Proteomic analysis of the biofilm and biofilm-associated phenotypes of *Pseudomonas aeruginosa* cultured in batch

by

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Submitted in partial fulfillment of the requirements for the degree
Philosophiae Doctor
in the Faculty of Natural and Agricultural Sciences
University of Pretoria
Pretoria

August 2005

ACKNOWLEDGEMENTS

Hereby I wish to express my sincere thanks and appreciation to:

Prof J Theron, for picking up the ball and running with it. Thank you for all the hard work and giving me a chance…

Prof VS Brözel, for getting me started and keeping me interested. You taught me so much…

National Research Foundation and **University of Pretoria**, for financial assistance.

Dr MC Oosthuizen, for her help in the laboratory, especially with establishing the 2D PAGE procedure, but more for her moral support and special friendship.

To my **fellow students and friends**, for their help, support, understanding, and just for being there, especially **Julian**, **Boet** and **Aida**.

Allan Hall, for helping with the glass wool photographs.

Everyone at **Stimuplant**, for their support and encouragement and for being so patient with me.

To my **mother**, for all her love, support, understanding, patience and so much more…

To my **Heavenly Father**, with whom everything is possible!

Dedicated to my father and brother

I wish that the two of you were still here… I miss you and love you always…

DECLARATION

I declare that the thesis, which I hereby submit for the degree, Philosophiae Doctor (Microbiology) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at another University.

Signed: …………………………………..   Date: …………………………..

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Pseudomonas aeruginosa is one of the most studied biofilm-forming organisms and has emerged as a model organism in the study of surface- and biofilm-induced gene expression. The transition from a planktonic to a biofilm mode of growth results in diverse changes in gene expression, which causes the attaching cells to become phenotypically and metabolically distinct from their planktonic counterparts. In this study, a proteomic approach was used to study differences in protein profiles obtained from 18-h old *P. aeruginosa* PAO1 (DSM 1707) planktonic, surface influenced planktonic (SIP) and biofilm populations grown in batch in the absence or presence of a glass wool substratum.

Glass wool as an attachment substratum not only supported growth of biofilms, but it also allowed for the separation of the biofilm biomass from the surrounding surface influenced planktonic (SIP) cells for further characterisation. Comparative analysis of the respective proteomes indicated striking differences in the protein patterns of planktonic, biofilm and SIP cells and several uniquely expressed proteins were seen on the 2-DE protein maps of the respective populations. Whereas a general down-regulation of protein expression was seen in the biofilm cells, in SIP cells, expression of the proteins was generally up-regulated. The results confirmed that the biofilm population differs from the planktonic population and indicated that the SIP population is not merely a mixture of planktonic and biofilm cells but rather a unique phenotype.

Several differentially expressed protein spots were selected and identified using a combination of N-terminal protein sequencing and peptide mass fingerprinting. The proteins comprised mostly of outer membrane or membrane-associated proteins. Based on these analyses, a mutant *P. aeruginosa* strain, deficient in outer membrane protein OprG, was generated and its ability to form biofilms on a glass wool substratum was compared with that of the wild-type *P. aeruginosa* strain. The mutant strain was attachment-proficient but biofilm-deficient, suggesting that OprG plays a role in *P. aeruginosa* biofilm development under the culturing conditions used in this study.

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LIST OF ABBREVIATIONS

%	percentage
°C	degrees Celsius
µg	microgram
µl	microlitre
µm	micrometre
2-DE	two-dimensional gel electrophoresis
A	ampere
AHL	acylated homoserine lactone
Amp^r	ampicillin resistance
bp	base pair
ca.	approximately
cfu	colony forming units
CH_3CN	acetonitrile
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
cm	centimetre
cm^2	square centimetre
CTAB	cetyltrimethylammonium bromide
DMSO	dimethyl sulfoxide
dNTP	deoxyribonucleoside-5'-triphosphate
DTE	dithioerythritol
DTT	dithiothreitol
e.g.	for example
EPS	exopolysaccharides
Fig.	figure
$\times g$	centrifugal force
Gm^r	gentamicin resistance
h	hour
IEF	iso-electric focusing
IPG	immobilized pH gradient
IPTG	isopropyl β-D-thiogalactoside
kb	kilobase pairs
kDa	kilodalton
L	litre

LB broth	Luria-Bertani broth
LPS	lipopolysaccharide
M	molar
MALDI-TOF	Matrix-assisted laser desorption ionization time-of-flight
min	minute
ml	millilitre
mM	millimolar
Mr	molecular mass
MSGY	modified mineral salts medium with glucose and yeast extract
NH_4HCO_3	ammonium bicarbonate
nm	nanometer
OD	optical density
OMP	outer membrane proteins
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pI	isoelectric point
pmol	picomole
PMSF	phenylmethylsulphonyl fluoride
PVDF	Immobilon-P polyvinylidene difluoride
rpm	revolutions per minute
s	second
SB3-10	N-decyl-N,N-dimethyl-3-ammonio-1-propane sulfonate
SDS	sodium dodecyl sulphate
SIP	surface influenced planktonic
TBP	tributyl phosphine
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
U	units
UHQ	ultra high quality
V	volts
v/v	volume per volume
Vh	Volt-hours
W	Watt
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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RESEARCH COMMUNICATIONS

Papers published:

1. **Steyn, B.**, Oosthuizen, M.C., MacDonald, R., Theron, J. and Brözel, V.S. (2001). The use of glass wool as an attachment surface for studying phenotypic changes in *Pseudomonas aeruginosa* biofilms by two-dimensional gel electrophoresis. Proteomics 1: 871-879.

2. Oosthuizen, M.C., **Steyn, B.**, Lindsay, D., Brözel V.S. and von Holy, A. (2001). Novel method for the proteomic investigation of a dairy-associated *Bacillus cereus* biofilm. FEMS Microbiol. Lett. 194: 47-51.

3. Oosthuizen, M.C., **Steyn, B.**, Theron, J., Cosette, P., Lindsay, D., von Holy, A. and Brözel, V.S. (2002). Proteomic analysis reveals differential protein expression by *Bacillus cereus* during biofilm formation. Appl. Environ. Microbiol. 68: 2770-2780.

Conference contributions:

National conferences:

1. **Steyn, B.**, Oosthuizen, M.C., Theron, J. and Brözel, V.S. Determination of the proteome of *Pseudomonas aeruginosa* growing planktonically and as a biofilm. BioY2K Conference, January 2000, Grahamstown, South Africa.

2. **Steyn, B.**, Oosthuizen, M.C., Theron, J., von Holy, A. and Brözel, V.S. Establishment of two-dimensional gel electrophoresis for the determination of bacterial proteomes. BioY2K Conference, January 2000, Grahamstown, South Africa.

3. Oosthuizen, M.C., **Steyn, B.**, Lindsay, D., Brözel, V.S. and von Holy, A. Determination of the proteome of a dairy-associated *Bacillus* growing planktonically and as a biofilm. BioY2K Conference, January 2000, Grahamstown, South Africa.

International conferences:

1. **Steyn, B.**, Oosthuizen, M.C., Cosette, P., Jouenne, T., Theron, J. and Brözel, V.S. Determination of the proteome of *Pseudomonas aeruginosa* growing planktonically and as a biofilm. Biofilms 2000 Conference, July 2000, Big Sky, Montana, USA.

2. Oosthuizen, M.C., **Steyn, B.**, Lindsay, D., Brözel, V.S. and von Holy, A. Proteome analysis of a dairy-associated *Bacillus cereus* growing planktonically and as a biofilm. Biofilms 2000 Conference, July 2000, Big Sky, Montana, USA.

3. **Steyn, B.**, Oosthuizen, M.C., Theron, J. and Brözel, V.S. Analysis of two surface-influenced phenotypes of *Pseudomonas aeruginosa* by two-dimensional gel electrophoresis. Pseudomonas 2001, September 2001, Brussels, Belgium.

4. Oosthuizen, M.C., **Steyn, B.**, Brözel, V.S., Lindsay, D. and von Holy, A. Dairy-associated *Bacillus cereus* growing as a biofilm has a distinct proteome. 88th Annual Meeting of the International Association for Food Protection, August 2001, Minneapolis, Minnesota, USA.

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
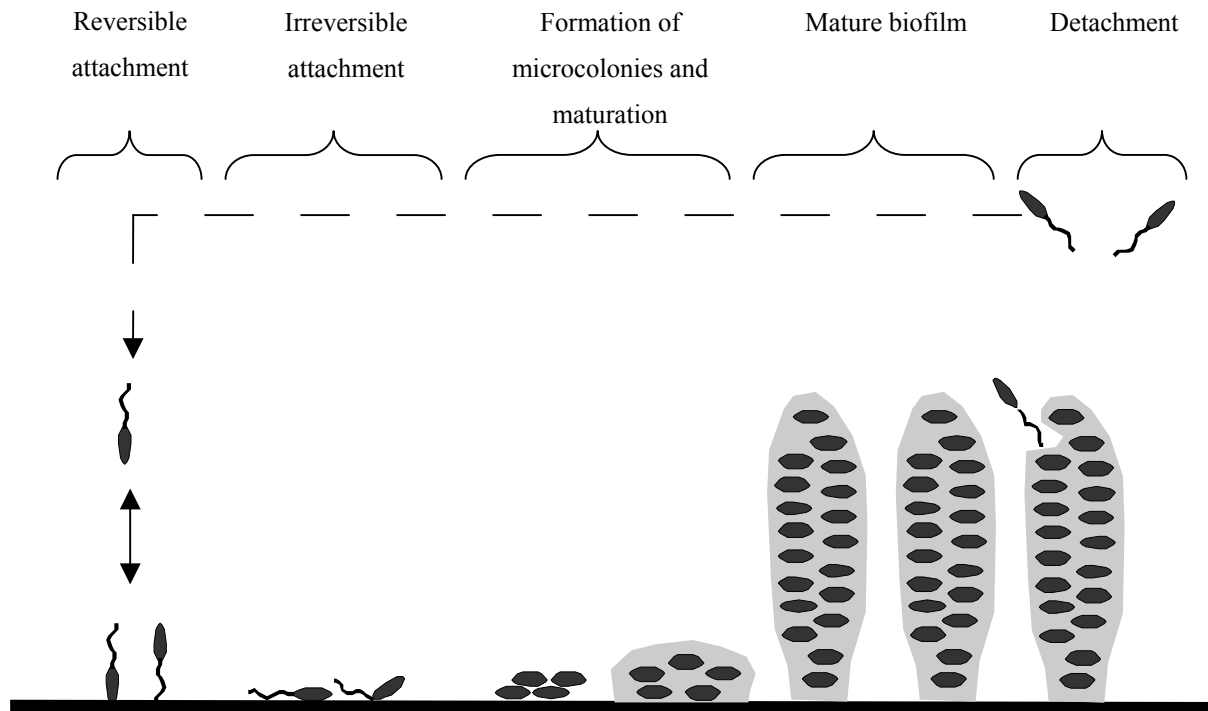
Fig. 1.1   **Model of biofilm development. In response to environmental cues, planktonic cells initiate cell-to-surface and cell-to-cell contacts resulting in the formation of microcolonies. In response to developmental signals, microcolonies undergo differentiation to form a well-developed biofilm characterised 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.

Bacterial cell pellet

SP1
Sodium phosphate buffer
Disrupt
Lysis buffer 1
94°C for 5 min

SP3
Tris-HCl
Disrupt
Lysis buffer B

SP2
Lysis buffer A
94°C for 5 min
Lysis buffer B

IEF1
400 volts for 16 h
800 volts for 1 h
(7 200 Vh)

IEF2
300 volts for 24 h
(7 200 Vh)

PET1
Post-equilibration treatment:
-70°C for at least overnight

S1
Coomassie

S2
Silver diamine stain

PET2
Post-equilibration treatment:
None
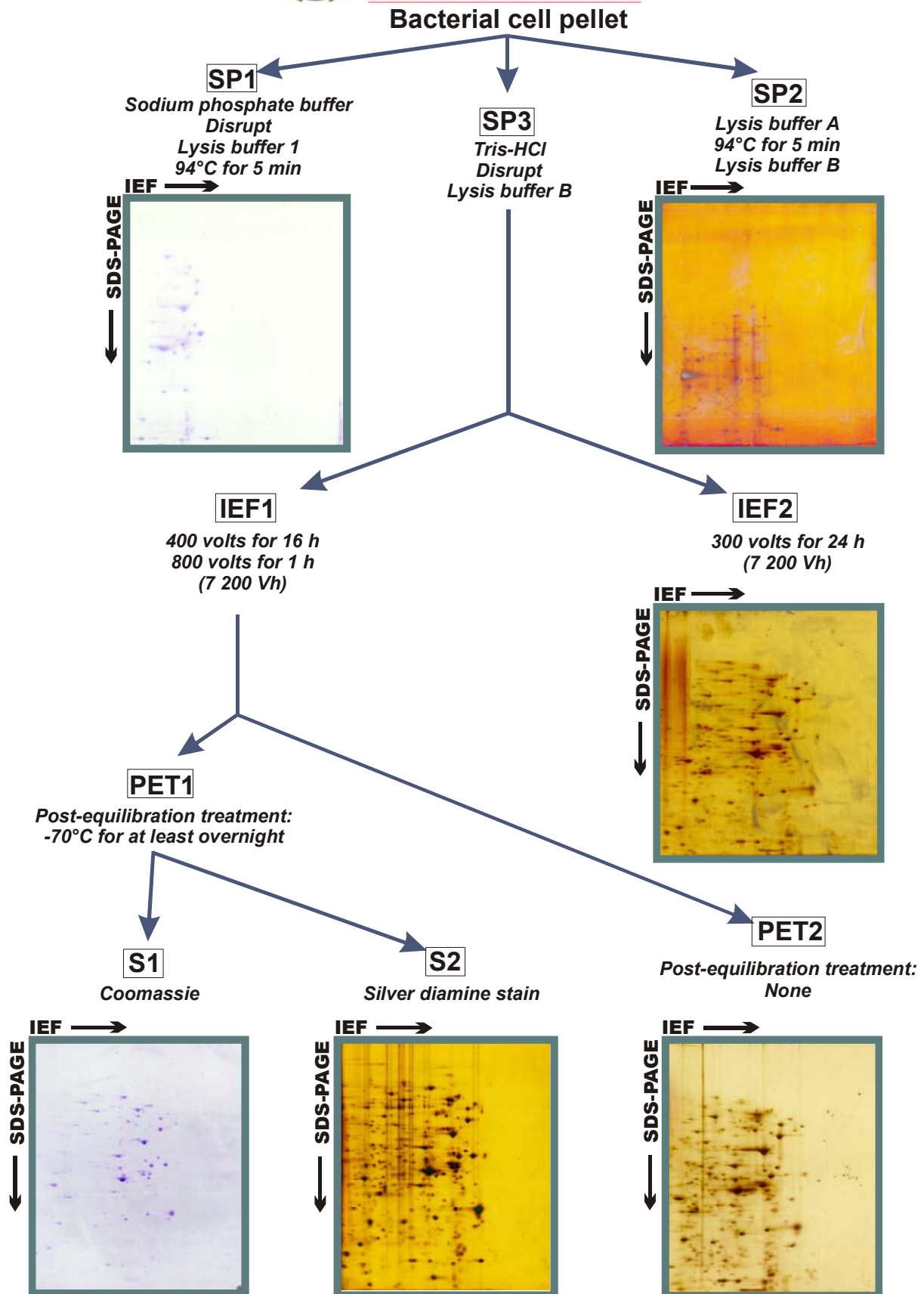
Fig. 2.1 **Flow-diagram depicting the 2-DE gels obtained using different sample preparation methods (SP), iso-electric focusing conditions (IEF), post-equilibration treatments of the IEF tube gels (PET) and staining methods (S).**

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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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CHAPTER THREE*

THE USE OF GLASS WOOL AS AN ATTACHMENT SURFACE FOR STUDYING PHENOTYPIC CHANGES IN *Pseudomonas aeruginosa* BIOFILMS BY TWO-DIMENSIONAL GEL ELECTROPHORESIS

3.1 INTRODUCTION

Pseudomonas aeruginosa, an opportunistic pathogen primarily infecting immuno-compromised persons (Burrows *et al*., 1996; Whitchurch *et al*., 1996), preferentially assumes a biofilm mode of growth (Costerton, 1984) by embedding itself in a gelatinous organic polymer matrix composed of alginate (Costerton *et al*., 1987). Biofilm formation occurs in response to a variety of environmental signals (Fletcher, 1991; Davies *et al.*, 1993; Dalton *et al.*, 1994) that leads to the expression of new phenotypes that distinguish the attached cells from their planktonic (free-flowing) counterparts. Most notably, biofilm bacteria have been shown to have a greatly enhanced tolerance to stresses and antimicrobial agents (Nickel *et al.*, 1985; Cochran *et al.*, 2000). Thus, biofilm bacteria are markedly different from planktonic bacteria with relation to gene expression and cellular physiology (Costerton *et al.*, 1995). Recent genetic studies, involving various different gram-negative bacteria, have identified genes involved in the formation and development of biofilms (Pratt and Kolter, 1999; Prigent-Combaret *et al.*, 1999; Watnick and Kolter, 1999). In *P. aeruginosa* expression of a number of genes is up-regulated in biofilm-growing cells, such as *algC* (Davies and Geesey, 1995), *algD* (Hoyle *et al.*, 1993) and *pilA* (O'Toole *et al.*, 2000a). Most biofilm-regulated genes have been identified by screening for mutants defective in biofilm formation (O'Toole *et al.*, 1999). Although this is a powerful approach, the information is limited to genes obligately related to biofilm formation, while those genes contributing to the biofilm phenotype, as well as genes regulated in this attached mode of growth, are omitted.

In order to extend the knowledge on the development of *P. aeruginosa* biofilms and its unknown underlying molecular mechanisms, we wanted to analyse global protein expression, *i.e.* to phenotypically characterise biofilm and the planktonic counterpart cells under defined culture conditions. High-resolution two-dimensional polyacrylamide gel electrophoresis (2-DE), that combines fractionation of proteins according to their charge and mass, is ideally suited for this purpose (Celis and Gromov, 1999; Herbert, 1999). To accomplish this, the culturing of copious amounts of biofilm is a prerequisite. Although various devices, such as the Robbins device (McLean *et al.*, 1999) and flow cells (Zinn *et al.*, 1999), have been developed to study biofilms formed under defined conditions, the surface area offered for biofilm development in these devices is too small to yield sufficient protein biomass for 2-DE. To overcome these limitations, recent studies have reported the use of gel-entrapped *Escherichia coli* cells (Jouenne *et al.*, 1994; Perrot *et al.*, 2000) and surface colonies off agar plates (Miller and Diaz-Torres, 1999) as models for immobilised (biofilm) cells. The use of glass wool as a substratum for cell attachment may, however, offer an useful alternative to these approaches. Glass wool affords a large surface-to-volume ratio (1 g with a mean diameter of 15 µm = 1 300 cm^2), supports the growth of biofilms under low shear conditions, thereby allowing for free movement of the cells between the inter-strand spaces, and it also facilitates the exchange of nutrients and oxygen.

The aims of this study were thus the development of a method to culture copious amounts of biofilm and to compare protein patterns of biofilm, surface influenced planktonic and true planktonic cells of *P. aeruginosa*. To accomplish this, *P. aeruginosa* was cultured in the presence of glass wool to yield biofilm cells. In order to find differences between the biofilm cells and their planktonic counterparts, phenotyping was accomplished by 2-DE of the whole-cell proteins using a non-linear carrier ampholyte pH gradient, ranging from pH 3.0 to 10.0, for the first dimension and the Laemmli (1970) SDS-PAGE system for the second dimension. Comparative analysis on the respective proteomes indicated striking differences between the protein patterns of planktonic and biofilm cells.

3.2   MATERIALS AND METHODS

3.2.1   Bacterial strain and medium

P. aeruginosa PAO1 (DSM 1707) was used in all studies. Preculture was performed in a 100-ml Erlenmeyer flask containing 50 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). The flask was incubated at 37°C on a rotary shaker (200 rpm) for 4 h until mid-exponential phase was reached (OD_{540} = 0.1). The culture was subsequently used to inoculate 100 ml MSGY broth in 500-ml Erlenmeyer flasks, with and without 2.5 g glass wool (mean diameter 15 µm, total surface area 3 250 cm^2) (Merck, Darmstadt, Germany), to a final inoculum of 4×10^6 cfu/ml.

3.2.2   Microscopy and analytical procedures

To evaluate whether glass wool served as a surface for the establishment of biofilms, bright-field microscopy (Zeiss Axioskop, Zeiss, Oberkochen, Germany) was performed on various samples of glass wool obtained at times 0 h, 4 h, 8 h, 18 h, 24 h and 48 h after inoculation. The glass wool was stained with 0.01% (w/v) crystal violet and immediately viewed by bright-field microscopy. Images were captured using a COHU monochrome CCD camera (RS-170, Cohn Inc., San Diego, CA, USA).

For analytical procedures, samples of planktonic cells cultured in the absence and presence of glass wool and biofilm cells were obtained after 18 h of incubation, as described in Sections 3.2.3.1 - 3.2.3.3. The planktonic cells taken from flasks containing glass wool were referred to as surface influenced planktonic (SIP) cells. The respective samples were diluted to a final volume of 100 ml prior to analysis. The culturable count was determined by plating 0.1-ml aliquots of serial dilutions onto triplicate plates of Luria Bertani (LB) agar and incubating for 24 h at 37°C. The optical density of cell suspensions was determined at 540 nm. Total protein concentrations were determined according to the method of Bradford (1976). Briefly, cell suspensions were disrupted by ultrasonication by applying 3×20 s pulses using a 4710 Series Ultrasonic Homogenizer (Cole-Palmer Instrument Co., Chicago, IL, USA) at an output of 40%. Lysates were boiled for 10 min, 50-µl aliquots mixed with 1.5 ml Coomassie Plus

Protein Assay Reagent (Pierce, Rockford, IL, USA) and the absorbance measured at 595 nm. The protein concentration was calculated using bovine serum albumin (BSA) as standard.

3.2.3 Extraction of whole-cell proteins

3.2.3.1 Planktonic biomass

After incubation for 18 h at 37°C, planktonic *P. aeruginosa* cells, cultured without glass wool, were collected by centrifugation at 13 000 $\times$ *g* for 10 min. The pellet was washed twice in 0.2 M sodium phosphate buffer (pH 6.8) and then resuspended in 10 mM Tris-HCl (pH 7.4). The suspension was heated to 95°C for 30 min and sonicated by six pulses of 15 s each using a 4710 Series Ultrasonic Homogenizer at an output of 40%. Lysis buffer B, composed of 9 M urea; 65 mM DTE; 65 mM CHAPS and 5% (v/v) ampholytes (pH 3.0 - 10.0) (Amersham-Pharmacia Biotech, Uppsala, Sweden)(Gravel and Golaz, 1996) was added. The protein sample was then stored at -70°C until required.

3.2.3.2 Biofilm biomass

The glass wool, cultured for 18 h with *P. aeruginosa*, was removed from the MSGY broth, rinsed twice in 0.2 M sodium phosphate buffer (pH 6.8) and then placed in a sterile flask containing 45 g of glass beads (mean diameter 6 mm). Ten ml of 10 mM Tris-HCl (pH 7.4) was added to the flask and it was shaken vigorously for 10 min to detach the bacterial cells from the glass wool surface. The bacteria were then collected by centrifugation (13 000 $\times$ *g*, 10 min) and samples were processed as described above for the planktonic bacterial cells.

3.2.3.3 Surface influenced planktonic (SIP) biomass

The *P. aeruginosa* cells remaining in the medium after removal of the glass wool were also collected by centrifugation at 13 000 $\times$ *g* for 10 min, and proteins were extracted as described in Section 3.2.3.1 for the planktonic bacterial cells. These bacterial cells are referred to as surface influenced planktonic (SIP) cells to indicate their origin.

3.2.4 Concentration of protein samples

All protein samples were concentrated using the method of Wessel and Flugge (1984). Since the biofilm biomass was less than both the planktonic and SIP biomasses, a larger volume of the biofilm sample was concentrated. One hundred and fifty µl of the planktonic and SIP

samples were concentrated to a final volume of 100 µl each. Three hundred µl of the biofilm sample was concentrated to a final volume of 40 µl. The protein content of each extract was determined by a Coomassie Plus Protein Assay Reagent (Pierce) and standardised to *ca.* 200 µg for each gel.

3.2.5 Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was performed according to the method of O'Farrell (1975). Briefly, iso-electric focusing (IEF) was carried out in 12-cm long, 1.0-mm diameter tube gels containing 6.7% (v/v) polyacrylamide, 15 M urea and 2% (v/v) ampholytes (Ampholine pH 5.0 - 7.0 and Pharmalyte pH 3.0 - 10.0; Amersham-Pharmacia Biotech) in a 4:1 ratio for 7 200 Vh. Upon completion of the 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). The gels were then sealed on top of a 10% SDS-PAGE gel (10% T, 2.7% C_{bis}) using a sealing solution (0.5 M Tris-HCl; 1% [w/v] SDS; 1% [w/v] agarose, pH 6.8). 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 5 h 35 min. A constant temperature of 18°C was maintained during electrophoresis. After electrophoresis, proteins were visualised by silver diamine staining (Dunn, 1996). The pH gradient was determined experimentally by using the 2-D SDS-PAGE Standard from BioRad (Cat. 161-0320). The molecular masses were determined by electrophoresis of a premixed molecular weight marker (Roche Diagnostics, Mannheim, Germany), covering the 14 - 98 kDa range, in the second dimension.

3.2.6 Image analysis

To account for experimental variations, at least three gels were prepared for each protein sample. The spot pattern of each gel was summarized in a standard after spot matching. Thus, one standard gel was obtained for each *P. aeruginosa* protein sample. These standards were then matched to yield information about up- and down-regulation of spots. Spots of interest on the gels were scanned with a GS 300 Transmittance/Reflectance Scanning Densitometer (Hoefer Scientific Instruments, San Francisco, CA, USA) and the data was processed with the Gelcompar 4 software program (Applied Maths BVBA, Kortrijk,

Belgium). The values of the planktonic spots were regarded as the spots of reference (0). The relative importance of the other spots compared to these reference spot levels was noted from -2 to 4. The highest value was assigned to the most intense spot.

3.3 RESULTS

3.3.1 Biofilm development on glass wool

Biofilm formation by *P. aeruginosa* PAO1 cells inoculated into MSGY broth containing glass wool was monitored at various time intervals by bright-field microscopy. Very few cells were visible on the glass wool immediately after inoculation (Fig. 3.1a). More cells became visible on the glass wool 4 h later (Fig. 3.1b) and microcolonies were clearly visible after 8 h (Fig. 3.1c). Dense biofilm structures formed within 18 h (Fig. 3.1d) and these became denser after 24 h (Fig. 3.1e) and 48 h (Fig. 3.1f) of incubation. The biofilms developed predominantly as colonies on the glass wool, leaving large sections uncovered. This led to a relatively open structure allowing for free movement of cells and liquid between the inter-strand spaces. It was therefore concluded that copious amounts of *P. aeruginosa* PAO1 biofilm biomass could be obtained after 18 h of growth in the presence of glass wool. Quantitative analysis of 18 h-old cultures revealed that cell parameters differed among the three modes of growth. Biofilm cells had a higher biomass-to-cell ratio than planktonic cells, both when measured by total cellular protein and by optical density (Table 3.1). This indicated that biofilm cells of *P. aeruginosa* were larger than planktonic cells and this has been confirmed by microscopic measurement of cell length and width (data not shown). In contrast, SIP cells appeared smaller than planktonic cells. Only *ca.* 15% of the biomass, corresponding to 5% of the number of cells, were attached to glass wool, the rest being in the SIP state.

3.3.2 2-DE maps

To characterise protein expression differences between the planktonic, SIP and biofilm cells, high-resolution 2-DE of whole-cell extracts was subsequently performed in parallel. Approximately 500 distinct protein spots in the pH range 3.0 - 10.0 were observed after silver staining. The reproducibility of separation of total proteins was high and Figures 3.2 to 3.4 show a representative example of planktonic, SIP and biofilm proteins, respectively. By matching and comparing the respective 2-DE maps, 41 proteins, the levels of which varied in a significant and reproducible way in the respective protein profiles, were selected.
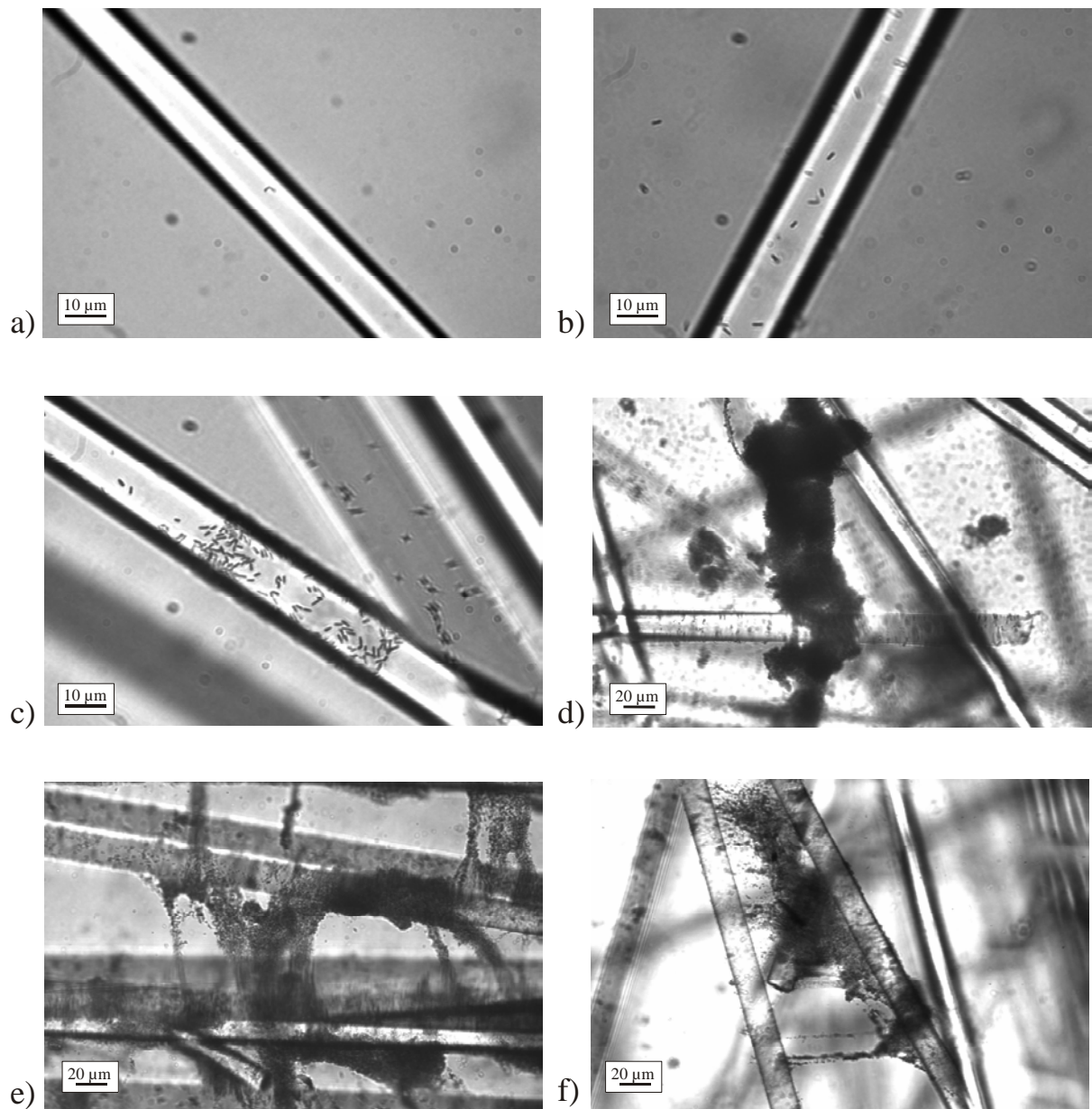
Fig. 3.1 **Development of *P. aeruginosa* biofilm on glass wool over time. One hundred milliliters of MSGY broth, containing 2.5 g of glass wool, was inoculated with *P. aeruginosa* PAO1 cells to a final inoculum of 4×10^6 cfu/ml. Bright-field microscopy was performed on samples of glass wool following staining with 0.01% (w/v) crystal violet at times (a) 0 h; (b) 4 h; (c) 8 h; (d) 18 h; (e) 24 h and (f) 48 h after inoculation.**

Table 3.1 **Biomass parameters of planktonic, SIP and biofilm cultures of *Pseudomonas aeruginosa* grown at 37°C for 18 h in MSGY broth (standard deviations are given in parentheses)**

	Planktonic [a]	SIP [b]	Biofilm [c]
Optical density (A_{540nm})	1.38 (0.01)	1.31 (0.02)	0.22 (0.07)
Protein concentration [d] (μM/ml)	248.8 (40)	231.3 (10)	45 (4)
Culturable count [e] (cfu/ml)	3.9×10^9 (3.3×10^8)	6×10^9 (5.8×10^9)	3.3×10^8 (2.5×10^7)
Culturable count [e] (cfu/cm^2)			1.02×10^7 (7.7×10^5)
A_{540nm}/Count $\times 10^{10}$	3.6	2.2	6.7
Protein/Count $\times 10^8$	6.4	3.9	13.6

a) Planktonic cells cultured in the absence of glass wool
b) Planktonic cells cultured in the presence of glass wool
c) Biofilm culture on glass wool
d) Protein concentration determined by the Bradford method
e) Culturable count on Luria Bertani agar after incubation for 24 h at 37°C

Table 3.2 summarizes the results obtained for the whole-cell protein extracts in comparison to that of planktonic cells. In general, four expression patterns were seen: (1) the spot is unique to a particular profile; (2) the spot is present in planktonic cells, is underexpressed in SIP cells, but overexpressed in biofilm cells; (3) the spot is present in planktonic cells, absent in SIP cells, but overexpressed in biofilm cells; (4) the expression of spots present in the planktonic cells remains unchanged in either SIP or biofilm cells. Pattern 2 was observed for most of the proteins expressed differentially.

3.3.3   Comparison of the proteome profiles

In the SIP cells, a general down-regulation of the spots was seen, but in biofilm cells expression of the spots was generally up-regulated. Altogether six unique proteins were seen in the planktonic cells, while the biofilm and SIP cells contained five and two unique proteins, respectively, *i.e.* they were not present or could not be detected by silver stain in the other cells in this pH range.

For 18 proteins, the levels were higher in the biofilm cells, and for five proteins, the levels were lower in the biofilm cells (Fig. 3.3) when compared to the planktonic cells (Fig. 3.2). The differences were usually more than 10 - 50% and in some cases a many-fold difference (700%) was observed (Table 3.2). A comparison of biofilm and SIP cells (Fig. 3.4) indicated that among the 18 proteins whose amounts increased in biofilm cells, 13 were common to SIP cells, but the other five were only expressed by biofilm cells (Table 3.2). Of these 13 spots, 10 were differentially down-regulated in the SIP cells, the expression levels of two spots were similar to that of their respective reference spots, while one was lower in the SIP cells than in the biofilm cells, but higher than that of the reference spot. With the exception of one protein, which was overexpressed in SIP cells, 17 spots were differentially down-regulated in the SIP cells by at least 10 - 200% in comparison with the planktonic cells. Interestingly, seven spots that were present in both planktonic and biofilm cells, were absent in the SIP cells.
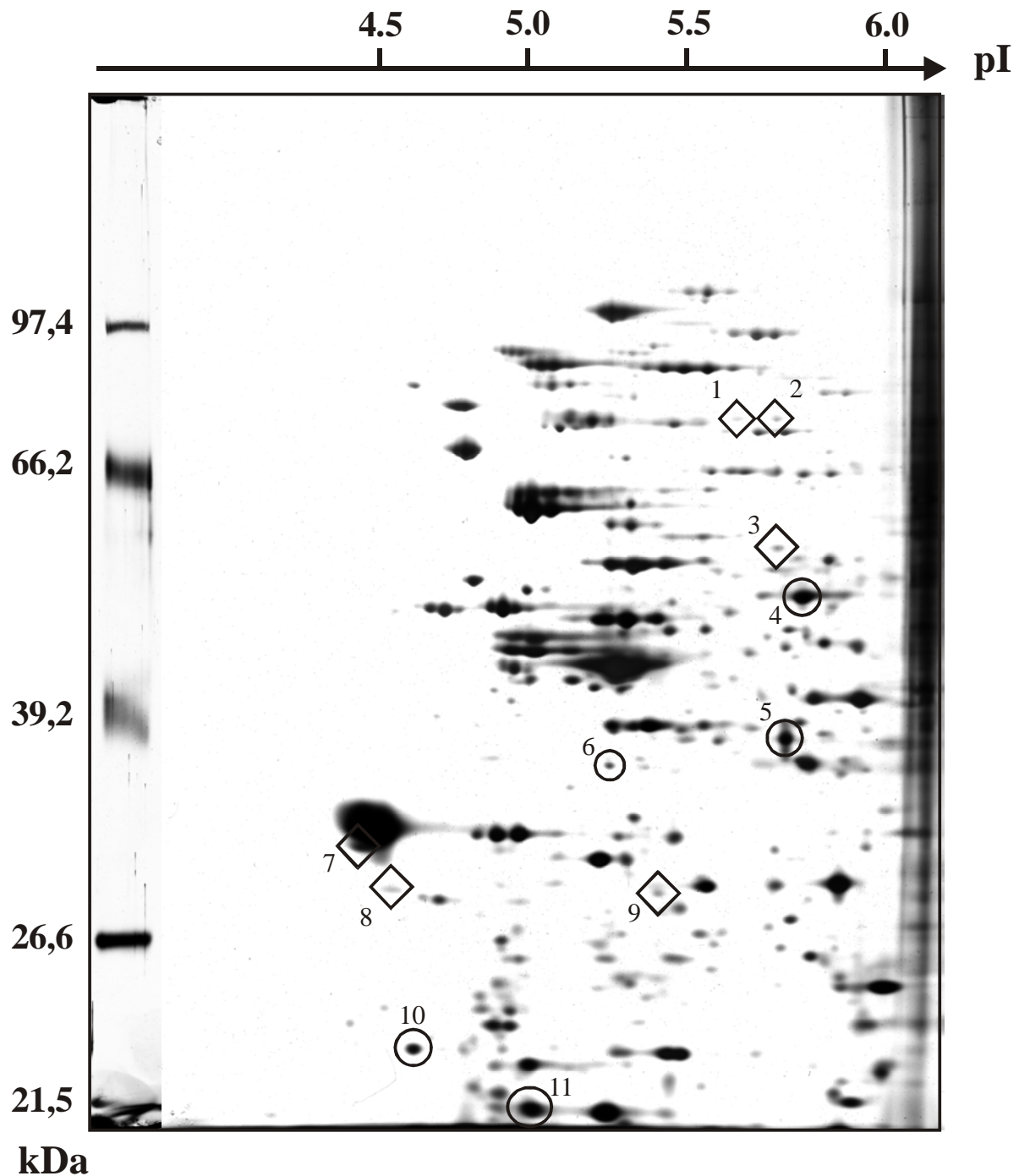
Fig. 3.2 **2-DE of whole-cell proteins of an 18 h planktonic *P. aeruginosa* PAO1 culture without glass wool. The proteins were focused in a non-linear pH gradient of 3.0 to 10.0. After iso-electric focusing, the sample was run on 10% SDS-PAGE and subsequently stained with silver diamine staining. The proteins that are differentially displayed between the planktonic and the other two phases (biofilm and SIP) are circled and assigned arbitrary numbers. Diamonds indicate proteins that are unique to the planktonic proteome. Molecular mass markers (in kDa) are indicated to the left of the gel.**
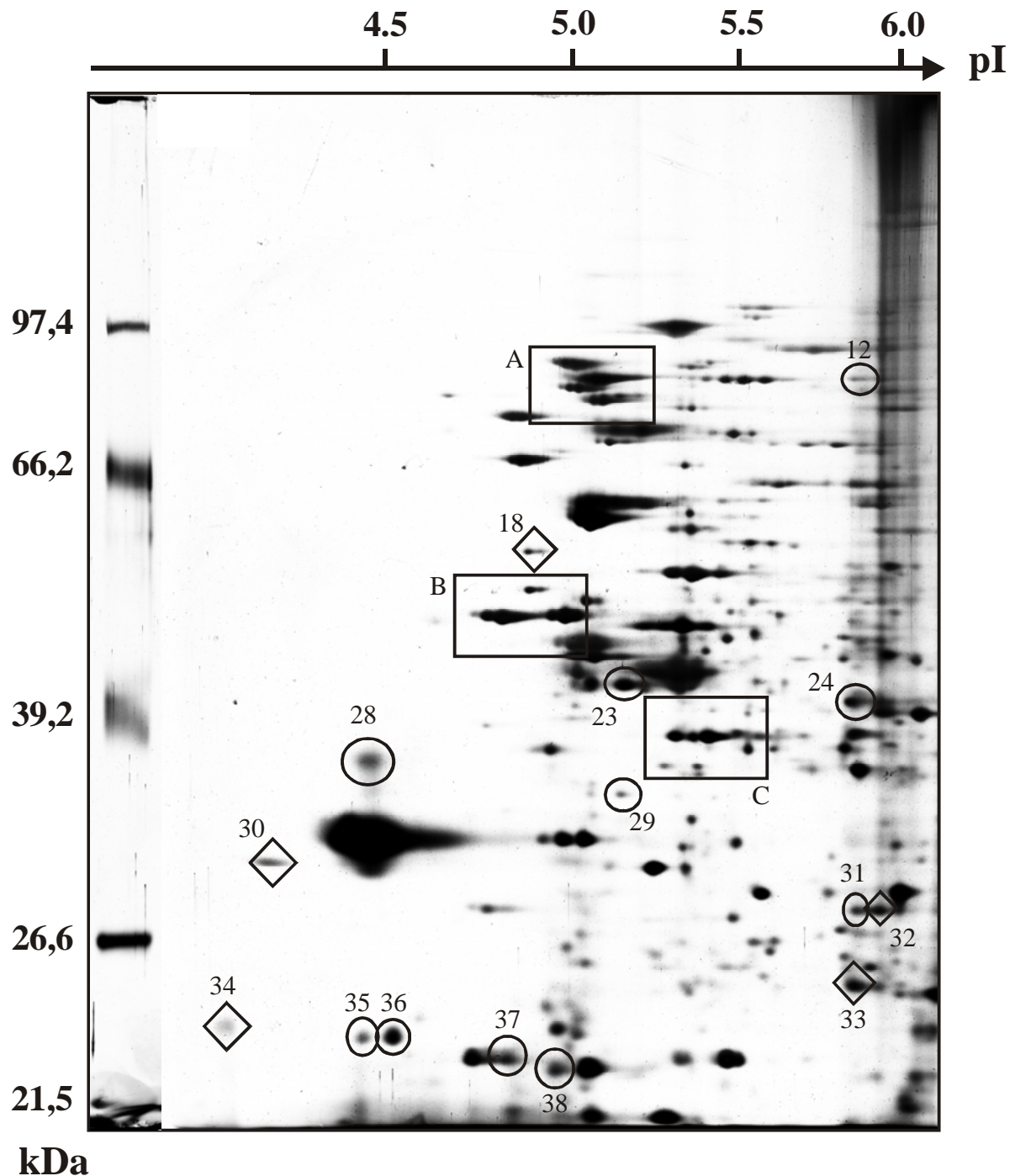
Fig. 3. 3 **2-DE of whole-cell proteins of an 18 h biofilm *P. aeruginosa* PAO1 culture grown on glass wool as attachment surface. The proteins that are differentially displayed between the biofilm and the other two phases (planktonic and SIP) are circled and assigned arbitrary numbers. Diamonds indicate proteins that are unique to the biofilm proteome. The framed areas A, B, and C are enlarged to enhance resolution of differences in that area (see following page). Molecular mass markers (in kDa) are indicated to the left of the gel.**
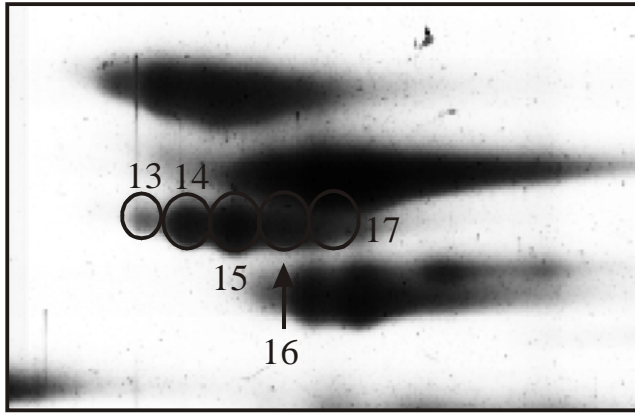
A
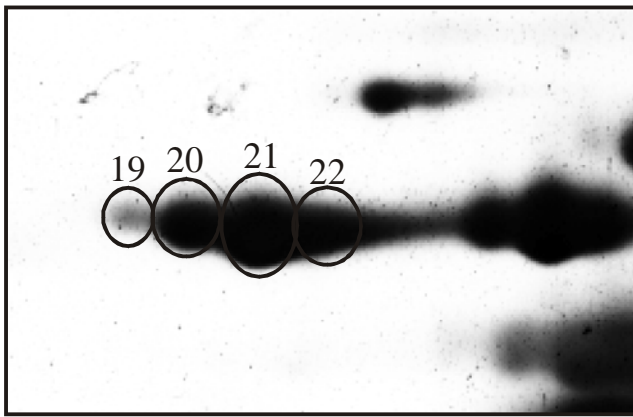
B
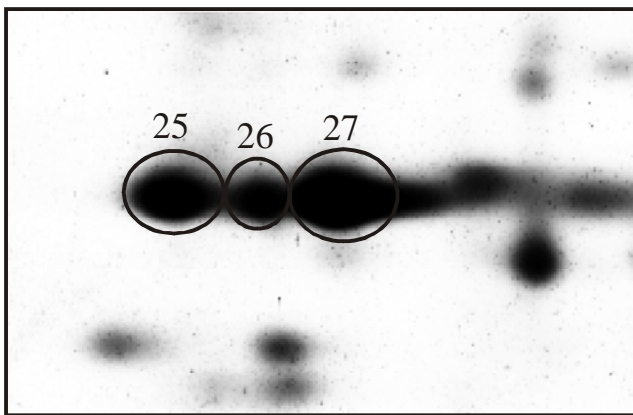
C
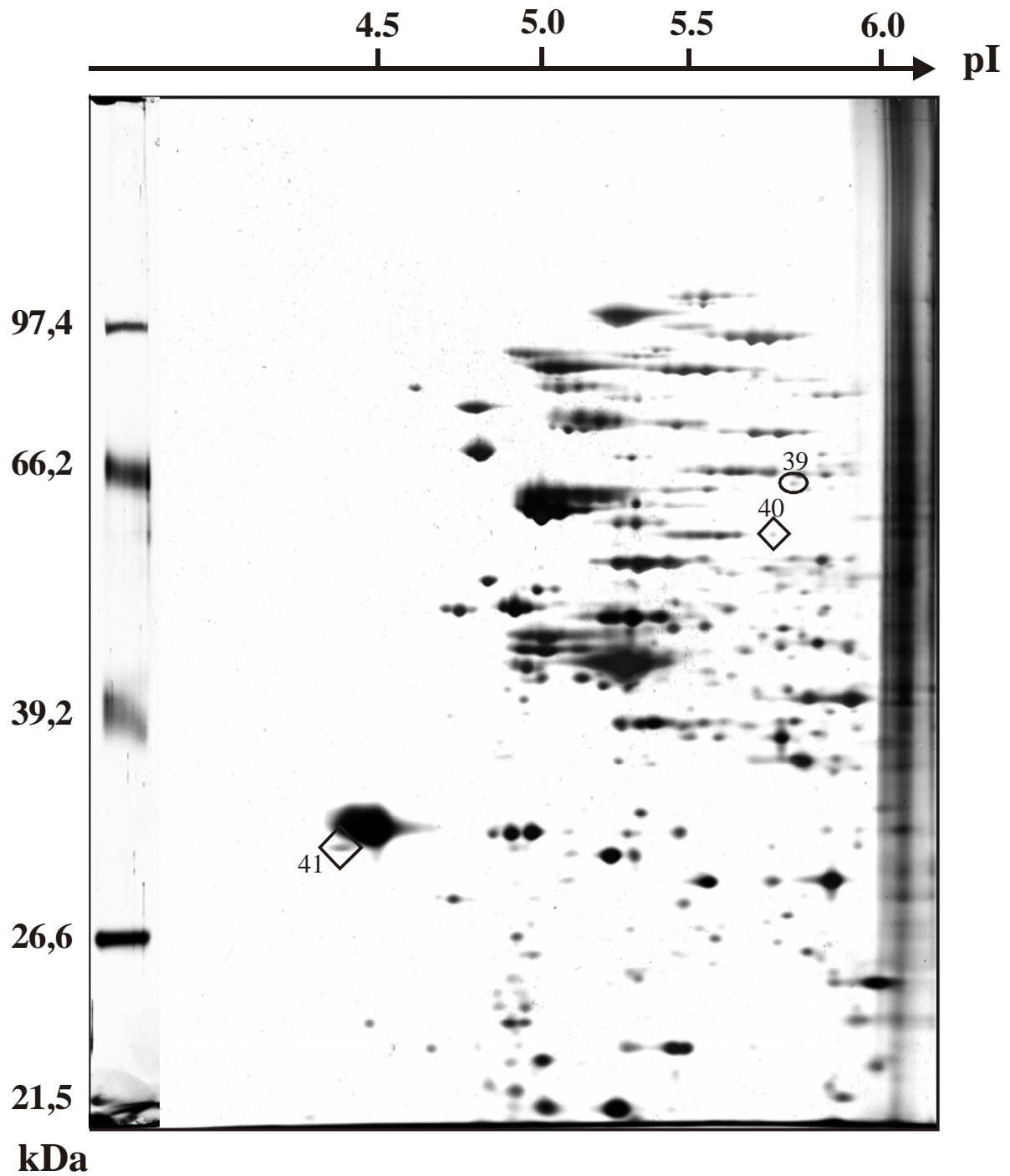
Fig. 3.4 **2-DE of whole-cell proteins of an 18 h SIP *P. aeruginosa* PAO1 culture. The protein that is differentially displayed between the planktonic and the SIP phase is circled and assigned an arbitrary number. Diamonds indicate proteins that are unique to the SIP proteome. Molecular mass markers (in kDa) are indicated to the left of the gel.**

Table 3.2 **Comparative analysis of 41 proteins selected from 2-DE profiles of 18 h planktonic, SIP and biofilm cells of *Pseudomonas aeruginosa***

Spot no.	Planktonic	SIP	Biofilm
1	U [a]	A [b]	A
2	U	A	A
3	U	A	A
4	0 [c]	-2	-2
5	0	0	-1
6	0	-1	-2
7	U	A	A
8	U	A	A
9	U	A	A
10	0	-2	0
11	0	-2	0
12	0	A	+2
13	0	A	0
14	0	A	+3
15	0	A	+2
16	0	A	+2
17	0	A	-1
18	A	A	U
19	0	-2	+2
20	0	-1	+1
21	0	0	+1
22	0	-1	+2
23	0	-1	+1
24	0	-1	+2
25	0	-1	+1
26	0	-1	+2
27	0	-1	0
28	0	-2	-2
29	0	0	+4
30	A	A	U
31	0	-1	+4
32	A	A	U
33	A	A	U
34	A	A	U
35	0	A	+3
36	0	+2	+4
37	0	-2	+2
38	0	-2	+2
39	0	-1	A
40	A	U	A
41	A	U	A

a) The protein spot is unique to the growth phase
b) Absence of the protein spot
c) Indicates a synthesis level similar to that of the planktonic growth phase
+ Up-regulation
- Repression/down-regulation
  1 = 10-50%; 2 = 51-200%; 3 = 201-700%; 4 = 700% +

3.4   DISCUSSION

Genetic studies of single-species biofilms have shown that they form in multiple steps (Watnick and Kolter, 1999; O'Toole and Kolter, 1998). Biofilms thus appear to be dynamic structures with cells leaving and re-colonizing elsewhere and this phenomenon should, therefore, be taken into account when studying bacterial biofilms.

In this study, we have expanded on a recently published method using glass wool as substrate for culturing biofilm biomass for proteomic analysis (Oosthuizen *et al.*, 2001) by not only comparing the planktonic and biofilm proteomes, but also the surface influenced planktonic (SIP) proteome. Microscopic examination of *P. aeruginosa* cells, cultured in the presence of glass wool, indicated that the cells attached to the substratum within 4 h, developed microcolonies within 8 h and mature biofilm structures were observed after 18 h of incubation. Nevertheless, only 15% of the total biomass was attached, with the remainder occurring in suspension (designated as SIP cells in this study). These results correspond well with those obtained by Rice *et al.* (2000) who demonstrated that a significant proportion of attached cells detach following the first and second division events. Neither has the factors affecting the ratio of attached to planktonic (SIP) cells, nor has the detached (SIP) cells been studied in any detail as most biofilm studies rely either on the use of continuous flow-through systems such as flow cells (Zinn *et al.*, 1999), or on entrapment procedures (Jouenne *et al.*, 1994; Gilbert *et al.*, 1998). Glass wool appears to be an ideal attachment surface where large amounts of biofilm biomass and SIP cells are required for separate proteomic analysis.

To investigate variations in protein expression between planktonic, SIP and biofilm *P. aeruginosa* cells, advantage was taken of the technique of 2-DE. For this initial study, 2-DE gels were produced that covered a broad pI range in order to provide a more accurate picture of all proteins being expressed. In addition to providing a high-resolution separation of a complex mixture of proteins, the degree of staining of individual protein spots represents a quantitative measurement of the relative amounts of the protein. Comparison of the respective 2-DE maps revealed noteworthy differences. These differences were observed across the proteome profiles and were not limited to specific protein sizes or pI values.

The planktonic, SIP and biofilm *P. aeruginosa* cells displayed distinct phenotypes, both by their 2-DE profiles and their respective deduced cell sizes (Table 3.1). A comparison of 28

protein spots that are differentially expressed in the planktonic and biofilm 2-DE maps indicated that the amounts of the majority of spots (18) appeared to be up-regulated in biofilm cells (Table 3.2). Spots no. 14, 29, 31, 35 and 36 were particularly overexpressed in biofilm cells. The amounts of five biofilm spots (4, 5, 6, 17, 28) decreased compared to the planktonic cells and the amounts of four spots (10, 11, 13, 27) remained unchanged. Five protein spots present in biofilm cells, were absent from the planktonic cells (18, 30, 32, 33, 34). The data demonstrate that biofilm cells of *P. aeruginosa* are not ordinary planktonic cells and the physiological differences between biofilm and planktonic cells are furthermore illustrated by the high expression levels of specific proteins in biofilm cells. Oosthuizen *et al.* (2001) reported that the biofilm and planktonic proteomes of *B. cereus* contained uniquely expressed proteins and Perrot *et al.* (2000) showed that the amounts of several proteins in gel-entrapped *E. coli* cells were significantly different from those in planktonic bacteria, indicating that biofilm-specific regulation of protein expression is not unique to *P. aeruginosa*. This supports several recent reports showing that a variety of genes are required for biofilm development (O'Toole *et al.*, 2000b).

The other main observation from this study was the down-regulation of a large number of SIP proteins (17) compared to planktonic cells. To our knowledge, this is the first 2-DE analysis of *P. aeruginosa* SIP cells. The SIP cells are a unique physiological state as five spots, up-regulated in the biofilm over their respective planktonic levels, were absent in these cells (12, 14, 15, 16, 35) (Table 3.2). Two other spots, one with expression levels in biofilm cells similar to that of planktonic cells (13) and the other down-regulated in biofilm cells (17), were also absent in the SIP cells. This indicated that certain proteins occurring during planktonic growth, and even more during biofilm growth, were shut down completely in the SIP state. Furthermore, ten spots down-regulated from planktonic to SIP state were up-regulated in the biofilm state (19, 20, 22, 23, 24, 25, 26, 31, 37, 38). The planktonic state displayed six spots absent in both biofilm and SIP profiles, indicating some commonality between the latter two states. Thus, it appears as if the SIP mode of growth constitutes a state distinct from both the planktonic and biofilm modes.

In conclusion, high-quality, reproducible displays of the patterns of proteins expressed by *P. aeruginosa* planktonic, SIP and biofilm cells by 2-DE were obtained. In addition, differences were also detected between proteins expressed of *P. aeruginosa* that have phenotypes associated with the planktonic, SIP and biofilm states. Subsequent studies should therefore

consist of further characterising these proteins so that the full significance of these differences with regards to biofilm formation and regulation can be completely understood. The details of these analyses are provided in the following Chapter (Chapter 4).

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CHAPTER FOUR

PROTEOME COMPARISON OF *Pseudomonas aeruginosa* PLANKTONIC, SURFACE INFLUENCED PLANKTONIC AND BIOFILM POPULATIONS BASED UPON COMPOSITE TWO-DIMENSIONAL ELECTROPHORESIS GELS

4.1   INTRODUCTION

It has long been recognised that bacteria can switch from planktonic unicellular organisms to sessile multicellular communities known as biofilms (Costerton *et al.*, 1987; 1995). The transition to surface-attached (biofilm) growth is known to result in diverse changes in gene expression, which causes the attaching cells to become phenotypically and metabolically distinct from their planktonic counterparts (Costerton *et al.*, 1999; Davey and O'Toole, 2000; Stewart and Costerton, 2001). *Pseudomonas aeruginosa*, a ubiquitous environmental gram-negative bacterium, is one of the most studied biofilm-forming organisms and has emerged as a model organism in the study of surface- and biofilm-induced gene expression.

P. aeruginosa biofilms have been studied predominantly in stagnant batch culture by using microtitre plates (O'Toole and Kolter, 1998) or under conditions of continuous flow using chemostats (Sauer and Camper, 2001; Sauer *et al.*, 2002) and various different flow cells (McLean *et al.*, 1999; Zinn *et al.*, 1999). In contrast, batch cultures grown in the presence of glass wool as substratum for cell attachment has been reported to not only support the growth of copious amounts of biofilms (Steyn *et al.*, 2001; Oosthuizen *et al.*, 2002), but it also allows for the easy separation of the biofilm cells from the surrounding suspended cells (referred to as surface influenced planktonic cells or SIP). Using this approach, it has been reported that the SIP growth phase constitutes a mode of growth that is distinct from the planktonic and biofilm modes of growth in both *P. aeruginosa* (Steyn *et al.*, 2001) and *Bacillus cereus* (Oosthuizen *et al.*, 2002).

Bacterial attachment to a surface is dictated by several factors, including surface composition (Marshall, 1985; Van Loosdrecht *et al.*, 1990), environmental factors (O'Toole *et al.*, 2000b; Stoodley *et al.*, 2002; Stanley and Lazazzera, 2004) and several different gene products (Allison *et al.*, 1998; O'Toole and Kolter, 1998; DeFlaun *et al.*, 1999; O'Toole *et al.*, 2000a; Parkins *et al.*, 2001; Vallet *et al.*, 2001; Finelli *et al.*, 2003).   In   addition   to

lipopolysaccharides, extracytoplasmic polymeric substances and cell surface appendages such as fimbriae, pili and flagella, outer membrane and membrane-associated proteins may also play a role in facilitating the attachment of bacteria to surfaces. Recently, outer membrane proteins (OMPs) such as OprF (Yoon *et al.*, 2002) and OpdF (Finelli *et al.*, 2003) have been reported to be required for biofilm development by *P. aeruginosa*. Furthermore, microarray (Whiteley *et al.*, 2001) and proteomic (Sauer *et al.*, 2002) studies have suggested that some OMPs of *P. aeruginosa* are up-regulated in biofilm versus planktonic cells. However, their functional role in biofilm development has yet to be characterised.

Although proteomic methods have been effective for characterising bacterial proteomes, the extraction efficiency of membrane proteins with conventional solubilisation reagents used for two-dimensional polyacrylamide gel electrophoresis (2-DE) is often poor. Consequently, many bacterial OMPs are missing from two-dimensional gel proteome maps (Molloy *et al.*, 1998; Wilkins *et al.*, 1998; Herbert, 1999). These problems can be overcome by pre-fractionating bacterial whole-cell proteins based on differences in the relative solubility of the proteins in a series of buffers (Molloy *et al.*, 1998). The proteins are typically extracted sequentially, first highly soluble proteins with Tris-base followed by more hydrophobic proteins using conventional reagents (8 M urea; 4% CHAPS; 100 mM DTT; 40 mM Tris), and finally more intractable proteins with an enhanced solubilisation solution incorporating thiourea, tributyl phosphine (TBP) and sulfobetaines (*e.g.* SB3-10) (Rabilloud *et al.*, 1997; Herbert *et al.*, 1998). Notably, protein separation occurs across three 2-DE gels, thereby simplifying the pattern in each gel and allows for arraying more proteins than possible with a single gel.

In the previous study, high resolution 2-DE of whole-cell proteins was used to demonstrate phenotypic differences between the protein patterns of *P. aeruginosa* planktonic, surface influenced planktonic (SIP) and biofilm cells grown in the absence or presence of glass wool (Steyn *et al.*, 2001). However, based on the involvement of OMPs in *P. aeruginosa* biofilm development and the improvements reported in the extraction and solubilisation of bacterial OMPs for 2-DE analysis, the aim of this study was to increase the resolution of protein spots on the 2-DE gels and thus allow for a more comprehensive description of the phenotypic differences between the respective *P. aeruginosa* populations. In this study, phenotyping was accomplished using 2-DE of sequentially extracted proteins from whole-cell extracts followed by image analysis of the proteins using PDQuest software. Several differentially expressed

protein spots were selected and identified using a combination of N-terminal protein sequencing and peptide mass fingerprinting. The majority of proteins identified were categorised as outer membrane or membrane-associated proteins.

4.2   MATERIALS AND METHODS

4.2.1   Bacterial strain and medium

P. aeruginosa PAO1 (DSM 1707) was used in all studies. Preculture was performed in a 100-ml Erlenmeyer flask containing 50 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). The flask was incubated at 37°C on a rotary shaker (200 rpm) for 4 h until mid-exponential phase was reached (OD_{540} = 0.1). The culture was subsequently used to inoculate 100 ml MSGY broth in 500-ml Erlenmeyer flasks, with and without 2.5 g glass wool (mean diameter 15 μm, total surface area 3 250 cm^2) (Merck, Darmstadt, Germany), to a final inoculum of 4×10^6 cfu/ml. All cultures were incubated for 18 h at 37°C on a rotary shaker (200 rpm).

4.2.2   Collection of biomass

Planktonic *P. aeruginosa* populations were obtained from cultures grown in the absence of glass wool, while cultures grown in the presence of glass wool were used as a source of biofilm and surface influenced planktonic (SIP) populations. The biomass was collected as described previously (Section 3.2.3). Briefly, planktonic biomass was collected by centrifugation of the planktonic culture at 13 000 × *g* for 10 min. To recover the biofilm biomass, the glass wool was removed from the culture, rinsed twice in 40 mM Tris-base (pH 9.5) and then placed in a sterile flask containing 45 g of glass beads (mean diameter 6 mm). Following addition of 10 ml 40 mM Tris-base (pH 9.5) to the flask, it was shaken vigorously for 10 min and the detached bacterial cells were collected by centrifugation (13 000 × *g*, 10 min). The SIP biomass, *i.e.* the *P. aeruginosa* cells remaining in the medium after removal of the glass wool, were collected by centrifugation at 13 000 × *g* for 10 min.

4.2.3   Extraction of whole-cell proteins through differential solubilisation

The sequential protein extraction method used in this study is indicated diagrammatically in Fig. 4.1, and was based on that described by Molloy *et al.* (1998), with the following modifications. The cell pellets of planktonic, biofilm and SIP cultures were washed twice in 40 mM Tris-base (pH 9.5) and then resuspended in the same buffer containing 0.25% (w/v) phenylmethylsulphonyl fluoride (PMSF). The suspensions were vortexed and sonicated by six pulses of 15 s each using a 4710 Series Ultrasonic Homogenizer (Cole-Palmer Instrument Co., Chicago, IL, USA) at an output of 40%. Following a second cycle of vortexing and sonication, the samples were centrifuged at 13 000 × *g* for 10 min. The resulting supernatants, containing water-soluble proteins, were collected (Extract 1). In the second step, proteins in the insoluble pellets derived from the Tris-base extractions were solubilised using conventional solubilisation solution (CSS), composed of 8 M urea; 4% (w/v) CHAPS; 100 mM DTE; 40 mM Tris (pH 9.5) and 0.5% (v/v) ampholytes (Pharmalyte pH 3.0 - 10.0; Amersham-Pharmacia Biotech, Uppsala, Sweden). The samples were subjected to vortexing, sonication and centrifugation, as described above, and the supernatants were collected (Extract 2). The pellets were then subjected to a further extraction with an enhanced extraction solution (EES), composed of 5 M urea; 2 M thiourea; 2% (w/v) CHAPS; 2% (w/v) SB3-10; 2 mM TBP; 40 mM Tris (pH 9.5) and 0.5% (v/v) ampholytes (Pharmalyte pH 3.0 - 10.0; Amersham-Pharmacia Biotech). Following vortexing, sonication and centrifugation, the supernatants enriched with hydrophobic proteins were collected (Extract 3). In the final step, the resultant pellets were resuspended in SDS sample solution (SDS-SS), composed of 1% (w/v) SDS; 375 mM Tris (pH 8.8); 50 mM DTE and 25% (v/v) glycerol, followed by boiling at 97ºC for 5 min (Extract 4). All protein samples were stored at -70ºC until needed.

4.2.4   Concentration of protein samples

All protein samples were concentrated with methanol, a non-polar solvent, using the method of Wessel and Flugge (1984). Since the biofilm biomass was less than both the planktonic and SIP biomasses, a larger volume of the biofilm sample was concentrated. One hundred and fifty µl of the planktonic and SIP samples were concentrated to a final volume of 100 µl each. Three hundred µl of the biofilm sample was concentrated to a final volume of 40 µl. The protein content of each extract was determined by a Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL, USA) and standardised to *ca.* 400 µg for each gel.
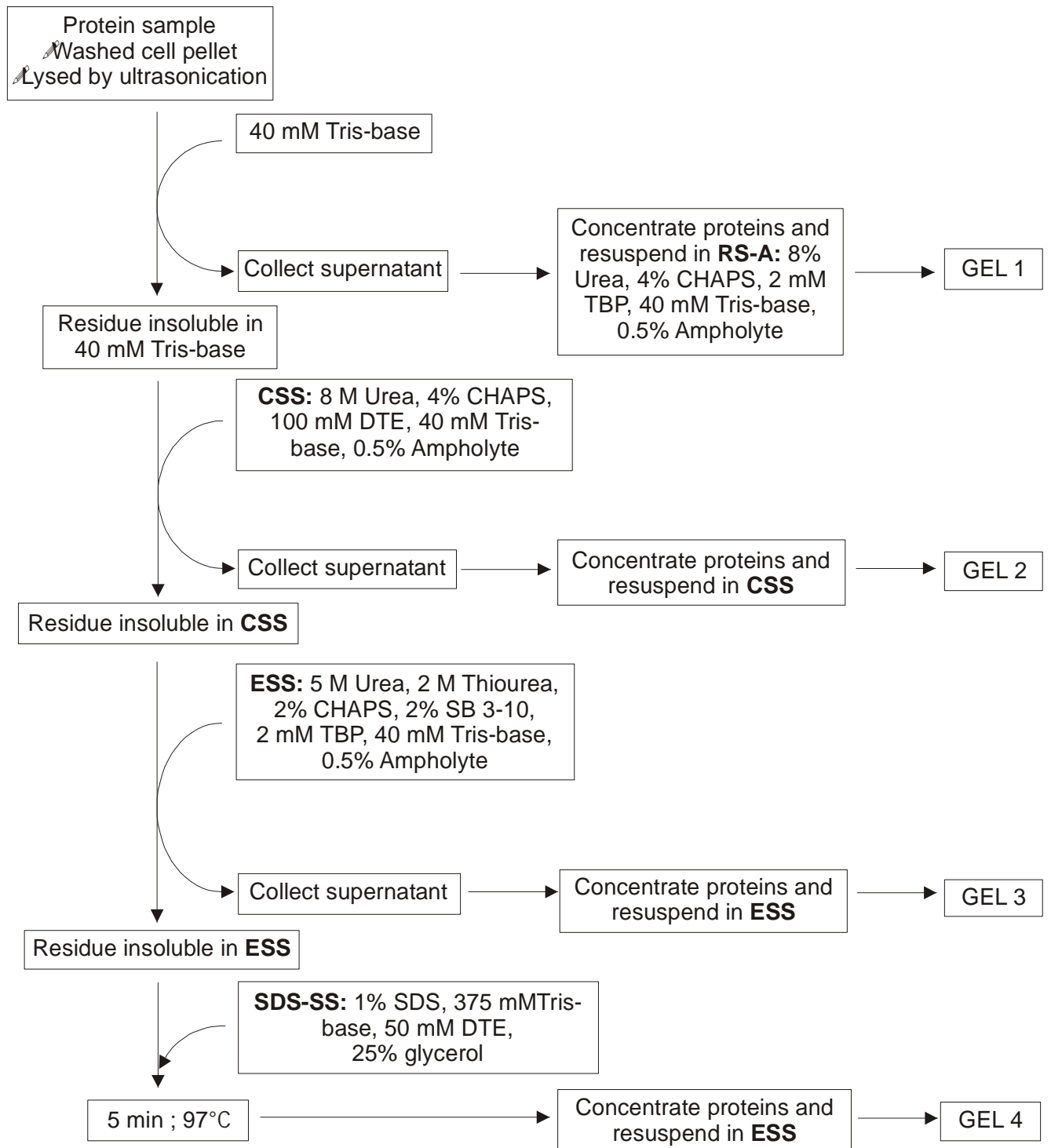
Fig. 4.1 **Schematic illustration of the differential extraction procedure that was performed on whole-cell protein samples from *P. aeruginosa* planktonic, SIP and biofilm populations. Abbreviations: RS-A, resuspension solution A; CSS, conventional solubilisation solution; ESS, enhanced solubilisation solution; SDS-SS, SDS sample solution.**

4.2.5 Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was performed according to the method of O'Farrell (1975). Briefly, iso-electric focusing (IEF) was carried out in 12-cm long, 1.0-mm diameter tube gels containing 6.7% (v/v) polyacrylamide, 15 M urea and 2% (v/v) ampholytes (Ampholine pH 5.0 - 7.0 and Pharmalyte pH 3.0 - 10.0; Amersham-Pharmacia Biotech) in a 4:1 ratio for 7 200 Vh. Upon completion of the 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). The gels were then sealed on top of a 10% SDS-PAGE gel (10% T, 2.7% C_{bis}) using a sealing solution (0.5 M Tris-HCl; 1% [w/v] SDS; 1% [w/v] agarose, pH 6.8). 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 5 h 35 min. A constant temperature of 18°C was maintained during electrophoresis. After electrophoresis, proteins were visualised either by silver diamine staining (Dunn, 1996) or by colloidal Coomassie G-250 staining (Sigma, St Louis, MO, USA) (Neuhoff *et al.*, 1988). The pH gradient was determined experimentally by using the 2-D SDS-PAGE Standard from BioRad (Cat. 161-0320). The molecular masses were determined by electrophoresis of a premixed molecular weight marker (Roche Diagnostics, Mannheim, Germany), covering the 14 - 98 kDa range, in the second dimension.

4.2.6 Image analysis

The gels were scanned with an ImageScanner (Amersham-Pharmacia Biotech). The image analysis, including spot quantification and matching between gels, was performed using the PDQuest Version 7.1.1 software program (BioRad Laboratories) according to the protocols provided by the manufacturer. To account for experimental variations, several gels from independent extractions were prepared. The gel spot pattern of 2-DE gels resulting from the analysis of prefractionated proteins of the *P. aeruginosa* planktonic, SIP and biofilm populations were summarised in a composite gel after spot matching. Thus, one composite gel was obtained for each of the respective *P. aeruginosa* populations and these were then matched to yield information about up- or down-regulation of spots. Only protein spots with a five-fold or greater change in signal intensity were taken into consideration.

4.2.7   Protein sequencing and identification

4.2.7.1   N-terminal amino acid sequencing and protein identification

The regions on the Coomassie blue-stained gels containing protein spots of interest were excised and electroblotted onto Immobilon-P polyvinylidene difluoride (PVDF) membranes (Millipore, Freehold, NJ, USA) using a 0.12 M Tris-0.04 M glycine buffer. The N-terminal sequence was determined by automated Edman degradation on a Procise 492 automatic sequencer (Applied Biosystems, Courtabeuf, France). The N-terminal sequences obtained were used for a BLASTP search to identify homologous amino acid sequences in the *P. aeruginosa* genome database (available at http://www.pseudomonas.com). Amino acid sequences were analysed for the presence of a signal sequence by the SignalP server (http://www.cbs.dtu.dk/services/SignalP), and the ExPASY compute pI/Mr tool (http://www.expasy.ch/tools/) was used for calculation of the theoretical molecular mass and pI of each identified protein.

4.2.7.2   Peptide mass fingerprinting and protein identification

Protein spots of interest were excised from the Coomassie blue-stained gels and sliced into small pieces using a sterile scalpel. Gel plugs were washed twice for 15 min with water and twice with H_2O/CH_3CN (1:1), and were then placed in 100% CH_3CN. Gel pieces were dried using a SpeedVac centrifuge for a few minutes before the trypsin solution, 10 µl of 20 ng/µl sequencing-grade trypsin (Roche Diagnostics) in 20 mM NH_4HCO_3 buffer, was added. After rehydration with the enzyme solution, buffer solution was added to cover gel pieces and digestion was allowed to proceed overnight at 37°C. Peptides were extracted using several volumes of a H_2O/CH_3CN/trifluoroacetic acid mixture (80:20:1). These fractions were pooled, dried in a vacuum centrifuge and then redissolved in 50 µl of 5% (v/v) formic acid. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) analyses were performed on a TOF-Spec-2E spectrometer (Micromass, Manchester, UK) equipped with a 337-nm nitrogen laser. The peptide fingerprints were matched against *in silico* digests using the MS-FIT software with the GenePept database restricted to *P. aeruginosa* (accessible at http://www.prospector.ucsf.edu). For confident spot identification, parameters including the number of peptides matched against the nonmatching number, sequence coverage, molecular mass and pI were used. The amino acid sequence of the identified proteins were analysed for the presence of a signal sequence, as described above.

4.3 RESULTS

4.3.1 "Composite map" creation of *P. aeruginosa* planktonic, SIP and biofilm populations

In a previous paper (Steyn *et al.*, 2001), 2-DE for whole-cell extracts from *P. aeruginosa* was established and it allowed for the reproducible separation of approximately 500 distinct protein spots in the pH range of 3.0 - 10.0 after silver staining. However, due to their hydrophobic nature, many bacterial outer membrane proteins (OMPs), membrane and cytoplasmic membrane proteins are missing from 2-DE gel proteome maps, thus hindering the analysis of complete proteomes (Molloy *et al.*, 1998; 2001; Herbert, 1999). Consequently, some technical improvements for 2-DE separation of bacterial OMPs have been reported. Major developments have concentrated on novel detergents, chaotropes and surfactants suitable for solubilising these proteins (Chevallet *et al.*, 1998; Herbert *et al.*, 1998; Santoni *et al.*, 1999; 2000). For successful 2-DE, standard IEF solubilisation solutions should purportedly be ameliorated to include thiourea, which has been reported to dramatically improve the separation of membrane proteins (Rabilloud *et al.*, 1997), and an amidosulfobetaine surfactant such as SB3-10, which has been reported to be a potent protein solubiliser (Molloy *et al.*, 1998). In addition, inclusion of phosphines such as TBP, an uncharged reducing agent (Ruegg and Rudinger, 1977), has been reported to improve resolution since it maintains reducing conditions for the entire IEF process, thereby minimising protein aggregation that could occur through disulfide bonding (Herbert *et al.*, 1998). Other reports have shown that a sequential series of extractions, removing highly soluble proteins first, is also an effective method for optimising the number of proteins seen on 2-DE gels (Molloy *et al.*, 1998; 2000; Hermann *et al.*, 2000).

To improve the existing methodology, proteins from whole-cell extracts of the *P. aeruginosa* planktonic, SIP and biofilm populations were sequentially extracted to prefractionate the proteins on the basis of their solubility in a series of buffers. The resultant "subproteomes", as defined by relative solubilities of the proteins, were then combined to create a "composite" protein map for each of the respective *P. aeruginosa* populations using the PDQuest software program. For this purpose, up to forty 2-DE subproteome gels were generated for each *P. aeruginosa* population using samples of several independent extractions. The 2-DE gels resulting from protein solubilisation in SDS sample buffer (SDS-SS) yielded gels containing either none or up to four protein spots and they were therefore excluded from the analysis.

Using the sequential extraction method, 692 unique protein spots could be resolved on the composite 2-DE protein maps in the pH range of 3.0 - 10.0. Compared to the single-step extraction method used previously, the visualised number of protein spots here represents an increase in resolution of approximately 38%. The increased resolution of proteins may be due to the use of sample preparation solutions that, in contrast to those used in single-step extraction methods, are capable of solubilising more and also poorly soluble proteins, especially hydrophobic membrane and membrane-associated proteins. In addition, prefractionating the sample proteins on the basis of their relative solubility in the different buffers may also have aided in minimising the overlap of less abundant proteins with abundant proteins, thus resulting in the resolution of many more proteins. The increased number of proteins resolved via 2-DE following sequential extraction of proteins solubilised from the planktonic, SIP and biofilm populations of *P. aeruginosa* is shown in Fig. 4.2 through Fig. 4.4. In each figure, highly soluble proteins are displayed in the first extract (A), followed by increasingly more hydrophobic proteins in the second (B) and third (C) extracts. The composite 2-DE protein maps derived from these three differential subproteome sets and representing approximately thirty 2-DE gels are also shown (D).

4.3.2 Proteome profile analysis

The composite proteome maps of the *P. aeruginosa* planktonic, SIP and biofilm populations after 18 h of growth in the absence or presence of a glass wool attachment substratum were subsequently investigated and compared to identify qualitative (*i.e.* protein spots not present or not detectable by silver stain in other proteomes), as well as quantitative (*i.e.* difference in concentration of individual protein spots) differences. For quantitative analysis, proteins exhibiting an increase or decrease by a factor of five or greater were chosen arbitrarily for further analysis. The results from these analyses are summarised in Table 4.1.

Comparative analysis of the 2-DE patterns obtained for the respective *P. aeruginosa* populations after 18 h of growth indicated the presence of five unique protein spots in the planktonic proteome, while the SIP and biofilm proteomes displayed 12 and 49 unique protein spots, respectively (Table 4.1a). Furthermore, 28 proteins were differentially up-regulated in the biofilm proteome, whilst 66 proteins were found to be up-regulated in the SIP proteome (Table 4.1b). The protein expression profiles also showed that approximately 10% of the proteins were always expressed to the same level in all populations (Table 4.1c). It was also
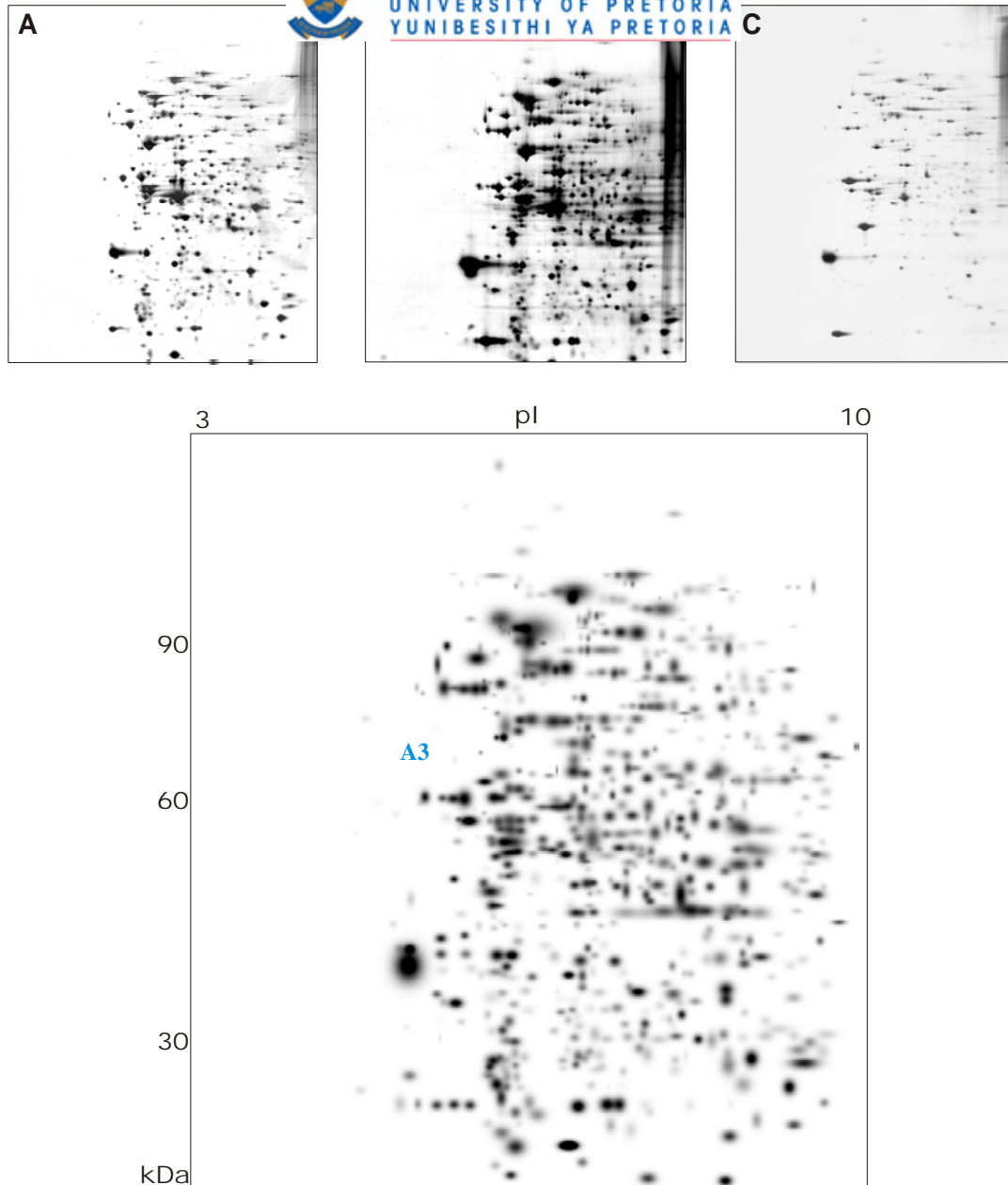
Fig. 4.2 **Sequential extraction of proteins from an 18 h planktonic *P. aeruginosa* PAO1 culture grown without glass wool. The proteins were focused in a non-linear pH gradient of 3.0 – 10.0, electrophoresed on 10% SDS-PAGE gels and subsequently stained with silver diamine staining. (A) Extract 1, proteins solubilised by 40 mM Tris-base; (B) Extract 2, proteins solubilised by 8 M urea, 4% CHAPS, 100 mM DTE, 40 mM Tris and 0.5% ampholytes; (C) Extract 3, proteins solubilised by 5 mM urea, 2 mM thiourea, 2% CHAPS, 2% SB3-10, 2 mM TBP, 40 mM Tris and 5% ampholytes; (D) Composite 2-DE gel representing sequentially extracted proteins from *P. aeruginosa* PAO1 planktonic cells. The proteins identified in this study are indicated by arrows. The red spots were identified by N-terminal sequencing (see Table 4.2), and the blue, numbered spots were identified by MALDI-TOF-MS analyses (see Table 4.3). Molecular mass markers (in kDa) are indicated to the left of the gels.**
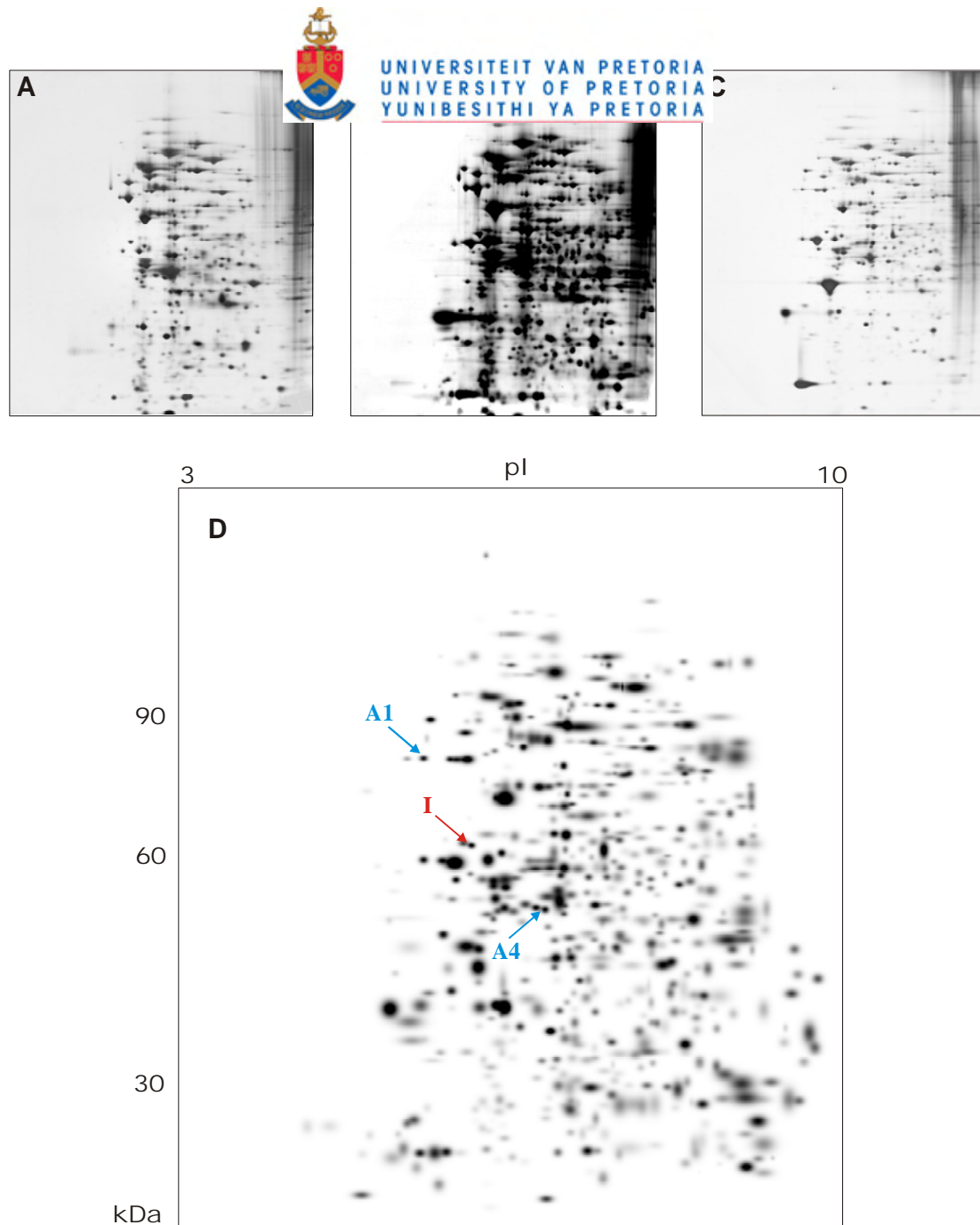
Fig. 4.3 Sequential extraction of proteins from an 18 h surface influenced planktonic (SIP) *P. aeruginosa* PAO1 culture. The proteins were focused in a non-linear pH gradient of 3.0 – 10.0, electrophoresed on 10% SDS-PAGE gels and subsequently stained with silver diamine staining. The proteins were sequentially extracted, as described in the legend to Fig. 4.2. (A) Extract 1; (B) Extract 2; (C) Extract 3; (D) Composite 2-DE gel representing sequentially extracted proteins from *P. aeruginosa* PAO1 SIP cells. The proteins identified in this study are indicated by arrows. The red spots were identified by N-terminal sequencing (see Table 4.2), and the blue, numbered spots were identified by MALDI-TOF-MS analyses (see Table 4.3). Molecular mass markers (in kDa) are indicated to the left of the gels.
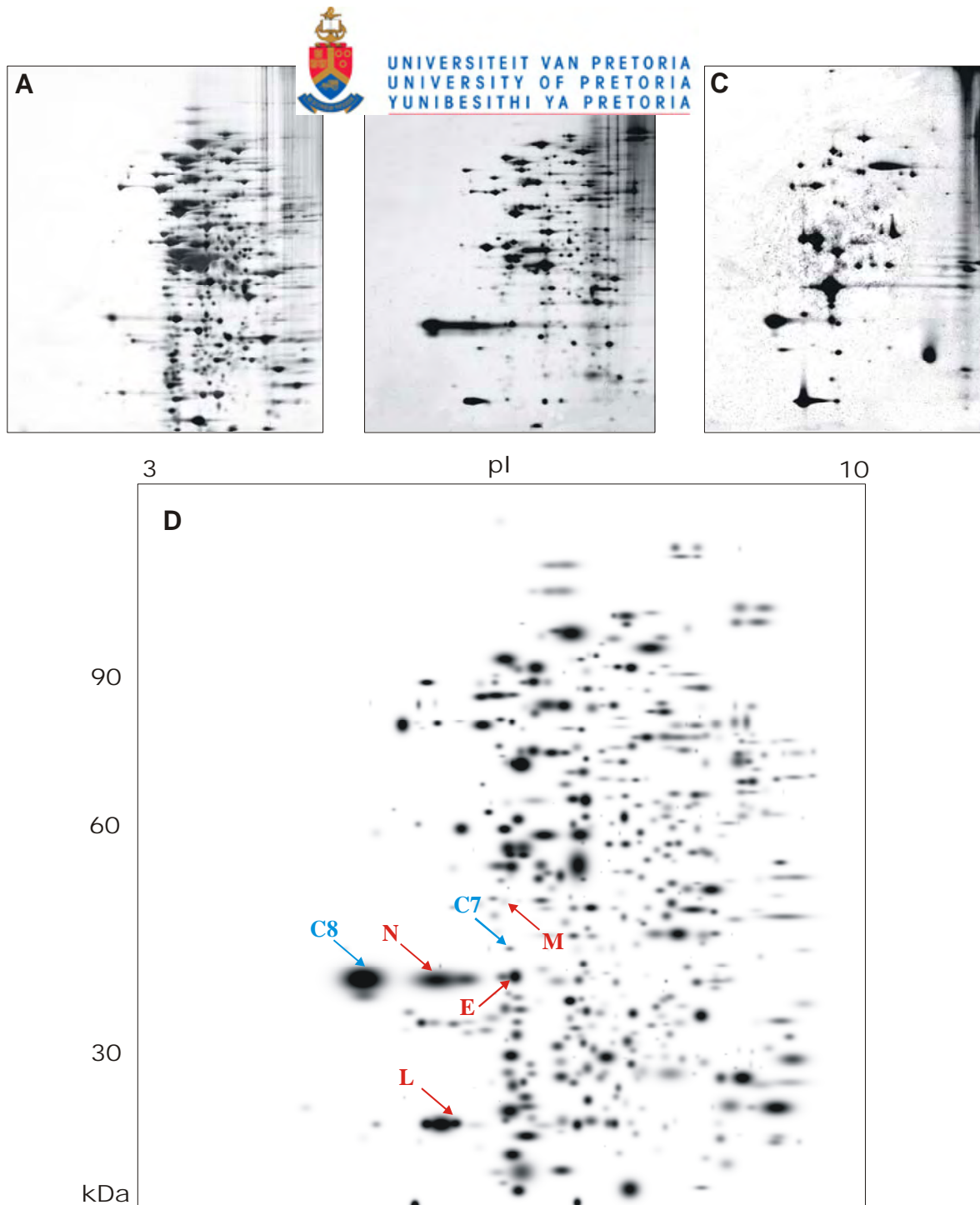
Fig. 4.4 Sequential extraction of proteins from an 18 h biofilm *P. aeruginosa* PAO1 culture grown on glass wool as attachment substratum. The proteins were focused in a non-linear pH gradient of 3.0 – 10.0, electrophoresed on 10% SDS-PAGE gels and subsequently stained with silver diamine staining. The proteins were sequentially extracted, as described in the legend to Fig. 4.2. (A) Extract 1; (B) Extract 2; (C) Extract 3; (D) Composite 2-DE gel representing sequentially extracted proteins from *P. aeruginosa* PAO1 biofilm cells. The proteins identified in this study are indicated by arrows. The red spots were identified by N-terminal sequencing (see Table 4.2), and the blue, numbered spots were identified by MALDI-TOF-MS analyses (see Table 4.3). Molecular mass markers (in kDa) are indicated to the left of the gels.

evident that the planktonic and SIP populations had 72 proteins in common, which were absent in the biofilm population (Table 4.1d). In contrast, the SIP and biofilm populations had 20 proteins in common that were absent in the planktonic cells, whereas the planktonic and biofilm populations displayed only nine uniquely shared proteins (Table 4.1e,f).

Cumulatively, the above results confirmed that the biofilm proteome differed from both the planktonic and SIP proteomes, and that the SIP cells displayed a distinct phenotype. The observed differences between the respective proteomes were not only limited to several spots being unique to either the planktonic, SIP or biofilm proteomes, but was also attributable to the up- and down-regulation of the expression of a multitude of proteins and may involve posttranslational modification of proteins.

4.3.3   Identity of differentially expressed proteins

In total, 34 protein spots were selected for protein identification. These spots were selected, because they varied reproducibly in their concentration as a consequence of changes in the growth conditions. The protein spots were analysed by N-terminal amino acid sequencing or peptide mass fingerprinting of tryptic digest fragments, following which the data was compared to the *P. aeruginosa* genome database in order to identify the proteins. Of the selected protein spots, six were identified by N-terminal sequence analysis (Table 4.2) and eight were identified by peptide mass fingerprinting (Table 4.3). A number of spots, including several uniquely expressed spots, could however, not be identified. In some cases, protein identification by peptide mass fingerprinting was compromised by unreliable peptide spectra (either few peptides or poor spectra). In addition, contaminants in the digest, probably resulting from insufficient washing of gel slices prior to digestion, resulted in high intensity of contaminant peaks that overshadowed the peptide peaks and thus led to a loss of data. These problems were most often associated with weakly stained protein spots. Similarly, the N-terminal amino acid sequence of some protein spots could also not be determined due to either low concentration of the protein spots or contamination of the excised protein spots with other proteins.

Overall, identifications were made to 14 protein spots representing 12 different proteins. The proteins could be divided into three main groups. The first group comprises proteins that could be categorised as outer membrane or membrane-associated proteins; the second group

Table 4.1 **Protein expression in *Pseudomonas aeruginosa* planktonic, surface influenced planktonic (SIP) and biofilm cells following 18 h of culturing in the absence and presence of glass wool**

Protein expression characteristic	Number of spots	% of total spots (n = 692)
a. Proteins unique to a specific growth phase:		
Planktonic cells	5	0.7
SIP cells	12	1.7
Biofilm cells	49	7.1
b. Proteins up-regulated five-fold in a specific growth phase above the levels of the corresponding spot in other phases:		
Planktonic cells	32	4.6
SIP cells	66	9.5
Biofilm cells	28	4.0
c. Proteins present in all growth phases:	68	9.8
d. Proteins present in planktonic and SIP cells, absent in biofilm cells:	72	10.4
e. Proteins present in SIP and biofilm cells, absent in planktonic cells:	20	2.9
f. Proteins present in planktonic and biofilm cells, absent in SIP cells:	9	1.3

comprises proteins that have sequence similarity to known OMPs; and the third group comprises cytoplasmic proteins, inclusive of two hypothetical proteins. The N-terminal amino acid sequence of protein spots E and M corresponded to hypothetical proteins PA0820 and PA5001, respectively. Comparison of these *P. aeruginosa* proteins to sequences contained in the GenBank database using the BLASTP tool indicated that the proteins displayed low levels of sequence identity to putative endonuclease enzymes (less than 40%) and glycosyltransferases (less than 30%), respectively. Notably, PA5001 contains a conserved RfaG domain, which is indicative of glycosyltransferase group 1 enzymes. Members of this family of enzymes transfer activated sugars (UDP-, ADP-, GDP- or CMP-linked sugars) to a variety of substrates, including glycogen, fructose-6-phosphate and lipopolysaccharides (Coutinho *et al.*, 2003).

4.3.3.1 Outer membrane proteins

Both protein spots B and L had the same molecular mass, but differed in their pI values. Whereas the level of expression of spot B was up-regulated in planktonic cells compared to biofilm cells, the inverse was observed for spot L and a higher level of expression was observed in biofilm cells compared to planktonic cells. The amino acid sequence derived from both protein spots corresponded to a single protein, outer membrane protein OprG precursor. To determine whether the two isoforms may represent posttranslationally modified versions of the same protein, the amino acid sequence of OprG was scanned using the ProScan function at the ExPASY Molecular Biology server. The results obtained indicated that the protein contains five potential phosphorylation sites for two different kinases. Thus, the spots may represent differentially phosphorylated versions of the same protein. For both proteins, a difference between the theoretical and observed molecular mass was also observed. It has been suggested that the OprG protein possesses a signal sequence (Gensberg *et al.*, 1999). The determined N-terminal sequence of protein spots B and L confirmed this suggestion, as the sequences obtained corresponded to residues starting at position 22 of the database sequence for OprG. Thus, the shift between the theoretical (25.19 kDa) and the observed (23 kDa) molecular mass may be due to proteolytic processing of the signal sequence (2.2 kDa), thereby resulting in the mature OprG protein being observed on the 2-DE gels. Although OprG is expressed constitutively at a low level, its expression is induced by an increase in iron concentration (Yates *et al.*, 1989). Subsequent reports have also indicated that differences in culturing conditions result in expression of varying concentrations of OprG

(Hancock *et al.*, 1990; Hancock and Brinkman, 2002). This broad regulation of OprG expression has therefore hampered efforts in assigning a function to the protein.

The amino acid sequence derived from protein spot N was determined to be outer membrane porin OprF precursor. Protein spots C7 and C8 were also identified as OprF after MALDI-TOF-MS analysis and database searches. Whereas protein spots N and C8 displayed a similar molecular mass, they differed in their pI values. Similar to OprG, this may be due to differential phosphorylation of the protein. Analysis of the OprF amino acid sequence using ProScan indicated that the protein contains 10 potential phosphorylation sites for three different kinases, and the spots may therefore represent differentially phosphorylated versions of the same protein. However, the observed molecular mass and pI of protein spots N and C8 differed significantly from that observed for protein spot C7. Analysis of the ORF coding for OprF indicated that a theoretical signal peptide is present in the database sequence. N-terminal sequence analysis of protein spot N revealed that the mature protein begins with the N-terminus " QGQNS…", some 24 residues after the predicted N-terminal amino acid in the *P. aeruginosa* database. Removal of the predicted N-terminal 24 amino acids gave the protein a predicted pI of 4.86 and molecular mass of 35.25 kDa, which is close to the observed pI and molecular mass of protein spots N and C8 on the 2-DE gels. The cleaved 24-residue N-terminal peptide has a predicted pI of 10 and molecular mass of 2.41 kDa and may therefore account for the shift observed in the molecular mass and pI of protein spots N and C8 relative to that of protein spot C7. Thus, whereas protein spots N and C8 correspond to the mature OprF protein, protein spot C7 may represent an unprocessed form of the protein. OprF is a major membrane protein in *P. aeruginosa* and has been studied extensively due to its proposed utility as a vaccine component (Hancock *et al.*, 1990; Knapp *et al.*, 1999). OprF is multifunctional, since it can function as a non-specific porin (Woodruff and Hancock, 1988), plays a role in maintenance of cell shape and is required for growth in low-osmolarity media (Hancock *et al.*, 1981; Gotoh *et al.*, 1989; Rawling *et al.*, 1998). Notably, OprF of *P. fluorescens* OE 28.3 has been reported to be involved in adhesion of these bacteria to plant roots (De Mot *et al.*, 1994). Similarly, the *P. aeruginosa* OprF protein has been reported to be involved in bacterial adherence to epithelial cells derived from the lung (Azghani *et al.*, 2002). Consequently, OprF may function as an adhesin to both biotic and abiotic surfaces. In addition, Yoon *et al*. (2002) reported that OprF is up-regulated in anaerobic *P. aeruginosa* biofilms and that its loss results in poor biofilm formation under such conditions. In this study, expression of OprF was up-regulated in biofilm cells (spots N and C7) or detected in

biofilm cells only (spot C8). Since it has been reported that oxygen transfer is limited in deeper layers of aerobic biofilms (Xu *et al.*, 1998), cells localised to the interior of biofilm structures are likely to experience decreased oxygen tension. Thus, after 18 h of culturing it is likely that a large proportion of the biofilm population, in contrast to the planktonic and SIP populations, is experiencing anaerobic conditions or reduced oxygen tension. Such anaerobic conditions could therefore result in the observed up-regulation of OprF synthesis in biofilm cells.

The database search for protein spot D4 from MALDI-TOF-MS analysis showed this protein to be outer membrane protein OprB. Following its initial identification by Hancock and Carey (1980), the OprB porin was shown to be selective for glucose (Trias *et al.*, 1988). Subsequent investigations have, however, reported that OprB facilitates the uptake of mannitol, glycerol and fructose, all of which are able to support the growth of *P. aeruginosa* (Wylie and Worobec, 1995). Thus, OprB may more appropriately be referred to as a carbohydrate-selective porin. Expression of OprB was up-regulated in planktonic and SIP cells compared to biofilm cells. In this study, *P. aeruginosa* was cultured in minimal medium supplemented with glucose as sole carbon source. Based on reports indicating that expression of OprB is induced in the presence of glucose (Hancock and Carey, 1980; Hancock and Brinkman, 2002), the up-regulated expression of OprB in planktonic and SIP cells compared to biofilm cells, may be due to these suspended cells having better access to the glucose present in the bulk culture liquid. In contrast, the down-regulated expression of OprB in biofilm cells may be due to limited glucose availability in the immediate biofilm environment.

The database search for protein spot A4 from MALDI-TOF-MS analysis yielded the cell surface flagellin protein FliC, a structural component of flagella. *P. aeruginosa* is motile via a single polar flagellum and flagella have been implicated as being important in the initial stages of host infection (Fleiszig *et al.*, 2001) and attachment to abiotic surfaces (O'Toole and Kolter, 1998). Flagellin proteins in *P. aeruginosa* can be designated type A or type B, based on differences in sequence, reaction with polyclonal antibodies and molecular mass (45.52 kDa for type A and 53 kDa for type B flagellins) (Wilson and Beveridge, 1993; Spangenberg *et al.*, 1996). The FliC protein of *P. aeruginosa* PAO1 is a type B flagellin (Montie and Anderson, 1988). Expression of FliC in biofilm cells was down-regulated, while high levels of expression was noted in planktonic and SIP cells. The results are in agreement with previous reports (McCarter and Silverman, 1990; Sauer and Camper, 2001; Sauer *et al.*, 2002)

indicating that flagella are required for motility by the planktonic cells, but their expression is down-regulated in biofilm cells following cell-to-surface contact, as they are not required for maintenance of a mature biofilm. Expression of FliC was, however, also observed in the 18-h old biofilm cells. In *Clostridium thermocellum* the onset of stationary phase has been correlated with increased detachment from the substratum (Lamed and Bayer, 1986), and it has been reported that starvation may lead to dispersal of *P. aeruginosa* biofilm cells to search for nutrient-rich habitats (O'Toole *et al.*, 2000b; Sauer *et al.*, 2004). Thus, expression of FliC in the 18-h old biofilm cells may signal that a proportion of cells in the biofilm population were preparing to leave the biofilm. The high level of FliC expression in SIP cells appears to be in agreement with a recent report by Sauer *et al.* (2004) indicating that dispersion of *P. aeruginosa* cells from biofilms is associated with increased expression of FliC and a corresponding decrease in expression of pilus (*pilA*) genes in the dispersed cells. Although the SIP population constitutes a distinct phenotype, it is conceivable that a proportion of the SIP population may comprise *P. aeruginosa* cells that have dispersed from the biofilm. Consequently, the up-regulation of FliC expression observed in SIP cells may allow these cells to swim to a new, more favourable niche for attachment.

4.3.3.2 Probable outer membrane proteins

The database search for protein spots A3 and C3 from MALDI-TOF-MS analysis showed these proteins to be probable outer membrane proteins. Spot A3 yielded OprD3 (now referred to as OpdT) and is predicted to be a porin based on its amino acid sequence similarity (57%) to porin OprD of *P. aeruginosa*. The OprD porin facilitates the diffusion of basic amino acids and peptides containing these residues (Trias and Nikaido, 1990; Ochs *et al.*, 1999), and has also been linked with gluconate transport across the outer membrane (Huang and Hancock, 1993). Spot C3 contained two different proteins and yielded a predicted porin OpdP (C3a), as well as a probable outer membrane protein precursor designated OpmH (C3b). Whereas OpdP displays 52% amino acid sequence similarity to OprD of *P. aeruginosa*, OpmH displays 54% amino acid sequence similarity to the TolC outer membrane protein of *Escherichia coli*. The TolC OMP of *E. coli* has been reported to be essential for *E. coli* to maintain organic solvent tolerance (Aono *et al.*, 1998). Curiously, *P. aeruginosa* was cultured in the absence of solvents in the growth medium and the results may suggest that OpmH is not expressed during solvent stress, but steady-state levels are rather retained to allow rapid adaptation to such environments. Expression levels of OpdT, OpdP and OpmH

were the highest in planktonic cells, and all three proteins were down-regulated in biofilm cells. The observed down-regulation of OpmH expression is in agreement with a report by Otto *et al.* (2001), indicating that expression of TolC is down-regulated following attachment of *E. coli* cells to a surface.

In addition to the above proteins, MALDI-TOF-MS analysis of protein spot C5 showed this protein to be a probable outer membrane protein designated FadL. The protein displays 47% amino acid sequence similarity to the long-chain fatty acid transport protein FadL of *E. coli*. Long-chain fatty acids represent an important source of metabolic energy and carbon for macromolecular synthesis and therefore must be specifically and efficiently transported across the cell envelope (Black and DiRusso, 1994; DiRusso *et al.*, 1999). In *E. coli*, FadL functions with an inner membrane-associated fatty acyl-coA synthetase (FACS, encoded by *fadD*) to facilitate the vectorial transport of long-chain fatty acids (DiRusso *et al.*, 1999). Both the *fadL* and *fadD* genes are normally expressed at basal levels under high-nutrient growth conditions. In the presence of long-chain fatty acids as the primary carbon source, the two genes become induced two- to three-fold (DiRusso *et al.*, 1993; 1999). In this regard, it has been proposed that FadL acts as an environmental sensor, which through activity of FACS, allow the cell to respond to environmental fatty acids. Expression of FadL was up-regulated in planktonic cells, while the lowest level of expression was observed in biofilm cells.

The function and potential substrates of all the above *P. aeruginosa* proteins are currently unknown, but it can be envisaged that potential roles for these putative proteins might include transport of small molecules, nutrients or metabolic products. Similar to OprB, the higher expression levels of these proteins observed in planktonic cells compared to biofilm cells may be in response to higher nutrient concentrations present in the bulk culture liquid, whilst the availability of nutrients in the immediate biofilm environment may have been limited.

4.3.3.3   Cytoplasmic proteins

The database search for protein spot A1 from MALDI-TOF-MS analysis showed this protein to be RpsA, which is identical to the S1 protein of the 30S subunit of prokaryotic ribosomes. The ribosomal proteins are named in accordance with the subunit of the ribosome that they belong to – small (S1 to S31) and the large (L1 to L44) – and they usually decorate the rRNA cores of their subunits (Maguire and Zimmermann, 2001). The proteins play an active role in

functions that may have evolved to streamline the process of protein synthesis (Maguire and Zimmerman, 2001). It has been suggested that the RNA-binding S1 protein helps polynucleotide phosphorylase (PNPase) to degrade mRNA, or it may serve as a helper molecule involved in other RNase activities (Danchin, 1997). Expression of RpsA was down-regulated in biofilm cells, but up-regulated in SIP cells. The low level of RpsA expression in biofilm cells may thus be a consequence of the general down-regulation of protein expression in these cells, whereas its increased expression in SIP cells could form part of a general up-regulation of protein expression observed in the SIP cells (Table 4.1).

The amino acid sequence derived from protein spot I corresponded to Trigger Factor, an ATP-independent chaperone that displays chaperone and peptidyl-prolyl-*cis-trans*-isomerase (PPIase) activities *in vitro* (Stoller *et al.*, 1995; Scholz *et al.*, 1997). In the cytosol, a fraction of the newly synthesised bacterial proteins requires the activity of molecular chaperones for folding to the native state. The major chaperones implicated in this folding process are the ribosome-associated Trigger Factor, and the DNaK and GroEL chaperones with their respective co-chaperones (Bukau *et al.*, 2000; Hartl and Hayer-Hartl, 2002). Since the Trigger Factor protein is positioned at the peptide exit channel (P-site) of ribosomes and is able to interact with nascent polypeptide chains as short as 57 residues, it is thought to be the first chaperone that binds to the nascent polypeptide chains (Valent *et al.*, 1995). The expression level of Trigger Factor was highest in SIP cells and was down-regulated in biofilm cells. Similar to RpsA, the decreased expression of Trigger Factor in biofilm cells can be attributed to the general down-regulation of protein expression observed in biofilm cells, whereas protein expression was generally up-regulated in SIP cells (Table 4.1).

4.4   DISCUSSION

Traditionally, bacteria have been regarded as individual organisms and have been grown and studied as homogenous planktonic populations. However, bacteria in natural environments are usually found as a community of sessile organisms organised in a biofilm (Davey and O'Toole, 2000). During the last decade, various strategies have been employed to study biofilm development by especially *P. aeruginosa*. Genetic approaches have been successful at identifying factors involved in the early steps of biofilm development (O'Toole *et al.*, 1998;

Table 4.2 Summary of the proteins identified through N-terminal sequencing from 2-DE gels of *P. aeruginosa* planktonic, SIP and biofilm protein extracts

Protein spot	PA no.	Concentration [a, b]	Protein identity	Protein function	N-terminal sequence	Estimated from gel		Calculated from sequence [c]		Signal peptide
						Size (kDa)	pI	Size (kDa)	pI	
B	PA4067	P>B>SIP	Outer membrane protein OprG precursor	Unknown, inducible in increased iron and increased Mg^{2+}	[RA]D[IQ]QGX[KT][AF]GD[FD]	23	4.6	25.19	4.85	Yes
E	PA0820	B>SIP>P	Hypothetical protein	Unknown	[MH][IQP]A[VE]I[KA]EN	33	5.0	30.07	6.46	No
I	PA1800	SIP>P>B	Trigger factor	Promotes posttranslational folding and proper assembly of unfolded proteins to native state	MQVS[VA]EST[QV]A	58	4.9	48.58	4.83	No
L	PA4067	B>P>SIP	Outer membrane protein OprG precursor	Unknown, inducible in increased iron and increased Mg^{2+}	[AY]D[IQ][QFL]G[HPL][KT][AF]GD[FD]	23	4.9	25.19	4.85	Yes
M	PA5001	B only	Hypothetical protein	Unknown	M[PKV][DNQ]YP[TL][VY]X[EK][VNER]VR	38	5.2	36.63	8.71	No
N	PA1777	B>P>SIP	Outer membrane porin OprF precursor	Major outer membrane protein, porin activity, maintenance of cell shape, required for growth in low-osmolarity medium	QGQNSVEIEAF[G]	35	4.7	37.64	4.98	Yes

a)   Growth phase-related differences in the concentration of protein spots selected for N-terminal protein sequencing
b)   P, planktonic cells cultured in the absence of glass wool; SIP, surface influenced planktonic cells growing in the presence of glass wool; B, biofilm cells
c)   Molecular mass and pI were calculated for full-length proteins in the *P. aeruginosa* database

Table 4.3 **Summary of the proteins identified through MALDI-TOF-MS from 2-DE gels of *P. aeruginosa* planktonic, SIP and biofilm protein extracts**

Protein spot	PA no.	Concentration [a, b]	Protein identity	Protein function	Matching peptides	Sequence coverage [c]	Estimated from gel		Calculated from sequence [d]		Signal peptide
							Size (kDa)	pI	Size (kDa)	pI	
A1	PA3162	SIP>P>B	30S ribosomal protein S1 (RpsA)	Binds to ribosome and unwinds mRNA structures before entry of the mRNA into ribosome	10	20	80	4.7	61.87	4.83	No
A3	PA2505	P>SIP>B	Probable porin (OprD3/OpdT)	Unknown	6	26	60	4.8	49.79	4.91	Yes
A4	PA1092	SIP>P>B	Flagellin type B (FliC)	Flagellar filament protein	3	18	50	5.4	49.24	5.4	No
C3a	PA4501	P>SIP>B	Probable porin (OpdP)	Unknown	8	18	58	5.8	53.03	5.61	Yes
C3b	PA4974	P>SIP>B	Probable outer membrane protein precursor (OpmH)	Unknown	10	18	58	5.8	53.41	5.80	Yes
C5	PA1288	P>SIP>B	Probable outer membrane protein precursor (FadL)	Unknown	3	18	50	5.6	45.56	5.73	Yes
C7	PA1777	B>P>SIP	Outer membrane porin OprF precursor	Major outer membrane protein, porin activity, maintenance of cell shape, required for growth in low-osmolarity medium	5	21	37	5.0	37.64	4.98	Yes
C8	PA1777	B only	Outer membrane porin OprF precursor	Major outer membrane protein, porin activity, maintenance of cell shape, required for growth in low-osmolarity medium	5	21	35	4.3	37.64	4.98	Yes
D4	PA3186	P>SIP>B	Outer membrane porin OprB precursor	Glucose/sugar uptake, inducible by glucose	10	20	55	5.3	50.75	5.48	Yes

a)   Growth phase-related differences in the concentration of protein spots selected for MALDI-TOF-MS analysis
b)   P, planktonic cells cultured in the absence of glass wool; SIP, surface influenced planktonic cells growing in the presence of glass wool; B, biofilm cells
c)   Coverage of the identified peptides on the protein
d)   Molecular mass and pI were calculated for full-length proteins in the *P. aeruginosa* database

Vallet *et al.*, 2001), whilst genomic and proteomic studies have examined gene and protein expression at all stages of biofilm formation (Steyn *et al.*, 2001; Sauer *et al.*, 2002; Whiteley *et al.*, 2001; Finelli *et al.*, 2003). In this study, a proteomic approach was used to study differences in protein profiles obtained from 18-h old *P. aeruginosa* planktonic, surface influenced planktonic (SIP) and biofilm populations grown in batch in the absence or presence of a glass wool substratum.

Although protocols for 2-DE analyses of *P. aeruginosa* planktonic and biofilm populations have been established previously and are based on isolating and examining the entire complement of cellular proteins (Steyn *et al.*, 2001; Sauer *et al.*, 2002), several reports have indicated that technical difficulties in both extracting and solubilising membrane proteins result in these proteins not being well represented on the 2-DE gels (Wilkins *et al.*, 1998; Molloy *et al.*, 1998; 2000; Herbert, 1999). Thus, to retrieve a maximum of information from the 2-DE analysis undertaken in this study, the proteomes of the respective *P. aeruginosa* populations were first separated into three subproteomes by prefractionating the protein samples on the basis of their relative solubility in enhanced solubilisation buffers. The resultant subproteome maps were subsequently combined to yield a single representative protein map for each population. Using this approach, approximately 692 unique protein spots were resolved in the pH range of 3.0 - 10.0. This represents a major improvement on the previous methodology in which approximately 500 silver-stained proteins were resolved.

Comparative analyses of the proteomes derived from the respective *P. aeruginosa* populations indicated that each population contained a number of unique protein spots. In addition, albeit in contrast to our previous results, a general down-regulation of protein expression was seen in the biofilm cells, whilst in SIP cells expression of the proteins was generally up-regulated (Table 4.1). Note should, however, be taken that in our previous study (Steyn *et al.*, 2001) only 41 protein spots whose expression levels varied significantly and reproducibly were selected and subsequently compared. In this study, all proteins visualised were analysed quantitatively and qualitatively using the PDQuest software program. Consistent with the results obtained in this study, Vilain *et al.* (2004) recently reported that agar-entrapped *P. aeruginosa* cells, which served as a source of biofilm cells, displayed a general down-regulation of protein synthesis after 18 h of incubation. Moreover, expression of RpsA and Trigger Factor, which are involved in protein synthesis and protein folding, respectively, were both up-regulated in SIP cells and down-regulated in biofilm cells (Tables 4.2 and 4.3). The

observed up-regulation in expression of these proteins coupled with a general up-regulation of protein expression in SIP cells, may also reflect on a difference in the growth rate between SIP and biofilm cells. The cell volume of bacteria is known to increase exponentially with the growth rate so that a decrease in cell volume indicates a decrease in growth rate, even in biofilm cells (Møller *et al.*, 1995). Comparison of the cell volumes of 18-h old *P. aeruginosa* SIP and biofilm cells indicated that the cell volume of biofilm cells (0.7 ± 0.039 µm^3) was smaller than that of SIP cells (0.898 ± 0.032 µm^3), suggesting that SIP cells were most likely growing at a faster rate compared to biofilm cells (Orlay and Oosthuizen, unpublished data). Despite the apparent contradiction, the results presented here is in agreement with our previous results, indicating that the biofilm population differs from the planktonic population and that the SIP population is not merely a mixture of planktonic and biofilm cells but rather a unique phenotype, as was evidenced by the differential expression of numerous proteins and the presence of several protein spots unique to this population.

Of the 34 protein spots selected from the differentially expressed proteins, 14 were identified using N-terminal sequencing and peptide mass fingerprinting. For a number of proteins identified, the theoretical and observed molecular mass and pI values differed (Tables 4.1 and 4.2), which may be due to proteolytic processing events and/or posttranslational modification of the proteins. In total, 12 different proteins were identified of which eight were annotated as membrane proteins, many previously missing from *P. aeruginosa* 2-DE gel maps (Sauer *et al.*, 2002; Nouwens *et al.*, 2000; Nouwens *et al.*, 2002). One explanation for the additional OMPs observed in this study, is the improvement in protein solubilisation and separation, although strain differences could also account for the observed variation in outer membrane proteins.

Membrane proteins have been reported to influence attachment of different bacteria to surfaces and may also play a role in biofilm development (Matthysse *et al.*, 1996; Espinosa-Urgel *et al.*, 2000; Yoon *et al.*, 2002; Finelli *et al.*, 2003). Differences in the expression of outer membrane proteins between planktonic and biofilm cells of *E. coli* (Otto *et al.*, 2001) and *P. aeruginosa* (Whiteley *et al.*, 2001; Sauer *et al.*, 2002) have also been reported. In this study, a number of OMPs have been identified whose expression was down-regulated in biofilm cells, *e.g.* OpdT, OpdP, OpmH, FadL and OprB. With the exception of OprB, which acts as a substrate-selective porin for a variety of sugars (Hancock and Carey, 1980; Wylie and Worobec, 1995), the function and potential substrates of these proteins are not known.

Based on their amino acid sequence homology to known porins and/or proteins, they may be involved in the uptake and transport of small molecules, nutrients or metabolic products. Consequently, it is possible that expression of these OMPs were down-regulated in biofilm cells in response to limited carbon source (glucose; OprB) and nutrient (OpdT, OpdP, OpmH and FadL) availability in the immediate biofilm environment. The two OprG isoforms displayed differential expression and was up-regulated in planktonic (spot B) and biofilm (spot L) cells, respectively. Although OprG is expressed constitutively at a low level, its expression is broadly regulated by various different environmental conditions (Yates *et al.*, 1989; Hancock *et al.*, 1990), and consequently the function of OprG is not known. It may be that different signals induce expression of OprG differently in planktonic versus biofilm cells.

In contrast to the above, expression of the major outer membrane porin OprF was up-regulated in biofilm cells. Since it has been reported that OprF is necessary for anaerobic growth of *P. aeruginosa* biofilms (Yoon *et al.*, 2002), the higher expression of OprF observed in biofilm cells may have been induced by anaerobic or reduced oxygen levels in the biofilm. Similarly, expression of OprE, whose expression is induced under anaerobic conditions (Yamano *et al.*, 1993), has also been reported to be up-regulated in biofilm cells (Whiteley *et al.*, 2001; Sauer *et al.*, 2002). Reporter gene fusion studies have shown that expression of genes that respond to reduced oxygen levels are induced in *E. coli* biofilms (Prigent-Combaret *et al.*, 1999). The results obtained thus suggest that the presence of anaerobic domains that induce expression of genes responsive to reduced oxygen concentration, such as OprF and OprE, may be an important signal in *P. aeruginosa* biofilm development.

Molecular structures often associated with surface-attached bacteria involve the increased synthesis of extracytoplasmic polymeric substances. Bacteria inhabiting biofilms usually produce one or more polysaccharides that provide a hydrated scaffolding to stabilise and reinforce the structure of the biofilm, mediate cell-to-cell and cell-to-surface interactions, and provide protection from biocides and antimicrobial agents (Costerton *et al.*, 1995; O'Toole *et al.*, 2000b; Mah and O'Toole, 2001; Jackson *et al.*, 2004). Historically, alginate has been considered the major exopolysaccharide of the *Pseudomonas aeruginosa* biofilm matrix and the alginate biosynthetic genes *algC* (Davies and Geesey, 1995) and *algD* (Davies *et al.*, 1993) were amongst the first genes reported to be up-regulated by adherence of *P. aeruginosa* to a solid surface. Using polyacrylamide gel electrophoresis followed by immune-detection of lipopolysaccharide (LPS) fractions, Giwercman *et al.* (1992) reported that the core LPS

fraction was more prominent in *P. aeruginosa* biofilm populations compared to planktonic populations. It has since been reported that the presence and composition of LPS contribute to the adhesiveness of *Pseudomonas* species (Williams and Fletcher, 1996), and mutations in the lipopolysaccharide core biosynthesis genes of *E. coli* (Genevaux *et al.*, 1999) and *P. fluorescens* (Rodriguez-Herva *et al.*, 1999) have been reported to reduce bacterial adhesion. In this regard, one of the more intriguing genes identified in this study was PA5001, which encodes a putative glycosyltransferase enzyme that functions in LPS core biosynthesis. Expression of PA5001 appears to be biofilm-induced, as it was not detected in either the planktonic or SIP populations. Thus, biofilm-induced expression of PA5001 may reflect the requirement for increased adhesiveness by the biofilm population.

In conclusion, the present work has highlighted several differences in the protein profiles of *P. aeruginosa* growing planktonically and as a biofilm on glass wool. The amounts of numerous proteins in biofilm and SIP cells were significantly different from those in planktonic cells. In addition, 12 proteins that are differentially expressed between the respective *P. aeruginosa* populations have been identified. Further functional studies are necessary to clarify the role of these and other proteins identified in this study in *P. aeruginosa* biofilm development and maintenance.

4.5   ACKNOWLEDGEMENTS

4.6   REFERENCES

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CHAPTER FIVE

CONSTRUCTION AND CHARACTERISATION OF AN OprG-DEFICIENT
MUTANT STRAIN OF *Pseudomonas aeruginosa* PAO1 (DSM 1707)

5.1   INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous gram-negative bacterium and an important opportunistic pathogen of humans, causing serious infections in immunocompromised patients such as those with cancer or AIDS, and patients suffering from cystic fibrosis and severe burns (Van Delden and Iglewski, 1998). The success of *P. aeruginosa* to grow in diverse environmental niches is attributed to its broad metabolic diversity and its many cell-associated and secreted virulence factors (Lazdunski *et al.*, 1990; Van Delden and Iglewski, 1998), as well as its ability to attach to both biotic and abiotic surfaces with the subsequent development of biofilms (Watnick and Kolter, 2000; Stoodley *et al.*, 2002). Over the past few years, much progress has been made towards understanding the development of *P. aeruginosa* biofilms. This progress has been largely due to the recent focus of analysing biofilms using genetic (O'Toole and Kolter, 1998; Whiteley *et al.*, 2001; Finelli *et al.*, 2003), proteomic (Steyn *et al.*, 2001; Sauer *et al.*, 2002) and molecular biological (Tolker-Nielsen *et al.*, 2000; Heydorn *et al.*, 2002; Klausen *et al.*, 2003) approaches.

The rate and extent of attachment of bacterial cells to a surface is influenced, amongst other, by cell surface hydrophobicity, presence of flagella, pili and adhesins, and production of extracytoplasmic polymeric substances (O'Toole and Kolter, 1998; DeFlaun *et al.*, 1999; Genevaux *et al.*, 1999; Espinosa-Urgel *et al.*, 2000). Furthermore, bacterial membrane proteins have been reported to influence attachment and may also play a role in early biofilm development. Mutations in surface and membrane proteins caused defects in the attachment of *P. putida* to corn (Espinosa-Urgel *et al.*, 2000), whilst attachment to abiotic surfaces was shown in *E. coli* to cause major changes in outer membrane composition (Otto *et al.*, 2001). Using a proteomic approach, Otto *et al.* (2001) demonstrated increased levels for 17 outer membrane proteins and decreased levels for 15. In *P. aeruginosa*, loss of porin OpdF has detrimental effects on the ability of the bacteria to form biofilms (Finelli *et al.*, 2003), whereas loss of the major outer membrane porin OprF results in poor biofilm formation under

anaerobic conditions (Yoon *et al.*, 2002). In addition to these two porins, several other outer membrane proteins (OMPs) have been reported to be differentially expressed in planktonic versus biofilm cells. Expression of OprE1 (Sauer *et al.*, 2002) and OprF (this study) has been shown to be up-regulated in *P. aeruginosa* biofilms, whilst expression of several other OMPs such as OprB, OpdT, OpmH and OpdP were down-regulated in the biofilm population (this study; Chapter 4).

The results obtained in the preceding Chapter indicated the presence of two isoforms of the outer membrane protein porin OprG of which the expression was up-regulated in the biofilm population and planktonic population, respectively. The appearance of OprG in the outer membrane is highly dependent on growth conditions. In particular, Yates *et al.* (1989) observed a direct relationship between the iron concentration in the medium and expression of OprG, and suggested that this OMP is involved in low-affinity iron uptake. Other conditions, including growth into the stationary phase, higher growth temperatures, Mg^{2+}-deficiency, certain lipopolysaccharide alterations and the presence of carbon sources, also result in the expression of varying concentrations of OprG (Hancock *et al.*, 1990; Hancock and Brinkman, 2002). Such apparently broad regulation of this OMP has hampered efforts to assigning a function to OprG.

Based on recent reports indicating a role for different OMPs in *P. aeruginosa* biofilm development (Yoon *et al.*, 2002; Finelli *et al.*, 2003), the aims of this study were to generate a *P. aeruginosa* PAO1 (DSM 1707) mutant strain deficient in OprG and to compare its ability to form biofilms on a glass wool substratum with that of the wild-type *P. aeruginosa* strain. OprG, encoded by PA4067, was selected, as it was the only other OMP that displayed up-regulation in biofilms, and, unlike OprF, has not yet been investigated for its involvement in biofilm formation.

5.2   MATERIALS AND METHODS

5.2.1   Bacterial strains, plasmids and culture conditions

Bacterial strains, plasmids and primers used in this study are listed in Table 5.1. *Escherichia coli* and *Pseudomonas aeruginosa* strains were routinely cultivated at 37°C on a rotary shaker (200 rpm) in Luria Bertani (LB) broth (0.5% [w/v] yeast extract; 1% [w/v] tryptone; 1% [w/v]

NaCl, pH 7.4). For comparative studies, *P. aeruginosa* PAO1 (DSM 1707) and mutant strains were grown in 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). The following antibiotics were used to maintain the plasmid DNA and chromosomal insertions in *P. aeruginosa* strains: gentamicin at 50 µg/ml, tetracycline at 10 µg/ml and carbenicilin at 200 µg/ml. For plasmid DNA selection and maintenance in *E. coli,* the concentration of antibiotics used was: 100 µg/ml for ampicillin and 10 µg/ml for gentamicin. All antibiotics were purchased from Sigma-Aldrich (St Louis, MO, USA).

5.2.2   Genomic DNA isolation

The genomic DNA of wild-type and mutant *P. aeruginosa* PAO1 (DSM 1707) strains was isolated using cetyltrimethylammonium bromide (CTAB), as described by Jansen (1995). Briefly, the cells from 500 µl of an overnight culture were collected by centrifugation at 8 000 $\times$ *g* for 3 min and suspended in 567 µl of 1 $\times$ TE buffer (10 mM Tris-HCl; 1 mM EDTA, pH 8.0). The cells were lysed by the addition of SDS to a final concentration of 0.5% (v/v) and the proteins were digested by addition of Proteinase K to a final concentration of 100 µg/ml in a total volume of 600 µl. Following incubation at 37°C for 1 h, 100 µl of 5 M NaCl and 80 µl of a CTAB/NaCl solution was added and incubation was continued for 10 min at 65°C. The CTAB-protein and -polysaccharide complexes were removed by extraction with an equal volume of chloroform/isoamyl alcohol (24:1), followed by centrifugation at 8 000 $\times$ *g* for 5 min. The supernatant, containing the genomic DNA, was recovered and transferred to a clean microfuge tube. The remaining CTAB was removed by addition of an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) followed by centrifugation (8 000 $\times$ *g*, 5 min). The chromosomal DNA was precipitated from the recovered supernatant by addition of 0.6 volume isopropanol. The precipitated chromosomal DNA was pelleted by brief centrifugation, rinsed with 70% ethanol, dried under vacuum and resuspended in 20 µl UHQ water. An aliquot of the DNA was analysed by electrophoresis on a 0.8% (w/v) agarose gel.

5.2.3 DNA amplification

5.2.3.1 Oligonucleotide primers

The oligonucleotide primers used in PCR assays to amplify the genomic region containing the PA4067 ORF from *P. aeruginosa* PAO1 (DSM 1707), are indicated in Table 5.1. The primers were designed on the basis of the complete genome sequence of *P. aeruginosa* strain PAO1 (Stover *et al.*, 2000). All primers were synthesised by Inqaba Biotechnical Industries (Pretoria, South Africa).

5.2.3.2 Polymerase chain reaction (PCR) amplification of DNA fragments

The reaction mixtures (50 μl) contained 100 ng of *P. aeruginosa* PAO1 (DSM 1707) genomic DNA as template, 20 pmol of each the FOR4067 and REV4067 primer, $1 \times$ PCR buffer (50 mM KCl; 10 mM Tris-HCl [pH 9.0]; 0.1% [v/w] TritonX-100), $MgCl_2$ at 1 mM, each deoxynucleoside triphosphate (dNTP) at a concentration of 0.2 mM, dimethyl sulfoxide (DMSO) at 5% (v/v) and 1 U of *Taq* DNA polymerase (Southern Cross Biotechnology, Cape Town, South Africa). The tubes were placed in a Perkin-Elmer GeneAmp 2400 thermal cycler and following an initial denaturation at 94°C for 5 min, the reactions were subjected to 30 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 55°C and elongation for 2 min at 72°C. After the last cycle, the reactions were kept for 5 min at 72°C to complete synthesis of all strands. For control purposes, an identical reaction mixture lacking template DNA was included. Aliquots of the PCR reaction mixtures were subsequently analysed by electrophoresis on a 0.8% (w/v) agarose gel in the presence of an appropriate DNA molecular weight marker.

5.2.4 Agarose gel electrophoresis

DNA was analysed by agarose gel electrophoresis (Sambrook *et al.*, 1989). For this purpose, horizontal 0.8% (w/v) agarose slab gels were cast and electrophoresed at 100 V in $1 \times$ TAE buffer (40 mM Tris-HCl; 20 mM NaOAC; 1 mM EDTA, pH 8.5). The agarose gels were supplemented with ethidium bromide (0.5 μg/ml) in order to allow visualisation of the DNA on an UV transilluminator. Where appropriate, the DNA fragments were sized according to their migration in the gel as compared to that of standard DNA molecular weight marker, namely GeneRuler™ DNA Ladder Plus (Fermentas AB, Germany).

Table 5.1   Bacterial strains, plasmids and oligonucleotide primers used in this study

Strain, plasmid or primer	Relevant properties	Reference
Strains:		
E. coli		
INFαF'	F´ *end*A1 *rec*A1 *hsd*R17 (r_k-, m_k+) *sup*E44 *thi*-1 *gyr*A96 *rel*A1 φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 λ-	Invitrogen
HB 101	F–, *thi*-1, *hsd*S20 (r_B–, m_B–), *sup*E44, *rec*A13, *ara*-14, *leu*B6, *pro*A2, *lac*Y1, *gal*K2, *rps*L20 (str^r), *xyl*-5, *mtl*-1	Promega
P. aeruginosa		
DSM 1707	Wild-type, Prototroph (PAO1)	DSM*
DSMOprG	DSM1707OprG::Gm^R	This study
Plasmids:		
pGEM®-T Easy	ColE1, Amp^R, LacZα peptide, cloning vector for PCR amplicons	Promega
pGEM-OprG	pGEM®-T Easy vector containing PA4067 amplicon	This study
pGEM-OprG-Gent	pGEM-OprG with a Gm^R cassette inserted at the *Kpn*I sites of the OprG-encoding ORF	This study
pSS125	pUC19 containing *oriT*	S.J. Suh (unpublished)
pGori-OprG-Gent	pGEM-OprG-Gent with *oriT*	This study
pU8G	Gm^R cassette cloned into pUC4K	J.B. Weyers (unpublished)
pRK600	Cm^R, ColE1, RK2-Mob⁺, RK2-Tra⁺, helper plasmid in triparental conjugations	Kessler *et al.* (1992)
Primers:		
FOR4067	5'-GGTTTAAAATGCTGCGCTCCCTGAC-3'	
REV4067	5'-CCAAATTTGCATGTTTGAACGCCCC-3'	
GenomicA	5'-CGAGCGACTATGAGTGGAGC-3'	
GenomicB	5'-CAGCACGCGGTGATGGTCTG-3'	
IntRp4067	5'-CGCGTGCTCGTTGAGCATG-3'	
Gentp1-1	5'-GCGGCGTTGTGACAATTTAC-3'	

*DSM – Deutche Sammlung von Mikroorganismen, Braunschweig, Germany

5.2.5 Purification of DNA fragments from agarose gels

DNA fragments were purified from 0.8% (w/v) agarose gels using a silica suspension, as described by Boyle and Lew (1995). Briefly, the DNA band of interest was excised from the agarose gel and mixed with 400 µl of a 6 M NaI solution. The agarose was dissolved by incubation at 55°C for 10 min, whereafter 8 µl of the silica suspension was added to the sample. The DNA was allowed to bind to the silica by incubation of the samples on ice for 30 min with intermittent vortexing. The DNA-silica complex was pelleted by centrifugation (8 000 × g for 30 s) and washed four times with Wash buffer (50 mM NaCl; 10 mM Tris-HCl [pH 7.5]; 2.5 mM EDTA; 50% [v/v] ethanol). The DNA was eluted from the silica matrix in a final volume of 7 µl UHQ water by incubation at 55°C for 10 min. The purified DNA fragments were analysed on a 0.8% (w/v) agarose gel to assess both their purity and concentration.

5.2.6 Nucleotide sequencing and sequence analysis

The nucleotide sequence of the gel-purified amplicon was determined using an ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). The primers used for PCR amplification were used in the nucleotide sequencing reactions (Table 5.1). Each reaction mixture contained 80-100 ng of gel-purified amplicon, 12 pmol of sequencing primer, 2 µl Terminator Ready Reaction Mix and water in a final volume of 5 µl. Cycle sequencing reactions were performed in a Perkin-Elmer GeneAmp 2400 thermal cycler with 25 of the following cycles: denaturation at 96°C for 10 s, primer annealing at 50°C for 5 s and extension at 60°C for 4 min. Following brief centrifugation, the extension products were precipitated by the addition of 4 µl UHQ water and 16 µl absolute ethanol and incubated at room temperature for 30 min in the dark. The tubes were then centrifuged at 13 000 × g for 30 min and the supernatants carefully aspirated. The pellets were rinsed with 50 µl of 70% ethanol, dried under vacuum for 10 min and stored at 4°C. Prior to electrophoresis, the purified extension products were resuspended in 3.5 µl Blue dextran/EDTA loading buffer, denatured for 2 min at 90°C and loaded onto a model 377 automated DNA sequencer (Perkin-Elmer). The nucleotide sequences obtained were compared against the *P. aeruginosa* PAO1 genome database (http://www.pseudomonas.com), as well as against sequences in the GenBank Database (http://www.ncbi.nlm.gov) by using BLASTN and BLASTP (Altschul *et al.*, 1997).

5.2.7 Restriction endonuclease digestions

All restriction endonuclease digestions were performed in clean microfuge tubes and contained the appropriate concentration of salt (using the 10× buffer supplied by the manufacturer) for the specific enzyme and 5-10 U of enzyme per µg of plasmid DNA. The reaction volumes were small (10-20 µl) and incubation was typically for 1-1.5 h at 37°C. All restriction enzymes were supplied by Roche or Promega (Promega Corp., Madison, WI, USA). The digestion products were typically analysed on a 0.8% (w/v) agarose gel in the presence of appropriate DNA molecular weight markers.

5.2.8 Cloning of DNA fragments into plasmid vectors

The pGEM®-T Easy vector system (Promega) was used for cloning of PCR amplicons. Ligation of the gel-purified amplicon and pGEM®-T Easy vector was performed at 4°C overnight in a final reaction volume of 10 µl. The reaction mixture contained 50 ng of vector DNA, 250 ng of insert DNA, 3 U of T4 DNA ligase (3 U/µl; Promega), 5 µl of a 2 × DNA ligase buffer and UHQ water. Ligation of specific DNA fragments and vector DNA was performed at 16°C overnight in a final reaction volume of 10 µl, which contained 1 µl of a 10 × DNA ligase buffer (660 mM Tris-HCl [pH 7.5]; 10 mM DTT; 50 mM MgCl$_2$; 10 mM ATP), 1 U of T4 DNA ligase (1 U/µl; Roche) and the ratio of vector to insert was typically in excess of 1:5.

5.2.9 Transformation of competent *E. coli* cells

Competent *E. coli* INFαF' cells (Invitrogen, Carlsbad, CA, USA) were transformed according to the manufacturer's instructions. Briefly, competent cells (50 µl) were mixed with 2 µl of the ligation reaction mixture in a sterile microfuge tube and incubated on ice for 30 min. The cells were subsequently incubated at 42°C for 2 min and then chilled on ice for 2 min. Following the addition of 250 µl of LB medium, the cells were allowed to recover by incubation at 37°C for 1 h. The transformed cells were selected by plating the transformation mixtures on LB agar supplemented with the appropriate antibiotic and incubated at 37°C overnight. When appropriate, the cells were plated together with 10 µl IPTG (100 mM stock solution) and 50 µl X-gal (2% [w/v] stock solution) to allow for blue/white colour selection, based on insertional inactivation of the *lacZ'* marker gene in the pGEM®-T Easy vector (Promega).

5.2.10 Extraction and purification of plasmid DNA

Plasmid DNA was isolated from transformants using the alkaline lysis method, as described by Sambrook *et al.* (1989). Single colonies were inoculated into 5 ml of LB broth containing the appropriate antibiotic and incubated at 37°C overnight. The cells from 3 ml of the cultures were then collected by centrifugation at 13 000 × *g* for 2 min. The bacterial cell pellets were suspended in 100 µl of Solution 1 (50 mM glucose; 25 mM Tris-HCl [pH 8.0]; 10 mM EDTA; 10 mg/ml lysozyme) and incubated at room temperature for 5 min. The resultant spheroplasts were lysed by the addition of 200 µl of freshly prepared Solution 2 (0.2 N NaOH; 1% [w/v] SDS), followed by incubation on ice for 5 min. Subsequently, 150 µl of Solution 3 (3 M sodium acetate, pH 4.8) was added and incubation was continued on ice for a further 10 min. The cell debris was removed by centrifugation at 13 000 × *g* for 10 min and the supernatant transferred to a clean microfuge tube. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added, mixed and the organic and aqueous phases separated by centrifugation at 13 000 × *g* for 5 min. The upper aqueous phase was recovered and extracted with an equal volume of chloroform. The plasmid DNA was finally precipitated from the aqueous phase by the addition of 2.5 volumes absolute ethanol, followed by incubation for 30 min at -70°C. The plasmid DNA was recovered by centrifugation, washed with 70% ethanol and dried under vacuum before being suspended in 30 µl UHQ water. The plasmid DNA was incubated with 1 µl RNase A (10 mg/ml) for 1 h at 37°C to remove any contaminating RNA, and stored at -20°C until needed.

5.2.11 Construction of allelic exchange vector

All molecular cloning techniques employed in the construction of the allelic exchange vector were performed according to the procedures described in the preceding sections. All plasmid constructs were confirmed by restriction endonuclease digestion using agarose gel electrophoresis and by nucleotide sequencing. The cloning strategy employed in the construction of the allelic exchange vector pGori-OprG-Gent is indicated diagrammatically in Fig. 5.1.

5.2.11.1 Construction of recombinant plasmid pGEM-OprG

PCR using oligonucleotide primer pair FOR4067 and REV4067, together with chromosomal DNA from *P. aeruginosa* PAO1 (DSM 1707), resulted in the amplification of a 2.734-kb

DNA fragment containing the full-length *oprG* gene. The amplicon was purified from an agarose gel and cloned into pGEM®-T Easy, resulting in the generation of the recombinant plasmid pGEM-OprG.

5.2.11.2 Construction of allelic exchange vector pGori-OprG-Gent

Recombinant plasmid pGEM-OprG was digested with *Kpn*I, which cuts twice in *oprG* only, thus excising a DNA fragment of 207 bp. The 5' protruding ends of the restricted vector DNA were subsequently blunt-ended at 37°C for 30 min after the addition of 2 U Klenow DNA polymerase (2 U/μl; Roche) and 1 μl of a dNTP mixture containing 250 μM of each dNTP. The gentamicin resistance cassette was recovered from plasmid pU8G by digestion with *Eco*RI and blunt-ended, as described above, before being ligated into the deletion site of plasmid pGEM-OprG to yield pGEM-OprG-Gent. To complete the construction of allelic exchange vector pGori-OprG-Gent, an *oriT*-containing DNA fragment was recovered from plasmid pSS125 by digestion with *Xba*I and cloned into pGEM-OprG-Gent, which had been linearised by digestion with *Spe*I.

5.2.12   Generation of mutant *P. aeruginosa* PAO1 (DSM 1707) strains

The allelic exchange vector pGori-OprG-Gent was introduced into *P. aeruginosa* PAO1 (DSM 1707) by triparental conjugation, as described by Kessler *et al*. (1992). A single colony of freshly streaked cultures of donor (*E. coli* INFαF' containing the allelic exchange vector), helper (*E. coli* HB 101 containing pRK600) and recipient (*P. aeruginosa*) strains were mixed on LB agar with a sterile inoculation needle and then incubated at 37°C overnight. Following incubation, the mixed growth was streaked onto LB agar supplemented with 50 μg/ml gentamicin and 10 μg/ml tetracycline. The agar plates were then incubated at 37°C for a further 24 to 48 h. Single colonies were subsequently replica-plated onto LB agar plates supplemented with 200 μg/ml carbenicilin and incubated at 37°C for 24 h. Colonies that were unable to grow on plates containing 200 μg/ml carbenicilin were selected from the replica plates and maintained on LB agar plates containing 50 μg/ml gentamicin. One of these, designated DSMOprG, was selected and used in subsequent investigations.
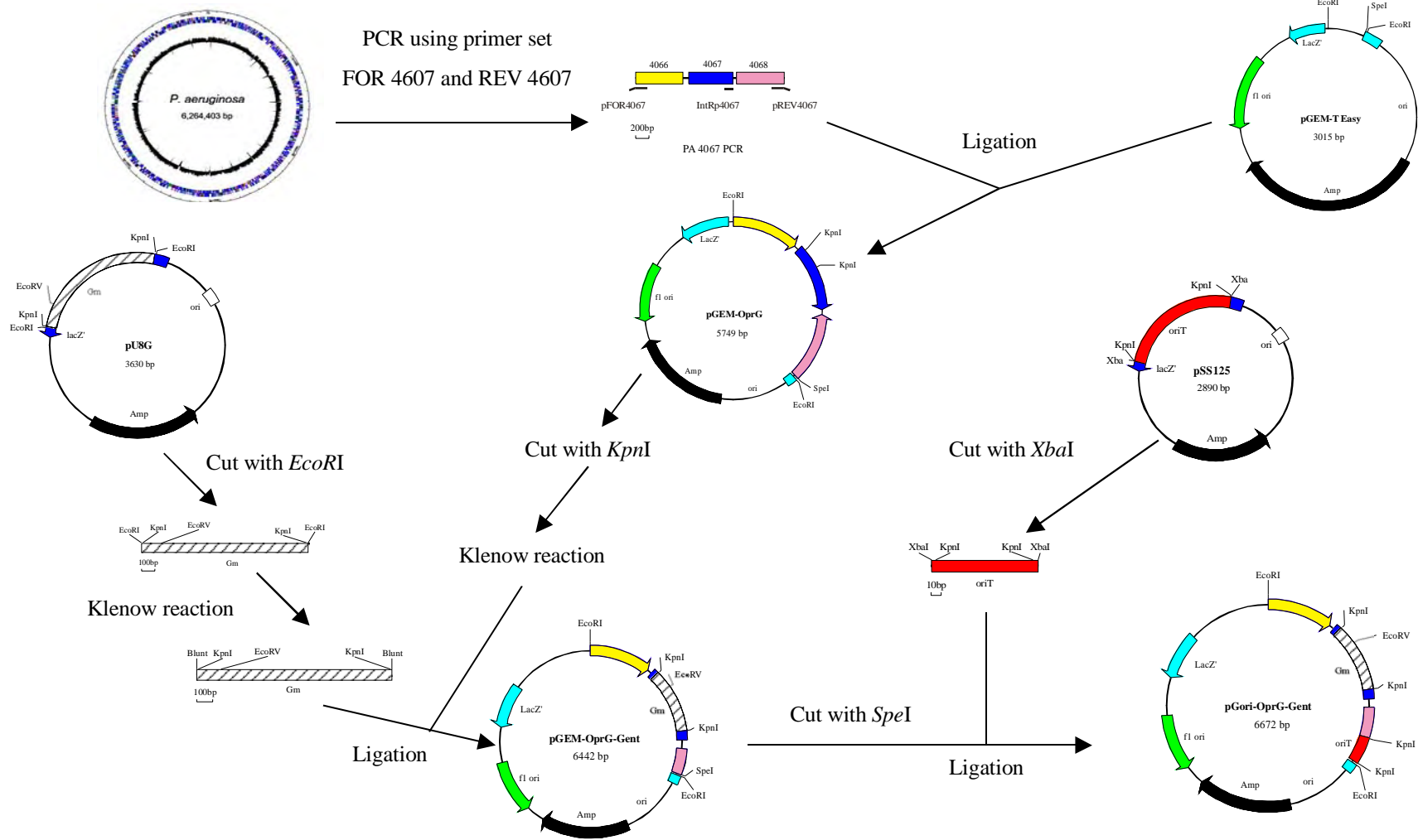
Fig. 5.1   Diagrammatic representation of the cloning strategy used to construct the allelic exchange vector pGori-OprG-Gent.

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5.2.13 Characterisation of mutant *P. aeruginosa* PAO1 (DSM 1707) strains

5.2.13.1 Oligonucleotide primers

The DSMOprG mutant strain was analysed for the presence of the gentamicin resistance cassette within the *oprG* gene by PCR analyses. Oligonucleotide primers GenomicB and Gentp1-1 (Table 5.1) were used to amplify a hybrid amplicon consisting of the 5' end of the gentamicin resistance cassette and the 3' end of the interrupted *oprG* gene, whereas oligonucleotide primers GenomicA and IntRp4067 (Table 5.1) were used to amplify a hybrid amplicon containing the 3' end of the gentamicin resistance cassette and the 5' end of the interrupted *oprG* gene. Oligonucleotide primer pair GenomicA and GenomicB (Table 5.1) were also used to amplify the *oprG* gene interrupted by the gentamicin resistance cassette in mutant chromosomal DNA.

5.2.13.2 PCR amplification

The PCR reaction mixtures (50 µl) contained 100 ng of chromosomal DNA, $1 \times$ PCR buffer (50 mM KCl; 10 mM Tris-HCl [pH 9.0]; 0.1% [v/v] TritonX-100), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 25 pmol of each the sense and antisense primer and 1 U of *Taq* DNA polymerase (Southern Cross Biotechnology). The PCR reaction mixtures were placed in a Perkin-Elmer GeneAmp 2400 thermocycler using the following temperature profile: initial denaturation for 5 min at 94°C, followed by 30 cycles of denaturation for 1 min at 94°C, primer annealing for 60 s at 55°C (primers GenomicB - Gentp1-1 as well as primers GenomicA - IntRp4067) or 60°C (primers GenomicA - GenomicB), and elongation for 3 min at 72°C. After the last cycle, the reactions were kept at 72°C for 5 min to complete synthesis of all strands. For all of the analyses, UHQ water served as a negative control, while chromosomal DNA extracted from the parental and mutant strains provided sample template DNA. Following PCR amplification, aliquots of the respective reaction mixtures were analysed by electrophoresis on 0.8% (w/v) agarose gels.

5.2.14 Batch assays of the *P. aeruginosa* DSMOprG mutant strain

5.2.14.1 Determination of bacterial growth curves

The *P. aeruginosa* wild-type PAO1 (DSM 1707) and mutant DSMOprG strains were cultured at 37°C overnight on a rotary shaker (200 rpm) in MSGY broth. The overnight cultures were diluted in fresh broth and incubated until mid-exponential phase was reached ($OD_{600} = 0.1$).

The cultures were subsequently used to inoculate 100 ml of MSGY broth in 250-ml Erlenmeyer flasks to an optical density of 0.03. The flasks were incubated at 37°C on a rotary shaker (200 rpm) and the optical density at 600 nm of each culture was determined at different time intervals.

5.2.14.2 Biofilm formation

The ability of the *P. aeruginosa* wild-type PAO1 (DSM 1707) and mutant DSMOprG strains to form biofilms was assayed using glass wool as an attachment substratum (Steyn *et al.*, 2001; Oosthuizen *et al.*, 2001). Preculture was performed in 100-ml Erlenmeyer flasks containing 50 ml of MSGY broth. The flasks were incubated at 37°C on a rotary shaker (200 rpm) until mid-exponential phase was reached ($OD_{600} = 0.1$) and then used to inoculate 50 ml of MSGY broth in 100-ml Erlenmeyer flasks, with and without 1.25 g glass wool (mean diameter 15 µm, total surface area 1 725 cm^2) (Merck, Darmstadt, Germany), to an optical density of 0.05. Biofilm formation was monitored on various samples of glass wool obtained at times 0 h, 2 h, 4 h, 8 h, 18 h and 26 h after inoculation. The glass wool was stained with 0.01% (w/v) crystal violet and immediately viewed by bright-field microscopy using a Zeiss Axiovert 200 fluorescent microscope (Zeiss Axioskop, Zeiss, Oberkochen, Germany). Images were captured using a Nikon charge-coupled device (CCD) camera (Nikon Instech Co., Kanagawa, Japan). In addition, the propensity of the wild-type and mutant *P. aeruginosa* strains to form biofilms on glass wool in MSGY broth was also investigated over a period of 26 h of culturing. For these assays, the culture medium of cultures grown in the presence of glass wool was aspirated, transferred to a new flask and referred to as surface influenced planktonic (SIP) cells. Following careful rinsing of the glass wool with a small volume of MSGY broth, the attached (biofilm) cells were then removed from the glass wool by adding 50 ml sterile MSGY broth and vortexing for 5 min. The supernatant was recovered and the removal of the biofilms cells from the glass wool substratum was verified by light microscopy. The optical density at 600 nm of both the attached (biofilm) and SIP populations was subsequently determined. These assays were performed in triplicate.

5.3   RESULTS

5.3.1   Construction of allelic exchange vector pGori-OprG-Gent

5.3.1.1   Construction of plasmid pGEM-OprG

Oligonucleotide primers FOR4067 and REV4067 were used in a PCR with chromosomal DNA of *P. aeruginosa* PAO1 (DSM 1707), as described under Materials and Methods (Section 5.2.3). To allow for a sufficient amount of sequence similarity with the chromosomal DNA that would permit homologous recombination between the *oprG* gene, following its inactivation by a marker gene, and chromosomal gene, primers FOR4067 and REV4067 were designed to anneal at the 5' ends of PA4066 (encoding a hypothetical protein) and PA4068 (encoding an epimerase), respectively. Following PCR, an aliquot of the reaction mixture was analysed by agarose gel electrophoresis and a single discreet amplicon of *ca.* 2.734 kb was observed (Fig. 5.2, lane 2). This corresponded in size to the full-length PA4066 (0.518 kb), *oprG* (0.698 kb) and PA4068 (0.929 kb) genes, together with intergenic regions totalling 0.589 kb. In contrast, no amplification product was observed in the negative control in which template DNA was omitted (Fig. 5.2, lane 3). The gel-purified amplicon was subsequently cloned into pGEM®-T Easy vector DNA and restriction endonuclease digestion of the derived recombinant plasmid DNA with *Eco*RI, which flanks the cloning site of the vector, resulted in the excision of a 2.734-kb DNA fragment (not shown). A recombinant clone, designated pGEM-OprG, was selected and the integrity of the cloned amplicon was verified by nucleotide sequencing of both terminal ends prior to it being used in subsequent DNA manipulations.

5.3.1.2   Construction of the allelic exchange vector pGori-OprG-Gent

Since double crossover events that incorporate a cloned gene from a plasmid into the chromosome of an organism are rare, it is not feasible to screen for such events if the cloned gene cannot be directly selected. However, by inactivating the cloned gene with a readily selectable marker, such as an appropriate antibiotic resistance gene, it is possible to directly screen for potential mutants based on their newly acquired resistance to the antibiotic. The gentamicin resistance cassette that was used in this study to disrupt the *oprG* gene of *P. aeruginosa* consists of the gene *aacC1*, which encodes the enzyme 3-*N*-aminoglycoside acetyltransferase, and is flanked by transcriptional and translational stop signals (Luckow *et al.*, 1993). By making use of the *aacC1* gene, it would thus be possible to rapidly and directly
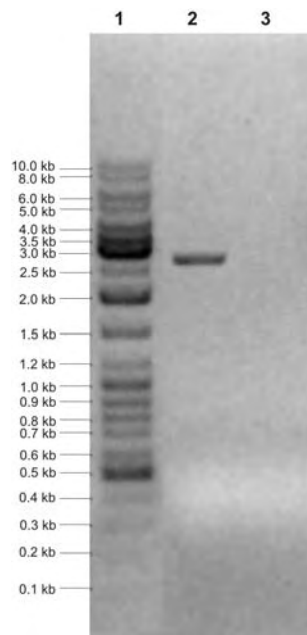
Fig. 5.2 **Agarose gel electrophoretic analysis of the amplicon obtained by PCR amplification using *P. aeruginosa* chromosomal DNA as template and primers FOR4067 and REV4068. Lane 1, DNA molecular weight marker; lane 2, sample of the reaction mixture following PCR; lane 3, negative control PCR reaction mixture lacking template DNA. The sizes of the DNA molecular weight marker, GeneRuler^{TM} DNA Ladder Plus, are indicated to the left of the figure.**

screen for *P. aeruginosa* mutant strains based on their newly acquired resistance to gentamicin.

Recombinant plasmids pGEM-OprG and pU8G served as sources for the construction of the allelic exchange vector pGori-OprG-Gent (Fig. 5.3a). Digestion of recombinant plasmid pGEM-OprG with *Kpn*I, which cuts twice in *oprG*, yielded two DNA fragments corresponding in size to 5.543 and 0.207 kb. The larger of the two DNA fragments was excised from the agarose gel and purified using a silica suspension. The full-length 900-bp gentamicin resistance cassette was recovered from plasmid pU8G by digestion with *Eco*RI. Since restriction of the vector and insert DNA yielded incompatible termini, the DNA fragments were converted to blunt-ended DNA fragments by treatment with Klenow DNA polymerase prior to ligation. The gel-purified DNA fragments were subsequently ligated and following transformation of competent *E. coli* INFαF' cells, plasmid DNA from gentamicin-resistant transformants were characterised by agarose gel electrophoresis and by restriction enzyme digestion. Digestion of the recombinant plasmid DNA with *Eco*RV, which cuts once at the 5' end of the gentamicin resistance cassette only, yielded a linear DNA fragment of 6.442 kb (Fig. 5.3b, lane 4). These results indicated that the gentamicin resistance cassette had been cloned successfully. One of the recombinant clones was selected for further use and designated pGEM-OprG-Gent.

To facilitate the conjugative transfer of the allelic exchange vector from *E. coli* to *P. aeruginosa*, an *oriT*-containing DNA fragment was cloned onto the pGEM-OprG-Gent vector backbone. Consequently, the recombinant vector was linearised by digestion with *Spe*I and the *oriT* was cloned from plasmid pSS125 as a 230-bp *Xba*I DNA fragment. Although *Spe*I and *Xba*I generate compatible termini, the resultant hybrid site does not constitute a target for either of the restriction endonuleases. Therefore, plasmid DNA from randomly selected gentamicin-resistant transformants was screened by digestion with *Kpn*I, which cuts twice in *oprG* and twice in the cloned *oriT* DNA fragment. Digestion of the recombinant plasmid DNA containing the cloned *oriT* yielded DNA fragments corresponding to 3.859, 1.683, 0.9 and 0.230 kb (Fig. 5.3b, lane 7). In contrast, recombinant plasmid DNA lacking the cloned *oriT* yielded two DNA fragments of 5.542 and 0.9 kb, corresponding in size to the recombinant vector backbone and the cloned gentamicin resistance cassette, respectively (Fig. 5.3b, lane 6). These results therefore confirmed that the *oriT* was successfully cloned. One of the recombinant clones was selected for further use and designated pGori-OprG-Gent.
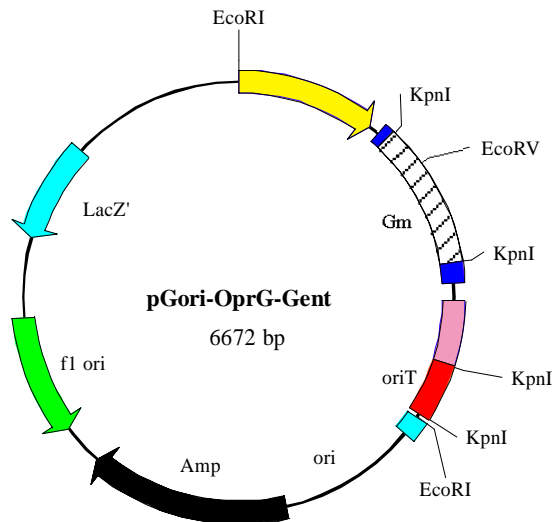
Fig. 5.3a **Plasmid map of the recombinant allelic exchange vector pGori-OprG-Gent.**
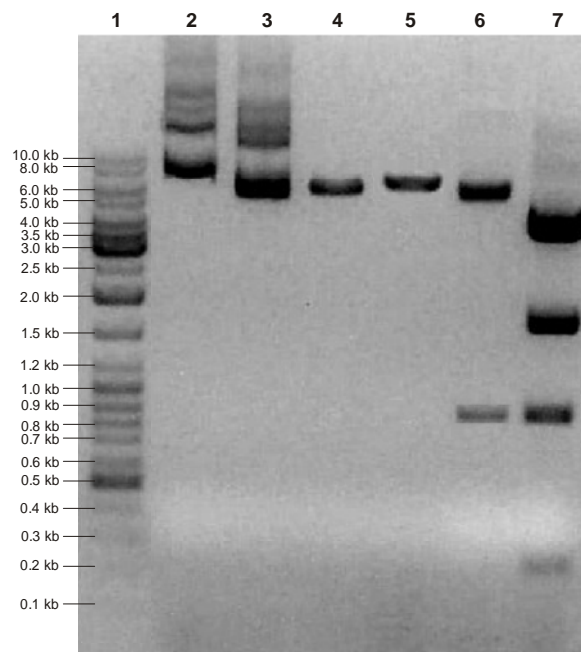
Fig. 5.3b **Agarose gel electrophoretic analysis of the recombinant plasmid pGori-OprG-Gent. Lane 1, DNA molecular weight marker; lane 2, uncut recombinant allelic exchange vector pGori-OprG-Gent; lane 3, uncut plasmid pGEM-OprG-Gent; lane 4, plasmid pGEM-OprG-Gent digested with *Eco*RV; lane 5, recombinant allelic exchange vector pGori-OprG-Gent digested with *Eco*RV; lane 6, plasmid pGEM-OprG-Gent digested with *Kpn*I; lane 7, recombinant allelic exchange vector pGori-OprG-Gent digested with *Kpn*I. The sizes of the DNA molecular weight marker, GeneRuler^TM DNA Ladder Plus, are indicated to the left of the figure.**

**5.3.2   Engineering of an OprG-deficient *P. aeruginosa* PAO1 (DSM 1707) strain**

5.3.2.1   Generation of mutant strains

Mutant strains of the wild-type *P. aeruginosa* PAO1 (DSM 1707) strain were generated by introducing the allelic exchange vector pGori-OprG-Gent into the wild-type PAO1 (DSM 1707) strain by triparental mating, and selecting for subsequent homologous recombination events between the *P. aeruginosa* DNA flanking the gentamicin resistance cassette in the vector and the wild-type locus on the genome. Recipient *P. aeruginosa* strains harbouring an integrated copy of the *oprG*::Gm^R null allele were selected by plating onto selective medium, as described under Material and Methods (Section 5.2.13).   A gentamicin-resistant *P. aeruginosa* strain was selected and designated DSMOprG.

5.3.2.2   PCR analysis of *P. aeruginosa* mutant strain DSMOprG

The presence of an integrated copy of the mutant allele in the DSMOprG strain was verified by PCR analyses using different pairs of oligonucleotide primers (Fig. 5.4a) to amplify hybrid products only if the gentamicin resistance cassette was located within the chromosomal-borne inactivated *oprG*. Moreover, primers were also used that annealed to genomic sequences flanking the region in which the mutant allele was integrated.

Primers GenomicA and IntRp4067 as well as GenomicB and Gentp1-1 were used to amplify a 2.101-kb and 2.714-kb hybrid product, respectively, if the gentamicin resistance cassette was located within the disrupted *oprG* gene. The respective products were produced when DSMOprG chromosomal DNA was used as template (Fig. 5.4b, lanes 3 and 4, respectively). As expected, when wild-type *P. aeruginosa* PAO1 (DSM 1707) chromosomal DNA was used as template in the PCR reactions a 1.201-kb product was amplified using primers GenomicA and IntRp4067 (Fig. 5.4b, lane 6), but no product was amplified using primers GenomicB and Gentp1-1 (Fig. 5.4b, lane 7).   In the final analysis, primers were used that annealed to genomic sequences upstream and downstream of the PA4067 ORF in which the mutant allele was integrated. Thus, primers GenomicA and GenomicB were used to amplify either a 2.798-kb product in the absence of the gentamicin resistance cassette, or a 3.698-kb product in the presence of the 0.9-kb gentamicin resistance cassette. As expected, a 3.7-kb product was produced when DSMOprG chromosomal DNA was used as template (Fig. 5.4b, lane 2). Template DNA from wild-type PAO1 (DSM 1707) generated the 2.8-kb product indicative of the absence of the gentamicin cassette within the *oprG* gene (Fig. 5.4b, lane 5).
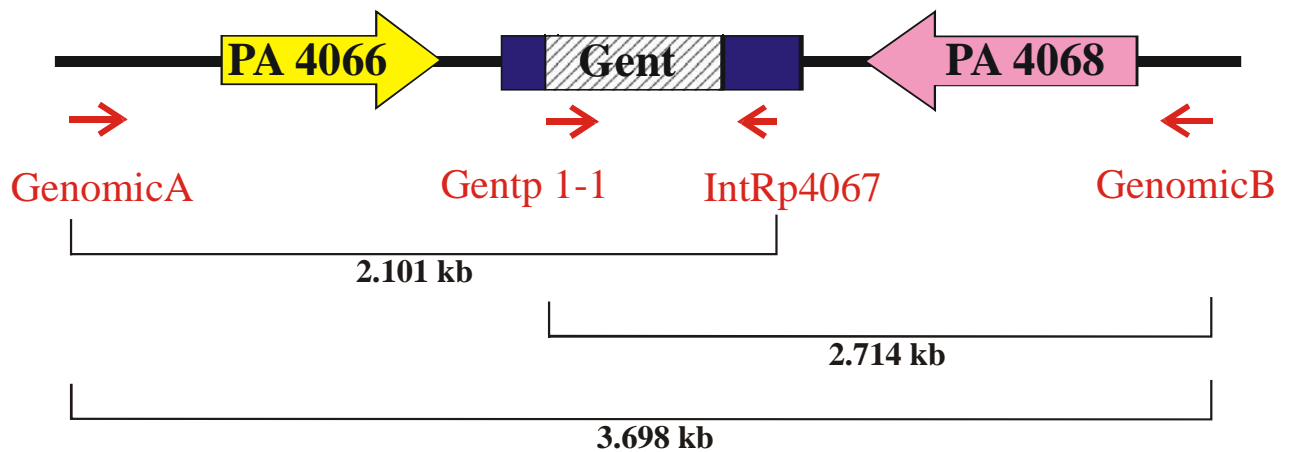
Fig. 5.4a Schematic presentation of specific primer annealing positions and direction of amplification in the mutant DSMOprG strain. The expected sizes of the different amplicons are indicated by brackets.
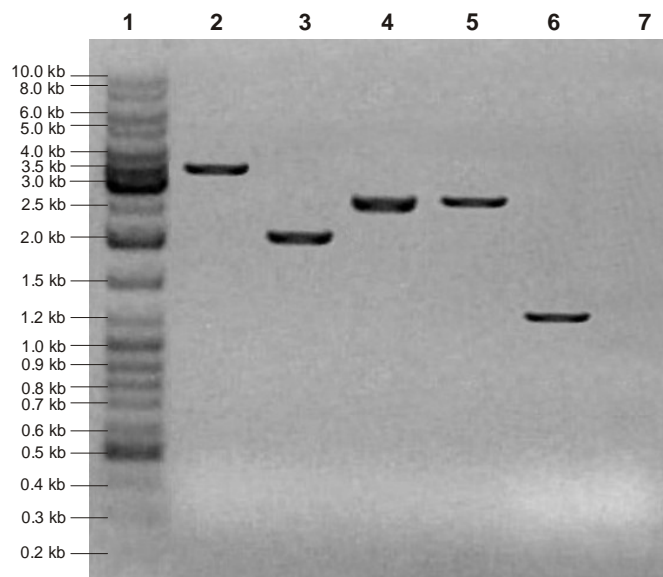
Fig. 5.4b Agarose gel electrophoretic analysis of the amplification products obtained following PCR analysis of DSMOprG and *P. aeruginosa* PAO1 (DSM 1707) using primers GenomicA and GenomicB (lanes 2 and 5), GenomicA and IntRp4067 (lanes 3 and 6), and GenomicB and Gentp1-1 (lanes 4 and 7). Lanes 2, 3 and 4 represent genomic DNA from mutant strain DSMOprG, while lanes 5, 6 and 7 represent genomic DNA from wild-type *P. aeruginosa* PAO1 (DSM 1707). The sizes of the molecular weight marker, GeneRuler[TM] DNA Ladder Plus, are indicated to the left of the figure.

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5.3.3 Characterisation of the DSMOprG mutant strain

5.3.3.1 Growth curves

Since reports have noted that insertion mutagenesis may influence the growth properties of a particular mutant strain (Kadurugamuwa *et al.*, 1993; Hoang *et al.*, 2000), it is possible that the observed effect following mutagenesis may be due to growth impairment of the strain rather than inactivation of a specific gene. Thus, to investigate whether the introduced mutation influenced the growth properties of the mutant strain, the wild-type *P. aeruginosa* PAO1 (DSM 1707) and mutant DSMOprG strains were cultured in MSGY broth and their growth was followed by taking optical density readings at 600 nm over a period of 26 h. The results that were obtained (Fig. 5.5) indicated that culturing of the wild-type PAO1 (DSM 1707) and mutant DSMOprG strains in MSGY broth yielded generation times of 130.60 min and 150.38 min, respectively. These results thus indicated that the mutant DSMOprG was slightly growth-impaired when compared to the wild-type strain.

5.3.3.2 Biofilm development on glass wool

To determine whether the DSMOprG mutant strain was capable of forming biofilms, biofilm development on glass wool in MSGY broth was monitored at various times by bright-field microscopy after staining of the glass wool with crystal violet. For this analysis, the wild-type *P. aeruginosa* PAO1 (DSM 1707) strain was included as a control. Although very few cells of both strains were visible on the glass wool up to 4 h after inoculation (Fig. 5.6a and b), more cells became visible on the glass wool after 8 h (Fig. 5.6c) and dense biofilm structures were formed within 18 h (Fig. 5.6d) that became denser after 26 h (Fig. 5.6e). However, some phenotypic differences between the biofilm structures of the wild-type PAO1 (DSM 1707) and mutant DSMOprG strain could be observed. Whereas the wild-type strain showed uniform colonisation of the glass wool surface punctuated with dense, thick multilayered structures, the DSMOprG strain showed sparser colonisation of the glass wool surface and the biofilm structures had the appearance of cell clumps (Fig. 5.6d and e).

To further investigate the propensity of the DSMOprG mutant cells to attach and grow as biofilms, the ratio of attached (biofilm) to surface influenced planktonic (SIP) biomass was calculated at various time intervals of culturing from cultures grown in MSGY broth with glass wool. Whereas the SIP cells were obtained by careful aspiration of the culture fluid, the
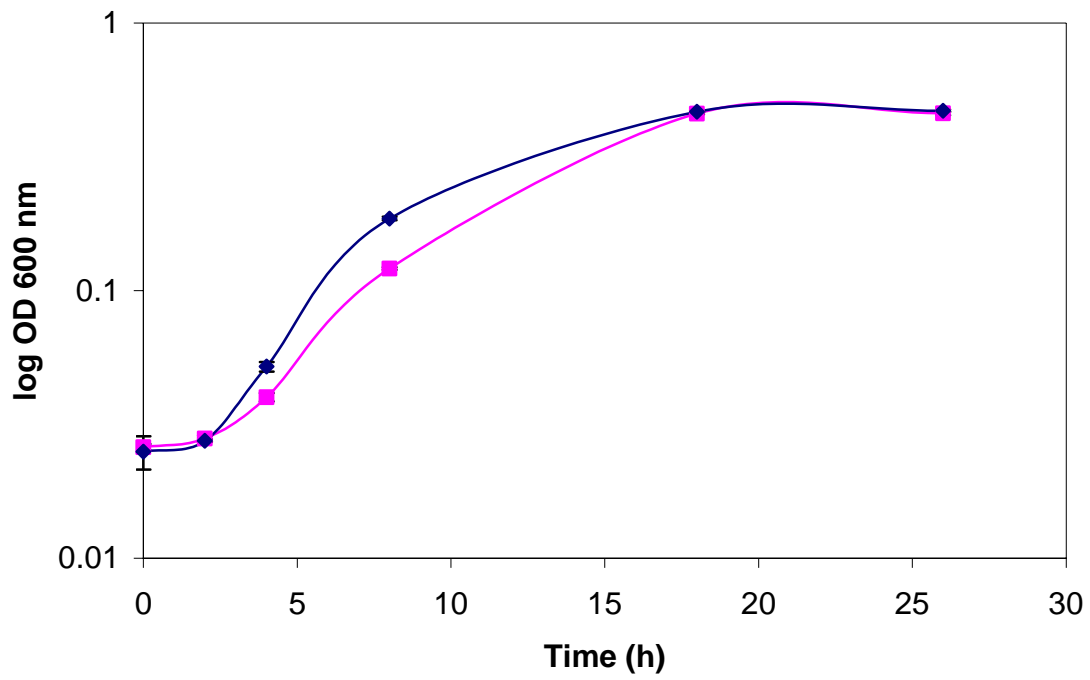
Fig. 5.5 **Growth curves of the wild-type *P. aeruginosa* (♦) and mutant DSMOprG strain (■) in MSGY broth.**

attached (biofilm) cells were recovered from the glass wool substratum by vortexing. The results obtained are presented in Fig. 5.7.

Comparative analyses of growth curves obtained for the planktonic, biofilm and planktonic surface influenced planktonic (SIP) populations of the wild-type PAO1 (DSM 1707) and mutant DSMOprG strains indicated that DSMOprG was impaired in its growth (Fig. 5.7a), thus confirming earlier results (Fig. 5.5). Regarding the propensity of DSMOprG to attach and grow as a biofilm, the results indicated that cells of both the wild-type PAO1 (DSM 1707) and mutant DSMOprG strains were capable of attaching to the glass wool substratum after 2 h of culturing, albeit that cells of the DSMOprG strain was more efficient in attaching to the glass wool substratum as was evidenced by a higher ratio of biofilm to SIP biomass (Fig. 5.7b). However, following attachment, cells of the DSMOprG strain were less prone to occur in a biofilm when compared to the wild-type PAO1 (DSM 1707) cells. This was evidenced by a decrease in the ratio of biofilm to SIP biomass of the DSMOprG strain compared to the wild-type PAO1 (DSM 1707) strain. From these results it was concluded that the DSMOprG strain was capable of attaching to the glass wool surface, but was impaired in its ability to grow as a biofilm.

5.4   DISCUSSION

Although *P. aeruginosa* encodes in excess of 160 known or predicted outer membrane proteins (Stover *et al.*, 2000), the functions of only a very few is known (Hancock and Brinkman, 2002). Outer membrane proteins (OMPs) have been reported to play an important role in antibacterial resistance, transport of nutrients, facilitation of cell-to-cell signalling and virulence in pathogenic bacteria (Ito *et al.*, 1999; Ochs *et al.*, 1999; Pearson *et al.*, 1999). In addition, several different OMPs have been reported to play a role in biofilm formation and development (Espinosa-Urgel *et al.*, 2000; Otto *et al.*, 2001; Yoon *et al.*, 2002). In this study, the role of outer membrane protein porin OprG in *P. aeruginosa* biofilm development was specifically investigated, since its expression was found to be up-regulated in an 18-h old *P. aeruginosa* biofilm population (Chapter 4). A frequently used approach whereby the role of proteins in biofilm formation has been determined relies on the use of isogenic mutant strains from which specific functions have been eliminated (Heilmann *et al.*, 1996; O'Toole and Kolter, 1998; Pratt and Kolter, 1998; Loo *et al.*, 2000). To generate such loss-of-function
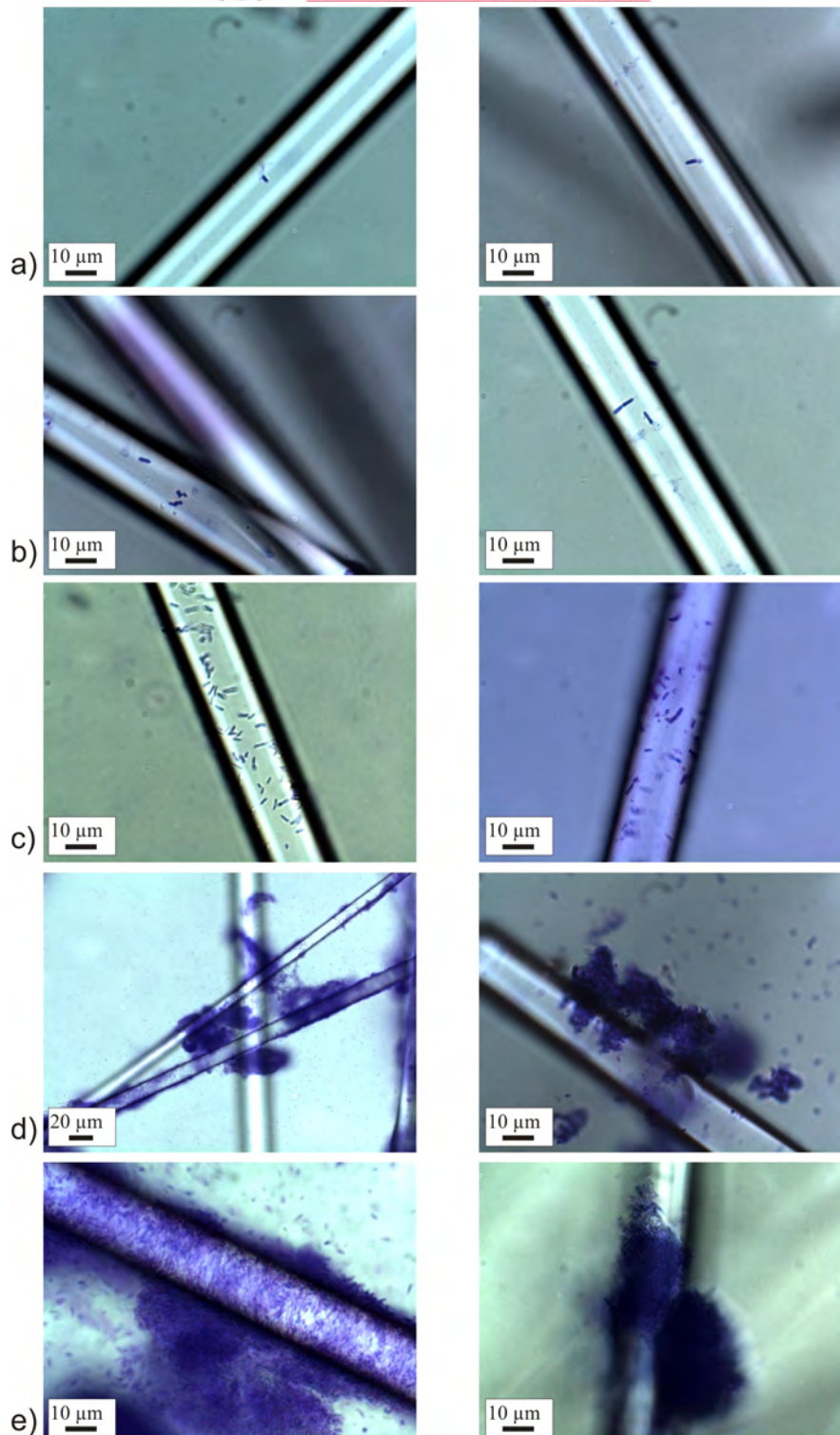
Fig. 5.6 **Biofilm development of *P. aeruginosa* wild-type and mutant strains on glass wool over time. Fifty milliliters of MSGY broth, containing 1.25 g of glass wool, was inoculated with either the *P. aeruginosa* wild-type PAO1 (DSM 1707) or mutant DSMOprG strain. Bright-field microscopy was performed on samples of glass wool following staining with 0.01% (w/v) crystal violet at times 2 h (a); 4 h (b); 8 h (c); 18 h (d) and 26 h (e) after inoculation.**

(a)
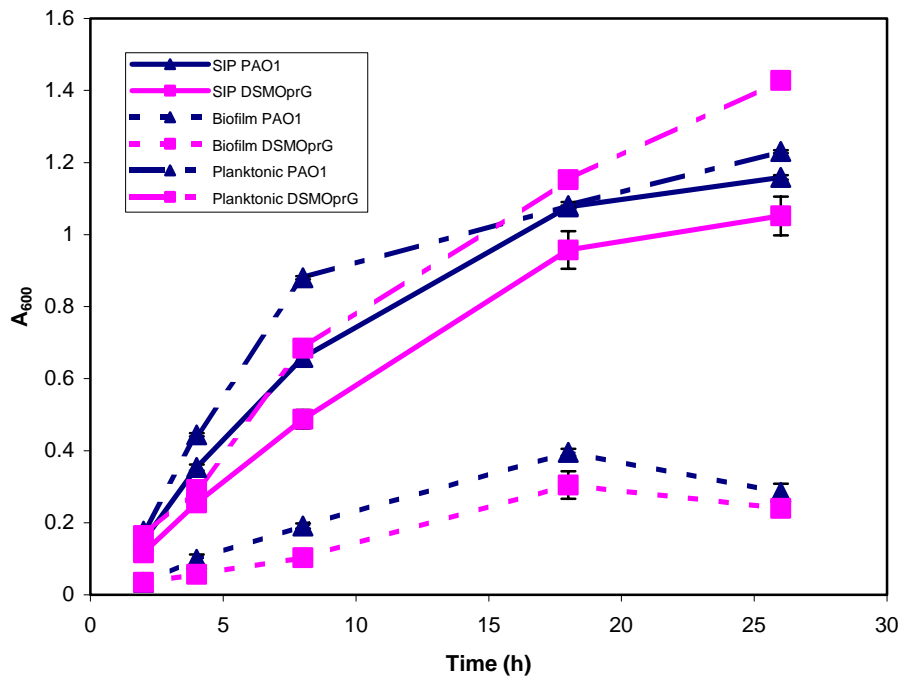
(b)
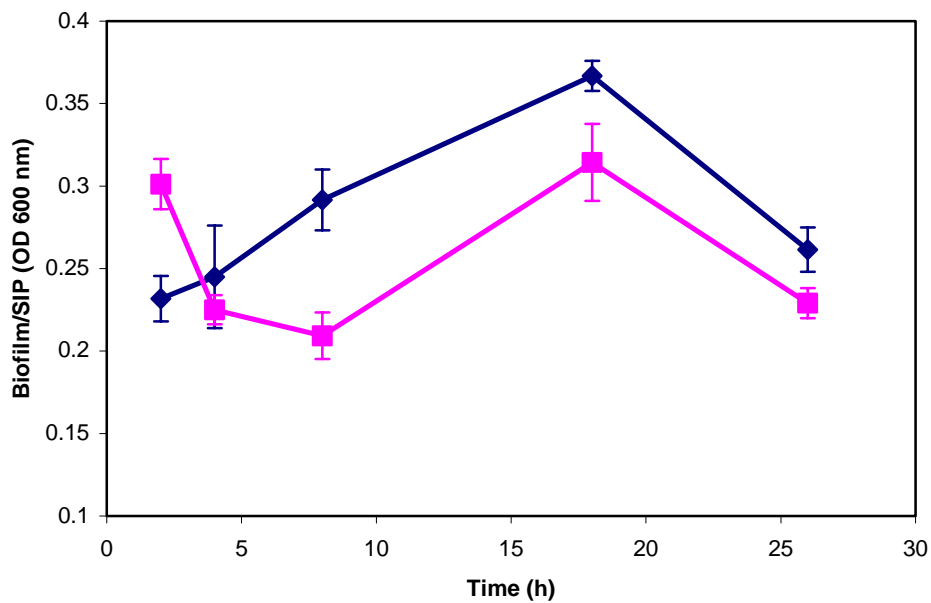
Fig. 5.7 **Propensity of DSMOprG cells to occur as a biofilm. (a) Optical density at 600 nm of planktonic, surface influenced planktonic (SIP) and attached (biofilm) populations of both wild-type *P. aeruginosa* PAO1 (DSM 1707) and mutant DSMOprG strains. (b) The ratio of attached (biofilm) to surface influenced planktonic (SIP) cells grown in MSGY broth in the presence of glass wool is indicated for wild-type *P. aeruginosa* PAO1 (DSM 1707) (♦) and mutant DSMOprG (■), as measured by optical density. Error bars denote standard error of the mean.**

mutations, both random transposon insertion mutagenesis and allelic exchange methods have been useful.

Transposons, being mobile genetic elements, have the capability of inserting themselves into genes on a bacterial chromosome or plasmid, thereby disrupting the gene itself and sometimes additional genes that are encoded downstream of the mutated gene. Nevertheless, this represents a powerful approach towards identifying genes involved in a specific function provided that an appropriate high-throughput screen is available (Hayes, 2003). Using such an approach, O'Toole and Kolter (1998) identified both pili and flagella as being important for the early stages of biofilm development. In addition, Espinosa-Urgel *et al.* (2000) also used this mutagenesis approach to identify several membrane and membrane-associated proteins that are required for attachment of *P. putida* to corn. In contrast to transposon insertion mutagenesis, allelic exchange involves using plasmids that are conditional for their replication in the studied strain ("suicide plasmids") to deliver an *in vitro*-inactivated or an *in vitro*-modified allele of a gene of interest in the chromosome (Toder, 1994; Suh *et al.*, 1999; Dasgupta *et al.*, 2000). Mutations made by allelic exchange are thus targeted, making it a more attractive method of mutagenesis than random transposon insertion mutagenesis. Towards determining the importance of OprG in *P. aeruginosa* biofilm development, allelic exchange was therefore preferred in this study for constructing an OprG-deficient mutant strain. Consequently, an allelic exchange vector harbouring a cloned copy of the *oprG* gene that had been inactivated through insertion of a gentamicin resistance cassette was constructed. The vector was subsequently introduced into *P. aeruginosa* PAO1 (DSM 1707) and presumptive mutant strains were characterised by PCR analyses. The results obtained from these analyses indicated that the insertional inactivation of the *oprG* ORF in the mutant DSMOprG strain occurred by means of a double crossover event and consequently resulted in the integration of only the mutant *oprG*::Gm^R allele.

To determine whether inactivation of the *oprG* gene of *P. aeruginosa* PAO1 (DSM 1707) affected its ability to form biofilm, the OprG-deficient mutant strain was tested for its ability to attach to and develop into a biofilm using glass wool as attachment substratum. Although the DSMOprG mutant strain was capable of forming biofilms on glass wool within 18 h of culturing, inspection of the biofilm phenotype revealed that in contrast to wild-type *P. aeruginosa* PAO1 (DSM 1707), the mutant DSMOprG strain displayed sparser colonisation of the glass wool surface and the cells were localised in clusters. More detailed analysis of

the defects conferred by inactivation of the *oprG* gene was obtained through determining the ratio of attached (biofilm) to surface influenced planktonic (SIP) biomass over a period of 26 h of culturing. Compared to the wild-type strain, cells of the DSMOprG strain attached more efficiently to the glass wool after 2 h, but the ratio of biofilm to SIP cells then dropped until 8 h after inoculation. Similar to results reported by Rice *et al.* (2000), these results may indicate that a greater proportion of the attached DSMOprG cells detached following the first and second division events compared to the wild-type PAO1 (DSM 1707) strain. The ratio of biofilm to SIP DSMOprG cells subsequently increased over time, albeit lower than the wild-type strain, indicating bacterial growth on the surface of the glass wool. After 18 h, the ratio of biofilm to SIP cells of both the wild-type and mutant *P. aeruginosa* strains declined sharply. These results coincide with results obtained earlier in this investigation by 2-DE analysis and those reported by Sauer *et al.* (2004), indicating that FliC is expressed in 18-h old *P. aeruginosa* biofilm populations (Sauer *et al.*, 2002; Sauer *et al.*, 2004; Chapter 4 of this study). Therefore, cells of both strains may have been actively detaching from the biofilm by reverting to a planktonic mode of growth. It is evident from these results that a common signal must have been responsible for detachment of the wild-type and mutant cells from the biofilm. Although it was beyond the scope of this study to determine the factors responsible for affecting the ratio of biofilm to surface influenced planktonic (SIP) cells, it may be that the onset of stationary phase or extended incubation could have resulted in increased detachment (Lamed and Bayer, 1986; Allison *et al.*, 1998). Alternatively, starvation could have resulted in increased detachment by an unknown mechanism to allow bacteria to search for nutrient-rich environments (O'Toole *et al.*, 2000; Sauer *et al.*, 2004).

Although the function of OprG is not known, further evidence supporting a role for this outer membrane protein in the *P. aeruginosa* biofilm developmental cycle has been provided by Sauer *et al.* (2004). Using microarray analysis, expression of OprG in four day-old biofilms was reported to be up-regulated 2.7-fold compared to dispersed cells. To identify homologues of the *P. aeruginosa* PAO1 OprG protein, a BLASTP search of the protein sequences in the GenBank Database was conducted. OprG displays 54% amino acid sequence similarity with the outer membrane protein OmpW of *E. coli*. The OmpW protein of *E. coli* has been reported to be a minor outer membrane protein that is localized to the poles of the cell (Lai *et al.*, 2004) and it serves as a receptor for colicin S4 (Pilsl *et al.*, 2004). Notably, colicin receptors have been reported to participate in other cellular functions such as the uptake of nutrients, *e.g.* iron complexes (Braun *et al.*, 1976), vitamin B_{12} (Di Masi *et al.*,

1973) and nucleosides (Hantke, 1976). Moreover, expression of Omp21 of *Comamonas acidovorans*, which displays 30% amino acid sequence identity to OmpW of *E. coli*, is induced by oxygen limitation (Baldermann *et al.*, 1998). Since a large portion of the mature *P. aeruginosa* biofilm population is under oxygen limitation (DeBeer *et al.*, 1994; Sauer *et al.*, 2002; Yoon *et al.*, 2002; Walters *et al.*, 2003; Werner *et al.*, 2004), it is tempting to speculate that OprG may be required for nutrient uptake, but under conditions of oxygen limitation, such as those experienced in a biofilm, its expression is up-regulated to enhance uptake of nutrients required for growth of the biofilm cells.

In conclusion, the results presented here suggest a role for the outer membrane protein OprG in *P. aeruginosa* biofilm development under the culturing conditions used in this study. This was evidenced by cells of an OprG-deficient *P. aeruginosa* strain, despite being attachment-proficient, were less prone to occur in a biofilm when compared to the wild-type PAO1 (DSM 1707) cells. It is as yet unclear whether OprG is indirectly, *e.g.* through structural alterations in the outer membrane, or directly responsible for this phenotype. Complementation studies, using a recombinant plasmid to provide the wild-type *oprG* gene *in trans*, may aid in clarifying the role of OprG in *P. aeruginosa* biofilm development. However, elucidating the role played by OprG during biofilm formation might not be a trivial task as it is also likely that the slower growth rather than inactivation of *oprG* may account for the altered phenotype displayed by the mutant DSMOprG strain.

5.5 ACKNOWLEDGEMENTS

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CHAPTER SIX

CONCLUDING REMARKS

Traditionally, microorganisms have primarily been characterised as planktonic, freely suspended cells and, as such, have contributed greatly to the understanding of a wide variety of basic physiological processes (Lengeler *et al.*, 1999). However, it was only with the rediscovery that microorganisms attach to and grow on exposed surfaces (Geesey *et al.*, 1977; Costerton *et al.*, 1999) that studies started to reveal that surface-associated microorganisms (biofilms) exhibit a distinct phenotype with respect to gene transcription and growth rate (Davey and O'Toole, 2000; O'Toole *et al.*, 2000a). Microbial biofilm formation is currently thought to represent a sequential bacterial development process (O'Toole *et al.*, 2000a; Stoodley *et al.*, 2002) and a series of genetic and phenotypic determinants involved in the different stages of biofilm development have been identified (O'Toole *et al.*, 2000b; Stoodley *et al.*, 2002; Sauer *et al.*, 2002; Davey *et al.*, 2003). Although classical genetic approaches have been successful in identifying genes involved in biofilm formation (Genevaux *et al.*, 1996; O'Toole and Kolter, 1998a, Pratt and Kolter, 1999), the detection of genome-wide changes of gene expression has become increasingly more important in identifying and examining functions of genes in relation to biofilm development, maintenance and regulation. Consequently, global approaches such as cDNA subtractive hybridization (Sauer and Camper, 2001), DNA microarrays (Whiteley *et al.*, 2001) and proteomics (Sauer and Camper, 2001; Sauer *et al.*, 2002; Oosthuizen *et al.*, 2002) have more recently been used to detect differences in gene expression that occur during the planktonic to biofilm phenotypic switch and to identify "biofilm" genes. In this study, a proteomic approach was used to study global changes in protein expression profiles obtained from 18-h old *P. aeruginosa* PAO1 (DSM 1707) planktonic, surface influenced planktonic (SIP) and biofilm populations grown in batch in the absence or presence of a glass wool substratum. The new information that has evolved during the course of this study is study is summarised briefly in the sections below.

Towards studying global changes in protein expression, a 2-DE technique was established in our laboratory whereby high-resolution 2-DE protein maps of *P. aeruginosa* planktonic and biofilm populations could be generated reproducibly, based on the use of ampholyte-containing polyacrylamide gels cast in tubes. Different sample preparation methods, iso-

electric focusing conditions and protein staining methods were investigated and compared (Chapter 2). Despite being technically demanding, it was possible to generate reproducible 2-DE protein maps of high resolution by making use of tube gels with carrier ampholyte-generated pH gradients. The optimised 2-DE method 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 a Lysis buffer 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 may be stained with either silver diamine or with Coomassie Brilliant Blue, depending on the resolution required.

Having established and optimised the 2-DE methodology, it was subsequently used to demonstrate phenotypic differences between *P. aeruginosa* biofilm cells and the planktonic counterpart cells grown in batch under defined culture conditions (Chapter 3). Glass wool was used as a substratum for cell attachment in these investigations, as it affords a large surface-to-volume ratio (1 g with a mean diameter of 15 μm = 1 300 cm^2), supports the growth of biofilms, allows for free movement of cells between the inter-strand spaces, and it facilitates the exchange of nutrients and oxygen. It also allowed for the separation of the biofilm biomass from the surrounding surface influenced planktonic (SIP) cells for further characterisation. Comparative analysis of the respective proteomes indicated striking differences in the protein patterns of planktonic, biofilm and SIP cells and several uniquely expressed proteins were seen on the 2-DE protein maps of the respective populations. These results not only indicated that glass wool is an ideal attachment surface for the study of biofilm development, but were also the first to suggest that SIP cells may represent a mode of growth distinct from that of planktonic and biofilm cells.

Based on the above results, the differences in protein profiles obtained from 18-h old *P. aeruginosa* planktonic, SIP and biofilm populations were studied in greater detail by making use of an improved protein extraction methodology. Phenotyping was accomplished using 2-DE of sequentially extracted proteins from whole-cell extracts followed by quantitative and qualitative image analysis of the proteins using the PDQuest software program (Chapter 4). Compared to the single-step extraction method used previously, the approach used in this part of the study resulted in an increase in resolution of *ca.* 38% in the visualised number of protein spots over a pH range of 3.0 - 10.0. Comparative analysis of the 2-DE patterns

indicated the presence of five unique protein spots in the planktonic proteome, while the SIP and biofilm proteomes displayed 12 and 49 unique protein spots, respectively. In the *P. aeruginosa* biofilm cells, a general down-regulation of protein expression was seen, but in SIP cells expression of the proteins was generally up-regulated. The results served to confirm that the biofilm population differs from the planktonic population and furthermore indicated that the SIP population is not merely a homogenous mixture of planktonic and biofilm cells but rather a unique phenotype. Much still needs to be learned, however, regarding the physiology of SIP cells, as well as the interplay of this phenotypically distinct population with the biofilm and planktonic populations during biofilm formation and development. Several differentially expressed protein spots were selected and identified using N-terminal protein sequencing and peptide mass fingerprinting. The proteins identified could be divided into three main groups. The first group comprises proteins that could be categorised as outer membrane or membrane-associated proteins (OprG, OprF, OprB); the second group comprises proteins that have sequence similarity to known outer membrane proteins (OpdT, OpdP, OpmH, FadL); and the third group comprises cytoplasmic proteins (RpsA, Trigger factor, FliC). Many of these proteins have been seen for the first time on *P. aeruginosa* proteome maps. The next goal should be to determine whether these proteins are indeed required for biofilm formation and to determine the specific stage in the development of a biofilm wherein these genes may be required.

Recent reports have indicated a role for different outer membrane proteins in *P. aeruginosa* biofilm development (Yoon *et al.*, 2002; Finelli *et al.*, 2003). Consequently, a *P. aeruginosa* PAO1 (DSM 1707) strain deficient in outer membrane protein OprG was generated and its ability to form biofilms on a glass wool substratum was compared to that of the wild-type *P. aeruginosa* strain (Chapter 5). OprG, encoded by PA4067, was selected for investigation since its expression was up-regulated in biofilm cells and it has not been investigated previously for its involvement in biofilm formation. Despite being slightly growth-impaired, the mutant DSMOprG strain was capable of forming biofilms on glass wool within 18 h of culturing in MSGY broth. However, inspection of the biofilm phenotype revealed that, in contrast to wild-type *P. aeruginosa* PAO1 (DSM 1707), the mutant DSMOprG strain displayed sparser colonisation of the glass wool surface and the cells were localised in clusters. More detailed analysis of the altered biofilm phenotype conferred by inactivation of the *oprG* gene was obtained through determining the ratio of attached (biofilm) to surface influenced planktonic (SIP) biomass over a period of 26 h of culturing. Compared to the

wild-type strain, cells of the mutant DSMOprG strain was attachment-proficient but biofilm-deficient. It is, as yet, unclear whether OprG is indirectly, *e.g.* through structural alterations in the outer membrane, or directly responsible for this phenotype.

It is interesting to note that despite several investigations having been directed at determining the degree to which gene regulation during *P. aeruginosa* biofilm development controls the switch from planktonic to attached growth, there appears to be little overlap between the reported results. O'Toole and Kolter (1998a), using a microtitre plate model to identify transposon mutants unable to initiate biofilm formation in *P. aeruginosa*, identified both pili and flagella as being important for the initial steps in biofilm formation. Subsequent studies of biofilm gene expression at the proteome and RNA levels have used biofilms grown in flow through systems. Whiteley *et al.* (2001), using DNA microarrays to compare gene expression of biofilm and planktonic *P. aeruginosa* PAO1 grown either in chemostats or in once-flow through tubing, reported that of the 73 genes (representing 1% of the *P. aeruginosa* genes) whose expression varied two-fold or greater, 34 were up-regulated and 39 were down-regulated in biofilm populations. 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. Sauer *et al.* (2002), using 2-DE analyses of *P. aeruginosa* grown in a once-flow through tubing system to follow changes in gene and protein expression throughout various stages of biofilm development, reported that, depending on the stage of biofilm maturation, up to 56% of resolvable proteins demonstrated altered levels of expression compared with those in chemostat-grown planktonic cells. Proteins detectable in mature biofilm samples and undetectable in planktonic bacteria fell into five major classes, *i.e.* metabolism, phospholipid and lipopolysaccharide biosynthesis, membrane transport and secretion, as well as adaptive and protective mechanisms. In this study, 4% of the resolvable proteins displayed altered levels of expression in 18-h old *P. aeruginosa* planktonic and biofilm cells grown in batch, and the expression of only two outer membrane proteins were up-regulated in biofilm cells. Therefore, the type, number and magnitude of expression changes detected appear to vary with the experimental system and experimental approach used. It seems likely that particular environmental stimuli (*e.g.* growth medium and/or the substratum) may trigger different developmental pathways, all culminating with the same end point, a biofilm (O'Toole and Kolter 1998b; O'Toole *et al.*, 2000b; Stanley and Lazazzera, 2004). Clearly, much work is still needed to characterise biofilms and to give a complete description of the suite of physiological changes that occur during biofilm

development. Nevertheless, by integrating the information obtained from multiple biofilm studies utilising a variety of approaches, such as those used in this investigation and those highlighted above, it may be possible to obtain a more complete view of *P. aeruginosa* biofilm development.

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