CHAPTER FOUR
CONCLUDING REMARKS

The majority of information regarding the physiology of bacteria is based on information obtained from cells grown in suspension. However, it has become increasingly apparent that bacteria have a remarkable ability to adhere to surfaces, and that these adherent bacteria have both a markedly different phenotype and characteristics than their planktonic counterparts. The ultimate goal of research into the molecular genetics of bacterial adhesion and growth is to gain insight into the molecular mechanisms underlying biofilm formation and maintenance. Towards achieving this goal, the objectives of this study were to characterize several attachment-induced genetic elements in *P. aeruginosa* and to investigate the transcriptional activity of one such element in the absence and presence of an attachment surface using reporter gene technology. The results that were obtained in the course of achieving these objectives have been discussed in the relevant chapters. In this conclusion, the new information that has evolved during the study are briefly summarized and some suggestions regarding future research will be made.

In the first part of the study, 24 clones that were previously shown to display up-regulation of a *lacZ* reporter gene upon attachment to a glass wool substratum were selected and their nucleotide sequences determined. Analysis of the obtained sequences led to the identification of 42 distinct putative regulatory elements which mapped to widely different positions on the *P. aeruginosa* genome. Notably, three of the sequences contained a recently described conserved quorum sensing-control element (Whiteley and Greenberg, 2001) which may be used in LasR-dependent expression of quorum sensing-controlled genes. These sequences mapped to the upstream regions of *flgE* encoding a flagellar hook protein, a gene encoding a leucine aminopeptidase and a hypothetical protein, respectively. Davies *et al.* (1998) have previously reported that *lasI*, encoding the inducer of LasR, plays an important role in maintaining the three-dimensional architecture of biofilms of *P. aeruginosa*. Although none of the obtained nucleic acid sequences displayed homology with σ^{70}-dependent promoter sequences, several of the sequences displayed significant homology with σ^{54}-dependent promoter sequences. Of the numerous genes that have been reported to be involved in biofilm formation, e.g. *algD* and flagella, many are known to be regulated by σ^{54}. The obtained results therefore indicate that σ^{54} may play an important role in the expression of genes involved in the formation of biofilms in *P. aeruginosa*. To date, no

direct link has, however, been made between σ^{54} and biofilm formation.

Since it appears that σ^{54} (RpoN) may play a role in regulating expression of genes important for biofilm formation, the influence of RpoN in biofilm formation may in future be investigated by comparison of the two-dimensional (2D) gel electrophoresis proteomic profiles of wild-type *P. aeruginosa* and an isogenic *rpoN*⁻ mutant strain under different growth conditions (grown both in the presence and in the absence of glass wool). The amino acid sequence of proteins differentially or uniquely expressed in the respective proteomes may then be determined, using either Edman-degradation or MALDI-TOF mass spectrophotometry, to produce a global picture of the influence of σ^{54} on gene expression in especially biofilm cells.

One of the sequenced attachment-induced genetic elements (clone 498) mapped upstream of a flagellar gene, *flgE*, and contained in addition to a consensus σ^{54} promoter sequence, an IHF binding site. This clone was selected and the minimum requirements for promoter activity was assessed by making use of truncated versions of the regulatory element. The respective clones were assayed in batch culture using *lacZ* as a reporter gene in ONPG hydrolysis assays following growth in the absence and presence of glass wool. The obtained results indicated that the σ^{54} promoter is induced by attachment to a surface and that the IHF binding site significantly enhances expression from the σ^{54} promoter. Although promoter activity from a construct containing only the putative σ^{54} promoter was observed in planktonic and PGW cells, it may have been due to non-specific binding of the activator proteins to the supercoiled DNA (Brahms *et al.*, 1995; Revet *et al.*, 1995). The low promoter activity of this construct in attached cells may have been the result of an altered DNA topology as a consequence of the difference in growth phase or nutrient availability in biofilm cells (Kusano *et al.*, 1996; Conter *et al.*, 1997; Schneider *et al.*, 1999). The use of glass wool as a substrate for attachment in this study offered many advantages as it allowed for a large surface-to-volume ratio (1 g with mean diameter of 15 μm = 1300 cm^2), supported growth of biofilms, allowed for free movement of cells between inter-strand spaces and it facilitates the exchange of nutrients and oxygen (Brözel *et al.*, 1995; Steyn *et al.*, 2001). Furthermore, glass wool also allowed for the easy separation of the biofilm cells from the surrounding suspended cells thereby facilitating analysis of the promoter activity in "true" biofilm cells.

Although the *lacZ* batch system proved to be useful in the initial characterization of the attachment-induced regulatory element, it, however, suffered several disadvantages. Notably, assaying the whole population for reporter activity may not reveal the heterogeneity of expression in a subset of the population, since only the average activity will be measured. In addition, being a plasmid-based system, the plasmid copy number may differ between cells and plasmid-based systems require antibiotic selective pressure to maintain the plasmid in the cell, which may in turn affect the physiology of the bacterial cells. For example, up-regulation in expression of the reporter gene may be due to the biofilm cells being more resistant to antibiotics, thus leading to a higher cell survival rate (more cells). Consequently, the observed increase in β-galactosidase levels may not be directly related to an increase in promoter activity. Furthermore, the up-regulation in expression may be in response to the antibiotic, rather than attachment to the glass wool *per se*. A difference in plasmid copy number may also result in false up-regulation as more plasmids per cell, in a specific growth phase, would yield a higher β-galactosidase activity. To overcome these problems, a pMiniCTX vector was used which allows for integration of a single copy of the transcriptional fusion at a defined, nonessential location on the *P. aeruginosa* genome. Since the integrated gene fusion is stably maintained, there is no need to use antibiotics to maintain selective pressure. In addition, *gfp* expression can be visualized at single cell level in a non-destructive manner using standard epifluorescence microscopy. Analysis of a *P. aeruginosa* strain harboring the integrated *flgE* promoter-*gfp* transcriptional fusion confirmed the up-regulation of expression of the GFP reporter molecule at single cell level upon attachment of the cells to glass wool.

To investigate the heterogeneity in expression of the *flgE* promoter in the biofilm population during the development of biofilms, expression of the *gfp* reporter gene was monitored by fluorescence microscopy over time in a continuous flow through system using a glass coverslip as attachment substratum. Fluorescence was observed as early as 2 h in some of the single cells attaching to the surface, but became less intense over time, presumably due to division of the cells. However, bright fluorescence was observed in cell clusters as well as single cells in 3- to 6-d old biofilms. The results indicated that flagella may be important not only in the initial attachment of bacteria to a surface, but also in the detachment of these bacteria from the biofilm. A model whereby *flgE* is proposed to play a role in the early events of attachment to a surface as well as detachment of the cells from the biofilm structures, has been proposed (Chapter 3).

According to this model, up-expression of *flgE* may lead to up-expression of the late flagellar genes and therefore complete synthesis of flagella. Flagella are important in the initial attachment of bacteria to a surface, presumably due to transport of the cells to the surface. However, once the biofilm is well-developed, or the environmental conditions unfavourable for growth in the biofilm, expression of *flgE* may once again favour the formation of flagella and cells or cell clusters may swim to the planktonic phase or to a new niche for attachment. The signal for the up-expression of *flgE* is as yet unknown, but may be related to quorum sensing.

With reference to the role of *flgE* in biofilm development, future studies in this regard may include the insertional inactivation of the *flgE* gene to determine the importance of *flgE* in the formation and development of *P. aeruginosa* biofilms. Depending on the ability of the mutant strain to form a biofilm in the absence of *flgE*, the role of *flgE* in detachment may be investigated by studying the mutant biofilm 3-d after inoculation. To further characterize regulation of the *flgE* promoter at single cell level, promoter fusions with unstable variants of the *gfp* gene may be integrated onto the *P. aeruginosa* genome and the expression monitored by time-lapse-video-microscopy. This should yield valuable information regarding the fate of cells expressing the reporter molecule as well as the timing of expression of the *flgE* promoter in biofilm cells.