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.

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Steyn, B., Oosthuizen, M.C., MacDonald, R., Theron, J., 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.

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 × *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 × *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 × *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.

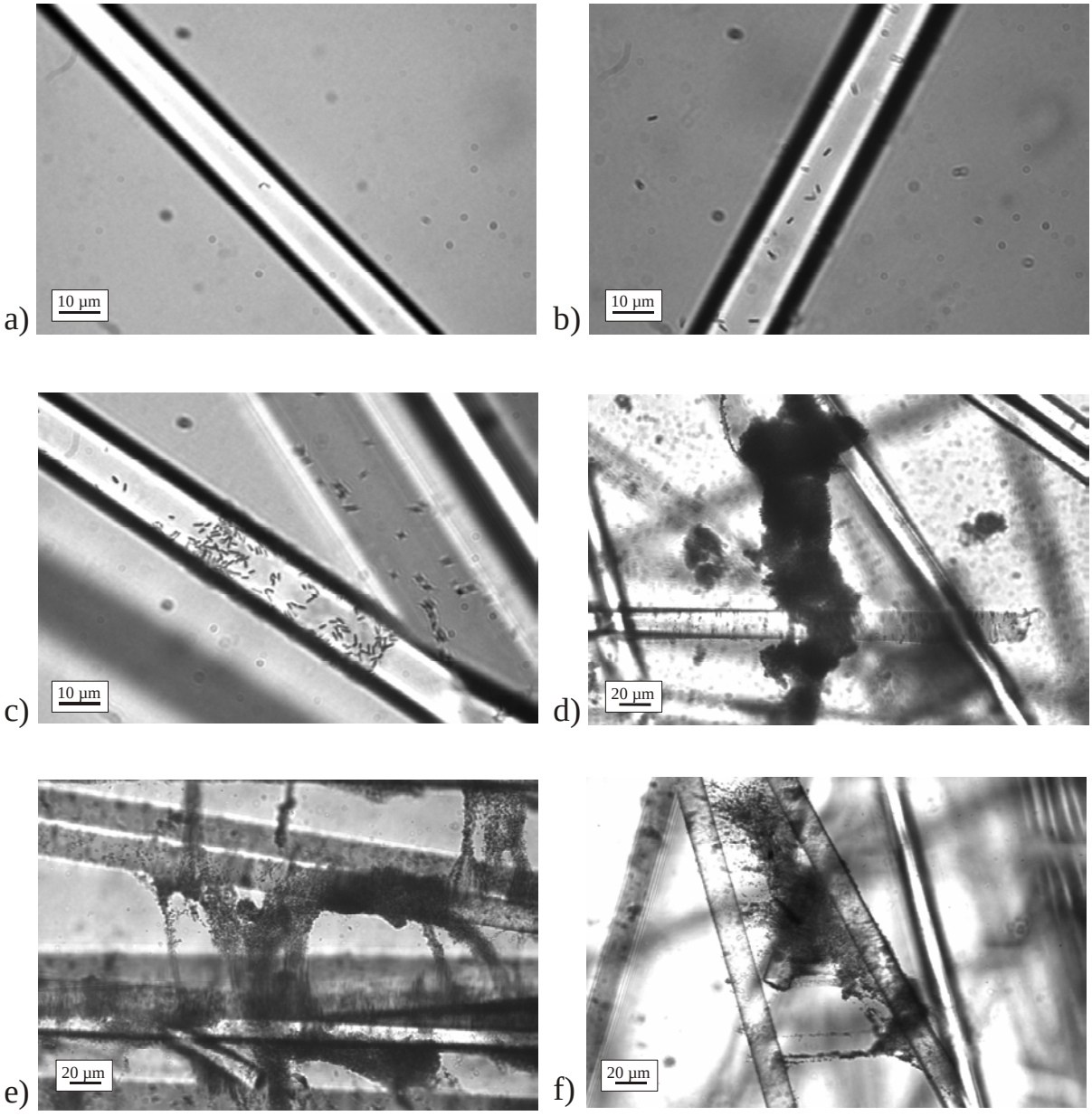


Fig. 3.1 **Development of *P. aeruginosa* biofilm on glass wool over time. One hundred milliliters of MSGY broth, containing 2.5 g of glass wool, was inoculated with *P. aeruginosa* PAO1 cells to a final inoculum of 4×10^6 cfu/ml. Bright-field microscopy was performed on samples of glass wool following 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.

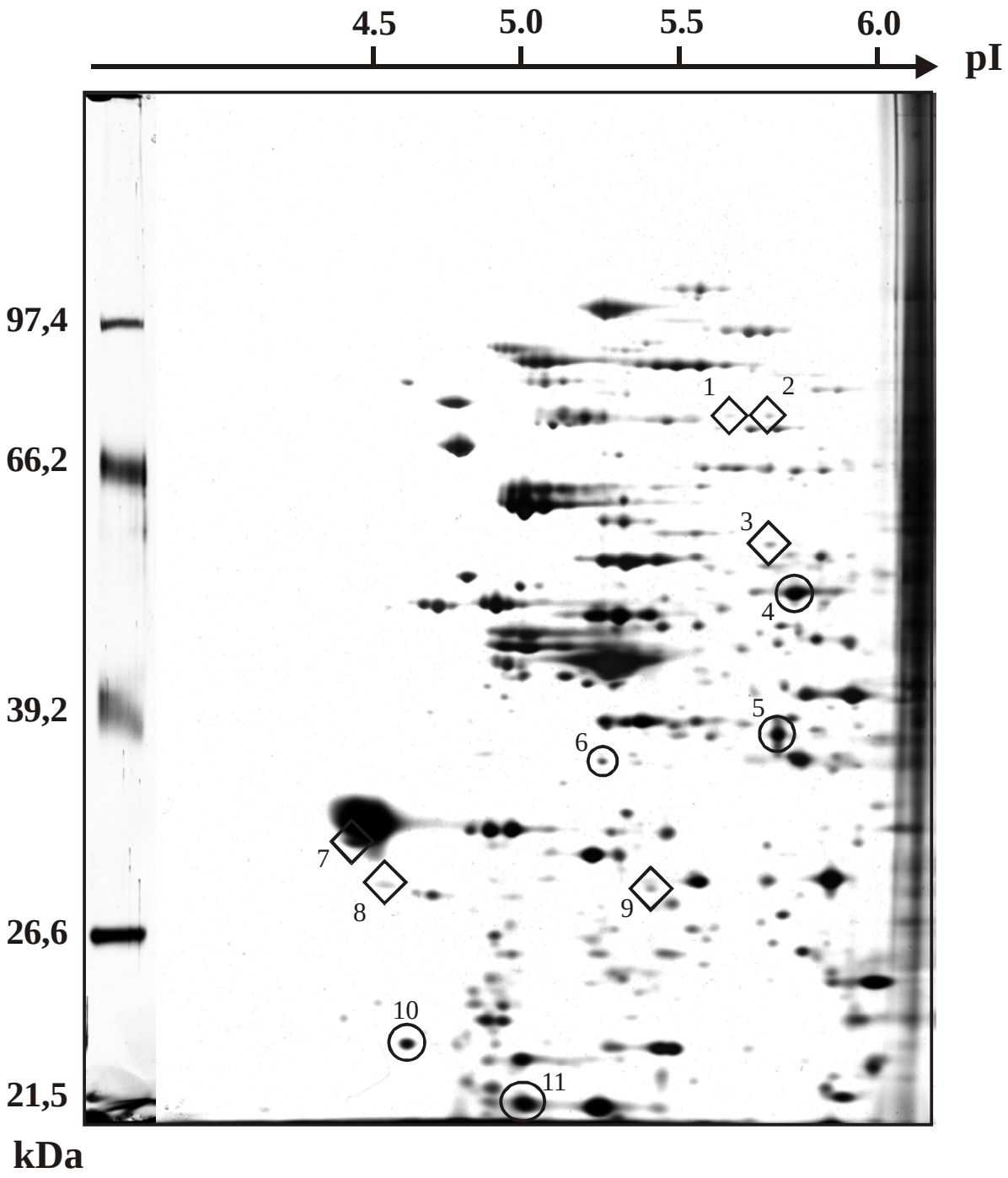


Fig. 3.2 **2-DE of whole-cell proteins of an 18 h planktonic *P. aeruginosa* PAO1 culture without glass wool. The proteins were focused in a non-linear pH gradient of 3.0 to 10.0. After iso-electric focusing, the sample was run on 10% SDS-PAGE and subsequently stained with silver diamine staining. The 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.**

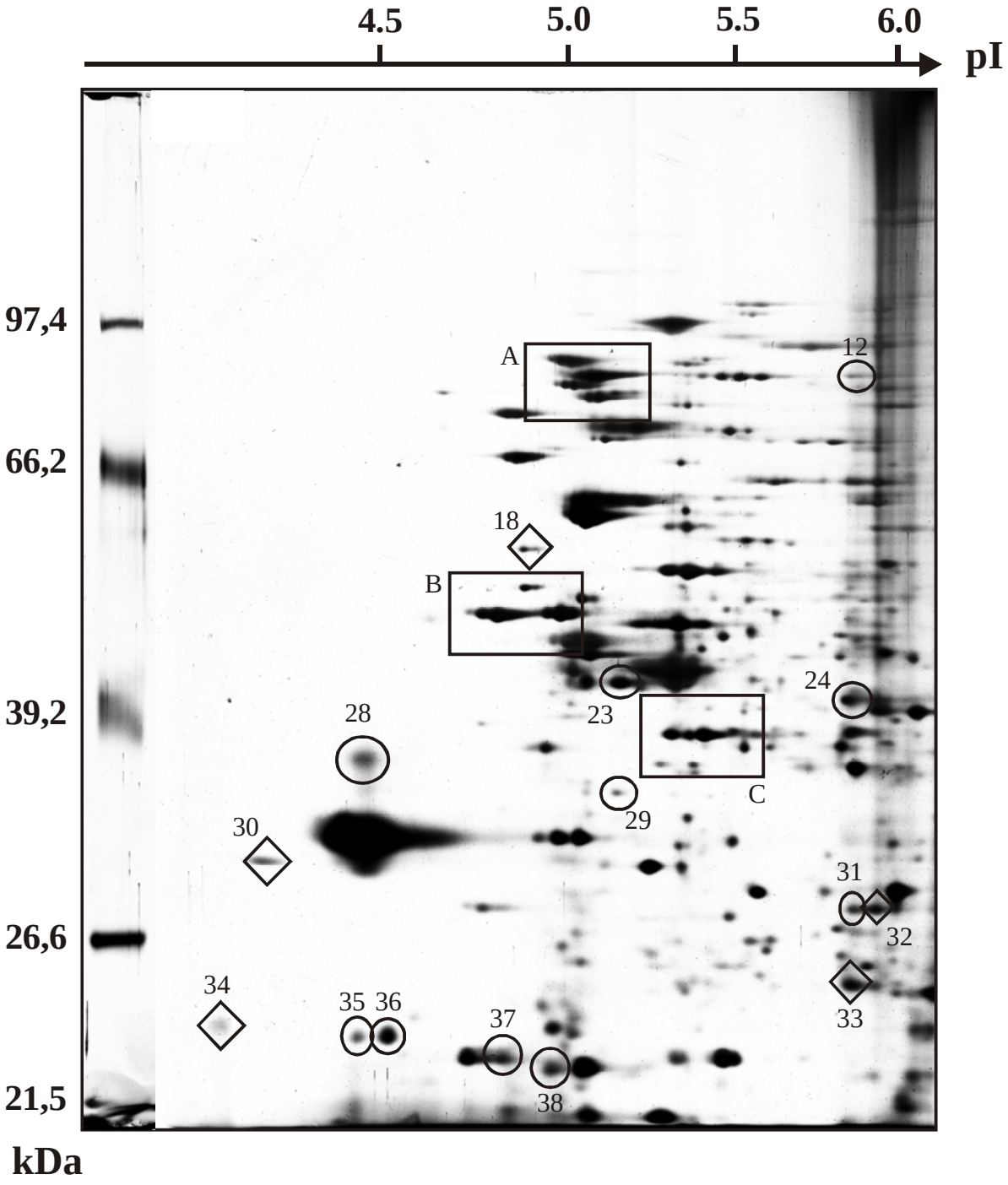
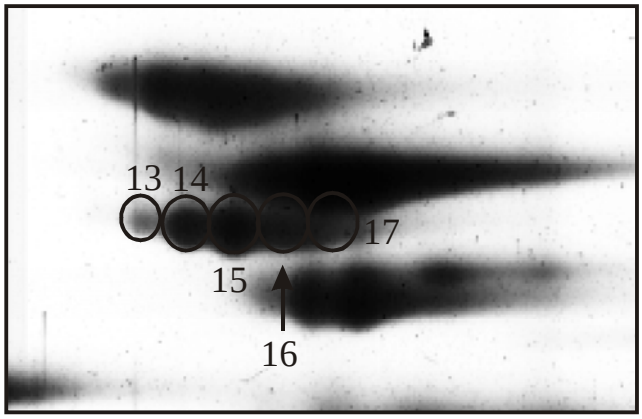
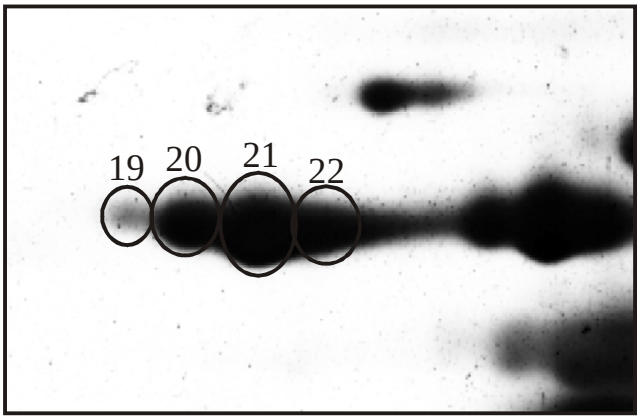


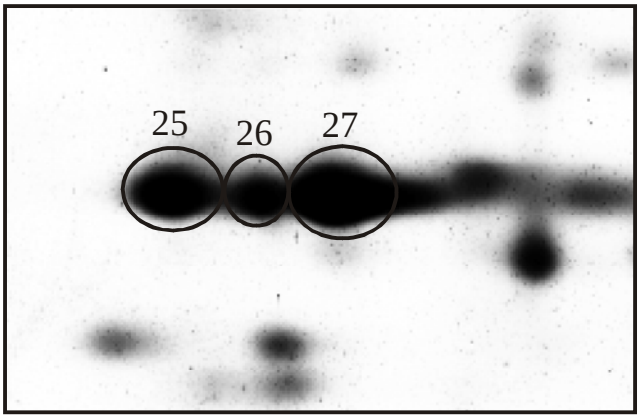
Fig. 3. 3 **2-DE of whole-cell proteins of an 18 h biofilm *P. aeruginosa* PAO1 culture grown on glass wool as attachment surface. The proteins that are differentially displayed between the biofilm and the other two phases (planktonic and SIP) are circled and assigned arbitrary numbers. Diamonds indicate 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

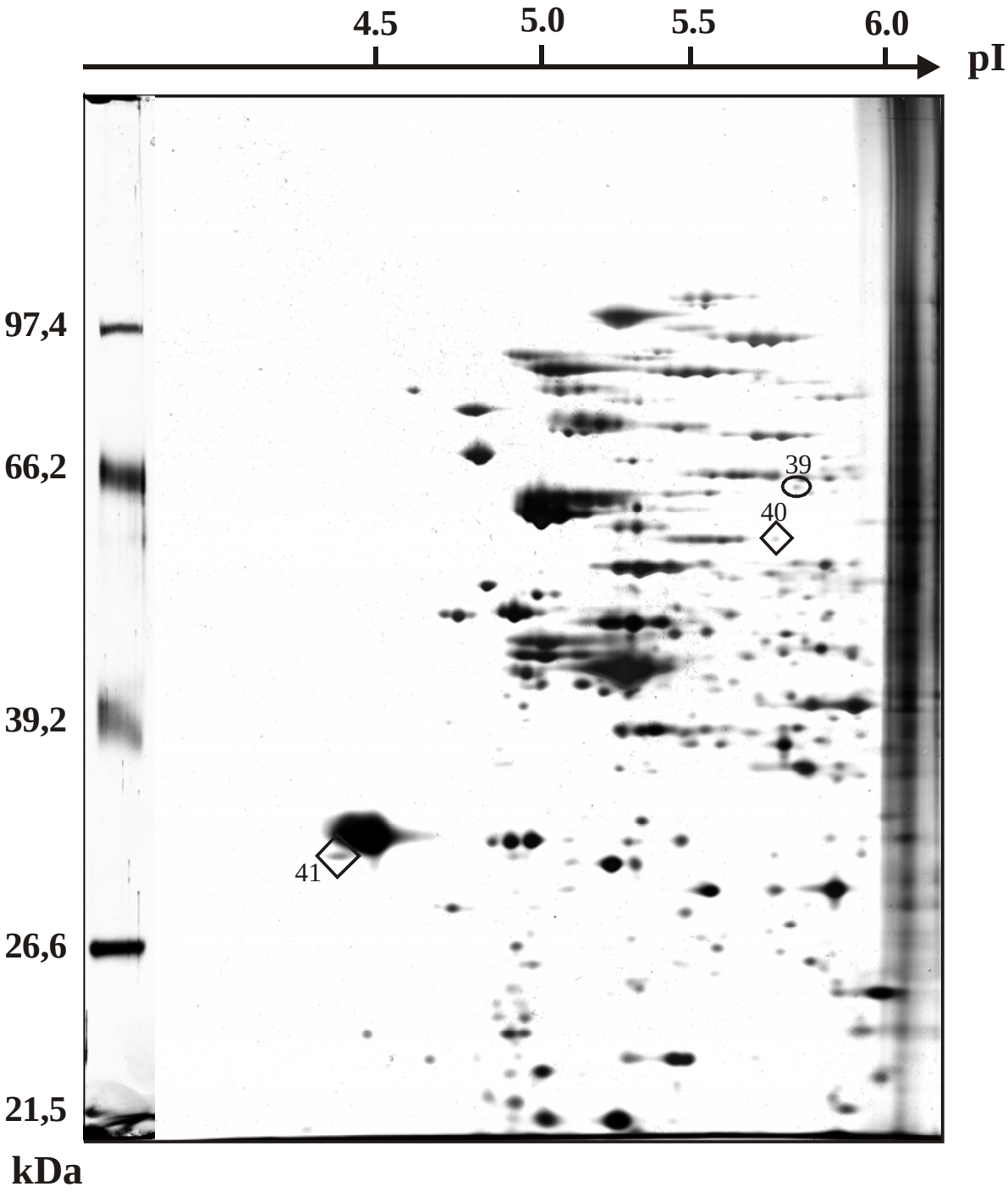


Fig. 3.4 **2-DE of whole-cell proteins of an 18 h SIP *P. aeruginosa* PAO1 culture. The protein that is differentially displayed between the planktonic and the SIP phase is circled and assigned an arbitrary number. Diamonds indicate proteins that are unique to the SIP proteome. Molecular mass markers (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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