of these genes is unclear as yet.

1.4.2 Factors regulating carbon metabolism

The *P. aeruginosa crc* gene, which encodes a global carbon metabolism regulator, has been shown to be required for biofilm formation (O'Toole *et al.*, 2000b). Crc is activated by tricarboxylic acid (TCA) cycle intermediates, the preferred carbon source for *P. aeruginosa* (O'Toole and Kolter, 1998a). In addition, Crc activates the transcription of *pilA* (O'Toole *et al.*, 2000b), which encodes the structural subunits required for type IV pili biosynthesis (Alm and Mattick, 1997). Strains of *P. aeruginosa* with mutations in *crc* are arrested at the surface-attached step of biofilm formation and do not form microcolonies (O'Toole *et al.*, 2000b). Since type IV pili-mediated twitching motility is required for *P. aeruginosa* biofilm formation by bringing cells together to form a microcolony (O'Toole and Kolter, 1998b), Crc has been proposed to link carbon source availability to the decision whether or not to enter a biofilm mode of growth.

The global carbon regulator CsrA was also recently reported to affect biofilm formation in *E. coli* (Jackson *et al.*, 2002). In contrast to *P. aeruginosa*, disruption of the *csrA* gene increased biofilm formation compared to the wild-type parental strain, whilst overexpression of CsrA was inhibitory in *E. coli* K-12 and in pathogenic isolates. The disruption of *csrA* enhanced biofilm formation even in the absence of extracellular appendages shown previously to be important for biofilm formation. The primary effect of CsrA appears to be as a regulator of glycogen metabolism and it was suggested that glycogen might be the principle carbon/energy source for stationary phase biosynthesis of adhesion factors such as pili, curli and fimbriae (Jackson *et al.*, 2002).

1.4.3 Phase-dependent regulators

The sigma factor RpoS, originally identified in *E. coli* (Hengge-Aronis, 1996), occurs at low levels in the exponential phase, but accumulate in *E. coli* (Lange and Hengge-Aronis, 1991) and in *P. aeruginosa* (Fujita *et al.*, 1994) at the onset of the stationary phase. In *E. coli*, RpoS appears to be obligatory for biofilm development (Adams and McLean, 1999), but other studies, however, have disputed the role of RpoS in biofilm formation (Corona-Izquierdo *et al.*, 2002). In *P. aeruginosa*, while RpoS regulates the general stress response as it does in *E. coli*, it does not appear to be obligatory for the initial stages of biofilm development (Suh *et al.*, 1999). However, *rpoS* mutant strains of *P. aeruginosa* form significantly thicker biofilm than the wild-type strains (Heydorn *et al.*, 2000; Whiteley *et al.*, 2001). Since RpoS production is regulated at multiple levels in response to stress conditions, including nutrient limitation (Venturi, 2003), it has been proposed that RpoS may regulate the depth of the mature biofilm structure to allow for maximal nutrient acquisition (Stanley and Lazazzera, 2004). As biofilms become sufficiently large, cells in the centre would have reduced access to nutrients, resulting in activation of RpoS, thereby signaling that nutrients are limiting in *P. aeruginosa* biofilms and that there is a growth disadvantage to being within a biofilm. Consequently, biofilm-associated cells may be released into the planktonic phase, thus allowing them to relocate to more favourable environments.

1.4.4 Quorum sensing

Recently, the role of rhamnolipid in the biofilm microcosm of *P. aeruginosa* has been investigated (Davey *et al.*, 2003). It was reported that rhamnolipid surfactant, of which the production is under the control of RhlR (Pearson *et al.*, 1997; Medina *et al.*, 2003), is required

for the maintenance of the pillar structures and water channel structures seen in biofilms, probably by reducing surface tension (Davey *et al.*, 2003). It has subsequently been proposed that rhamnolipid may prevent invading bacteria from colonising open spaces in the biofilm and it may play a role in maintaining the nutritional balance of the biofilm (Espinosa-Urgel, 2003). Consistent with quorum sensing being required for surfactant production and the maintenance of biofilm architecture, *lasI* mutant strains of *P. aeruginosa* have been found, under some environmental conditions, to lack the complex differentiated structure seen in biofilms formed by the wild-type strain (Davies *et al.*, 1998; De Kievit *et al.*, 2001a). This is likely to result, at least in part, from lower levels of rhamnolipid production in the quorum sensing mutant strains.

1.5 THE BIOFILM PHENOTYPE

Previously, biofilms were thought to be composed of cells with similar phenotypic characteristics. It has, however, become increasingly clear that, at least in pure culture biofilms, biofilms are composed of phenotypically different cells working together in order to build and maintain the biofilm. This might not be surprising, as at any one time free-floating cells may be recruited from the bulk fluid to the biofilm (Tolker-Nielsen *et al.*, 2000), whilst biofilm-associated cells may divide (Tolker-Nielsen *et al.*, 2000), die (Webb *et al.*, 2003b) or detach from the biofilm (Sauer *et al.*, 2004). In addition, the channel-and-pillar architecture of mature biofilms creates physicochemical gradients in terms of nutrients, oxygen, pH and osmolarity (DeBeer *et al.*, 1994; Xu *et al.*, 1998; Sternberg *et al.*, 1999). It is therefore to be expected that, under such conditions, expression of different sets of genes would result in phenotypes that differ significantly from those of planktonically grown cells (Sauer *et al.*, 2002; Oosthuizen *et al.*, 2002). Most notably, biofilms are resistant to antimicrobial agents and it has been reported that bacteria existing in a biofilm can become up to 1000-times more resistant to antimicrobial agents than planktonic cells (Nickel *et al.*, 1985; Gristina *et al.*, 1987). Although the increased resistance to antimicrobial agents has been suggested to be a consequence of the distinct phenotypic changes associated with growth on a surface (Mah and O'Toole, 2001; Drenkard, 2003), the molecular basis of biofilm resistance remains elusive.

1.5.1 Phenotypic differentiation during biofilm development

In *P. aeruginosa*, molecular and microscopic evidence have suggested the existence of a succession of biofilm phenotypes (Sauer *et al.*, 2002; Stoodley *et al.*, 2002). Two-dimensional polyacrylamide gel electrophoresis of cells representing each of the distinct stages during the development of a *P. aeruginosa* biofilm (Fig. 1.1), have indicated that the average difference in protein production between each developmental stage was 35% of detectable proteins (Sauer *et al.*, 2002). The transition from planktonic growth to the stage of irreversible attachment resulted in a 29% change in the production of detectable proteins. The transition from irreversibly attached cells to the stage of mature biofilms caused a change in the protein production of 40%, with the majority of proteins showing an increase in concentration. In contrast, the transition from mature-stage biofilm to the dispersion stage resulted in a reduction in 35% of detectable proteins. Cells during this stage of development had protein profiles that were more similar to planktonic cells than to mature-stage biofilm cells. The most profound differences were, however, observed when planktonic cells were compared to mature biofilm cells, with more than 800 detectable proteins showing more than a six-fold change in concentration (Sauer *et al.*, 2002).

1.5.2 Antimicrobial resistance of *P. aeruginosa* biofilms

P. aeruginosa has an inherent resistance to numerous antimicrobial agents that is even more pronounced when this organism is found growing in a biofilm (Costerton *et al.*, 1999). Antimicrobial resistance is a trait typical of most of biofilm organisms and it has been speculated that biofilms are the causative agent of up to 65% of bacterial infections (Potera, 1999), including native valve endocarditis, osteomyelitis, dental caries, medical device-related infections, ocular implant infections and chronic lung infections in cystic fibrosis patients (Costerton *et al.*, 1987; 1999; Hall-Stoodley *et al.*, 2004). Consequently, much research is being directed towards studying the effects of antimicrobial agents on biofilms and the mechanisms underlying the recalcitrance of *P. aeruginosa* biofilms to antimicrobial agents.

1.5.2.1 Mechanisms of biofilm resistance

The induction of a biofilm-specific phenotype has been proposed to lead to the activation of mechanisms that are critical for the development of resistance to antimicrobials (Mah and O'Toole, 2001; Drenkard, 2003). Using DNA microarrays, Whiteley *et al.* (2001) reported a set of 20 genes that were differentially expressed in *P. aeruginosa* biofilms exposed to high

levels of the antibiotic tobramycin compared to untreated biofilms. Among them, expression of *dnaK* and *groES*, both of which are involved in stress responses, and two probable efflux systems were up-regulated. However, further studies are required to identify the resistance mechanisms associated with the induction of specific genes in *P. aeruginosa* biofilms. In addition to the induction of a biofilm-specific phenotype, several other mechanisms have been suggested to account for biofilm resistance to antimicrobial agents. These include the presence of an exopolysaccharide matrix that can slow the diffusion of antibiotics (Stewart, 1996), slow growth (Brown *et al.*, 1988; Gilbert *et al.*, 1990; Evans *et al.*, 1991) owing to nutrient and oxygen limitation (Tack and Sabath, 1985; DeBeer *et al.*, 1994; Xu *et al.*, 1998) or owing to activation of the general stress response initiated by growth in a biofilm (Brown and Barker, 1999; Cochran *et al.*, 2000), and the presence of multidrug efflux pumps (Maira-Litran *et al.*, 2000; Poole and Srikumar, 2001). How each of these different proposed mechanisms contributes to the overall resistance displayed by bacterial biofilms is, however, unclear. For example, with the exception of that of aminoglycosides, the exopolysaccharide matrix has not been found to notably retard diffusion of fluoroquinolones (Shigeta *et al.*, 1997; Ishida *et al.*, 1998; Walters *et al.*, 2003) and since most antibiotics target primarily metabolically active cells it may therefore not be surprising that slow-growing and non-growing bacteria could contribute considerably to a decrease in biofilm susceptibility to antimicrobial agents (Lewis, 2001; Gilbert *et al.*, 2002). Moreover, it has also been reported that four different multidrug resistance efflux pumps (MexAB - OprM, MexCD - OprJ, MexEF - OprN and MexXY) do not play a role in *P. aeruginosa* biofilm resistance to antimicrobial agents (Brooun *et al.*, 2000; De Kievit *et al.*, 2001b).

1.5.2.2 Persister cells, phenotypic variants and mutant cells

In a recent study of *P. aeruginosa* biofilms, Brooun *et al.* (2000) reported that the majority of cells were killed by low concentrations of antibiotics and despite further increases in antibiotic concentration, a small fraction of biofilm cells remained that were invulnerable to killing. It was subsequently shown that these cells, termed persister cells, were largely responsible for the high tolerance of *P. aeruginosa* biofilms to antimicrobial agents (Spoering and Lewis, 2001). Unlike resistant mutants, persisters are phenotypic variants of the wild-type cells that upon reinoculation produce a culture with a similar amount of persister cells (Keren *et al.*, 2004). It has been proposed that the function of persister cells is thus to ensure the survival of the population in the presence of lethal factors (Lewis, 2001). Based on the

premise that antimicrobial agents are not directly responsible for cell death but that they cause cell damage that indirectly triggers programmed cell death, Lewis (2001) proposed that persister variants ensure survival of the biofilm by having a defective programmed cell death program. More recently, the gene expression profile of persister cells of an *E. coli* culture was determined and it was suggested that the formation of persister cells is dependent on chromosomally encoded toxin-antitoxin modules (Keren *et al.*, 2004). Whilst overexpression of both RelE and HipA toxins caused an increase in multidrug tolerant persister cells, deletion of the *hipBA* module caused a 10- to 100-fold decrease in persister cells in stationary and biofilm cultures. Based on the results obtained, a revised model of persister cell production and antibiotic tolerance was proposed. Due to random fluctuations in the ratio of toxin-antitoxin in a population, it was proposed that some bacterial cells (*ca.* 1% of the population) would express high levels of a toxin, thus giving rise to persister cells. Since bactericidal antibiotics function by binding to a target protein and corrupting its function thereby generating a lethal product that results in cell death, it may be that in persister cells the target protein is blocked by binding of a toxin protein and its function is thus inhibited. Although the antibiotic can bind to the blocked target protein, it can no longer corrupt its function and the result is antibiotic tolerance, allowing the cells to survive (Keren *et al.*, 2004). Moreover, Drenkard and Ausubel (2002) also reported the presence of phenotypic variants in *P. aeruginosa* PA14 populations, at frequencies of *ca.* 10^{-6}, that are resistant to high concentrations of antibiotics. It was suggested that phenotypic variants present in the biofilm population are partially responsible for the elevated levels of resistance to antimicrobial agents observed in *P. aeruginosa* biofilms. A regulatory protein, PvrA, that controls the conversion between antibiotic-resistant variants and antibiotic-susceptible forms was subsequently shown to regulate biofilm formation and resistance to antibiotics, suggesting that there is a link between phenotypic variation and biofilm resistance (Drenkard and Ausubel, 2002).

In addition to the phenotypic variants reported above, Mah *et al.* (2003) reported the identification of a mutant of *P. aeruginosa* PA14 that was capable of forming mature biofilms but do not develop high-level biofilm-specific resistance to antibiotics. The locus identified, designated as *ndvB*, encodes for a glucosyltransferase that is required for the synthesis of cyclic glucans. The glucans are circular polymers of glucose that are located in the periplasm and are also secreted into the extracellular media. Based on the physical interaction of these glucans with the antibiotic tobramycin, it was suggested that these glucose polymers might be

responsible for sequestering antimicrobial agents in the periplasm and therefore prevent them from reaching their sites of action in the cytoplasm. Alternatively, it was also proposed that the periplasmic glucans may contribute to antibiotic resistance of biofilm cells by slowing diffusion of antibiotics into the cell, thereby allowing the bacteria additional time to adapt to the antibiotic influx (Mah *et al.*, 2003).

1.6 THE STUDY OF BACTERIAL BIOFILMS

As highlighted previously (Section 1.4), recent advances in the study of biofilm development have indicated that the formation of surface-attached communities requires complex regulatory systems to sense and respond to environmental- and bacterial-derived signals. It is not surprising, therefore, that diverse changes in gene expression are necessary to develop the complex architecture and unique physiological properties of a mature biofilm. Towards understanding the global effects triggered during the formation of biofilms, a variety of complementary approaches have been used to monitor changes in gene expression.

1.6.1 Culturing systems

The study of biofilms and biofilm-associated gene expression has been facilitated greatly by the development of various tools for the culturing and analysis of biofilm communities. Biofilms of *P. aeruginosa* have been studied under conditions of flow, primarily by using chemostats (Sauer *et al.*, 2002), a pebble reactor (Whiteley *et al.*, 2001), silicone tubing (Sauer and Camper, 2001; Finelli *et al.*, 2003) and flow cells (Zinn *et al.*, 1999; McLean *et al.*, 1999), or in stagnant batch culture using microtitre plates (O'Toole and Kolter, 1998a).

Whereas flow cells supporting biofilm growth have enabled real-time microscopic investigations of the biofilm and cell behaviour (Palmer, 1999; Heydorn *et al.*, 2000; Klausen *et al.*, 2003; Werner *et al.*, 2004), flow-through systems, such as chemostats and once-flow through silicon tubing, have been used to positively select mutants with increased biofilm formation capacities (Vidal *et al.*, 1998) and to study biofilm gene expression at either the proteome (Sauer and Camper, 2001; Sauer *et al.*, 2002) or RNA (Whiteley *et al.*, 2001) levels. Unfortunately, such flow-through systems are not readily amenable to high throughout analysis.

In contrast to the above, microtitre plates have been very useful in studies aimed at high throughout screening of transposon mutants unable to initiate biofilm formation in both *E. coli* (Pratt and Kolter, 1998; Prigent-Combaret *et al.*, 1999) and *Pseudomonas* spp. (O'Toole and Kolter, 1998a; 1998b). These studies are, however, limited by the use of a static mode of growth. Under such growth conditions, gradients of oxygen and other factors may form across the biofilm, thus influencing the range of genes identified. Consequently, the main outcome of this approach has been to identify a variety of surface organelles (flagella, pili, fimbriae, adhesins) that participate in the initial stages of biofilm formation. Several studies aimed at investigating biofilm-specific gene expression using a proteomic approach have reported the use of agar-embedded bacterial colonies (Jouenne *et al.*, 1994; Tresse *et al.*, 1997; Perrot *et al.*, 2000; Vilain *et al.*, 2004), as well as colonies growing on the agar surface (Miller and Diaz-Torres, 1999) as a source of biofilm cells. However, "biofilms" obtained in this manner also develop under static growth conditions and it is doubtful as to whether this mode of "biofilm" growth is capable of inducing the full spectrum of genes involved in biofilm development and maintenance.

Recently, an alternative culturing system has been described whereby the yield of biofilm biomass can be increased for subsequent analysis. This system relies on culturing of the bacteria in batch cultures in the presence of glass wool as an attachment surface (Oosthuizen *et al.*, 2001; 2002). The glass wool provides a large surface-to-volume ratio and allows for easy separation of the biofilm biomass from the surrounding planktonic cells for further characterisation. Although cells cultured in this manner develop under batch conditions, it is possible to introduce flow by varying the size of the culture flask.

1.6.2 Approaches to studying biofilm-specific gene expression

Although classical genetic approaches based on the use of random transposon insertion mutagenesis have been useful in identifying genes that are required for biofilm formation, major differences in gene expression is, however, thought to occur during switching from the planktonic to biofilm mode of growth (Sauer *et al.*, 2002; Stoodley *et al.*, 2002). Indeed, in *E. coli* a study of biofilm gene expression showed that of 446 transposon-mediated *lacZ* fusions examined, 38% were differentially expressed by at least two-fold between biofilm and planktonic cells (Prigent-Combaret *et al.*, 1999). Similarly, Oosthuizen *et al.* (2002) reported that 6% of the 345 protein spots analysed were up-regulated in 18-h old biofilms of *Bacillus*

cereus DL5 compared to planktonic cells. These results suggest that a significant fraction of the bacterial genome could be involved or affected during biofilm formation. Consequently, global genome-wide profiling approaches, as highlighted below, have become increasingly more popular as a means to extend current knowledge regarding the identity of biofilm-specific genes and changes in gene expression during biofilm development.

1.6.2.1 Reporter gene-based approaches

Reporter gene technology has proved to be extremely valuable in the study of environmental control of gene expression (DeVault *et al.*, 1990; Davies *et al.*, 1993; Sternberg *et al.*, 1999; De Kievit *et al.*, 2001a; Sauer *et al.*, 2004) and in the isolation of promoters that are induced under particular physiological conditions (Burne *et al.*, 1997; Weyers, 1999). Recently, Finelli *et al.* (2003) described an approach based on the use of *in vivo* expression technology (IVET) as a means to identify genes up-regulated in *P. aeruginosa* grown to a mature biofilm. A reporter gene library of *P. aeruginosa* was screened for clones in which gene expression is turned on during biofilm development. The *P. aeruginosa* IVET system used was based on the complementation of *de novo* adenine biosynthesis by randomly cloned promoters able to drive expression of a promoterless *purEK* operon. Using this approach, three new genes (PA3782, PA3701 and PA0240 [designated *opdF*]) were identified that do not affect planktonic growth but are required for biofilm formation, development and fitness (Finelli *et al.*, 2003). A disadvantage of this technology, however, is the inability to identify genes that are repressed in the biofilm environment.

1.6.2.2 Proteomic approaches

The proteome is defined as the complete protein complement of a genome (Wasinger *et al.*, 1995; Wilkins *et al.*, 1996). It should, however, be kept in mind that the proteome is dynamic and therefore the proteome of an organism will reflect the immediate environment in which it is studied. Consequently, a comprehensive description of the proteome of an organism not only provides a catalogue of all proteins encoded by the genome but also data on protein expression under defined conditions, the occurrence of post-translational modifications, protein-protein interactions and the distribution of specific proteins within an organism (Graves and Haystead, 2002).

Proteome analysis has been used to investigate biofilm-specific gene expression in biofilms of both *P. putida* (Sauer and Camper, 2001) and *P. aeruginosa* (Sauer *et al.*, 2002). The results obtained from this approach indicated that a large number of genes are differentially expressed during biofilm development, possibly correlating with the expression of different biofilm phenotypes (Sauer *et al.*, 2002). In *P. putida* biofilms, proteomic analysis and subtractive cDNA libraries showed that protein patterns changed soon after the initial adhesion on a surface. More than 45 proteins displayed differential expression, suggesting that bacteria undergo physiological changes as early as in the first 6 h after contact with a surface. Of the ten proteins identified, three corresponded to unassigned functions and the others related to functions such as amino acid metabolism, extracellular polymer synthesis, organelle structure and transport processes (Sauer and Camper, 2001). The issue of biofilm-specific changes in protein and gene expression during the later stages of *P. aeruginosa* biofilm formation was subsequently addressed in a study by Sauer *et al.* (2002) in which proteome analysis was carried out under careful microscopic monitoring of the different stages of biofilm development. In contrast to the *P. putida* study, only a few differences in protein levels were observed 8 h after attachment, whilst very different protein patterns were detected after one day and reached a maximum after six days. A total of 57 biofilm-associated proteins were identified that differed from the planktonic profile. Of these, 90% were found to be overexpressed, 23 of which were found to be involved in oxidative damage, EPS production, amino acid and carbon metabolism, and lipid biosynthesis. Following the mature biofilm stage, dispersion of biofilm-associated cells occurred, which coincided with these cells reverting to the planktonic state and most of the differentially expressed genes being repressed (Sauer *et al.*, 2002).

Notably, only a limited number of *P. aeruginosa* outer membrane proteins (three) were identified in the above study (Sauer *et al.*, 2002). The authors speculated that this might have been a consequence of the typically higher hydrophobicity of membrane proteins, thus resulting in poor solubilisation of these proteins during sample preparation and iso-electric focussing, and that the presence of lipids in the protein samples may have interfered with solubilisation in the rehydration buffer. Although many advances have been made in the preparation and solubilisation of membrane and membrane-associated proteins (Molloy *et al.*, 1998; Herbert, 1999; Rabilloud *et al.*, 1997; Molloy *et al.*, 2000; Santoni *et al.*, 2000), proteomics is still faced with a number of challenges. A major challenge is the study of low-abundance proteins. Many important classes of proteins such as transcription factors, protein

kinases and regulatory proteins are low-copy proteins and may not be observed in the analysis of cell lysates without some purification (Cordwell *et al.*, 2000; Nouwens *et al.*, 2000). Furthermore, despite recent advances (Molloy *et al.*, 1998; 2000), further methodological improvements are required to allow visualisation of near-to-total proteomes in order to allow retrieval of a maximum amount of information from functional proteomes (Cordwell *et al.*, 2000; Jungblut, 2001; Graves and Haystead, 2002).

1.6.2.3 Transcriptomic approaches

The comprehensive analysis of transcriptomes is of great value for amongst other, studying gene function and regulation, and gene expression patterns can provide information about the dynamic changes in physiological states and functional activities of a cell under different environmental conditions (Marshall and Hodgson, 1998; Brent, 1999). However, mRNAs are not the functional entities within the cell but just the transmitters for synthesising proteins. Consequently, there may potentially be several problems associated with using mRNA co-expression profiles for understanding gene function and regulation. First, although there is a strong connection between co-expression and gene function, some functionally unrelated genes might have similar expression patterns. Thus, any measurement based on mRNA expression levels may result in misleading interpretations. Second, not all functionally related genes are expressed together, and thus, approaches based on mRNA expression profiles may miss important, functionally related genes. Finally, translational regulation and post-translational modification are also important in determining gene function and regulation, and these cannot be determined using transcriptomics (Freeman *et al.*, 2000; Vasil, 2003).

To date, the only transcriptome analysis comparing *P. aeruginosa* planktonic and biofilm cells has been reported by Whiteley *et al.* (2001) and led to the conclusion that only 1% (73 genes) of the *P. aeruginosa* genes showed differential expression in these two modes of growth. Half of these genes were overexpressed and half were repressed. The genes that were identified were assigned into classes such as motility, attachment, translation, metabolism and regulation. Since only a minor fraction of genes are strongly differentially expressed in biofilms, these results appear to argue against the proposal that living in a biofilm results in dramatic differences in gene expression and regulation (Sauer *et al.*, 2002; Stoodley *et al.*, 2002). In interpreting these results, it should be kept in mind, however, that DNA microarrays provide a sensitive but transient snapshot of gene expression that does not

truly measure translation. In addition, transcription analyses are essentially informative about metabolically active cells and some reports have speculated as to whether such cells are truly representative of biofilms, where a significant fraction of the matrix-embedded bacteria is thought to be almost quiescent (Lewis, 2000; Werner *et al.*, 2004). Alternatively, the low number of biofilm-affected genes identified may also be a consequence of the stringent approach used in order to avoid a plethora of candidates. In the study of Whiteley *et al.* (2001) only genes whose expression varied by a factor of two or more were taken into account, but it has been reported that genes with a less than two-fold variation in expression can be significantly over- or underexpressed and an increase in transcription down to 1.4-fold was shown to be significant (Arfin *et al.*, 2000). Therefore, although the stringent approach might have avoided false-positive candidates, it may have led to underestimating the number of genes that are involved in biofilm formation (Ghigo, 2003).

1.7 AIMS OF THIS INVESTIGATION

As early as 1933, Henrici recognised the phenomenon that marine bacteria grow for most part on submerged surfaces, rather than being free-floating. With the re-discovery that bacteria are found predominantly attached to surfaces in aquatic systems (Geesey *et al.*, 1977; Costerton, 1995), much attention has been paid to unravelling the molecular mechanisms underlying the formation and regulation of biofilms. In the last decade, advances in molecular and microscopic techniques have made in-depth investigations in the field of biofilm physiology more attainable. It has become increasingly clear that the natural assemblage of bacteria within the biofilm functions as a cooperative consortium in a complex and coordinated manner (Davey and O'Toole, 2000; O'Toole *et al.*, 2000a; Stoodley *et al.*, 2002). However, although the physiological commitment necessary for planktonic bacteria to adopt the biofilm phenotype is under intense scrutiny, there are few examples of biofilm-specific regulatory networks. Clearly much is still to be learned regarding the development, maintenance and dissolution of biofilms even in the model organisms, the gram-negative *E. coli* and *P. aeruginosa*.

Detecting genome-wide changes of gene expression under different conditions has become an important aspect of examining functions of genes, as well as to identify regulatory processes and factors involved in biofilm development. Many DNA- and RNA-based technologies,

including microarrays (Whiteley *et al.*, 2001), have been developed to conduct such research. However, the mRNA levels may not always correlate well with the levels of translated proteins (Anderson and Anderson, 1998). Proteome analysis is an alternative approach to analyse differential gene expression at the protein level by comparing the two-dimensional electrophoresis patterns of proteomes under different conditions (Otto *et al.*, 2001; Sauer and Camper, 2001; Sauer *et al.*, 2002; Oosthuizen *et al.*, 2002; Vilain *et al.*, 2004). Since the biological processes are directly executed by proteins, which are dynamically modified and processed at multiple levels during or after maturation, the state of an organism is essentially reflected in its proteome. Consequently, proteome analysis has become a popular method of choice to examine differentially expressed proteins in biofilm bacteria.

Therefore, the aims of this investigation were the following:

- To establish a two-dimensional gel electrophoresis (2-DE) technique in our laboratory whereby high-resolution 2-DE maps of *P. aeruginosa* PAO1 (DSM 1707) could be generated reproducibly, based on the use of ampholyte-containing tube gels.

- To develop a method to culture copious amounts of *P. aeruginosa* PAO1 (DSM 1707) biofilm and to compare protein patterns of the biofilm and biofilm-associated phenotypes.

- To obtain a comprehensive description of the phenotypic differences between biofilm, surface influenced planktonic (SIP) and planktonic cells of *P. aeruginosa* PAO1 (DSM 1707) using 2-DE of sequentially extracted proteins from whole-cell extracts, followed by image analysis using PDQuest software and identification of several differentially expressed protein spots using a combination of N-terminal protein sequencing and peptide mass fingerprinting.

- To generate a *P. aeruginosa* PAO1 (DSM 1707) mutant strain deficient in the outer membrane protein OprG and to compare the capacity of the *P. aeruginosa* wild-type and OprG-deficient mutant strains to form biofilm on glass wool.

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CHAPTER TWO

ESTABLISHMENT OF TWO-DIMENSIONAL GEL ELECTROPHORESIS FOR DETERMINATION OF THE *Pseudomonas aeruginosa* PROTEOME

2.1 INTRODUCTION

In natural and artificial habitats, most bacteria, including *Pseudomonas aeruginosa*, have a strong tendency to adhere to surfaces within microbial consortia called biofilms (Costerton *et al.*, 1995). The formation of a well-developed biofilm is believed to occur in a sequential process of transport of microorganisms to a surface, initial attachment of the microorganisms to the surface, formation of microcolonies and formation of well-developed biofilms (Marshall, 1985; Van Loosdrecht *et al.*, 1990). However, little is known regarding the events following adhesion than about the adhesion process itself. Previous studies have indicated that biofilm cells differ significantly from their planktonic counterparts in terms of their physiology (Anwar *et al.*, 1990) and more recent studies regarding gene expression have indicated the differential expression of numerous genes in biofilm cells when compared to their planktonic counterparts (Sauer and Camper, 2001; Whiteley *et al.*, 2001; Stanley *et al.*, 2003; Beenken *et al.*, 2004).

P. aeruginosa is among the best-studied biofilm formers, due in large part to the long history of study of these organisms, their genetic tractability and the availability of the genome sequence of the laboratory strain PAO1 (Stover *et al.*, 2000). *P. aeruginosa* maintains a particularly large genetic capacity (*ca*. 5 500 genes), including an unusually high number of signal transduction pathways, and is thought to be amongst the most evolved prokaryotes yet discovered (Spiers *et al.*, 2000). Although the completion of the genome sequence for *P. aeruginosa* PAO1 provides a plethora of novel genetic information, the genomic sequence alone may be of limited value in obtaining a complete understanding of gene function or cellular physiology (Van Bogelen *et al.*, 1999a). At best, it provides only the potential framework for the response to a given stimulus, and expression analyses are needed to determine the genetic dynamics of such responses. Since proteins are essentially these units of response, global analysis of protein expression profiles will be invaluable for obtaining a more complete understanding of the biology of *P. aeruginosa*, including its ability to adhere

to surfaces and develop into mature biofilms. Proteomics, the term used to describe global examination of proteins expressed by a genome (Anderson and Anderson, 1996; Wilkins *et al.,* 1996; Klose, 1999; Van Bogelen *et al.*, 1999b; Graves and Haystead, 2002), allows the characterisation of proteins *en masse* and combines high resolution two-dimensional gel electrophorectic separation of proteins with sensitive protein identification, thereby providing a qualitative and quantitative view of gene expression. These proteomic methods have been very effective for characterising the proteomes of many different bacteria (Van Bogelen *et al.*, 1997; Jungblut *et al.*, 2000; Nouwens *et al.*, 2000; Buttner *et al*., 2001; Hecker *et al*., 2003; Liao *et al.*, 2003; Eymann *et al*., 2004).

Two-dimensional gel electrophoresis (2-DE), originally described by O'Farrell (1975) and Klose (1975), involves the separation of proteins according to their iso-electric points (pI) in the first dimension and their molecular mass (Mr) in the second dimension. In the original description of the technique, the first-dimension separation was performed in carrier ampholyte-containing polyacrylamide gels cast in narrow tubes after which the gel rods were removed from their tubes, equilibrated and placed on vertical SDS-polyacrylamide gels for the second-dimension separation. 2-DE is now commonly performed utilizing immobilised pH gradients (IPG) and gel strips supported by a plastic film backing instead of the tube gels (Görg *et al.*, 1985; Görg *et al.*, 1988; Bjellqvist *et al*., 1993). This approach, however, is reliant on specialised equipment and requires a sizeable initial financial investment. In addition to the aforementioned technical improvements, 2-DE has also undergone numerous refinements regarding methods for sample preparation (Rabilloud *et al*., 1997; Rabilloud, 1998; Harder *et al.*, 1999; Herbert, 1999; Pridmore *et al*., 1999), protein staining and analysis of gels (Neuhoff *et al*., 1985; Blum *et al*., 1987; Steinberg *et al*., 1996), and identification of individual proteins (Celis and Gromov, 1999).

Towards understanding and unravelling the molecular mechanisms underlying biofilm formation and maintenance, this study was aimed at using proteomics to investigate global changes in protein expression that take place when planktonic cells of *P. aeruginosa* make the transition to the biofilm mode of growth. However, at the time of its inception, expertise in 2-DE was not only lacking at the University of Pretoria but also nationally. In addition, funding and budgetary constraints precluded the use of immobilised pH gradients and acquisition of the necessary equipment. Consequently, the aim of this part of the investigation was to establish a 2-DE technique in our laboratory whereby high-resolution 2-DE maps of *P.*

aeruginosa could be generated reproducibly, based on the use of ampholyte-containing tube gels, as originally described by O'Farrel (1975). To this end, different sample preparation methods, iso-electric focusing conditions and protein staining methods were investigated and compared.

2.2 MATERIALS AND METHODS

2.2.1 Bacterial strain and culture conditions

P. aeruginosa PAO1 (DSM 1707) was used in all studies. The culture was grown under agitation at 37°C for 18 h in 100 ml of a modified mineral salts medium with glucose and yeast extract (MSGY) of the following composition (in distilled water): 1.74 g/L $NaNH_4HPO_4.4H_2O$; 0.54 g/L $NaH_2PO_4.H_2O$; 0.2 g/L $MgSO_4.7H_2O$; 0.2 g/L yeast extract; 0.04 g/L KCl; 0.005 g/L $FeSO_4.7H_2O$; 5.0 g/L glucose and 1% (v/v) trace mineral solution (2.86 g/L H_3BO_3; 1.81 g/L $MnCl_2.4H_2O$; 0.22 g/L $ZnSO_4.7H_2O$; 0.08 g/L $CuSO_4.5H_2O$; 0.06 g/L $CoCl_2.6H_2O$; 0.025 g/L $Na_2MoO_4.2H_2O$) (Atlas, 1993).

2.2.2 Whole-cell protein extractions

After incubation for 18 h at 37°C, planktonic *P. aeruginosa* cells were collected by centrifugation at 9 000 × *g* for 10 min. The cell pellets were washed twice in 0.2 M sodium phosphate buffer (pH 6.8) after which the excess fluid was blotted away and the pellets used in three different protein extraction methods, as described below.

2.2.2.1 Sample preparation method 1 (SP1)

The cell pellet was suspended in 300 µl 0.2 M sodium phosphate buffer (pH 6.8) and sonicated by three pulses of 15 s each using a 4710 Series Ultrasonic Homogenizer (Cole-Palmer Insrument Co., Chicago, IL, USA) at an output of 40%. Following sonication, 200 µl of Lysis buffer 1 (0.5 M Tris-HCl [pH 6.8]; 5% [v/v] 2-mercaptoethanol; 10% [v/v] glycerol; 2% [w/v] SDS) (Laemmli, 1970) was added to the sample, mixed and incubated at 94°C for 5 min. The sample was then centrifuged at 9 000 × *g* for 10 min to remove the cellular debris. The clarified supernatant was subsequently recovered and stored at -70°C until required.

2.2.2.2 Sample preparation method 2 (SP2)

The cell pellet was suspended in 10 µl of Lysis buffer A (10% [w/v] SDS; 150 mM DTE) (Gravel and Golaz, 1996). Following incubation at 94°C for 5 min, the sample was cooled at room temperature for 2 min after which 485 µl of Lysis buffer B (9 M urea; 65 mM DTE; 65 mM CHAPS; 5% [v/v] ampholytes pH 3.0 - 10.0) (Gravel and Golaz, 1996) was added to the sample and then stored at -70°C until required.

2.2.2.3 Sample preparation method 3 (SP3)

The cell pellet was suspended in 250 µl 10 mM Tris-HCl (pH 7.4) and sonicated by three pulses of 15 s each using a 4710 Series Ultrasonic Homogenizer (Cole-Palmer Insrument Co.) at an output of 40%. Following sonication, 250 µl of Lysis buffer B (9 M urea; 65 mM DTE; 65 mM CHAPS; 5% [v/v] ampholytes pH 3.0 - 10.0) was added to the sample and then stored at -70°C until required.

2.2.3 Two-dimensional polyacrylamide gel electrophoresis (2-DE)

2.2.3.1 Preparation of the ampholyte-containing tube gels for iso-electric focusing

For iso-electric focusing, a gel solution was prepared using 5.5 g urea; 2.5 ml double distilled water; 1.0 ml 40% (w/v) acrylamide/bisacrylamide (40% T, 5% C_{bis}); 2.0 ml 10% (v/v) Nonidet P-40 (NP-40); 0.4 ml ampholyte pH 5.0 - 7.0; 0.1 ml ampholyte pH 3.0 - 10.0; 7.0 µl N,N,N',N-tetramethyl-ethylenediamine (TEMED) and 10 µl 10% (w/v) ammonium persulfate. Gels were cast in 15-cm long glass tubes and left for 1 h at room temperature to polymerise, yielding gels of 12 cm in length.

2.2.3.2 First-dimension iso-electric focusing (IEF)

After gel polymerisation, the tubes were placed in the upper chamber of a Hoefer SE 600 Series electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA, USA) and the bottoms of the tubes were filled with anode solution (10 mM phosphoric acid). The upper chamber was placed inverted into the lower buffer chamber filled with anode solution. The upper chamber was filled with degassed cathode solution (10 mM histidine). Five microlitres of sample overlay solution (9 M urea; 2% [w/v] ampholytes pH 3.0 - 10.0 and pH 5.0 - 7.0) was added on top of each tube. The gels were pre-electrophoresed as follow: (i) 200 V for 15 min, (ii) 300 V for 30 min, and (iii) 400 V for 30 min. The power was turned off, the sample

overlay solution was removed and 15 µl of the protein extracts, mixed with 5 µl sample buffer (9.5 M urea; 2% [v/v] Nonidet P-40; 2% [v/v] ampholytes pH 3.0 - 10.0 and pH 5.0 - 7.0; 5% [v/v] 2-mercaptoethanol), were loaded. Five microlitres sample overlay solution was added on top of each tube. The gels were then electrophoresed at 400 V for 16 h, followed by electrophoresis for 1 h at 800 V (7 200 Vh) (IEF1) or for 24 h at 300 V (7 200 Vh) (IEF2).

2.2.3.3 Second-dimension protein separation (SDS-PAGE)

Upon completion of the iso-electric focusing time, the tube gels containing focused proteins were equilibrated immediately for 20 min in 5 ml treatment buffer (62.5 mM Tris-HCl; 2% [w/v] SDS; 10% [v/v] glycerol; 5% [v/v] 2-mercaptoethanol, pH 6.8) (Dunbar *et al.*, 1990) and then either frozen in the treatment buffer at -70°C overnight (PET1), or directly embedded onto a uniform 10% SDS-polyacrylamide separating gel (10% T, 2.7% C_{bis}) using an agarose sealing solution (0.5 M Tris-HCl; 1% [w/v] SDS; 1% [w/v] agarose, pH 6.8) (PET2). The buffer used was the standard Laemmli buffer used for SDS-PAGE (Laemmli, 1970). The second dimensional separation was performed using a Protean II electrophoresis unit (BioRad Laboratories, Hercules, CA, USA) at 5 Watt for 15 min, followed by 10 Watt for 4 h 45 min. A constant temperature of 18°C was maintained during electrophoresis.

2.2.4 Visualisation of proteins on 2-DE gels

2.2.4.1 Coomassie R250 staining (S1)

After electrophoresis, gels were stained in a Coomassie R250 solution (0.3% [w/v] Coomassie Brilliant Blue R250; 50% [v/v] methanol; 10% [v/v] acetic acid) for 2 h with agitation. Destaining was performed with agitation in a solution consisting of 25% (v/v) methanol and 10% (v/v) acetic acid (Neuhoff *et al.*, 1985). The destaining solution was changed regularly until the background of the gels was clear and the gels were then preserved in double distilled water.

2.2.4.2 Silver diamine staining (S2)

2.2.4.2.1 Preparation of silver diamine staining and spot development solutions

The silver diamine solution (Dunn, 1996) was prepared by adding 21 ml of 0.36% (w/v) NaOH to 1.4 ml of 35% (w/v) ammonia, followed by the dropwise addition of 4 ml of 20% (w/v) silver nitrate to the solution whilst stirring gently. In the event of the staining solution

not clearing due to the formation of a brown precipitate, more ammonia was added until complete dissolution of the precipitate was achieved. The solution was then made up to 100 ml with deionised distilled water and used within 5 min after its preparation. For the development of protein spots in the gels, a developing solution was prepared that consisted of 1% (w/v) citric acid in deionised distilled water to which 0.26 ml of 36% (v/v) formaldehyde was added. The solution was then made up to 500 ml with deionised distilled water and 2.5 ml of the solution was used for each gel. All solutions were freshly prepared using clean glassware and deionised distilled water.

2.2.4.2.2 Staining method

After electrophoresis, proteins were visualised by silver diamine staining, as described by Dunn (1996), and all of the incubation steps were performed at room temperature. The gels were incubated overnight in 200 ml of 20% (w/v) trichloroacetic acid (TCA) and the proteins were then fixed by incubating the gels in 200 ml of a fixing solution composed of 40% (v/v) ethanol and 10% (v/v) acetic acid. After 30 min, the fixing solution was replaced and incubation was continued for a further 30 min. The gels were washed twice for 20 min each wash in excess water and then sensitised in 10% (w/v) gluteraldehyde for 30 min. After washing the gels three times, as above, the silver reaction was performed by soaking the gels in the freshly prepared silver diamine solution for 30 min. After washing the gels three times for 5 min each wash in excess water, protein spots were developed with 0.005% citric acid and 0.02% formaldehyde (final concentrations) until the spots were clearly visible. Development of protein spots was stopped by incubation of the gels in 40% (v/v) ethanol, 10% (v/v) acetic acid for 10 min, after which the gels were rinsed thoroughly and then preserved in double distilled water.

2.3 RESULTS AND DISCUSSION

2.3.1 Sample preparation

Sample preparation is considered to be of critical importance for good 2-DE results and ideally the process should result in efficient lysis of the sample material and in the complete solubilisation, disaggregation, denaturation and reduction of the proteins in the sample (Harder *et al*., 1999; Shaw and Riederer, 2003). Three different sample preparation methods were investigated in this study. Each of these methods was performed at least twice and on

separate occasions. Two-dimensional gel electrophoresis was performed in duplicate on samples from each of the preparations. Thus, a minimum of four gels per sample preparation method was generated whereby the gel-to-gel reproducibility and resolution could be evaluated objectively.

2.3.1.1 Cell lysis

Many different mechanical and chemical methods for cell disruption have been reported. The methods used to disrupt bacterial cells include freeze-thaw lysis (Souzu, 1980; Svensäter *et al*., 2001) and enzymatic lysis using lysozyme (Cull and McHenry, 1990; Dykes *et al*., 2003), but more-vigorous lysis methods such as sonication (Teixeira-Gomes *et al.*, 1997; Benov and Al-Ibraheem, 2002; Trémoulet *et al*., 2002a; 2002b), glass bead homogenisation (Cull and McHenry, 1990; Svensäter *et al*., 2001; Benov and Al-Ibraheem, 2002) and the use of a French press (Molloy *et al*., 2000; Buttner *et al*., 2001; Benov and Al-Ibraheem, 2002) appears to be most frequently used. In this study, the *P. aeruginosa* cells were disrupted either by sonication (SP1 and SP3) or by boiling in lysis buffer containing SDS and DTE (SP2). Sonication was performed in short bursts to prevent heating and foaming of the sample, which may decrease the efficiency of lysis (Teixeira-Gomes *et al.*, 1997). Since proteases liberated during cell lysis may degrade the sample proteins and thus complicate analysis of the resultant 2-DE maps, the samples were prepared and kept on ice at all times as proteases are less active at low temperatures. Furthermore, proteases were also inhibited by using lysis buffers that either contained a strong denaturant such as 2% SDS (Lysis buffer 1 of SP1) or 9 M urea (Lysis buffer B of SP2 and SP3) (Rabilloud, 1996; Harder *et al*., 1999; Shaw and Riederer, 2003).

2.3.1.2 Protein solubilisation

In order to characterise proteins in a complex protein mixture, first-dimension IEF must be performed under denaturing conditions. In order to achieve a well-focused IEF separation, the sample proteins must therefore be completely denatured and fully soluble to ensure that aggregation and intermolecular interactions are minimised and that each protein is present in only one configuration. To ensure complete solubilisation and denaturation of the proteins, sample solutions generally include a denaturant, detergents and reducing agents.

Urea, a neutral chaotrope, is frequently used as denaturant in 2-DE and almost always at a concentration of at least 8 M (Herbert, 1999). Urea disrupts hydrogen bonding, leading to protein denaturation and unfolding to their fully random conformation, with all ionizable groups exposed to solution (Herskovits *et al.*, 1970). Nonionic zwitterionic detergents are included in the sample solution to ensure complete solubilisation and prevent aggregation through hydrophobic interactions that may occur through chaotrope-generated exposure of hydrophobic domains. The zwitterionic detergent CHAPS has been reported to be more effective than nonionic TritonX-100 or NP-40 in preventing protein aggregation (Perdew *et al.*, 1983; Herbert, 1999; Shaw and Riederer, 2003). To ensure full sample solubilisation, the anionic detergent SDS, which is a very effective protein solubiliser that disrupts almost all noncovalent protein interactions, may also be included in the sample solutions (Wilson *et al.*, 1977; Harder *et al.*, 1999). Thiol reducing agents are frequently included in the sample solution to break intra- and intermolecular disulfide bonds and to maintain all proteins in their fully reduced state (Righetti *et al.*, 1982; Rabilloud, 1996; Shaw and Riederer, 2003). The most commonly used reducing agents are dithiothreitol (DTT) or dithioerythritol (DTE), but 2-mercaptoethanol is also used (Herbert, 1999). These reagents are used in excess to favour an equilibrium shift toward oxidation of the reducing agent with concomitant reduction of protein disulfides. Carrier ampholytes are also frequently included in the sample solution (Molloy *et al*., 1998; Shaw and Riederer, 2003). They enhance solubility of individual proteins by minimising protein aggregation due to charge-charge interactions as they approach their isoelectric points and they also produce an approximately uniform conductivity across a pH gradient without affecting its shape (Khoudoli *et al*., 2004).

Since the effectiveness of protein solubilisation depends on the choice of detergents and composition of the sample solution, different sample solutions were investigated and compared in this study. Lysis buffer 1 used in sample preparation method 1 (SP1) consisted of Tris-HCl, 2-mercaptoethanol, glycerol and SDS, and the sample proteins were denatured by heating the samples. In the case of sample preparation method 2 (SP2), the cells were disrupted by boiling in buffer containing SDS and DTE after which Lysis buffer B was added, which consisted of urea, DTE, CHAPS and ampholytes. Although the same Lysis buffer B was used in sample preparation method 3 (SP3), the cells were, however, disrupted by sonication. Where samples were heated (SP1 and SP2), urea was omitted from the buffers to prevent modification of the proteins by carbamylation at these high temperatures.

The results that were obtained (Fig. 2.1) indicated that 2-DE of samples prepared according to SP1 and SP2 generated 2-DE maps of which the spots were localised to one side of the gel only. In contrast, the 2-DE maps of samples prepared using SP3 resulted in protein spots that were evenly distributed over a much broader surface area of the gels. Since the buffers used in SP3 lacked SDS, the poor separation of protein spots observed in 2-DE maps from SP1 and SP2 may therefore have been due to the high concentration of SDS present in the sample solutions. Final concentrations of SDS higher than 0.25% in IEF sample buffers have been reported to have a deleterious effect on IEF (Harder *et al.*, 1999; Shaw and Rieder, 2003). Although the SDS in samples prepared according to SP2 were diluted to 0.2% (final concentration) by the addition of Lysis buffer B to the samples, poor separation of the proteins was nevertheless observed. This may have been due to displacement of the SDS with an excess of less efficient detergents for solubilising the proteins, thereby leading to the reaggregation of some proteins present in the sample (Harder *et al.*, 1999). Based on these results, all protein samples used in subsequent investigations were prepared according to sample preparation method 3 (SP3).

2.3.2 First-dimension IEF

IEF is a method whereby proteins are separated according to their iso-electric points (pI), *i.e.* the specific pH at which the net charge of a protein is zero. The presence of a pH gradient is therefore critical to the IEF technique. In a pH gradient, under the influence of an electric field, a protein will move to the position in the gradient where its net charge is zero and allows proteins to be separated on the basis of very small charge differences. The degree of resolution is determined by the slope of the pH gradient and the electric field strength (Berg *et al.*, 2002).

In this study, carrier ampholytes were used to generate pH gradients in the polyacrylamide tube gels. The carrier ampholytes are small, soluble, amphoteric molecules with a high buffering capacity near their pI. When a voltage is applied across the carrier ampholyte mixture, the carrier ampholytes with the lowest pI (and the most negative charge) move towards the anode, and the carrier ampholytes with the highest pI (and the most positive charge) move towards the cathode. The other carrier ampholytes align themselves between the extremes, according to their pIs, and buffer their environment to the corresponding pHs. The result is a continuous pH gradient (Klose, 1975; O'Farrell, 1975).

Typically, IEF generally proceeds through a series of voltage steps that begins at a relatively low value to minimise sample aggregation (Gravel and Golaz, 1996). The voltage is then gradually increased to the final desired focusing voltage, where it is held for several hours. Since IEF conditions vary with the nature of the sample (Hochstrasser *et al*., 1988; Dunbar *et al.*, 1990), the amount of protein loaded (Görg *et al*., 2000) and the presence of small ions, which move to the ends of the gel before protein focusing can occur (Görg *et al*., 2000), the IEF conditions must be determined empirically. Two IEF conditions were thus compared in this study, *i.e.* focusing at 400 V for 16 h and then at 800 V for 1 h (IEF1) and, alternatively, focusing at 300 V for 24 h (IEF2).

The results that were obtained (Fig. 2.1) indicated that focusing of the proteins at a higher voltage for a shorter period of time (IEF1) generated gels of which the resolution of individual protein spots was much higher compared to those generated from proteins focused at a lower voltage for a longer period of time (IEF2). Because the mode of action of reducing agents is an equilibrium reaction, loss of the reducing agent through migration in the pH gradient (DTT/DTE are weakly acidic) can allow reoxidation at protein disulfides and contribute to horizontal streaking, as was observed in the 2-DE maps obtained under IEF2 conditions. Therefore, to maintain high resolution when thiol reducing agents are used, IEF should be conducted for the shortest possible focusing times. Consequently, IEF was performed at 400 V for 16 h and then at 800 V for 1 h (IEF1) in all subsequent experiments.

2.3.3 Second-dimension SDS-PAGE

After IEF and prior to SDS-PAGE, the IEF tube gels were equilibrated in this study using a treatment buffer containing Tris-HCl, SDS, glycerol and 2-mercaptoethanol (Dunbar *et al.*, 1990). Whereas the Tris-HCl maintains the gel pH in a range appropriate for electrophoresis, SDS denatures the proteins and forms negatively charged protein-SDS complexes, glycerol improves transfer of proteins from the first dimension to the second dimension, and 2-mercaptoethanol preserves the reduced state of denatured proteins, thus ensuring that separations are exclusively on the basis of molecular mass.

The second-dimension separation was subsequently performed in polyacrylamide gels containing SDS to allow for the electrophoretic separation of the focused proteins according to their molecular mass. The presence of SDS in the gel negates the intrinsic electrical charge

of the sample proteins so that it does not play a role in the separation. Since SDS has a high propensity to bind to proteins, at *ca.* one SDS molecule per every two amino acids (Nelson, 1971), the bound SDS masks the charge of the proteins, thereby forming anionic complexes with a constant net negative charge per unit mass (Nelson, 1971). The SDS also disrupts hydrogen bonds, blocks hydrophobic interactions and partially unfolds the protein molecules (Maizel, 2000), thus minimising differences in molecular form by eliminating the secondary and tertiary structures (Maizel, 2000). During electrophoresis a constant temperature of 18°C was maintained to prevent heat from building up and to improve gel-to-gel reproducibility.

Although 20 IEF tube gels could be prepared simultaneously, it was possible only to run four gels based on the equipment available. Thus, it was important to be able to store the IEF tube gels without subsequent loss in resolution. To investigate, the IEF tube gels were either stored at -70°C overnight in treatment buffer (PET1) or used immediately following equilibration (PET2). Comparison of the 2-DE maps obtained (Fig. 2.1) indicated that storage overnight led to an improvement in the resolution of protein spots compared to those obtained when second-dimension SDS-PAGE was immediately performed. Consequently, IEF gels were either used immediately or stored, depending as the need arose.

2.3.4 Staining of 2D-PAGE gels

After electrophoresis, gels were either stained with Coomassie Brilliant Blue R250 (S1) or with a silver diamine stain (S2). Although there are numerous published versions of the silver staining protocols (Heukeshoven and Dernick, 1988; Rabilloud, 1992; Dunn and Crisp, 1994; Jin *et al*., 2004), silver stains can generally be classified into two families according to the nature of the silver reagent used for binding silver to the proteins. The first and simplest type of stain is the silver nitrate stain in which the gel is soaked in a solution of silver nitrate, and the colour is developed by reduction with formaldehyde at alkaline pH (Merril *et al.*, 1981; Heukeshoven and Dernick, 1988; Nesterenko *et al*., 1994). The second, more sensitive, silver stain is the diamine stain in which the silver is complexed with ammonia (Merril *et al*., 1979; Switzer *et al*., 1979; Oakely *et al*., 1980; Hochstrasser *et al.*, 1988). Whereas silver staining can be used to detect proteins with relative sensitivity and exhibits a linear range of about 0.5 - 20 ng/mm^2, Coomassie blue is significantly less sensitive with a linear range of about 50 ng/mm^2 - 1 µg/mm^2 (Candiano *et al*., 2004). However, Coomassie staining, as is reductive silver provided that no gluteraldehyde is used, has advantages of providing good quantitative

data and is also compatible with peptide mass mapping procedures used for protein spot identification.

Approximately 503 distinct protein spots in the pH range 3.0 - 10.0 was observed after silver diamine staining, compared to the approximately 216 protein spots observed after Coomassie staining (Fig. 2.1). Consequently, the high sensitivity silver diamine stain was used in analytical analysis, whilst Coomassie staining was used in routine analysis of the generated 2-DE maps.

2.4 CONCLUDING REMARKS

The primary objective of this investigation was to study the global changes in protein expression that occur when planktonic cells of *P. aeruginosa* make the transition to the biofilm mode of growth. Two-dimensional gel electrophoresis (2-DE) is currently the only method available for quantitative and qualitative arraying of complex protein mixtures such as the bacterial proteome (Wilkins *rt al.,* 1996; Klose, 1999; Corthals *et al*., 2000; Hille *et al.*, 2001). However, the elucidation of a bacterial proteome is a time-consuming procedure and thus, it was necessary to first standardise on the specific 2-DE protocol to be employed. The need for high resolution and reproducibility in proteomics has stimulated advances in gel technology for IEF separation (Görg *et al*., 2000), improvements in methods for enhancing protein solubility (Rabilloud, 1996; Herbert *et al*., 1998; Molloy *et al*., 1998; Herbert, 1999; Molloy *et al*., 1999; Rabilloud, 1999; Molloy *et al*., 2000; Shaw and Riederer, 2003) and methods for protein visualisation (Patton, 2002; Candiano *et al*., 2004). Consequently, all of these parameters have to be taken into account and optimised prior to undertaking proteomic investigations.

With respect to the 2-DE technology, the majority of studies use immobilized pH gradient (IPG) strips for the first-dimension separation and SDS-PAGE for the second dimension. Although IPGs offer superior reproducibility, it requires specialised equipment, which was not available at the time of this investigation. Despite being technically demanding, it was, however, possible to generate 2-DE protein maps of high resolution and reproducibility by making use of tube gels with carrier ampholyte-generated pH gradients.

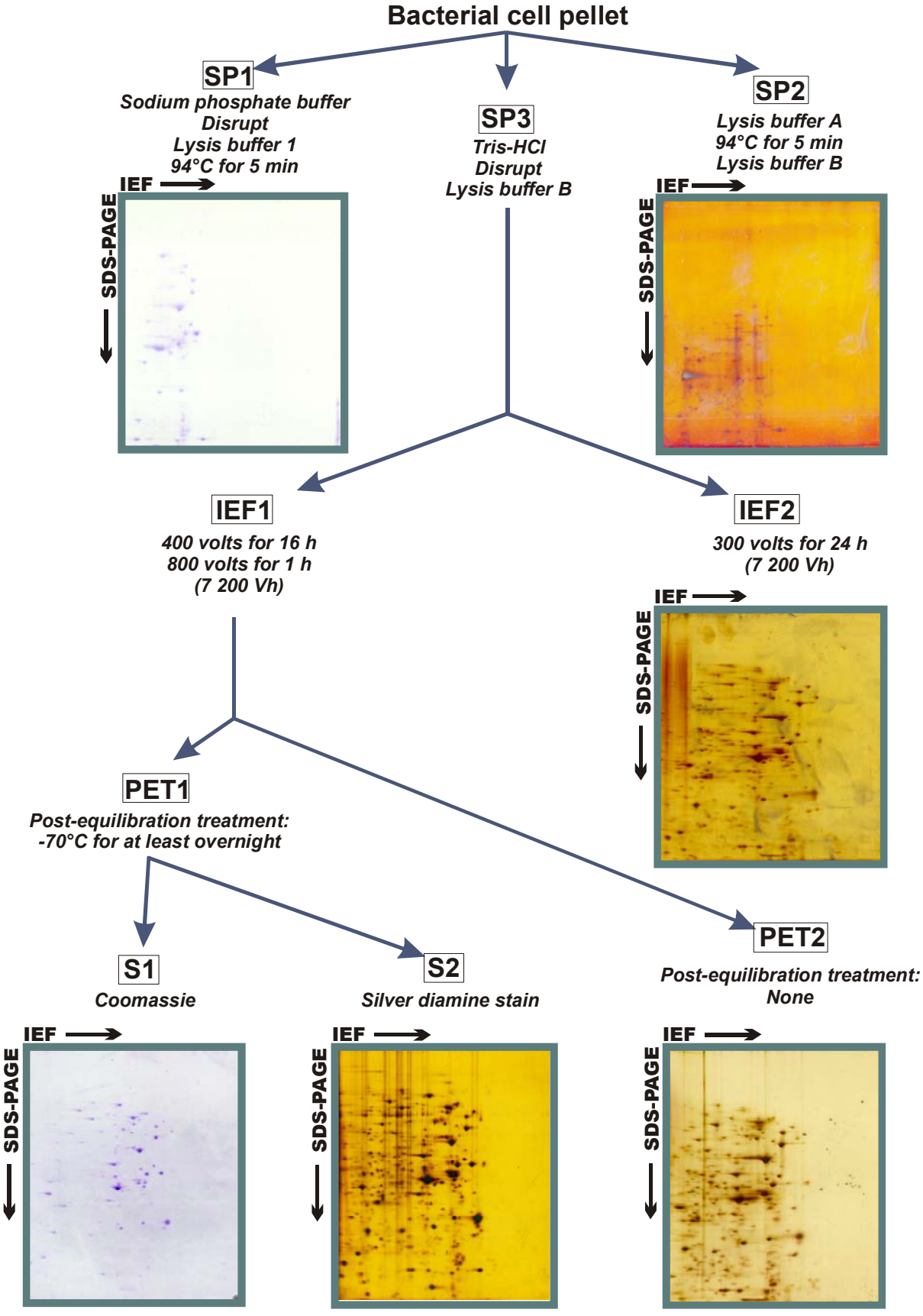

Fig. 2.1 **Flow-diagram depicting the 2-DE gels obtained using different sample preparation methods (SP), iso-electric focusing conditions (IEF), post-equilibration treatments of the IEF tube gels (PET) and staining methods (S).**

For efficient protein solubilisation and high resolution, it is necessary to achieve complete disruption of molecular interactions, to obtain single polypeptides, and to maintain this state throughout the separation process. Of the different protein solubilisation solutions investigated in this study, protein samples prepared in Lysis buffer B (urea, DTE, CHAPS, Tris-HCl, carrier ampholytes) yielded the best results, provided that the cells were disrupted by sonication and not by boiling in a SDS-containing buffer. The presence of SDS in the sample solutions resulted in horizontal streaking of the protein spots, thereby severely compromising the resolution.

Despite being expensive and time-consuming, silver diamine staining was more sensitive than Coomassie staining in detecting protein spots, and in excess of 500 protein spots could be resolved on the gel. High purity reagents and precise timing were found to be necessary for reproducible high-quality results and poor staining results were obtained when water containing impurities was used for preparing the staining reagents. In contrast, staining of the gels with Coomassie R250 yielded only 216 protein spots, but the simplicity of the staining procedure makes it amenable for routine analyses of the gels.

In conclusion, having investigated and compared different sample preparation methods, iso-electric focusing conditions and protein staining methods, the optimised 2-DE method used in this investigation consisted of disrupting the *P. aeruginosa* cells by sonication in 10 mM Tris-HCl (pH 7.4), followed by solubilisation of the sample proteins in Lysis buffer B composed of 9 M urea, 65 mM DTE, 65 mM CHAPS and 5% (v/v) ampholytes (pH 3.0 - 10.0). Iso-electric focusing was carried out at 400 V for 16 h and then at 800 V for 1 h (7 200 Vh) and following second-dimension 10% SDS-PAGE separation, the gels can be stained with either silver diamine or with Coomassie Brilliant Blue, depending on the resolution required.

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