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.

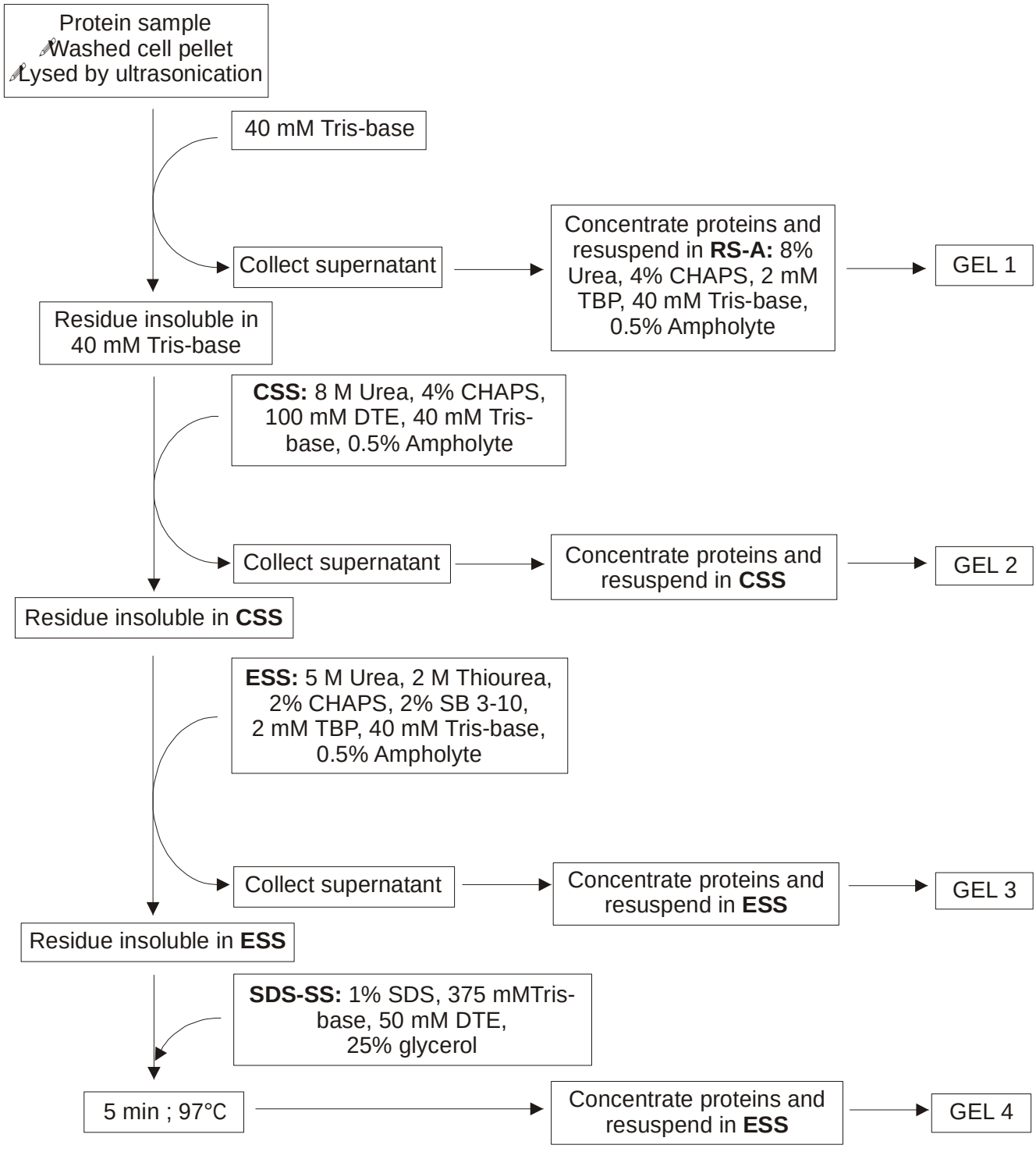

Fig. 4.1 **Schematic illustration of the differential extraction procedure that was performed on whole-cell protein samples from *P. aeruginosa* planktonic, SIP and biofilm populations. Abbreviations: RS-A, resuspension solution A; CSS, conventional solubilisation solution; ESS, enhanced solubilisation 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

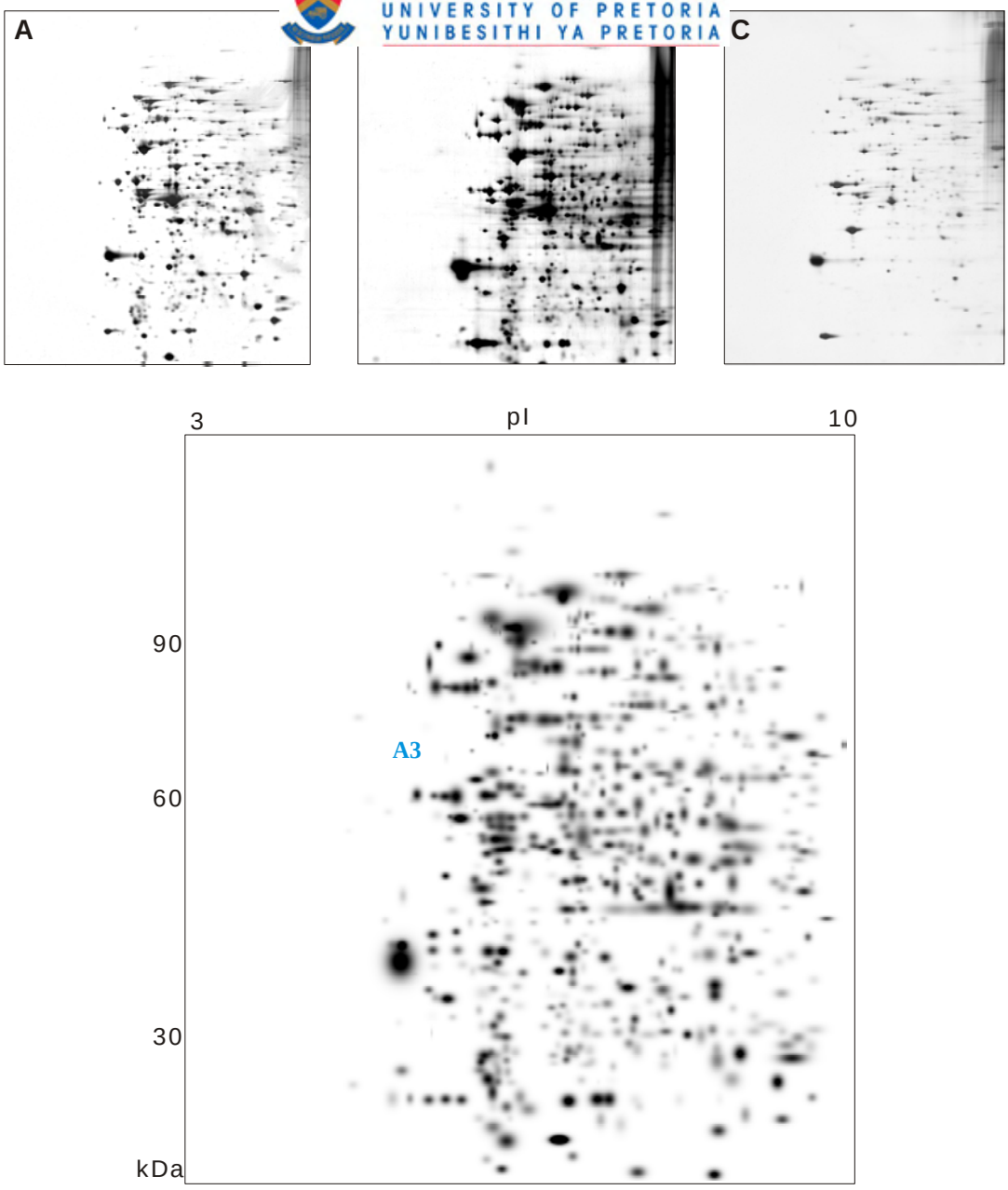

Fig. 4.2 **Sequential extraction of proteins from an 18 h planktonic *P. aeruginosa* PAO1 culture grown without glass wool. The proteins were focused in a non-linear pH gradient of 3.0 – 10.0, electrophoresed on 10% SDS-PAGE gels and subsequently stained with silver diamine staining. (A) Extract 1, proteins 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.**

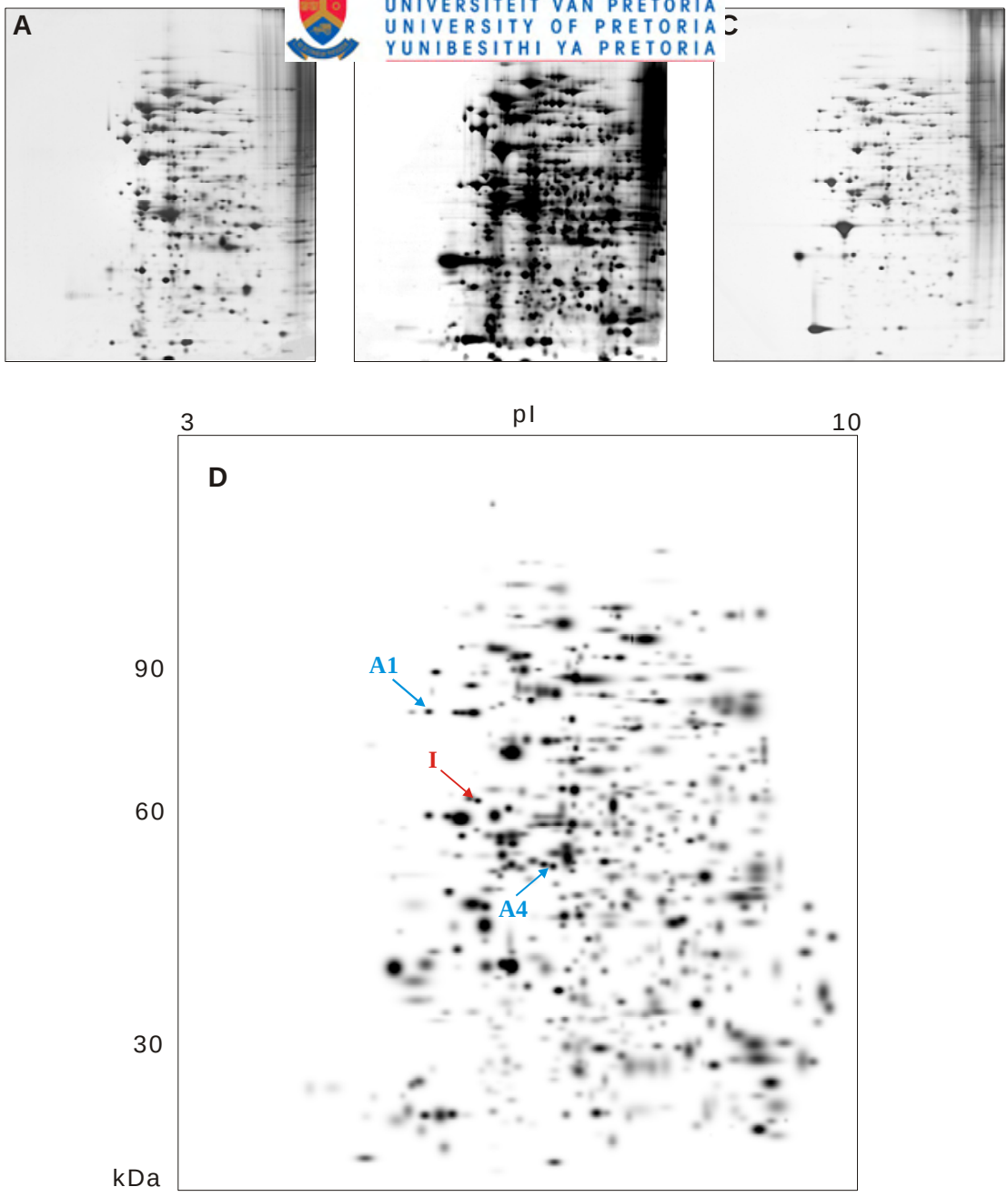

Fig. 4.3 **Sequential extraction of proteins from an 18 h surface influenced planktonic (SIP) *P. aeruginosa* PAO1 culture. The proteins were focused in a non-linear pH gradient of 3.0 – 10.0, electrophoresed on 10% SDS-PAGE gels and subsequently stained with silver diamine staining. The proteins were 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.**

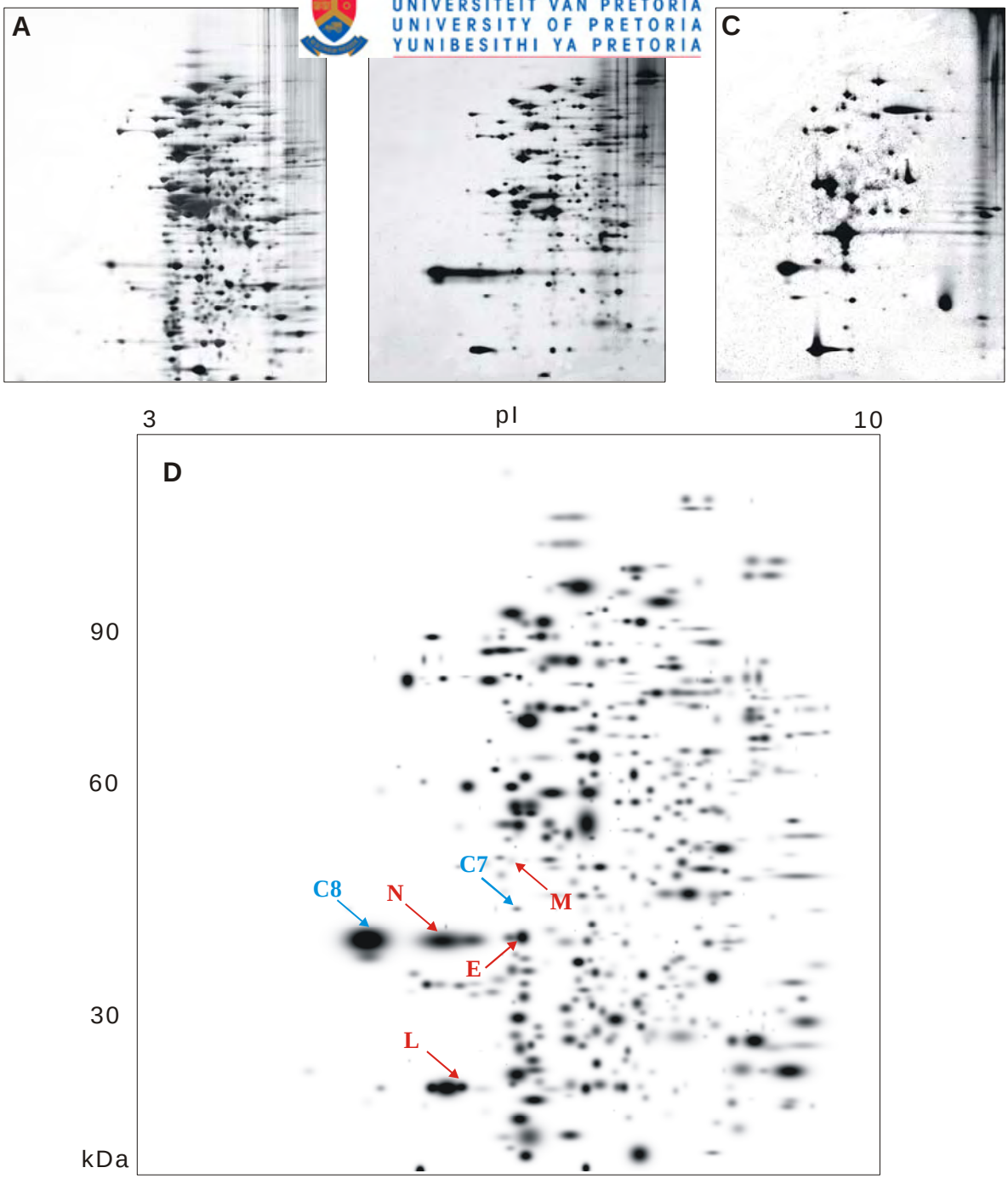

Fig. 4.4 **Sequential extraction of proteins from an 18 h biofilm *P. aeruginosa* PAO1 culture grown on glass wool as attachment substratum. The proteins were focused in a non-linear pH gradient of 3.0 – 10.0, electrophoresed on 10% SDS-PAGE gels and subsequently stained with silver diamine staining. 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.

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