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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