Characterization of genetic elements up-regulated in *Pseudomonas aeruginosa* PAO biofilms and transcriptional activity of the flagellar hook protein gene, *flgE*, during biofilm development

by

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Submitted in partial fulfilment of the requirements of the degree

Master of Science

In the Faculty of Natural and Agricultural Sciences

University of Pretoria

Pretoria

JULY 2002

DECLARATION

I declare that the dissertation, which I hereby submit for the degree Magister Scientiae at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at another university.

_____   _____21/10/2002_____
MARIA SUSANNA MEIRING   DATE

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to the following people:

Dr J Theron for his willingness to guide me and for his encouragement and support throughout this study.

Prof. V.S. Brözel for his input and encouragement.

The National Research Foundation of South Africa for financial support.

The personnel and students in the Department of Microbiology and Plant Pathology for their interest and advice during this study.

The personnel in the Laboratory of Microscopy and Micro-analysis for their friendliness and helpfulness.

My friends for standing by my side through the easy times, but also the hard times. Thank you for your encouragement, help and friendship.

My parents, brothers and grandparents for their love, interest, support and encouragement.

My heavenly Father for His grace, love and the gift of the ability to study.

SUMMARY

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Pseudomonas aeruginosa, an opportunistic human pathogen, can grow in association with surfaces. Biofilm formation occurs in response to a variety of environmental signals and studies involving various different Gram-negative bacteria have shown that biofilms form in multiple steps, require intercellular signalling and demonstrate a profile of gene expression and cellular physiology that is distinct from that of planktonic cells. Although much progress has been made in the elucidation of genes and molecules necessary for bacterial attachment to surfaces and subsequent biofilm formation, much work is still needed to completely characterize the developmental process of biofilm formation. To investigate, previous attempts in our laboratory have relied on the isolation of genetic elements in *P. aeruginosa* PAO (DSM1707) that are up-regulated by attachment to a glass wool substratum. In this study, several of these attachment-induced genetic elements were genetically characterized and the transcriptional activity of one

of these regulatory elements was further investigated by reporter gene technology.

The nucleotide sequence of 24 attachment-induced genetic elements was determined and the results indicated that several of the genetic elements contained putative sigma 54 (σ^{54}) recognition sequences. Thus, σ^{54} may play an important role in biofilm formation as σ^{54} is known to regulate the expression of numerous genes under a wide range of different environmental conditions. One of the regulatory elements was selected and further characterized. Reporter gene technology, using the gene encoding the green fluorescent protein (GFP), was used to investigate the transcriptional activity of the regulatory element of the flagellar biosynthesis gene, *flgE*, in developing and well-developed *P. aeruginosa* biofilms under conditions of continuous flow using glass as substratum. *In vivo* detection of *flgE* expression in biofilms was performed by using fluorescent microscopy coupled with detailed inspection and comparison of images. The results indicated that transcription from the *flgE* promoter is up-regulated in specific single cells in the early stages of biofilm development as well as in cell clusters in 3-day old biofilms. Thus, flagella may not only play a role in the initial attachment of *P. aeruginosa* cells to the substratum, but also in the detachment of bacterial cells from the biofilm structures.

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LIST OF ABBREVIATIONS

A	absorbance
α	alpha
amp^r	ampicillin resistance
A/P ratio	ratio of β-galactosidase activity for the attached (A) cells to that of the planktonic (P) cells
ATP	adenosine triphosphate
β	beta
bp	base pair
ca.	approximately
°C	degrees Celsius
dATP	2'-deoxyadenosine-5'-triphoshate
dCTP	2'-deoxycitidine-5'-triphoshate
ddH$_2$O	deionized distilled water
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside-5'-triphosphate
DTT	dithiothreitol
e.g.	for example
Fig.	figure
GFP	green fluorescent protein
h	hour
IHF	integration host factor
IPTG	isopropyl β-D-thiogalactosidase
kb	kilobase pairs
lacZ	β-galactosidase gene
LB-medium	Luria-Bertani-medium
M	molar
mA	milliampere
MCS	multiple cloning site
mg	milligram

min	minute
ml	millilitre
mM	millimolar
NH₄OAc	ammonium acetate
OD	optical density
ONPG	2-nitrophenyl-β-D-galactopyranoside
ORF	open reading frame
PCR	polymerase chain reaction
PGW cells	planktonic cells grown in the presence of glass wool
RBS	ribosome binding site
RNA	ribonucleic acid
RPM	revolutions per minute
σ	sigma
SDS	sodium dodecyl sulphate
t	transcriptional terminator
T	translational terminator
TE	Tris-EDTA
tetʳ	tetracycline resistance
TN-medium	tryptone-nitrate-medium
U	units
μg	microgram
μl	microlitre
UHQ	ultra-high quality
UV	ultraviolet
V	volts
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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CHAPTER ONE
LITERATURE REVIEW

1.1   GENERAL INTRODUCTION

Pseudomonas aeruginosa is a motile, rod-shaped Gram-negative bacterium that is found in a variety of environments. In addition to causing disease in insects, plants and animals, it is also an important opportunistic pathogen of humans, causing serious infections in immuno-compromised patients such as those with cancer or AIDS, as well as patients suffering from cystic fibrosis and severe burns (Van Delden and Iglewski, 1998). The success of *P. aeruginosa* to grow and cause infections in these diverse environments is attributed to its broad metabolic versatility and its many cell-associated and secreted virulence factors (Van Delden and Iglewski, 1998). In the vast majority of ecological niches, *P. aeruginosa* can grow in association with surfaces (Costerton *et al.*, 1995). Such surface-associated growth leads to the formation of biofilms that are defined as organized communities of cells embedded in an extracellular polysaccharide matrix attached to a biological or abiological surface (Costerton *et al.*, 1987, 1994; Palmer and White, 1997).

More often than not, biofilms are unwanted and may result in various problems. These may include dental plaque, medical implant-associated infections and corrosion of pipes in the oil industry (Palmer and White, 1997). In addition, biofilms may harbor pathogens in drinking water distribution systems, and food spoilage and pathogenic bacteria on food contact surfaces (Czhechowski, 1990; Matilla *et al.*, 1990). By contrast, some biofilms may also be beneficial to humans, e.g. in sewage treatment plants, biofilms are needed for the efficient degradation of xenobiotics (Wolfaardt *et al.*, 1994), while biofilms of lactic acid bacteria form part of the normal indigenous microbiota in humans and their maintenance is essential in the prevention of disease (Gottenbos *et al.*, 1999). Given the medical and economic consequences of biofilm formation, it is important to understand the formation of these complex communities in order to develop strategies whereby their formation can be prevented.

Previous studies have indicated that biofilm cells differ significantly from their planktonic counterparts in terms of their physiology (Anwar *et al.*, 1990). More recent studies regarding gene expression have indicated the differential expression of numerous genes in biofilm cells when compared to their planktonic counterparts (Prigent-Combaret *et al.*, 1999; Steyn *et al.*, 2001; Sauer and Camper, 2001; Whiteley *et al.*, 2001). One of the major limitations in investigating the regulation of gene expression during growth on solid surfaces is the lack of appropriate methods to properly monitor changes of gene expression *in situ*. However, these limitations can be overcome by making use of reporter gene technology. Consequently, relevant literature regarding the initial stages of biofilm formation, regulation of gene expression and reporter molecules whereby gene expression can be monitored in biofilm-growing bacteria, will be reviewed in this chapter.

1.2   BACTERIAL BIOFILMS

In most natural and artificial habitats, bacteria have a propensity to adhere to wet surfaces (Costerton *et al.*, 1987; 1994). Bacterial colonization of both biotic and abiotic surfaces is regarded as a fundamental aspect of their pathogenesis and ecology. Survival of environmental bacteria under starvation conditions, and of bacterial pathogens outside of their host has led to the development of two main bacterial survival strategies: entry into a dormant state, including spores and stationary phase cells (Grossman, 1995; Givskov *et al.*, 1994; Klotz and Hutcheson, 1992; Kolter *et al.*, 1993; Sarniguet *et al.*, 1995) or development of sessile communities in close association with surfaces, i.e. biofilms (Costerton *et al.*, 1994; O'Toole *et al.*, 2000b). Despite general agreement that biofilm bacteria are phenotypically different from their planktonic counterparts, the molecular mechanisms implicated in the biofilm formation process is still under intense investigation.

1.2.1   Formation of biofilms

The formation of a well-developed biofilm (Fig. 1.1) is believed to occur in a sequential process of transport of microorganisms; initial attachment; formation of microcolonies; and formation of well-developed biofilms (Marshall, 1985; Van Loosdrecht *et al.*, 1990). Prior to surface

colonization, a preconditioning film, composed of proteins, glycoproteins and organic nutrients, is believed to be formed on the attachment surface upon its immersion in liquid (Marshall *et al.*, 1971). Bacteria may be transported to the attachment surface through Brownian motion, sedimentation, liquid flow or active bacterial movement (Quirynen *et al.*, 2000). Initial attachment of bacteria to the preconditioned surfaces is then facilitated by Van der Waals forces, electrostatic interactions and specific interactions, or by a combination of these, depending on the proximity of the organisms to the attachment surface (Zottola and Sasahara, 1994; Razatos *et al.*, 1998). Initial attachment is followed by a phase during which production of bacterial exopolysaccharides (EPS) results in a more stable attachment by forming organic bridges between the cells and the substratum (Notermans *et al.*, 1991). Subsequent growth and multiplication of firmly attached primary colonizing organisms lead to the formation of microcolonies. Cells which are loosely attached may detach and these cells together with offspring of other biofilm cells may recolonize previously uncolonized surfaces (Korber *et al.*, 1989), extending the spatial boundaries of the biofilm. The well-developed biofilm is finally characterized by mushroom- or pillar-like structures surrounded by EPS and interspersed with fluid-filled channels (Kuchma and O'Toole, 2000). However, even established biofilms display dynamic behaviour, with periodic detachment and growth in new areas (Heydorn *et al.*, 2002). These dynamics may also be in response to environmental conditions (Nielsen *et al.*, 2000; Tolker-Nielsen *et al.*, 2000; Wolfaardt *et al.*, 1994).
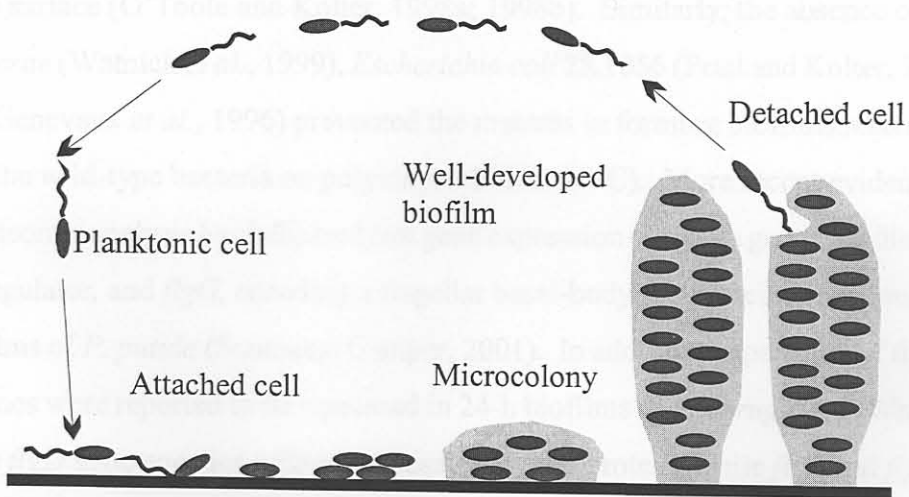
Fig. 1.1   Schematic presentation of the steps in biofilm formation (Modified from O'Toole *et al.*, 2000b).

1.2.2 Structural components required for initial attachment

Less is known about the cascade of events following adhesion than about the adhesion process itself. Of the processes leading to a well-developed biofilm, bacterial structural components required for initial attachment have been best characterized. These include flagella and pili, but membrane proteins and extracellular polysaccharides may also influence initial attachment of bacteria to surfaces.

1.2.2.1 Flagella, pili and adhesins

Although earlier studies have suggested that simple chemical models could account for the bacterial behaviour during the initial stages of attachment (Marshall *et al.*, 1971; McEldowney and Fletcher, 1986), subsequent studies, mainly through transposon mutagenesis, have shown that structural components such as flagella, pili and adhesins, play a critical role in bacterial interaction with the surface. The primary function of flagella in biofilm formation is assumed to be in transport and in initial cell-to-surface interactions. These assumptions have been based on observations that the absence of flagella impaired *Pseudomonas fluorescens* and *Pseudomonas putida* in colonization of potato and wheat roots (DeFlaun *et al.*, 1994; De Weger *et al.*, 1987) and reduced cellular adhesion of *P. aeruginosa* and *P. fluorescens* WCS365 to a polystyrene surface (O'Toole and Kolter, 1998a; 1998b). Similarly, the absence of flagella in *Vibrio cholerae* (Watnick *et al.*, 1999), *Escherichia coli* 2K1056 (Pratt and Kolter, 1998) and *E. coli* K-12 (Genevaux *et al.*, 1996) prevented the mutants in forming biofilms resembling those formed by the wild-type bacteria on polyvinylchloride (PVC). More recent evidence obtained through proteomic analysis has indicated that gene expression of *fleN,* a gene encoding a flagellar synthesis regulator, and *flgG,* encoding a flagellar basal-body rod protein were down-regulated in 6-h biofilms of *P. putida* (Sauer and Camper, 2001). In addition, expression of the *flgD, fliD* and *flgE* genes were reported to be repressed in 24-h biofilms of *P. aeruginosa* (Whiteley *et al.,* 2001). The *flgD* gene encodes a flagellar basal-body rod protein, while *fliD* and *flgE* encode a flagellar capping protein and flagellar hook protein, respectively. These observations seem to indicate that after initial cell-to-surface contact, the flagella become dispensable for further biofilm development.

Pili and pilus-associated adhesins have also been shown to be important for the adherence to and colonization of surfaces. Expression of *sfaA*, the gene encoding S-fimbrial adhesins, of a pathogenic strain of *E. coli* has been reported to be up-regulated upon attachment (Schmoll *et al.*, 1990). In *P. aeruginosa* PA14, type IV pili mutants were reduced in their ability to form microcolonies (O'Toole and Kolter, 1998a). In *E. coli*, attachment is reduced by mutations in the *csgA* gene, a biosynthetic curlin gene (Dorel *et al.*, 1999; Vidal *et al.*, 1998), and in the type I pili biosynthetic gene *fimH*, which encodes the mannose-specific adhesin (Pratt and Kolter, 1998). Vallet *et al.* (2001) reported that a *cupA* mutant was defective in biofilm formation in *P. aeruginosa*. The *cupA* gene cluster encodes the components of a chaperone/usher pathway that is involved in assembly of fimbrial subunits such as P pili and type I pili, but not type IV pili (Soto and Hultgren, 1999).

Mutations in the mannose-sensitive hemaglutinin pilus of *V. cholerae* El Tor (Watnick *et al.*, 1999), in *ica*, the gene for the polysaccharide intercellular adhesin of *Staphylococcus epidermidis*, and in *atlE*, the gene for autolysin of *Staphylococcus aureus* (Heilmann *et al.*, 1997; Mack *et al.*, 1994; Rupp *et al.*, 1999) all have been reported to reduce adhesion to surfaces. The importance of pili in the initial stages of biofilm formation has been supported by proteomic analysis indicating the up-regulation of gene expression of *pilR*, *pilC* and *pilK* in 6-h old biofilms of *P. putida* (Sauer and Camper, 2001). Whereas *pilR* encodes a two-component response regulator, both *pilC* and *pilK* encode for proteins involved in the biogenesis of type IV pili. In well-developed biofilms of *P. aeruginosa*, expression of the *pilA* gene, which encodes the type IV pilin subunit, was repressed. This may indicate that although type IV pili are involved in the initial steps of biofilm formation, they may not be required for maintenance of the well-developed biofilm (Whiteley *et al.*, 2001).

1.2.2.2 Membrane proteins and extracellular polysaccharides

Membrane proteins have been reported to have a substantial influence on attachment and may also play a role in early biofilm development. Mutations in surface and membrane proteins, including a calcium-binding protein, a hemolysin, a peptide transporter, and a potential glutathione-regulated K^+ efflux pump caused defects in attachment of *P. putida* to corn seeds

(Espinosa-Urgel *et al.*, 2000). The expression of genes encoding several membrane proteins has also been shown to be up-regulated in *P. putida* biofilm cells grown on silicone tubing. These included *nlpD,* the gene encoding an outer membrane lipoprotein, *potB*, the gene encoding a component of the polyamine ABC transporter, *mexA,* the gene for a resistance/nodulation/cell division/multidrug efflux pump, *ybaL*, the gene encoding a probable K$^+$ efflux transporter and *xcpS*, the gene encoding the general secretion pathway protein F. In *P. aeruginosa*, the expression of *tatA* and *tatB*, genes encoding translocation proteins, *tolA*, the gene encoding a product which affects lipopolysaccharide (LPS) structure, and the *omlA* gene, which encodes an outer membrane protein, were up-regulated in well-developed biofilm cells compared to their planktonic counterparts (Whiteley *et al.*, 2001).

Bacterial extracellular polysaccharides may also influence attachment and initial biofilm development, since these factors contribute to cell surface charge, which affects electrostatic interactions between bacteria and the substratum (Van Loosdrecht *et al.*, 1989). Adhesiveness of *Pseudomonas* species has been reported to be related to the presence and composition of polysaccharides (Williams and Fletcher, 1996). Substantially reduced attachment to biotic and abiotic surfaces was observed in O-polysaccharide-deficient *Pseudomonas* spp. (DeFlaun *et al.*, 1999; Dekkers *et al.*, 1998), while changes in *P. aeruginosa* lipopolysaccharide (LPS) resulted in an altered attachment behaviour (Makin and Beveridge, 1996). For example, a *P. aeruginosa* strain containing a mutant B-band LPS showed reduced attachment to hydrophilic surfaces and increased attachment to hydrophobic surfaces. *E. coli* W3110 strains with mutations in the LPS core biosynthetic genes *rfaG*, *rfaP* and *galU* also displayed reduced attachment to surfaces (Genevaux *et al.*, 1999a). The extracellular polysaccharide alginate appears to be required for formation of thick three-dimensional *P. aeruginosa* biofilms and has been shown to be the intercellular material of *P. aeruginosa* microcolonies (Nivens *et al.*, 2001).

1.2.3 Gene expression following attachment

Following attachment of bacteria to a surface, numerous changes in gene expression are initiated which enable the bacteria to adapt to the changing environment. In *P. aeruginosa*, expression of the *algC* and *algD* genes has been reported to be up-regulated following bacterial adhesion

(Davies *et al.*, 1993; Davies and Geesey, 1995; Hoyle *et al.*, 1993). Both the *algC* and *algD* genes are involved in the biosynthesis of the exopolysaccharide alginate. In addition, *algC* is also involved in LPS core biosynthesis. By contrast, expression of *mucC*, a negative regulator of alginate synthesis, was found to be up-regulated in biofilm cells of *P. putida* (Boucher *et al.*, 2000; Nunez *et al.*, 2000; Sauer and Camper, 2001), indicating that alginate expression is down-regulated in biofilm cells of *P. putida* following attachment. Following adhesion of *P. putida* to a surface, the expression of genes involved in carbon and energy metabolism and co-factor biosynthesis (e.g., *gltR*, *rbsK*, *leuS* and *thiE*) as well as *recB* and *ksgA* are repressed (Sauer and Camper, 2001). The *gltR* gene encodes a two-component response regulator, the *rbsK* gene encodes a ribokinase, *leuS* encodes a leucyl-tRNA synthase and *thiE* encodes a phosphate pyrophosphorylase. The *recB* and *ksgA* genes encode an exoribonuclease and a rRNA dimethyltransferase, respectively. Several Pf1 bacteriophage genes as well as genes involved in translation and metabolism were shown to be differentially expressed in *P. aeruginosa* biofilm cells when compared to planktonic cells (Whiteley *et al.*, 2001). Changes in gene expression that correlate with attachment to surfaces have also been described for antibiotic resistance in *P. aeruginosa* (Giwercman *et al.*, 1991), and for antibiotic production such as phenazine synthesis in *P. aureofaciens* (Wood *et al.*, 1997). After attachment of *E. coli* to a surface, up-regulation was observed for colanic acid exopolysaccharide production, a nickel high-affinity transport system (*nikA*), which is known to be up-regulated by anaerobiosis, an aminotripeptidase (*pepT*), induced by autoinducer PAI-1, and three genes induced by high osmolarity (*ompC, proU and wcaB*) (Prigent-Combaret *et al.*, 1999). The *ompC* gene encodes a porin protein, while the *proU* operon encodes a high-affinity glycine betaine transport system and *wcaB* encodes for a protein involved in the synthesis of colanic acid.

Recent studies have also linked quorum sensing and biofilm formation (Davies *et al.*, 1998). *P. aeruginosa* possesses two complete, semi-independent quorum sensing systems, designated *las* and *rhl*. These two quorum sensing systems are inter-related in that LasR activates the expression of the *rhlR* and *rhlI* genes (Latifi *et al.*, 1996; Pesci *et al.*, 1997; Whiteley *et al.*, 1999). Each quorum sensing system consists of a transcriptional activator, LasR (Gambello *et al.*, 1993) or RhlR (Brint and Ohman, 1995), and an autoinducer synthetase, LasI or RhlI. LasI directs the synthesis of the autoinducer PAI-1, N-(3-oxododecanoyl)-$_L$-homoserine lactone

(Pearson *et al.*, 1994), while RhlI directs the synthesis of the autoinducer PAI-2, N-butyryl-$_L$-homoserine lactone (Pearson *et al.*, 1995). Whereas a *P. aeruginosa lasI* mutant lacked the three-dimensional architecture of the wild-type biofilm and was susceptible to treatment with SDS, a *rhlI* mutant biofilm closely resembled the wild-type biofilm, suggesting that the *las* quorum sensing system, but not the *rhl* quorum sensing system, is important for *P. aeruginosa* biofilm development into three-dimensional structures (Davies *et al.*, 1998). In addition, reporter gene studies by De Kievit *et al.* (2001) indicated that up-regulation of the *lasI* gene corresponded with the metamorphosis of microcolonies to the three-dimensional architecture characteristic of well-developed biofilms. By contrast, *rhlI* expression fluctuated very little during biofilm development. However, only about 5 to 15% of the cells expressed *rhlI*, and these cells were concentrated around the base of the biofilm.

1.2.4   Regulation of gene expression in biofilms

From the above, it is evident that numerous genes and proteins have been identified that are involved in the initial attachment of bacteria to surfaces and the subsequent development of well-developed biofilms. However, in only a few cases has regulation of their expression been investigated. Proteins that have associated regulatory functions have been identified by transposon mutagenesis. These include the two-component sensor systems CpxA/CpxR and EnvZ/OmpR, which are involved in the regulation of curli synthesis in *E. coli* (Dorel *et al.*, 1999; Vidal *et al.*, 1998), and two proteins acting at a posttranslational level, DsbA in *E. coli* (Genevaux *et al.*, 1999b) and ClpP in *P. fluorescens* (O'Toole and Kolter, 1998b). DsbA is a disulfide isomerase that facilitates subunit folding and is required for pilus biogenesis (Jacob-Dubuisson *et al.*, 1994), while ClpP is a protease, suggesting that there is a protease-sensitive regulator involved in biofilm development, although the target of this protease is not known. The observation adds biofilm formation to the list of bacterial systems that are regulated by proteases (Damerau and St.John, 1993; De Crecy-Lagard *et al.*, 1999; Jenal and Fuchs, 1998; Lazazzera and Grossman, 1997; Msadek *et al.*, 1998). Expression of the *P. aeruginosa pilA* gene, which encodes the pilin subunit, has been reported to be regulated by the global regulator of carbon metabolism, Crc (O'Toole *et al.*, 2000a). The mechanism of regulation is still unclear, but is though to occur in response to nutritional signals (O'Toole *et al.*, 2000a). Analysis of the

regulation of *algD* gene expression, a gene up-regulated by attachment, indicates a possible role for two different sigma factors, although this regulation may not necessarily be ascribed to attachment *per se*. Boucher *et al.* (2000) indicated that two different pathways of conversion to mucoidy exist in *P. aeruginosa*; AlgU (AlgT, σ^E)-dependent *algD* expression and σ^{54} (RpoN)-dependent *algD* expression followed by alginate production. Earlier, Totten *et al.* (1990) and Mohr *et al.* (1990) reported that *algD* transcription was not regulated by RpoN in mucoid strains, whereas Kimbara and Chakrabarty (1989) reported that *algD* transcription was regulated by RpoN in non-mucoid strains. In general, σ^{54}-RNA polymerase transcribes genes with diverse roles in various organisms as well as diverse roles within a single organism (see Section 1.5.1).

The *fleS, fleR* and *fleQ* genes are also involved in the regulation of mucin adhesion and flagellar expression in *P. aeruginosa*. Products of *fleS* and *fleR* are homologous to members of the two-component systems involved in transcription regulation of a number of genes with σ^{54}-dependent promoters. Promoter fusion experiments have indicated that the *fleSR* operon is regulated by σ^{54} and FleQ, a transcriptional regulator (Arora *et al.*, 1997). The involvement of σ^{54} in the synthesis of flagella, pili and adhesins, supports the hypothesis that σ^{54} may be involved in the up-expression of biofilm-specific regulatory elements.

Earlier, Martin *et al.* (1994) have indicated that the *algR, algD* and *algU* genes are under control of *algU*. The *algR* gene encodes a regulatory protein, *algD* encodes a protein involved in the biosynthesis of alginate while *algU* encodes a sigma factor. Garret *et al.* (1999) showed by mutational analysis that the alternative sigma factor σ^{22} (AlgT / AlgU) modulates gene expression which leads to expression of the alginate operon, and it appears to inhibit flagellum synthesis and motility. This σ^{22}-mediated inhibition occurs through transcriptional control of *fliC,* a flagellar filament protein, most probably by controlling the expression of a negative effector of flagellum synthesis (Garret *et al.*, 1999). It is still unclear which quorum sensing-controlled genes are important for biofilm maturation. In addition, both quorum sensing systems in *P. aeruginosa* are involved in the regulation of twitching motility, a flagellum-independent mode of surface translocation which requires functional type IV pili (Glessner *et al.*, 1999). While the *rhl* system was shown to affect pilin export and assembly, it was proposed that the *las* quorum sensing system is required for maintaining optimal spacing between cells which migrate together in rafts.

1.3 GENE EXPRESSION

1.3.1 Importance of sigma factors

The term gene expression refers to the entire process whereby the information encoded in a particular gene is decoded into a particular protein (Lodish *et al.*, 1995). Although it being a complex process involving many different steps, transcription initiation through promoter clearance and release from the RNA polymerase is the most important control point in determining whether or not most genes are expressed (Ishihama, 1988; Lodish *et al.*, 1995; Reznikoff *et al.*, 1985). However, before genes can be transcribed from specific DNA promoter sequences, the bacterial core RNA polymerase (with a subunit composition of $\alpha_2\beta\beta'$) must combine with a dissociable sigma subunit (σ) to form a RNA polymerase holoenzyme ($\alpha_2\beta\beta'\sigma$). The σ factors are thus central to the function of the RNA polymerase holoenzyme (Burgess *et al.*, 1969; Helmann and Chamberlin, 1988; McClure, 1985) (Table 1.1). The reversible binding of various different alternative σ factors, which are expressed under specific conditions or may be sequestered by complementary anti-sigmas until needed (Arthur and Burgess, 1998), allows formation of different holoenzymes able to distinguish groups of promoters required for different cellular functions (Stragier and Losick, 1990; Gross *et al.*, 1992; Blattner *et al.*, 1997). In addition to double-stranded DNA promoter recognition and binding, σ proteins also play a role in promoter melting (DeHaseth and Helmann, 1995; Fenton *et al.*, 2000; Guo and Gralla, 1998; Marr and Roberts, 1997; Wösten, 1998), inhibition of non-specific transcription initiation and they are often targets for activators (Gribskov and Burgess, 1986; Gross *et al.*, 1998; Helmann and Chamberlin, 1988).

Based on structural and functional criteria, the different σ factors identified in bacterial cells can be grouped into two classes. One class contains σ^{54} (also referred to as σ^N) as the only member, while the remainder of σ factors belong to the σ^{70} class (Merrick *et al.*, 1987; Sasse-Dwight and Gralla, 1990; Kustu *et al.*, 1989; Thöny and Hennecke, 1989; Lonetto *et al.*, 1992). The σ^{70} class of sigma factors can be further classified into primary, e.g. σ^{70}, and alternative sigma factors, e.g. σ^{32}, σ^{28}, etc. (Lonetto *et al.*, 1992). Despite the lack of any significant sequence similarity and vast differences in their transcription mechanism, both classes of σ factors bind the same core

RNA polymerase, but result in holoenzymes with very different properties. In *E. coli*, both σ^{70} and σ^{54} are always present, but the synthesis of other minor σ^{70} class members is induced under certain stress conditions such as high osmolarity or low temperature (Jishage and Ishihama, 1997). Although σ^{54} and σ^{70} are unrelated by primary amino acid sequence, they both share a significant overall structural similarity as indicated by X-ray scattering data (Svergun *et al.*, 2000), as well as by protein footprinting studies (Traviglia *et al.*, 1999).

Table 1.1 Functions of *E. coli* core RNA polymerase subunits

Subunit	Gene	Function
α	*rpoA*	Association of β and β' subunits Promoter recognition, by binding to upstream (UP) elements in some promoters Indirectly involved in some promoters by providing a contact point for the activator protein
β	*rpoB*	Catalysis of RNA synthesis (initiation and elongation) Recognition of terminators Binding of substrate ribonucleotide 5'-triphosphates Binding of product RNA Stringent control Autogenous regulation of ββ' synthesis Binding of rifampicin and streptolidogen
β'	*rpoC*	Binding of template DNA Binding of sigma subunits

(Compiled from Ishihama, 1988; Record *et al.*, 1996)

1.3.2 Sigma 54-dependent gene transcription

1.3.2.1 Occurrence and function of σ^{54}

By contrast to σ^{70} factors which are involved in expression of most genes during exponential growth (Lonetto *et al.*, 1992), σ^{54} appears to play a role in many different apparently unrelated biological activities across the eubacteria (Merrick, 1993; Barrios *et al.*, 1999). Genes encoding σ^{54} have been identified in many proteobacteria (Merrick, 1993) as well as in *Bacillus subtilis*, where σ^{54} is involved in utilization of arginine and ornithine (Gardan *et al.*, 1997) and transport of fructose (Debarbouille *et al.*, 1991). Among the proteobacteria, the functions carried out by the products of σ^{54}-dependent transcription include utilization of various nitrogen and carbon sources, energy metabolism (Lenz *et al.*, 1997), RNA modification (Genschik *et al.*, 1998), chemotaxis, development, flagellation, electron transport, response to heat and phage shock (Weiner *et al.*, 1991), and expression of alternative σ factors (Barrios *et al.*, 1999; Kustu *et al.*, 1989; Studholme and Buck, 2000). In *P. aeruginosa*, σ^{54} is important for transport of dicarboxylic acid, catabolism of toluene and xylene, nitrogen fixation, flagella and pilus synthesis, glutamine prototrophy, non-pilus adhesin synthesis and osmoprotection (Ishimoto and Lory, 1989; Totten *et al.*, 1990; Ramphal *et al.*, 1991; Merrick, 1993; Sage *et al.*, 1997).

The lack of any obvious theme in the diverse functions carried out by products of σ^{54}-dependent transcription, points to σ^{54} being biologically important and advantageous. The ability of a single σ^{54} polypeptide to mediate transcriptional responses to such diverse physiological needs is due to the independent regulation of σ^{54}-RNA polymerase at a wide variety of genes by means of sequence-dependent enhancer proteins with promoter-specific binding sites (Collado-Vides *et al.*, 1991). Since initiation of transcription at a σ^{54}-dependent promoter requires the activity of the cognate activator protein, transcription can therefore be tightly regulated, with low levels of leaky expression (Wang and Gralla, 1998). It furthermore allows the capability to vary transcriptional efficiency at a given promoter over a wide range without the use of a separate repressor. Thus, genes can be either silent or highly expressed, depending on the physiological or environmental conditions (Buck *et al.*, 2000).

The σ^{54}-RNA polymerase recognizes promoter sequences with conserved GG and GC elements located -24 and -12 nucleotides upstream from the transcriptional start site (Thöny and Hennecke, 1989; Cases and De Lorenzo, 2001; Barrios *et al.*, 1999). However, several exceptions do exist, e.g. the σ^{54} promoters of the *nifH* gene of *Rhizobium leguminosarum* biovar *viciae* and the *glnB* gene of *Rhodospirillum rubrum* both contain GG-N_{10}-GA, while the σ^{54} promoters of the *E. coli glnH* gene and the *P. aeruginosa oprE* gene contain sequences of GG-N_{10}-TC and GG-N_{10}-CC, respectively (Yamano *et al.*, 1998; Nohno and Saito, 1987; Roelvink *et al.*, 1990; Johansson and Nordlund, 1996). Optimal spacer length in σ^{54}-dependent promoters are 10 nucleotides, while deletions of one or more nucleotides in the region between the -24/-12 elements abolish promoter function (Keseler and Kaiser, 1995; Buck, 1986; Mullin and Newton, 1989; Mullin and Newton, 1993).

1.3.2.3 Regulation of transcription from σ^{54}-dependent promoters

On its own, the σ^{54}-RNA polymerase forms stable, closed complexes with promoters and does not result in melting of the DNA (Sasse-Dwight and Gralla, 1988; Popham *et al.*, 1989). Basal, unactivated transcription from the closed complex is intrinsically very low, consistent with the lack of repressors associated with σ^{54}-dependent promoters (Collado-Vides *et al.*, 1991). Transcription initiation from these complexes is dependent on one or several activator proteins (Morett and Segovia, 1993) that bind to upstream enhancer sequences (UAS), because hydrolysis of ATP, a process catalyzed by the activator proteins (Wedel and Kustu, 1995), is required for the formation of an open complex. These activators, which include NR1/NtrC, FhlA and NifA, are also referred to as prokaryotic enhancer binding proteins (EBPs) (Cases and De Lorenzo, 2001). Whereas the binding sites of the NR1/NtrC activators occur in a number of positions between -90 and -160, binding sites for FhlA and NifA can, in addition to these positions, also occur at much greater distances upstream from the promoter region. For example, a functional FhlA site was recently found more than 700 bp upstream of the transcription start site and a NifA site has been mapped to a position 250 bp upstream of the transcription start site (Gralla and Collado-Vides, 1996). Once an activator that has bound to the UAS receives a specific signal, an upstream nucleoprotein complex is formed which loops round and contacts the σ^{54} factor of the holoenzyme prebound to the -12/-24 sequences of the promoter. ATP hydrolysis is then

channelled through an as yet unknown mechanism into DNA strand separation and subsequent transcription initiation (Vicente *et al.*, 1999). At some promoters, the looping-out of the intervening DNA is facilitated by integration host factor (IHF), a DNA bending protein (Hoover *et al.*, 1990).

Integration host factor (IHF) is a heterodimeric protein that bends DNA by approximately 160° and binds to the promoter region of many σ^{54}-dependent promoters (Wassem *et al.*, 2000) as well as some σ^{70}-dependent promoters (Goosen and Van de Putte, 1995; Bertoni *et al.*, 1998). Earlier reports have indicated that IHF may directly stimulate transcription from the Pe promoter of bacteriophage Mu (Goosen en Van de Putte, 1984; Krause and Higgins, 1986), the pL1 promoter of bacteriophage λ (Giladi *et al.*, 1990) and the P_G2 promoter of the *ilvGMEDA* operon of *E. coli* (Pagel and Hatfield, 1991), without the involvement of any other transcription factor. Although the mechanism whereby IHF may directly stimulate transcription is still unclear, it is thought that IHF binds to a site located just upstream from the promoter, and appears to function in enhancing closed-complex formation (Krause and Higgins, 1986; Giladi *et al.*, 1992). In most cases, however, IHF functions by facilitating the loop formation that is required to bring together the activator protein and the σ^{54}-holoenzyme (Carmona and Magasanik, 1996; Carmona *et al.*, 1997) (Fig. 1.3). The outcome of looping is an activator-dependent isomerization of the closed complex into an open one, which subsequently leads to initiation of transcription (Bertoni *et al.*, 1998). This mechanism is not observed for the σ^{70}-holoenzyme, where both highly stable closed complexes and looping are rarely used for activation (Gralla, 1996). However, IHF can also function as a repressor by occluding the binding of an activator by directly occupying its specific binding site (Bewley *et al.*, 1998). EBPs can also form stable complexes on non-specific DNA (Popham *et al.*, 1989; Wedel *et al.*, 1990) and non-sequence-specific DNA such as superhelical regions of a supercoiled plasmid that may then function as a generalized enhancer sequence (Brahms *et al.*, 1995; Revet *et al.*, 1995). In these cases, IHF generally acts as an inhibitor of activation from σ^{54} promoters from non-specific sites by generating a bend which prevents EBPs from making productive contact with the σ^{54}-RNA polymerase (Claverie-Martin and Magasanik, 1991; Austin *et al.*, 1994). Therefore, IHF may be bi-functional; enhancing transcription when the EBP binds to specific, appropriate sites and repressing transcription when the activator binds to non-specific, inappropriate sites.
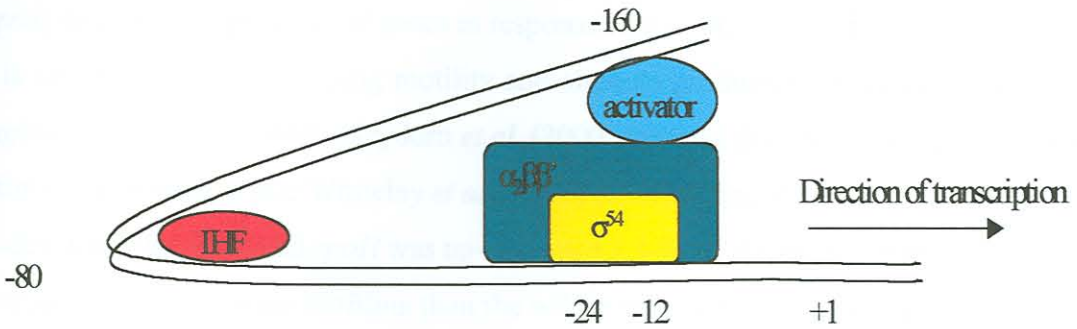
Fig. 1.3   Regulation of the σ^{54}-holoenzyme by activator proteins. Activators of the σ^{54}-holoenzyme bind to sites pre-upstream of the promoter, and are brought into close contact with the σ^{54}-holoenzyme, pre-bound to the -24 -12 promoter elements, by a sharp bend in the DNA that is induced by the integration host factor (IHF). The activator catalyzes the isomerization of the closed complex between the σ^{54}-holoenzyme and the promoter to an open complex, through a process that requires ATP hydrolysis (Modified from Hoover *et al.*, 1990).

1.3.3.   Sigma 70-dependent gene transcription

1.3.3.1 Occurrence and function of σ^{70}

Sequence conservation among the σ^{70} family of proteins suggests that this group of proteins may be divided into three groups: the primary sigmas which are responsible for most RNA synthesis (group 1), closely related but non-essential sigmas (group 2), and alternative sigmas which are responsible for transcription of specific regulons (group 3). While the primary sigma factors are phylogenetically related, the alternative sigma factors are functionally related (Lonetto *et al.*, 1992). The major sigma factor, σ^{70}, encoded by *rpoD*, is required for transcription of genes involved in the housekeeping functions of the cell, e.g. metabolism and biosynthesis during exponential growth (Record *et al.*, 1996). Sigma factors are able to regulate regulons in response to particular environmental stimuli by regulating the availability or activity of sigma factors alternative to the housekeeping σ^{70}. For example, the heat-shock response is mediated by the expression of σ^{H} (σ^{32} encoded by *rpoH*) and directs the expression of a large number of genes

involved in the heat-shock response, while the starvation response sigma factor, σ^S (σ^{38} encoded by *rpoS*) directs the expression of genes in response to starvation (Cases and DeLorenzo, 2001) and is also required for twitching motility and alginate production in a mucoid variant of *P. aeruginosa* (Suh *et al.*, 1999). Heydorn *et al.* (2000) reported that *rpoS* may serve some role in biofilm development, while Whiteley *et al.* (2001) reported that *rpoS* was repressed 2.3-fold in well-developed biofilms and *rpoH* was up-regulated 2.3-fold. An *rpoS* mutant of *P. aeruginosa* was reported to form larger biofilms than the wild-type strain (Heydorn *et al.*, 2000; Whiteley *et al.*, 2001). The expression of flagellum genes is mediated by σ^{28}, encoded by *rpoF/fliA* (Starnbach and Lory, 1992). Both AlgU and PvdS have been grouped with the extracytoplasmic function (ECF) sigma factors, for most of their activities deal with extracytoplasmic functions (Missiakas and Raina, 1998). In *P. aeruginosa*, the alternative sigma factor PvdS is not only required for the production of pyoverdine, which enables iron uptake under conditions where little free iron is available (Wilson *et al.*, 2001), but is also required for the synthesis of exotoxin A, a virulence factor, and PrpL, an extracellular proteinase (Wilson *et al.*, 2001).

1.3.3.2 Domain structure of the σ^{70} protein and σ^{70}-dependent promoter sequences

Based on sequence alignments, σ^{70} has been divided into four regions (Region 1 to 4) by sequence conservation (Gribskov and Burgess, 1986; Helmann and Chamberlin, 1988; Stragier *et al.*, 1989; Lonetto *et al.*, 1992) (Fig. 1.4). Region 1 is conserved only among primary sigma factors, while the remainder of the protein is conserved among all σ^{70}-type sigma factors (Lonetto *et al.*, 1992). Region 2 is involved in core binding (Lesley and Burgess, 1989), in recognition of the -10 region of the promoter (Daniels *et al.*, 1990; Kahn and Ditta, 1991; Siegele *et al.*, 1989; Tatti *et al.*, 1991; Waldburger *et al.*, 1990; Zuber *et al.*, 1989), and possibly in DNA strand melting (Helmann and Chamberlin, 1988). There is some evidence that part of Region 3 may also be involved in core binding in some sigma factors (Zhou *et al.*, 1992). The helix-turn-helix motif (Brennan and Matthews, 1989; Gribskov and Burgess, 1986; Helmann and Chamberlin, 1988; Stragier *et al.*, 1989) in Region 4 is involved in the recognition of the -35 region of the promoter (Kenny and Moran, 1991; Kenney *et al.*, 1989; Siegele *et al.*, 1989; Waldburger *et al.*, 1990). By contrast to Regions 2 and 4, which are highly conserved and tend to be very basic, Regions 1 and 3 exhibit lower conservation and are acidic.
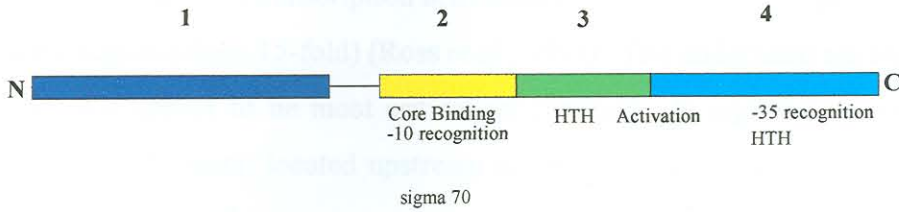
Fig. 1.4   Domain organization of the sigma 70 protein (Modified from Lonetto *et al.*, 1992; Record *et al.*, 1996). Region 1 is conserved among primary sigmas, Region 2 is involved in core binding and recognition of the -10 region of the promoter, Region 3 contains a helix-turn-helix (HTH) motif and may be involved in core binding, while Region 4 is involved in recognition of the -35 region of the promoter.

The σ^{70} family of RNA polymerases recognize diverse promoter sequences (Table 1.2) with conserved hexamers centred at -35 and -10 nucleotides upstream from the transcriptional start site (Hawley and McClure, 1983). In general, the *in vivo* strength of a promoter correlates with its qualitative homology with the consensus sequence and the favoured spacing of 17 bp between the -35 and -10 sequences (Record *et al.*, 1996; Reznikoff *et al.*, 1985). Although the optimal spacer length in σ^{70} promoters is 17 +/-1 nucleotide, functional promoters with a spacing between 15 and 20 non-conserved nucleotides have been reported (Hawley and McClure, 1983; DeHaseth and Helmann, 1995). The role of spacer length is to orientate the positions of the -35 and -10 hexamers (Record *et al.*, 1996) for recognition by the RNA polymerase enzyme. The level of transcription of genes under control of σ^{70}-dependent promoters may be affected by signals located upstream, downstream or between the core promoter elements. Some promoters appear to lack the -35 sequence and mutational studies have indicated that in these cases the sequence immediately upstream of the -10 region affects promoter activity. The optimal sequence for extended -10 sequences appears to be TnTGn followed by the -10 region (Record *et al.*, 1996). In the case of the *rrnB* P1 promoter, an A + T-rich region located at -40 to -60 relative to the transcription start site, the upstream (UP) element, has been shown to increase promoter activity, possibly by stabilizing the initial closed complex (Record *et al.*, 1996). The UP element contains two conserved regions, a 11 bp distal region [-57 to -47: AAA(a/t)(a/t)T(a/t)TTTT] and a 4 bp proximal region [-44 to -41: AAAA]. Each region can function independently, but the proximal

region confers a higher degree of transcription activation on the *rrn*B P1 core promoter (>100-fold) than the distal region (about 15-fold) (Ross *et al.*, 1998). The underlined nucleotides (-51 to -53 and -41 to -43) appear to be most critical for function. In addition, curved or bent sequences (e.g., rich in A tracts) located upstream of the -35 hexamers generally lead to an increase in the promoter strength.

Table 1.2 Conserved promoter sequences for different holoenzymes belonging to the σ^{70} family of *E. coli*

Holoenzyme & synonyms	Biological role	Promoter sequence	
		-35	-10
$E\sigma^{70}$, σ^{D}	Housekeeping functions	TTGACA	TATAAT
$E\sigma^{24}$, σ^{E} AlgU[#]	Stress response Alginate production	GAACTT	TCTGA
$E\sigma^{32}$, σ^{H}	Heat shock regulation	TCTCNCCCTTGAA	CCCCATNTA
$E\sigma^{28}$, σ^{F}	Motility and chemotaxis	CTAAA	CCGATAT
$E\sigma^{38}$, σ^{S}	Stationary phase regulation	?[$]	TATACT
$E\sigma^{19}$, FecI PvdS*	Iron uptake Pyoverdine biosynthesis	(G/C)(G/C)TAAAT (T/A)(G/C)	

[#]AlgU in *P. aeruginosa*

[$]No defined -35 sequence

*PvdS in *P. aeruginosa*

(Compiled from Lodish *et al.*, 1995; Reznikoff *et al.*, 1985; Martin *et al.*, 1994; Lonetto and Gross, 1996; Gross, 1996; Wilson *et al.*, 2001; Park *et al.*, 2001)

1.3.3.3 Regulation of transcription from σ^{70}-dependent promoters

Transcription initiation by σ^{70}-RNA polymerases can be regulated by the action of regulatory proteins that inhibit (repress) or stimulate (activate) transcription initiation, as well as by regulation of the DNA topology and the modification of DNA sequences within the promoter sequence (Reznikoff *et al.*, 1985). Of these mechanisms, repression of transcription initiation is the most common means of regulation of *E. coli* σ^{70} promoters (Gralla and Colado-Vides,

Repressors bind to operator sequences which overlap the region contacted by the RNA polymerase holoenzyme bound to the promoter, and either sterically block polymerase binding, inhibit open complex formation or prevent transcription occurring from the promoter region (Lodish *et al.*, 1995). By contrast, activators of σ^{70}-RNA polymerase generally bind to DNA sequences on opposite sides of the double helix from the RNA polymerase in the region from -20 to -50, or just upstream of the RNA polymerase near -60 (Fig. 1.5). There are no indications as yet that other members of the σ^{70} family use fundamentally different mechanisms to control promoter function. This is to be expected as activators appear to work primarily through the α and σ subunits of the RNA polymerase. RNA polymerases within the σ^{70} family contain identical α and similar σ subunits and thus are in principle subject to similar mechanisms of activation (Gralla and Collado-Vides, 1996).
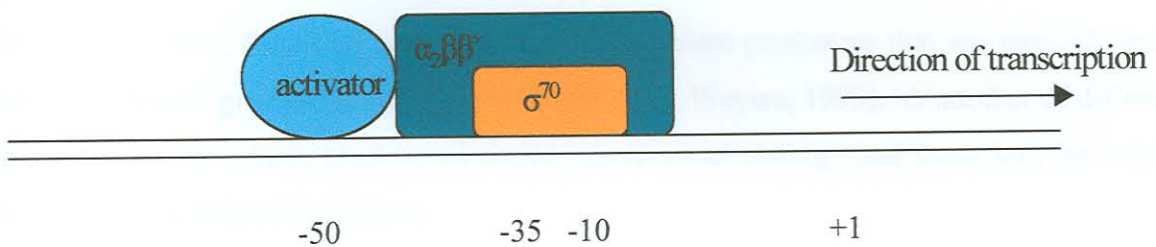
Fig. 1.5   Regulation of the σ^{70}-holoenzyme by activator proteins (Modified from Busby and Ebright, 1994). An activator protein binds to a site adjacent to the σ^{70} RNA polymerase-holoenzyme that is bound to the -35 -10 promoter elements and enhances transcription.

The superhelical character of the template DNA can affect the promoter activity, with many promoters being stimulated by negative superhelicity while others are inhibited (Balke and Gralla, 1987). Whereas DNA supercoiling in bacteria can be influenced by environmental factors (Higgins *et al.*, 1988), other factors, such as growth phase and nutritional limitation, have also been shown to affect DNA topology (Dorman *et al.*, 1988; Kusano *et al.*, 1996; Conter *et al.*, 1997; Schneider *et al.*, 1999). Methylation of *dam* sites has been reported to decrease transcription, by apparently inhibiting the recognition of σ^{70} promoters by the RNA polymerase holoenzyme (Reznikoff *et al.*, 1985).

1.4   ANALYSIS OF GENE EXPRESSION IN BIOFILM BACTERIA USING REPORTER GENES

Direct biochemical measurements of the activity or abundance of gene products are often sufficient to monitor gene expression in biofilm populations (Romani and Sabater, 2000). However, not all gene products are readily assayable and frequently such measurements are not adequately sensitive or readily adaptable to kinetic studies in adherent populations. Quantitative data about gene expression has been obtained by *in situ* reverse transcription followed by hybridization with fluorescently-labeled oligonucleotides (Møller *et al.*, 1998), but an appropriately equipped microscope is required and some method to permeabilize the biofilm cells is needed. A number of investigators have circumvented these limitations by making use of reporter gene technology. The use of reporter genes has proved to be extremely valuable in the study of environmental control of gene expression (Belas *et al.*, 1986; Davies *et al.*, 1993; De Kievit *et al.*, 2001; Sternberg *et al.*, 1999) and to isolate promoters that are induced under particular physiological conditions (Burne *et al.*, 1997; Weyers, 1999). A number of different reporter genes have been used in microbial genetics and ecology and these will be briefly discussed in the following sections.

1.4.1   Genes encoding chromogenic substrate cleavage enzymes

A number of genes encoding metabolic enzymes that are capable of cleaving chromogenic substrates have been described and used as reporter genes, e.g., *xylE* (Curcic *et al.*, 1994), *phoA* (Reuber *et al.*, 1991) and *celB* (Sessitsch *et al.*, 1996). The β-galactosidase (*lacZ*) gene of *E. coli* was one of the first genes of this class to be used as a reporter gene (Drahos *et al.*, 1986). Its popularity has been due to the ease by which the *lacZ* gene activity can be quantitatively assayed using a variety of relatively inexpensive chromogenic (o-nitrophenyl-β-D-galactoside [ONPG]) and fluorescent substrates (fluorescein-di-β-D-galactopyranoside [FDG] and methylumbelliferyl-β-D-galactoside [MUG]). The primary advantages of using the *lacZ* metabolic marker are that rapid visual screening is possible and the enzyme activity assay can be performed in cuvettes or in microtiter plates, and the samples can be read on an enzyme-linked immunosorbent (ELISA) plate reader, spectrophotometer, luminometer or fluorimeter, depending on the product of the

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reaction (Burne and Chen, 2000; Schmoll *et al.*, 1990). Alternatively, epifluorescence or confocal laser scanning microscopy (CLSM) can be used, in combination with fluorescent substrates, to study expression of the reporter gene at a single cell level (Davies *et al.*, 1993; Davies and Geesey, 1995). Although *lacZ* is a versatile reporter, its utility may be hindered by the presence of endogenous microbial β-galactosidases (Bronstein *et al.*, 1994) and thus necessitates deletion of the native *lacZ* gene prior to its use. Further disadvantages of using *lacZ* are the stability of the enzyme, preventing the study of temporal gene expression, and the denaturation of the enzyme at pH values below neutrality (Burne and Chen, 2000).

1.4.2 Antibiotic resistance genes

A number of antibiotic resistance genes have been used as reporter genes in a variety of Gram-negative and Gram-positive bacteria. The genes conferring chloramphenicol (Cm^r or *cat*) resistance upon their host have been particularly useful, owing to the fact that the expression of these reporter genes can not only be selected for in growth medium, but also quantified via enzyme assays for chloramphenicol acetyltransferase (CAT) activity. The *cat* gene has been employed to monitor qualitative and quantitative gene expression in a wide variety of bacteria (Osbourne *et al.*, 1987). Although assays for CAT activity are not as easy or as inexpensive as those for LacZ activity, and the fluorescent substrates used in CAT assays are not able to penetrate cells, it may offer distinct advantages for studying biofilm bacteria. CAT seems fairly stable in cells growing at pH values between 4.0 and 7.5. It has also been reported that CAT, at least in a number of streptococci, has a short half-life, thereby enabling the assay of temporal gene expression (Burne and Chen, 2000). CAT activity may be measured by various methods, including kinetic assays and ELISAs with anti-CAT antibodies and anti-CAT substrates. The use of fluorescent CAT substrates may be adaptable to biofilms which have been gently fixed, but thus far no publication has reported such a method (Burne and Chen, 2000).

1.4.3 Bioluminescence genes

Luciferase activity (bioluminescence) is frequently used as a reporter system in both eukaryotic and prokaryotic cells (Gould and Subramani, 1988; De Wet *et al.*, 1985; Palomares *et al.*, 1989;

Greener *et al.*, 1992; Stewart and Williams, 1992) as well as in biofilm studies (King *et al.*, 1990; Denyer *et al.*, 1991; Mittelman *et al.*, 1992; Rice *et al.*, 1995). Luciferase is an oxidase that catalyzes the oxidation of reduced flavin ($FMNH_2$) to form an intermediate peroxide, which then reacts with a long-chain aldehyde to give blue-green luminesence emitting at 490 nm and oxidized flavin plus the corresponding long-chain fatty acids as products (Hastings, 1996). The genes for this light-producing reaction have been obtained from the firefly (*Photorhabdus luminescens*; *luc*) or bacteria (*Vibrio fisheri*; *lux*). Although bioluminescence represents a powerful tool for genetic analysis, it may be problematic to use, since the reaction requires the addition of an exogenous substrate for the light reaction to occur. In the case of using the *luxAB* genes as reporter, an aldehyde substrate, usually *n*-decanal, must be supplied to the cells during the assay procedure. Although the substrate penetrates the cells readily, it can be toxic at relatively low doses. To circumvent the addition of the aldehyde substrate, investigators have cloned all five genes of the *lux* operon (*luxCDABE*), which allows a completely independent light-generating system that requires no additional substrate (Applegate *et al.*, 1998; Hay *et al.*, 2000). However, cells containing the entire *lux* operon exhibit reduced viability compared to cells containing only *luxAB* (De Weger *et al.*, 1991; Amin-Hanjani *et al.*, 1993). Often the assays also use extracts from samples, rather than whole cells, and therefore destructive sampling is required (Møller *et al.*, 1995). Bioluminescent reporter genes can be directly monitored by visual or microscopic observations (Masson *et al.*, 1993; Flemming *et al.*, 1994), by measuring the light input in a luminometer (Rattray *et al.*, 1990) or a scintillation counter in chemiluminescence mode (Belas *et al.*, 1986). The light output is indicative of a metabolically active population of cells, since the luciferase enzymes are dependent on cellular activity reserves, or reducing equivalents for bioluminescence. However, after long-term incubation, microbial cells often become starved or stressed and the light production from luciferase enzymes declines as a response to the change in cellular energy status (Duncan *et al.*, 1994). Therefore, *in situ* bioluminescence may not be a reliable indicator and limits its utility.

1.4.4 Green fluorescent protein genes

The *gfp* gene, encoding green fluorescent protein (GFP), has been isolated and cloned from the jellyfish *Aequorea victoria* (Prasher *et al.*, 1992). Through an autocatalytic reaction, GFP forms

a cyclic peptide that is highly fluorescent and stable (Cody *et al.*, 1993). An advantage of GFP, over other reporters is the fact that no other energy source or substrate addition is required, other than oxygen during initial formation of the chromophore (Chalfie *et al.*, 1994; De Weger *et al.*, 1994; Inouye and Tsuji,1994; Stewart and Williams, 1992). In addition, GFP is stable in the presence of many denaturants and proteases, and persists at high temperatures (65°C) and pH values (6-12) (Ward *et al.*, 1980). It is, however, not yet clear how reliable GFP would be in the oxygen-restricted conditions that may develop in deep biofilms. A potential disadvantage of using *gfp* as a reporter gene is the extreme stability of the GFP protein (Tombolini *et al.*, 1997). Whereas stability of the protein is advantageous for the environmental monitoring of GFP-tagged cells, it can be problematic in studies regarding temporal changes in gene expression, since, once the reporter protein is synthesized, it may persist. To overcome this problem, various unstable variants of GFP have been constructed that are more susceptible to degradation by ClpXP-type protease complexes and therefore have shorter half-lives (Andersen *et al.*, 1998; Cormack *et al.*, 1996; Heim *et al.*, 1994; Keiler *et al.*, 1996). Additionally, various groups have obtained GFP mutants exhibiting diverse spectral properties that may allow simultaneous analysis of gene expression from a number of different promoters (Delgrave *et al.*, 1995; Ehrig *et al.*, 1995). Depending on the nature of the study, GFP fluorescence can be monitored by exposing bacterial colonies to UV light (Tresse *et al.*, 1998), by fluorometric detection (Burlage *et al.*, 1996), epifluorescence microscopy (Chalfie *et al.*, 1994; De Kievit *et al.*, 2001), confocal laser scanning microscopy (Eberl *et al.*, 1997) or flow cytometry (Tombolini *et al.*, 1997). Numerous *gfp*-based reporter systems have been developed for use in especially environmental applications to study GFP-tagged bacteria in soils, water systems, rhizospheres, activated sludges, root nodules and biofilms (Eberl *et al.*, 1997; Chalfie *et al.*, 1994). Recently, *gfp*-based reporter systems have also been used successfully to study gene expression in bacteria (De Kievit *et al.*, 2001; Heydorn *et al.*, 2002; Sternberg *et al.*, 1999).

1.5   AIMS OF THIS STUDY

As early as 1933, Henrici recognized the phenomenon that marine bacteria grow for the most part on submerged surfaces, rather than being free-floating. With the rediscovery that bacteria are found predominantly attached to surfaces in aquatic systems (Geesey *et al.*, 1977), much

attention has been paid to unravelling the molecular mechanisms underlying the formation and regulation of biofilms. To investigate, a number of regulatory elements from P. aeruginosa PAO (DSM1707) have been isolated in our laboratory. These are thought to control the expression of genes which may play roles in the attachment of *P. aeruginosa* to a surface, subsequent biofilm formation and/or detachment of cells from the biofilms.

In an attempt to isolate genetic elements up-regulated by attachment, Weyers (1999) constructed a chromosomal library of *P. aeruginosa* DSM1707 in the broad-host-range reporter vector, pALacZsd, by cloning genomic fragments upstream of the promoterless *lacZ* reporter gene. One thousand clones, representing *ca.* 3% of the genome, were screened for up-expression of β-galactosidase activity upon attachment of the transformed *P. aeruginosa* cells to a glass wool substratum. The results were expressed as a ratio between the β-galactosidase activity assayed for the cells grown in the presence of glass wool (attached; A) and for the cells grown in the absence of glass wool (planktonic; P). The clones were divided into four main groups according to their A/P ratio and basal level of activity of planktonic growing cells; Group I consisted of 337 clones and had an A/P ratio $\geq$ 3, Group II consisted of 338 clones and had an A/P ratio of between 2 and 3, Groups III and IV consisted of 325 clones, had an A/P ratio $<$ 2 and had low (<0.5) and high (>0.5) basal levels of activity of planktonic cells, respectively. The clones in Group I was further differentiated and a total of 131 clones (Group IAi) were regarded as being significantly affected by attachment, since they displayed a high level of β-galactosidase activity in the attached state (A$>$ 0.5) and a low level of β-galactosidase activity in the planktonic state (P$<$0.5). A total of 24 clones, consisting of 20 clones from Group IAi and 4 selected clones from Group II, were chosen for further characterization in this investigation.

Therefore, the aims of this investigation were the following:

a)   To characterize selected attachment-induced genetic elements obtained from a *P. aeruginosa* genomic library.

b)   To investigate the transcriptional activity of one such element under different growth conditions using reporter gene technology.

The research strategies for obtaining the primary objectives involved the following:

- Nucleotide sequencing of the genetic elements and analysis of the obtained sequence data in order to identify putative promoter sequences and to determine which genes may be expressed by these regulatory elements (Chapter 2).

- Preparation of *lacZ* and *gfp* transcriptional fusions, and analysis of the transcriptional activity of a selected attachment-induced regulatory element under different growth conditions as well as in developing and well-developed biofilms, respectively (Chapter 3).

CHAPTER TWO

SEQUENCE ANALYSIS OF *Pseudomonas aeruginosa* DSM1707 ATTACHMENT-INDUCED GENETIC ELEMENTS

2.1   INTRODUCTION

Biofilms are complex, highly organized communities of microorganisms attached to a surface and their formation occurs in response to a variety of environmental signals (Costerton *et al.*, 1995; O'Toole and Kolter, 1998a, 1998b; Pratt and Kolter, 1998; Wimpenny and Colasanti, 1997) which lead to the expression of new phenotypes that distinguish the attached cells from their planktonically growing counterparts (O'Toole *et al.*, 2000b; Steyn *et al.*, 2001; Sauer and Camper, 2001). Most notably, biofilm bacteria have been reported to have a greatly enhanced tolerance to stresses and antimicrobial agents (Nickel *et al.*, 1985; Cochran *et al.*, 2000).

In recent years, a number of different approaches have been used to identify genes involved in the formation, development and maintenance of biofilms. Most biofilm-regulated genes have been identified by screening for mutants defective in biofilm formation (O'Toole *et al.*, 1999). By screening a library of *Tn* insertion mutants of *P. aeruginosa* for altered adhesion abilities, O'Toole and Kolter (1998a) identified 13 surface attachment defective (*sad*) mutants. The majority of these mutants were affected in motility, indicating that flagella and type IV pili play an important role in the early events of biofilm development in *P. aeruginosa*. Although it represents 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 the attached mode of growth, may be omitted if not obligately required for biofilm development *per se*.

Early studies by Brözel *et al.* (1995), in which global gene expression patterns in attached *P. aeruginosa* cells were investigated, have indicated that the expression levels of 11 proteins were altered during various stages of attachment. Proteomic studies involving the examination of the expression pattern of cellular proteins under different conditions have consequently become a popular method whereby global gene regulation can be studied (Miller and Diaz-Torres, 1999).

Two-dimensional gel electrophoresis of proteins extracted from both attached (biofilm) and planktonic cells, followed by amino acid sequencing of selected spots using either Edman-degradation or Matrix-assisted laser desorption / ionisation-time of flight mass spectrometry (MALDI-TOF MS), has lead to the identification of numerous proteins that are differentially expressed, up-regulated and/or repressed in *Pseudomonas* spp. growing as a biofilm (Steyn *et al.*, 2001; Sauer and Camper, 2001). Using a proteomic approach, in combination with cDNA subtractive hybridization of mRNA, Sauer and Camper (2001) recently identified several proteins involved in amino acid metabolism, membrane proteins involved in transport processes and proteins involved in the production of extracellular polymers that are differentially expressed in *P. putida* biofilm cells.

The use of DNA microarrays to study gene expression in *P. aeruginosa* biofilms has also been reported (Whiteley *et al.*, 2001). Differential expression of 73 genes in *P. aeruginosa* biofilm cells, in comparison to planktonic cells, was noted. These included, amongst other, bacteriophage genes, genes involved in motility, genes involved in translation and metabolism as well as genes encoding for membrane and secretory proteins.

From the above, it is apparent that numerous genes are differentially expressed by *P. aeruginosa* growing as a biofilm. However, in most cases, the mechanism(s) that regulate this differential gene expression remain to be elucidated. In an earlier study undertaken by Weyers (1999), attempts were made to isolate genetic elements which may be important in regulating the expression of genes required for the attachment of *P. aeruginosa* to a surface and the subsequent formation of well-developed biofilms. The approach was based on the use of *lacZ* reporter gene technology to identify genomic sequences responsible for up-regulated expression of the reporter gene upon attachment to a glass wool substratum. These studies resulted in the isolation of 131 elements displaying significant up-regulation of the reporter gene expression. To identify and to characterize the genetic elements, 24 of these putative regulatory elements were selected for use in this study.

The aims of this part of the investigation were therefore (i) to determine the nucleotide sequence of the selected genetic elements, (ii) to identify putative promoter sequences and (iii) to map the

DNA fragments to the *P. aeruginosa* genome in order to determine which genes might be expressed by these regulatory elements.

2.2   MATERIALS AND METHODS

2.2.1   Bacterial strains and plasmids

Escherichia coli JM109 was routinely propagated in Luria-Bertani broth (LB: 1% tryptone; 0.5% yeast extract; 1% NaCl; pH 7.4) with shaking for 16 to 18 h at 37°C or maintained on LB-agar plates. The recombinant pALacZsd plasmids used in this study have been described previously (Weyers, 1999) and were kindly supplied by J.B. Weyers (Department of Microbiology and Plant Pathology, University of Pretoria).

2.2.2   Preparation of competent *E. coli* JM109 cells

Competent *E. coli* JM109 cells were prepared essentially as described by Sambrook *et al*. (1989). An overnight culture of *E. coli* JM109 cells was prepared by inoculating 3 ml LB-broth with a single colony of the bacterial strain and shaking overnight at 37°C. One ml of this culture was used to inoculate 100 ml of preheated (at 37°C) sterile LB-broth after which the cells were grown at 37°C with agitation to mid-logarithmic phase (OD_{550} of 0.4 to 0.5) and then placed on ice for 20 min. The cells from 30 ml of the culture were pelleted in a Corex tube by centrifugation at 4 000 rpm for 10 min at 4°C in a pre-cooled Sorvall HB 4 rotor. The pellet was suspended in 10 ml filter-sterilized ice-cold 50 mM $CaCl_2$, incubated on ice for 1 h, pelleted as before and gently resuspended in 1 ml of the $CaCl_2$ solution. The cells were incubated on ice for at least 1 h before being used in transformation experiments.

2.2.3   Transformation of competent *E. coli* cells

The prepared competent *E. coli* JM109 cells were transformed with recombinant pALacZsd plasmid DNA using the heat shock-method as described by Sambrook *et al*. (1989). The competent cells (200 µl) were mixed with 200 ng plasmid DNA in a sterile transformation tube

and maintained on ice for 30 min. The cells were then incubated at 42°C for 90 s and immediately chilled on ice for 2 min. After addition of 800 µl prewarmed (37°C) LB-broth, the transformation mixtures were incubated at 37°C for 1 h to allow the cells to recuperate and express the tetracycline resistance gene. As controls, the competent cells were either transformed with 10 ng pUC18 plasmid DNA to determine the transformation efficiency, or directly plated onto LB-agar plates to check for contamination. The cells were plated in aliquots of 100-200 µl onto 1.2% LB-agar plates supplemented with 20 µg/ml tetracycline or 100 µg/ml ampicillin (for the transformation of the pUC plasmid DNA) and incubated overnight at 37°C. Transformants were selected for further characterization and grown overnight at 37°C in 10 ml LB-broth supplemented with 20 µg/ml tetracycline.

2.2.4 Plasmid DNA extraction

The recombinant plasmid DNA was isolated using a modified alkaline lysis method (Birnboim and Doly, 1979). The cells from 3 ml of the overnight cultures were harvested by centrifugation for 1 min at 15 000 rpm. The bacterial pellets were suspended in 400 µl of a solution containing 50 mM glucose; 25 mM Tris-HCl (pH 8.0); 10 mM EDTA and 100 µg RNaseA and incubated at room temperature for 10 min. The resultant spheroplasts were lysed following the addition of 400 µl of a freshly prepared solution containing 1% SDS and 0.2 M NaOH. The mixture was neutralized by the addition of 300 µl of 7.5 M NH_4OAc (pH 7.6), resulting in the precipitation of chromosomal DNA, high molecular weight RNA and proteins. After incubation on ice for 10 min and centrifugation for 10 min at 15 000 rpm, the plasmid DNA was precipitated from the recovered supernatants by the addition of 650 µl isopropanol and incubated at room temperature for 10 min. The precipitated DNA was collected by centrifugation and resuspended in 100 µl 2 M NH_4OAc (pH 7.4). After incubation for 10 min on ice, the excess proteins were removed by centrifugation at 15 000 rpm for 10 min and the plasmid DNA in the supernatant precipitated by the addition of 110 µl isopropanol. The plasmid DNA was pelleted by centrifugation, rinsed with 1 ml of 70% ethanol, dried under vacuum and resuspended in 25 µl UHQ water.

2.2.5 Agarose gel electrophoresis

An aliquot of each plasmid DNA preparation was analyzed by agarose gel electrophoresis. For this purpose, horizontal 1% (w/v) agarose slab gels were cast and electrophoresed at 100 V in 1 × TAE buffer (40 mM Tris-HCl; 20 mM NaOAc; 1 mM EDTA; pH 8.5) using a Biorad Mini Sub™ electrophoresis unit. The agarose gels were stained with ethidium bromide (EtBr) at a final concentration of 1 mg/ml and the DNA visualized by UV-induced fluorescence on a transilluminator.

2.2.6 Nucleic acid sequencing

The recombinant plasmid DNA was of sufficient quality to allow its direct use in nucleic acid sequencing reactions. The recombinant plasmid DNA was sequenced using an ABI PRISM™ Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Perkin Elmer). The forward primer JB-1 (5'-GAATTCGAGCTGGGTAC-3') and the reverse primer JB-2 (5'-GTTTTCCCAGTCACGAC-3') were used in the sequencing reactions. The JB-1 and JB-2 primers anneal to the 5' and 3' ends of the multiple cloning site of pALacZsd, respectively. Each reaction mixture contained 200-500 ng of template DNA, 1.6 pmol sequencing primer, 2 µl Terminator Ready Reaction Mix, 3 µl 5 × buffer and UHQ water in a final reaction volume of 20 µl. Cycle sequencing was performed using the following program for 25 cycles: denaturation at 96°C for 30 s; annealing at 50°C for 15 s and extension at 60°C for 4 min. Following brief centrifugation, the extension products were precipitated by the addition of 20 µl 80% ethanol. The tubes were incubated at room temperature for 20 min in the dark, centrifuged at 15 000 rpm for 30 min and the supernatant carefully aspirated. The pellets were rinsed twice with 50 µl 70% ethanol, vacuum-dried for 10 min and then stored at -20°C. Prior to electrophoresis, the purified extension products were resuspended in 3.5 µl Blue dextran/EDTA loading buffer, denatured for 2 min at 90°C and loaded onto a model 377 automated DNA sequencer (Perkin-Elmer). The obtained nucleotide sequences were edited using the ABI PRISM Sequencing Analysis 3.1 and Sequencing Navigator 1.0.1 software programs (PE Applied Biosystems).

2.2.7   Computer analysis

The non-redundant GenBank database as well as the *P. aeruginosa* genome sequence (Stover *et al.*, 2000) was searched for nucleotide sequences similar to the obtained sequences using the BLAST alignment program (Altschul *et al.*, 1997). Multiple alignments were carried out with ClustalW (Thompson *et al.*, 1994). Putative integration host factor (IHF) and sigma 54 (σ^{54}) recognition sites were identified using SEQSCAN, while DNAMAN was used to identify both repeat sequences and σ^{70} promoters within the obtained sequences. The origin and location of the programs used in the above analysis are indicated in Table 2.1.

Table 2.1 Web-based and other computer programs used in this study

Name	Address or distributor
BLAST	http://www.ncbi.nlm.nih.gov/BLAST
Pseudomonas genome project	http://www.pseudomonas.com
ClustalW	http://www.dot.imgen.bcm.tmc.edu:9331/multi-align
SEQSCAN	http://www.bmb.psu.edu/seqscan/seqform1.htm
DNAMAN	Linchon Biosoft

2.3   RESULTS

2.3.1 Identification of attachment-induced regulatory elements

A chromosomal library of *P. aeruginosa* DSM1707 had been previously constructed by digestion of the isolated chromosomal DNA with *Sau* 3AI, and DNA fragments of approximately 200-300 bp in length were cloned into the promoterless broad-host-range reporter vector pALacZsd (Weyers, 1999). Recombinant plasmids displaying promoter activity, as indicated by *lacZ* gene expression, were subsequently analyzed for up-regulation of the reporter gene expression following attachment to a glass wool substratum. From these, a total of 24 clones were selected and further characterized in this study. Recombinant plasmid DNA corresponding to the selected

clones was transformed into competent *E. coli* JM109 cells and following plasmid DNA extraction, the nucleotide sequence of the cloned insert DNA was determined by automated sequencing procedures as described under Materials and Methods (Section 2.2.7). The length of the cloned DNA fragments varied between 61 bp (clone 278) to 445 bp (clone 65). Sequence analysis of these clones furthermore indicated that 8 of the 24 recombinant clones (clones 30, 56, 65, 205, 267, 461, 569 and 899) contained more than one cloned DNA fragment. The formation of such chimeric DNA sequences was not surprising, since *Sau* 3AI digestion of the genomic DNA results in DNA fragments with sticky ends which would be able to concatemerize prior to ligation into the reporter vector molecule. Thus, the nucleotide sequence of a total of 42 distinct putative regulatory elements were determined. These sequences are supplied at the back of this dissertation (Appendix 1).

A BLAST search against all nucleotide sequences contained in the GenBank database revealed significant identity between the obtained sequences and sequences originating from *P. aeruginosa*. A subsequent BLAST search of the *P. aeruginosa* genome (www.pseudomonas.com) indicated that all of the obtained sequences, except one (clone 498), displayed 98-100% sequence identity to sequences located in the *P. aeruginosa* genome. In the case of clone 498, the sequence displayed 51.5% identity to a sequence located in the *P. aeruginosa* genome. As expected, the nucleotide sequences mapped to regions all over the *P. aeruginosa* PAO1 genome. The obtained sequences could, however, be classified into two broad groups. The first group (23/42) consisted of cloned DNA fragments mapping to regions within genes encoding hypothetical proteins (8/23), genes encoding proteins with functions in the transport of molecules, translation, amino acid biosynthesis and metabolism as well as genes encoding for probable two component sensors (10/23) and probable transposases (5/23). The second group (17/42) consisted of cloned DNA fragments mapping to regions upstream of genes encoding hypothetical proteins (5/17), probable transcription regulators (3/17), proteins involved in motility (2/17) or genes encoding different enzymes and membrane proteins (7/17). In addition to the above, one sequence each mapped to a region adjacent to a gene encoding HisM, which is responsible for the transport of small molecules, and to a region downstream of a gene encoding protein PA1136, a probable transcription terminator. The location of these fragments in the *P. aeruginosa* genome is summarized in Table 2.2.

Table 2.2 Location of the sequenced attachment-induced regulatory elements in the *Pseudomonas aeruginosa* genome

Fragment	Position on fragment	Position on *Pseudomonas* genome map	Open reading frame and encoded protein
~30(1)	1-80	3 157 028-3 156 948	Inside PA2800, conserved hypothetical protein
30(2)	77-343	4 006 350-4 006 616	From 159 bp upstream to 107 bp of PA3574, probable transcription regulator
30(3)	339-437	2 365 733-2 365 831	275 bp upstream from PA2151, conserved hypothetical protein
55	1-391	4 627 158-4 627 548	Inside PA4137, probable porin
56(1)	1-65	5 783 132-5 783 068	Inside PA5134, probable carboxyterminal protease (Translation, post translational modification, degradation)
56(2)	62-278	5 896 475-5 896 259	330 bp upstream from PA5238, probable O-antigen acetylase (Cell wall/LPS/capsule)
56(3)	275-377	609 388-609 286	Inside PA0550, hypothetical protein
65(1)	1-226	2 781 439-2 781 214	601 bp upstream from PA2463, hypothetical protein
65(2)	223-445	4 288 237-4 288 459	483 bp upstream from PA3831, leucine aminopeptidase (Translation, post-translational modification, degradation)
73	1-95	6 090 565-6 090 471	Inside PA5412, hypothetical protein
205(1)	1-196	3 890 086-3 890 281	368 bp upstream from PA3477, transcriptional regulator RhlR
205(2)	193-260	4 068 865-4 068 934	Inside PA3635, enolase
267(1)	1-79	3 216 518 -3 216 596	Inside PA2864, conserved hypothetical protein
267(2)	76-238	967 242-967 080	Inside PA0892, AotP, transport of small molecules
278	1-61	2 209 087-2 209 147	Inside PA2018, RND (mex) multidrug efflux transporter

457	1-115	3 661 781-3 661 895	Inside PA3271, probable two component sensor
461(1)	1-120	3 281 560-3 281 441	Next to PA2925, HisM, transport of small molecules
461(2)	116-258	4 219 431-4 219 572	268 bp upstream from PA3764, conserved hypothetical protein
461(3)	255-330	2 746 448-2 746 373	Inside PA2445, amino acid biosynthesis and metabolism
498	285-303	1 165 803-1 165 821	62 bp upstream from PA1080, FlgE, flagellar hook protein
560	1-90	4 538 347-4 538 436	252 bp upstream from PA4058, hypothetical protein
561	1-148	4 589 529-4 589 676	Inside PA4106, conserved hypothetical protein
569(1)	1-100	4 578 510-4 578 609	900 bp upstream from PA4097, probable alcohol dehydrogenase
569(2)	94-166	542 818-542 890	Inside PA0482, carbon compound catabolism
570	1-97	3 868 548-3 868 644	826 bp upstream from PA3461, hypothetical protein
614(a)	1-70	500 902-500 833	Inside PA0445, probable transposase
614(b)	1-70	4 473 840-4 473 909	Inside PA3993, probable transposase
614(c)	1-70	3 843 071-3 843 002	Inside PA3434, probable transposase
614(d)	1-70	5 383 593-5 383 524	Inside PA4797, probable transposase
614(e)	1-70	2 557 166-2 557 235	Inside PA2319, probable transposase
~633(1)	1-157	1 572 726-1 572 882	669 bp upstream from PA1444, FliN, motility and attachment
~699	1-441	5 277 262-5 277 702	Inside PA4699, hypothetical protein
~703	1-429	3 254 326-3 253 897	808 bp upstream from PA2897, probable transcription regulator
707	1-106	524 761-524 866	603 bp upstream from CreD (PA0465), a membrane protein

877	1-193	1 228 563-1 228 371	Downstream from PA1136, probable transcriptional regulator
~889(1)	1-188	1 933 112-1 933 302	Inside PA1785, hypothetical protein
889(2)	183-259	942 036-941 960	Inside PA0861, hypothetical protein
889(3)	254-414	1 227 597-1 227 440	Inside PA1136, probable transcriptional regulator
~921	1-147	4 456 550-4 456 404	829 bp upstream from PA3974, probable two-component sensor
~930(1)	1-64	2 052 889-2 052 825	215 bp upstream from PA1881, probable oxidoreductase
930(2)	60-164	1 925 695-1 925 799	Map start from PA1779, assimilatory nitrate reductase, opposite direction
930(3)	160-261	4 172 627-4 172 526	42 bp upstream from PA3725 (RecJ), single stranded - DNA - specific exonuclease (DNA replication, recombination, modification and repair)

~: Indicates that the sequence is incomplete at the 5' end
( ): The numbers and letters in brackets denote chimeric DNA fragments and fragments located more than once on the genome, respectively

2.3.2 Characterization of the obtained nucleic acid sequences

2.3.2.1 Identification of conserved motifs and repeat sequences

The obtained sequences were characterized in order to identify conserved motifs and inverted repeat sequences. Although multiple sequence alignment analysis may provide a powerful tool for identifying conserved domains in homologous sequences, ClustalW alignment analysis of the sequences obtained in this study did not reveal any significant conserved regions or domains. This may have been due to the low levels of sequence identity observed between the respective DNA fragments as they originated from widely different regions within the *P. aeruginosa* genome.

Previous reports have indicated that inverted repeat sequences within the upstream region of the *algD* gene of *P. aeruginosa* enhance transcription of the gene (Mohr *et al.*, 1990). *AlgD* is involved in alginate synthesis and is also up-regulated by attachment of the cells to a surface (Hoyle *et al.*, 1993; Rice *et al.*, 1995). Consequently, the sequences of the elements were analysed using DNAMAN to identify direct and/or inverted repeat sequences. Although several of the clones contained direct and/or inverted repeat sequences of varying lengths, none of these displayed a resemblance to the types found upstream of the *algD* promoter. The significance, if any, of the identified repeat and inverted repeat sequences will have to be experimentally verified through further mutational analysis.

2.3.2.2 Comparison of the sequences to promoter specificity elements in quorum sensing-controlled genes

P. aeruginosa possesses two quorum sensing systems, the LasR-LasI system and the RhlR-RhlI system, which are globally regulatory elements that control the expression of numerous genes (Fuqua *et al.*, 1996; Whiteley *et al.*, 1999). Davies *et al.* (1998) have reported that the *lasI* system is required for maturation of *P. aeruginosa* biofilms. Recently, Whiteley and Greenberg (2001) described LasR- and RhlR-dependent determinants in promoters of several quorum sensing-controlled (qsc) genes. The promoters possessed inverted repeat sequences, termed lux-

box-like sequences, that display considerable dyad symmetry (16 to 18 bp of 20 bp) and extensive sequence variation. A minimal consensus sequence for the lux-box-like element of *P. aeruginosa* qsc promoters was defined as NNCT-N_{12}-AGNN following sequence analysis of the promoters of several *P. aeruginosa* qsc genes.

Analysis of the sequences obtained from the attachment-induced regulatory elements revealed the presence of the lux-box-like element in the sequences derived from clones 65(2), 498 and 560 (Fig. 2.1). These sequences mapped to the upstream regions of genes encoding a leucine aminopeptidase, FlgE and a hypothetical protein of unknown function, respectively (Table 2.2). Induction of promoters containing an A at position 8 and a complementary T at position 13 of the lux-box-like sequence (Fig. 2.1) have been reported to require a functional LasI and a functional RhlR for full induction, while promoter regions lacking the 8A-13T motif respond to LasR specifically (Whiteley and Greenberg, 2001). Although the sequences of the respective attachment-induced regulatory elements contained the lux-box-like element, they possessed either a 8C-13T or 8G-13A motif, but not the 8A-13T motif. This may therefore suggest that LasR could play a role in the induction of these putative promoter elements.

2.3.2.3 Identification of consensus promoter sequences

To identify consensus promoter sequences, the respective sequences were analyzed using the DNAMAN and SEQSCAN programs. None of the sequences revealed homology with the typical sigma 70 (σ^{70}) family of promoter sequences of *E. coli*. However, a number of the clones were found to contain sequences corresponding to that of σ^{54} promoters (Fig. 2.2). Although the dinucleotides GG-N_{10}-GC are generally highly conserved in σ^{54} promoters (Cases and De Lorenzo, 2001; Barrios *et al.*, 1999), note should be taken that exceptions do occur. The σ^{54} promoters of the *nifH* gene of *Rhizobium leguminosarum* biovar *viciae* and the *glnB* gene of *Rhodospirillum rubrum* both contain GG-N_{10}-GA, while the σ^{54} promoters of the *E. coli glnH* gene and the *P. aeruginosa oprE* gene contain sequences of GG-N_{10}-TC and GG-N_{10}-CC, respectively (Yamano *et al.*, 1998; Nohno and Saito, 1987; Roelvink *et al.*, 1990; Johansson and Nordlund, 1996). By taking these exceptions into account, 6 clones were found to contain putative σ^{54} promoter sequences (Fig. 2.2).

However, when the putative σ^{54} promoters were analyzed for their orientation and proximity relative to *P. aeruginosa* open reading frames (ORFs) (Table 2.2), only two of these were located within 400 bp upstream of *P. aeruginosa* genes. The respective genes encode a probable O-antigen acetylase (clone 56(2)) and FlgE, a flagellar hook protein (clone 498). A third putative σ^{54} promoter was located approximately 600 bp upstream of a gene encoding a hypothetical protein of unknown function (clone 65(1)). The remainder of the putative σ^{54} promoter sequences were located within the open reading frames of a probable transposase, a hypothetical protein and a probable transcriptional regulator. Since *P. aeruginosa* has a G + C content of 66.6% (Stover *et al.*, 2000), the identification of putative σ^{54} consensus sequences in these latter sequences may have been fortuitous.

2.3.2.4 Identification of motifs associated with σ^{54} promoters

Transcription initiation from σ^{54} promoters is a multistep process involving the recognition of the promoter by σ^{54}, binding of the core RNA polymerase to the σ^{54} to form a closed complex, and subsequent activation to an open complex following binding by an enhancer binding protein (EBP) (Dworkin *et al.*, 1997). In most cases, the EBP is brought into contact with the σ^{54}-RNA polymerase complex by DNA looping, an event mediated by the integration host factor (IHF) (Hoover *et al.*, 1990). Since IHF binding sites are therefore often found in the proximity of σ^{54} promoters, the above sequences were examined for putative IHF binding sites by searching for the consensus WATCAA-N4-WTR sequence (Hoover *et al.*, 1990; Taylor *et al.*, 1996). A single IHF binding site was identified only in the upstream sequence of clone 498, 70 nucleotides upstream of the putative σ^{54} promoter.

Gene/fragment	Nucleotide sequence	Dyad symmetry
*gsc*102	A C **C T** G C C C G G A A G G G C **A** G G T	16
*rsa*L	A A **C T** A G C A A A T G A G A T **A** G A T	12
*las*I	A T **C T** A T C T C A T T T G C T **A** G T T	12
*qsc*117	C A **C T** G C C A G A T C T G G C **A** G T T	18
*phz*A	A C **C T** A C C A G A T C T T G T **A** G T T	16
*las*B OP1	A C **C T** G C C A G T T C T G G C **A** G G T	18
*hcn*A	A C **C T** A C C A G A A T T G G C **A** G G G	12
498	G C **C T** G G G C A G C A T C C C A G C A	12
65(2)	G A **C T** G C G G C C G G A C A T **A** G A C	12
560	A G **C T** C G G C G G T C T C G A **A** G G T	10

Fig. 2.1 An alignment of the lux-box-like elements (NNCT-N_{12}-AGNN) in quorum sensing-controlled genes (Whiteley and Greenberg, 2001) and the attachment-induced regulatory elements (this study). Highly conserved nucleotides are indicated in bold, while specificity determinants are boxed. Nucleotides of dyad symmetry are indicated to the right.

Fragment	Nucleotide sequence
56(2)	T **G G** C G C C G A A G G T **G C** T
65(1)	T **G G** G A C T G C T C C T **G C** C
498	T **G G** C A A G G T C A C T T **C** A
614	T **G A** C A A G C A C G A T **G C** A
699	C **G G** C G C G C G A C C T **G C** T
889(3)*	T **G G** T A G C G G C C T T **G C** G
889(3)*	T **G G** C G C G G G C A T T **C C** T

Fig. 2.2 Identification of putative σ^{54} promoter sequences (GG/A-N_{10}-G/T/C) in six attachment-induced regulatory elements using the SEQSCAN software program. Highly conserved nucleotides are indicated in bold. An asterisk (*) denotes a single DNA fragment containing two putative σ^{54} promoter sequences.

2.4 DISCUSSION

Although significant advances have been made toward identifying environmental conditions, surface properties and bacterial surface structures that may affect bacterial attachment to surfaces, the molecular mechanisms underlying the switch(es) from planktonic to attached growth and regulation of such mechanisms, have yet to be completely elucidated. To investigate, initial approaches in our laboratory relied on the isolation of regulatory genetic elements from *P. aeruginosa* in response to surface growth. It was envisaged that this may assist in the identification of genetic elements required for bacterial attachment and would yield information on how these elements are regulated.

In this part of the investigation, the nucleotide sequence of 24 clones selected from a group of clones that displayed up-expression of a *lacZ* reporter gene upon attachment of *P. aeruginosa* to a glass wool substratum (Weyers, 1999), was determined. As a consequence of the cloning strategy used, several of the obtained sequences were found to consist of ligated DNA fragments originating from different locations within the *P. aeruginosa* genome. Thus, although 24 clones were initially selected, a total of 42 distinct putative regulatory elements could be identified following sequence analysis (Table 2.2). Since it was not known which of the cloned elements within the chimeric DNA sequences was responsible for the observed up-regulated expression of the *lacZ* reporter gene, all of the obtained sequences were included in the subsequent analysis of the sequence data.

The identity of the attachment-induced genetic elements was determined by BLAST homology searches against the *P. aeruginosa* PAO1 genome sequence. With the exception of the sequence obtained from clone 498, all of the obtained sequences displayed 98-100% sequence identity to sequences within the *P. aeruginosa* genome (Table 2.2). The sequence obtained from clone 498 did however reveal identity to a region upstream of the *P. aeruginosa flgE* gene. The low level of sequence identity may be due to differences in the genomic DNA sequences of *P. aeruginosa* strain DSM1707 used in this study and strain PAO1 of which the genome has been completely sequenced. For example, the genomes of the these two clonally derived *P. aeruginosa* prototype strains have been shown to differ by the presence of a 2.19 Mbp inversion between the *rrnA* and

rrnB operons including *oriC* (Stover *et al.*, 2000). It is therefore possible that other sequence differences may exist between these two *P. aeruginosa* strains. A rather surprising finding was that approximately half of the attachment-induced genetic elements are localized to regions within the ORF of genes. The significance of these sequences being responsible for up-expression of the reporter gene, is therefore unclear. However, a recent report has indicated the presence of a functional promoter within the coding region of another gene. Ochsner and co-workers (1999) reported that the proximal *furP1* promoter of *P. aeruginosa* was located within the *omlA* coding sequence. The encoded proteins had no influence on each other's expression, excluding *trans*-acting cross-regulation between *fur* and *omlA*. The significance of this type of promoter structure awaits future experimentation by for example primer extension analysis.

Recently, the promoters of several *P. aeruginosa* quorum sensing-controlled genes were analyzed and shown to possess conserved quorum control elements that were shown to be important in quorum sensing-controlled transcription (Whiteley and Greenberg, 2001). It has been suggested, that the quorum control elements may serve as activator binding sites and that the activators function by making contact with the RNA polymerase α C-terminal domain and with some other part of the RNA polymerase (Whiteley and Greenberg, 2001). Analysis of the sequences indicated the presence of a quorum control element, $NNCT-N_{12}-AGNN$, in the sequence obtained from three different clones. These mapped to the upstream regions of *flgE* (clone 498) encoding a flagellar hook protein, and a gene encoding a leucine aminopeptidase (clone 65(2)). Clone 560 mapped to the upstream region of a gene encoding a hypothetical protein. These genes do not correspond to the quorum sensing-controlled genes described by Whiteley *et al.* (1999). Since a saturation mutagenesis was performed and there may be conditions other than those used by Whiteley *et al.* (1999) that can reveal other genes not detected in their screen, it may be possible that other quorum sensing-controlled genes exist. In addition, Whiteley and Greenberg (2001) indicated that some genes with an upstream $NNCT-N_{12}-AGNN$ motif as well as an A at position 8 and a T at position 13, required both the N-(3-oxododecanoyl-$_L$-homserine lactone ($3OC_{12}$-HSL) and N-butyryl-$_L$-homoserine lactone (C_4-HSL) signals for full induction, but that they can respond to either of the signal receptors LasR and RhlR. Thus, the 8A-13T element appears to relax specificity of the quorum sensing-controlled promoters so that it will respond to either LasR or RhlR. By contrast, quorum sensing-controlled genes which lacked the 8A-13T motif responds

only to LasR. Since none of the sequences analyzed contained the 8A-13T motif, it is tempting to speculate that these genes may be regulated by LasR. However, detailed analysis is required before a final conclusion can be drawn.

Analysis of the sequences for consensus promoter sequences indicated that 6 of the 24 clones contained putative σ^{54} promoter sequences (Barrios *et al.*, 1999; Cases and De Lorenzo, 2001). Although the RpoN sigma factor (σ^{54}) was originally discovered in *E. coli* as being essential for nitrogen metabolism (Hirschman *et al.*, 1985), it has since been shown to be involved in various functions in other organisms, amongst other flagellar assembly, pilin synthesis (Arora *et al.*, 1997; Kinsella *et al.*, 1997), alginate production (Zielinski *et al.*, 1992) and rhamnolipid production (Pearson *et al.*, 1997). Bacterial genes transcribed by σ^{54}-RNA polymerases are invariably regulated at a distance by a class of transcription regulators known as prokaryotic enhancer binding proteins (EBP). These regulatory proteins, which catalyze the isomerization of the enzyme-σ^{54} promoter complex from the closed to the open state, bind to upstream activator sequences (UAS) located 80 to 200 bp upstream of the promoter and act through DNA looping to contact the holoenzyme (Dworkin *et al.*, 1997). DNA looping may, however, also be mediated by intrinsic DNA bends. The putative σ^{54} promoter sequence identified in clone 498 was the only promoter sequence identified containing an IHF consensus sequence. Notably, this sequence mapped to the upstream region of the *P. aeruginosa flgE* gene, which encodes a flagellar hook protein, one of the structural components of flagella. In several bacteria, flagella and pili have been described as major structures required for either stable cell-to-surface attachment and/or cell-to-cell interactions required in the formation of microcolonies (DeFlaun *et al.*, 1994; De Weger *et al.*, 1987; O'Toole and Kolter, 1998a; 1998b; Watnick *et al.*, 1999; Pratt and Kolter, 1998; Genevaux *et al.*, 1996). Recently, Sauer and Camper (2001) proposed a role for flagella in the detachment of cells from well-developed biofilms as flagella could be detected in 3- to 7-day old biofilms. Of the other clones containing consensus σ^{54} promoter sequences, clone 56(2) mapped to the upstream region of a gene encoding a probable O-antigen acetylase. This enzyme may O-acetylate polysaccharides, e.g. alginate (Gacesa *et al.*, 1998). Adhesiveness of *Pseudomonas* species has been reported to be related to the presence and composition of polysaccharides (Williams and Fletcher, 1996) and substantially reduced attachment to biotic and abiotic surfaces has been observed in O-polysaccharide-deficient *Pseudomonas* spp. (DeFlaun

et al., 1999; Dekkers *et al.*, 1998). None of the other sequences containing consensus σ^{54} promoter sequences mapped to regions upstream of *P. aeruginosa* genes and as *P. aeruginosa* has a high G + C content, their identification may have been fortuitous.

In conclusion, sequence analysis of *P. aeruginosa* genomic DNA fragments responsible for up-regulating the expression of a reporter molecule upon attachment to a glass wool substratum indicated no homology with σ^{70}-dependent promoter sequences. By contrast, several of the sequences displayed significant homology with σ^{54}-dependent promoter sequences. This may indicate that σ^{54} plays a role in the formation of biofilms in *P. aeruginosa* by regulating transcription of specific genes whose products are required for biofilm formation. The sequence of one of the DNA fragments (clone 498) mapped upstream of a flagellar gene and contained in addition to a consensus σ^{54} promoter sequence, an IHF binding site. This clone was selected and the transcriptional activity from this regulatory element was characterized at a single cell level in both biofilm and planktonic cells using reporter gene technology, as described in the following chapter.

CHAPTER THREE

TRANSCRIPTIONAL ACTIVITY OF A *P. aeruginosa* ATTACHMENT-INDUCIBLE REGULATORY ELEMENT UNDER DIFFERENT GROWTH CONDITIONS

3.1   INTRODUCTION

P. aeruginosa has been shown to assume a biofilm mode of growth, embedding itself in a gelatinuous organic polymer matrix composed of alginate (Costerton *et al.*, 1995). In addition, the biofilm bacteria have been reported to markedly differ from their planktonic counterparts with relation to gene expression and cellular physiology. Recent genetic (O'Toole and Kolter, 1998a; O'Toole *et al.*, 2000a; Davies *et al.*, 1998) and proteomic (Steyn *et al.*, 2001; Sauer and Camper, 2001) studies have identified several genes involved in the formation and development of biofilms. In *P. aeruginosa*, expression of a number of genes is up-regulated in biofilm-growing cells, e.g. *algC* (Davies *et al.*, 1993; Davies and Geesey, 1995), *algD* (Hoyle *et al.*, 1993; Rice *et al.*, 1995), *pilA* (O'Toole *et al.*, 2000a), *tolA*, *mreC*, *omlA*, *tatA* and *tatB* (Whiteley *et al.*, 2001).

Despite numerous genes having been identified that appear to play a role in the biofilm phenotype, the regulation of only a few of these genes has been reported. Whereas the transcription of *algC* is dependent on the alternative sigma factor, σ^{54} (Zielinski *et al.*, 1992), and the response regulator AlgR (Fujiwara *et al.*, 1993), the transcription of *algD* has been reported to be modulated by two different pathways and can be either σ^{54}- or σ^{22} (AlgU/AlgT)-dependent (Boucher *et al.*, 2000). Also, transcription of the *pilA* gene of *P. aeruginosa* requires, in addition to σ^{54}, the regulatory factors *pilR* and *pilS* (Ishimoto and Lory, 1989; 1992; Johnson *et al.*, 1986). Furthermore, genes encoding proteins which aid in biofilm development such as those required for flagellar assembly (Kinsella *et al.*, 1997), pilin synthesis (Arora *et al.*, 1997) and rhamnolipid production (Pearson *et al.*, 1997) have all been reported to be σ^{54}-dependent. Thus, σ^{54} appears to play an important role in the transcription of genes required for biofilm formation.

The understanding of how genes are regulated has been greatly facilitated by the rapid developments in reporter gene technology (Prigent-Combaret *et al.*, 1999). By cloning a DNA

fragment containing the putative promoter into a plasmid vector containing a promoterless reporter gene (e.g., genes encoding β-galactosidase, the green fluorescent protein or luciferase), the reporter protein activity can be readily quantified using a variety of different approaches (Tresse *et al.*, 1998; Burlage *et al.*, 1996; Chalfie *et al.*, 1994; Eberl *et al.*, 1997; Tombolini *et al.*, 1997). In the last few years, the green fluorescent protein gene (*gfp*) encoding green fluorescent protein (GFP) of the bioluminescent jellyfish *Aequorea victoria* (Prasher *et al.*, 1992) has attracted considerable attention as a reporter for the visualization of gene expression and protein subcellular localization (Chalfie *et al.*, 1994). Various studies have described the use of GFP for studying multiple-species bacterial communities in biofilms (Møller *et al.*, 1998; Skillman *et al.*, 1998) and adhesion of bacteria to flocs in activated sludge (Eberl *et al.*, 1997; Olofsson *et al.*, 1998) as well as for studying gene expression in biofilm cells (De Kievit *et al.*, 2001; Heydorn *et al.*, 2002; Sternberg *et al.*, 1999).

Biofilms have been studied predominantly in stagnant batch culture by using microtitre plates (O'Toole and Kolter, 1998a; 1998b; Watnick and Kolter, 1999; Vidal *et al.*, 1998; Dorel *et al.*, 1999) or under conditions of continuous flow using various different flow cells (Christensen *et al.*, 1999; Zinn *et al.*, 1999). Whereas batch systems are inexpensive and well suited for genetic studies where a high throughput is required, it is, however, not possible to investigate gene expression in single cells or the heterogeneity in gene expression amongst various members within a specific population. These limitations may be overcome by making use of flow through systems, which, coupled with the use of an appropriate reporter gene and fluorescence or confocal laser scanning microscopy, allow for monitoring of gene expression in biofilms at single cell level over time (Bloemberg *et al.*, 1997; Gottenbos *et al.*, 1999; Korber *et al.*, 1999).

From the preceding sections it appears that transcription of a number of genes up-regulated in biofilm-growing cells is dependent on the alternative sigma factor, σ^{54}. In addition, bacterial structures such as flagella and pili have been reported to play important roles during the initial stages of biofilm formation. In the previous chapter, a DNA sequence mapping to the upstream region of the *flgE* gene encoding a flagellar hook protein, and which contains both an IHF binding site and a σ^{54} promoter consensus sequence, was identified (clone 498). Thus, the aims of this part of the study were (i) to determine the minimum requirements for attachment-induced

promoter activity by preparing *lacZ* transcriptional fusions with truncated versions of the regulatory element and (ii) to investigate the transcriptional activity of the *flgE* regulatory element in bacterial cells under different growth conditions using a *gfp* reporter gene construct.

3.2   MATERIALS AND METHODS

3.2.1   Bacterial strains, plasmids and media

The bacterial strains and plasmids used in this study are listed in Table 3.1. *E. coli* and *P. aeruginosa* DSM1707 strains were routinely cultured at 37°C in LB-broth or on LB-agar plates. When required, the medium was supplemented with the required antibiotics as indicated in the text. β-galactosidase assays and fluorescent microscopy were performed in FABG medium (0.2% $(NH_4)_2SO_4$; 0.6% $Na_2HPO_4.2H_2O$; 0.3% KH_2PO_4; 0.3% NaCl; 0.0001% $MgCl_2$; 0.00001% $CaCl_2$; 0.000366% Fe-EDTA [Sigma E-6760]; 0.5% glucose) (Heydorn *et al.*, 2000). Plasmids pJBA27(*gfp*mut3) and pMiniCTX were kindly supplied by J.B. Andersen (Department of Microbiology, The Technical University of Denmark) and H.P. Schweizer (Department of Microbiology, Colorado State University), respectively.

Table 3.1 Bacterial strains, plasmids and primers used in this study

Strain, plasmid or primer	Relevant properties	Reference or source
Strains		
E. coli JM109	F' *traD36 lacI*q Δ*(lacZ)M15 proA*+*B*+/e14-(McrA-) Δ*(lac*-*proAB) thi gyrA96* (Nal^r) *endA1 hsdR17* (r_k-m_k+) *relA1 supE44 recA1*	Promega
P. aeruginosa DSM1707	Prototroph (PAO)	DSM^a
DSM1707::pMiniCTX-GFP	pMiniCTX-GFP integrated onto DSM1707	This study
DSM1707::pMiniCTX-498-GFP	pMiniCTX-498-GFP integrated onto DSM1707	This study
DSM1707::pMiniCTX-IP-GFP	pMiniCTX-IP-GFP integrated onto DSM1707	This study

Strain, plasmid or primer	Relevant properties	Reference or source
Plasmids		
pALacZsd	Tet^r, IncQ/RSF1010, t, T, promoterless *lacZ* gene	Weyers, 1999
pALacZsdIP	pALacZsd containing the *lac* promoter from pBluescript (co-ordinates 719-1719)	Weyers, 1999
pALacZsd$_{(DSM1707)}$498	pALacZsd containing a fragment up-regulated by attachment (contains putative IHF and σ^{54} sites)	Weyers, 1999
pALacZsd$_{(DSM1707)}$498A	subclone A from pALacZsd498 (putative IHF site)	This study
pALacZsd$_{(DSM1707)}$498B	subclone B from pALacZsd498 (putative σ^{54} site)	This study
pGEM®-T-Easy	Amp^r, cloning vector for PCR products	Promega
pGEM®-T-Easy-498	pGEM®-T-Easy containing a fragment up-regulated by attachment (contains putative IHF and σ^{54} sites)	This study
pJBA27(*gfp*mut3)	Amp^r, pUC18Not-$_{PA1/04/03}$-RBSII-*gfp*mut3*-T$_0$-T$_1$	Andersen *et al.*, 1998
pMiniCTX	Tet^r,self-proficient integration vector with *tet*, *FRT-attP-MCS, ori, int*, and *oriT*	Hoang *et al.*, 2000
pMiniCTX-GFP	pMiniCTX containing promoterless *gfp* gene from pJBA27	This study
pMiniCTX-498-GFP	pMiniCTX containing cloned 498-*gfp* fusion	This study
pMiniCTX-IP-GFP	pMiniCTX-GFP containing the *lac* promoter from pBluescript (co-cordinates 719-1719)	Van Schalkwyk, 2001
pRK2013	ColE1*mob$^+$tra$^+$*(RK2)Kan^r	Figurski & Helinski, 1979
pUC19	Amp^r, cloning vector	Promega
pUC498	pUC19 containing cloned 498 fragment	This study
pUC498A	pUC19 containing 498A fragment from pUC498	This study
pUC498B	pUC19 containing 498B fragment from pUC498	This study
Primers		
EBF	5'-GCATCACCTTCACCCTCTCC-3'	This study
EBR	5'-CCGGCGCGCGTAATACG-3'	This study
JB-1	5'-GAATTCGAGCTGGGTAC-3'	Weyers, 1999
JB-2	5'-GTTTTCCCAGTCACGAC-3'	Weyers, 1999

a: DSM - Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany

3.2.2   PCR amplification of the insert DNA contained in clone 498

PCR amplification of the cloned DNA fragment in clone 498 was used to obtain a sufficient amount of the insert DNA to facilitate further cloning procedures. The PCR reaction mixture contained 1 × PCR buffer (50 mM KCl; 10 mM Tris-HCl (pH 9.0); 0.1% Triton® X-100), MgCl$_2$ at 1.5 mM, 50 pmol of each the JB-1 and JB-2 oligonucleotide primers (Table 3.1), each deoxynucleoside triphosphate (dNTP) at a concentration of 0.2 mM, 25-50 ng template DNA and 1 U of Taq DNA polymerase (Promega) in a final volume of 50 µl. The tubes were placed in a GeneAmp 9600 thermal cycler (Perkin Elmer). Following an initial denaturation of 95°C for 2 min, the reaction was subjected to 30 cycles of amplification using the following conditions: denaturation at 94°C for 1 min, annealing at 52°C for 30 s and elongation at 72°C for 1 min. After the last cycle, the reaction was kept at 72°C for 5 min to complete synthesis of all strands. For control purposes, a reaction mixture containing distilled water and all other reagents but no template DNA was included. Aliquots of the PCR reaction mixtures were subsequently analyzed by electrophoresis on 1% (w/v) agarose gels in the presence of an appropriate DNA molecular weight marker.

3.2.3   Purification of DNA fragments from agarose gels

Amplicons or restriction digestion fragments were purified from agarose gels by the Geneclean™ procedure according to the instructions of the manufacturer (Bio 101, Inc.). Briefly, the appropriate DNA fragment was excised from the agarose gel with a scalpel blade, placed in a microfuge tube, weighed and mixed with 3 volumes of a 6 M NaI solution. The agarose was melted at 55°C after which 7 µl glassmilk® was added to the suspension. After incubation for 20 min on ice (fragments > 500 bp) or incubation for 20 min at 55°C (fragments < 500 bp), the silica-bound DNA was pelleted by brief centrifugation and washed four times with ice-cold NEW Wash (a solution containing NaCl; Tris and EDTA in ethanol and water). The DNA was then eluted from the silica matrix at 55°C for 2 to 3 min in a final volume of 7 µl UHQ water.

3.2.4   Cloning of the purified amplicon into the pGEM®-T-Easy vector

The pGEM®-T-Easy vector system (Promega) was used to clone the gel-purified amplicons.

(a)

Fig. 3.1   Construction of the recombinant reporter vectors pALacZsd$_{(DSM1707)}$498A and pALacZsd$_{(DSM1707)}$498B containing putative IHF and σ^{54} consensus sites, respectively. (a) Cloning of the 498 DNA fragment into pUC19.
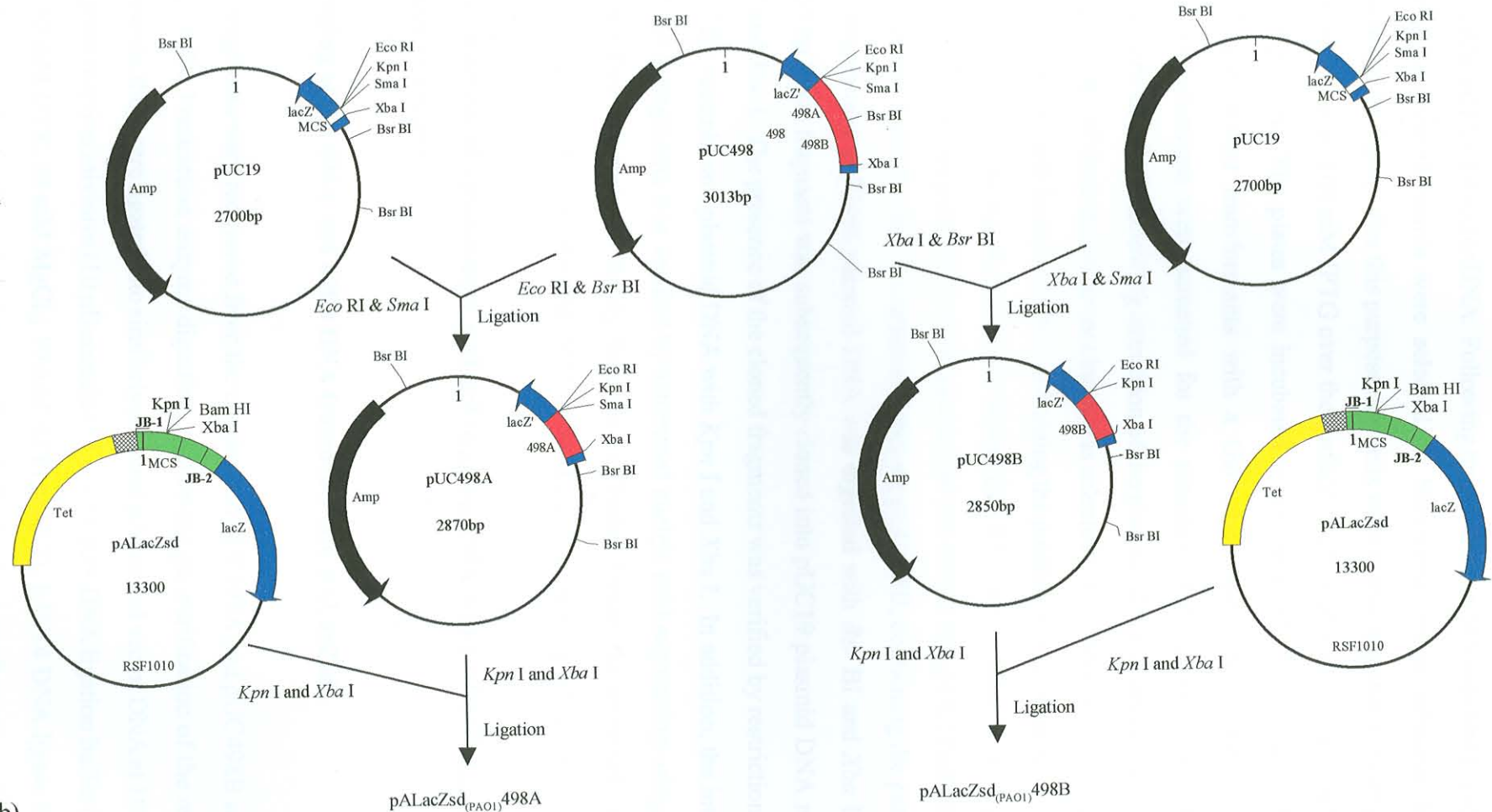
(b)

Fig. 3.1   Construction of the recombinant reporter vectors pALacZsd$_{(DSM1707)}$498A and pALacZsd$_{(DSM1707)}$498B containing putative IHF and σ^{54} consensus sites, respectively. (b) Subcloning of the 498A and 498B DNA fragments into pALacZsd.

identically restricted pUC19 vector DNA. Following transformation of competent *E. coli* JM109 cells, recombinant transformants were selected by blue/white colour selection based on inactivation of the *lacZ* gene. For this purpose, the cells were spread together with 40 μl of 2% (w/v) X-gal and 10 μl of 100 mM IPTG over the surface of LB-agar plates supplemented with 100 μg/ml ampicillin. The plates were incubated overnight at 37°C and observed for the presence of recombinant transformants with a Gal⁻ phenotype. A number of putative recombinant transformants were screened for the presence of the cloned insert DNA by restriction enzyme digestion following extraction of the plasmid DNA as previously described (Section 2.2.4). One of these recombinant clones was selected for further use and designated as pUC498. To construct subclone pUC498A, containing the putative IHF sequence, recombinant pUC498 plasmid DNA was digested with *Bsr* BI and *Eco* RI. The excised DNA fragment of approximately 170 bp was purified from the agarose gel and cloned into pUC19 plasmid DNA restricted with *Sma* I and *Eco* RI. To construct subclone pUC498B, containing the putative σ⁵⁴ promoter, recombinant pUC498 plasmid DNA was digested with *Bsr* BI and *Xba* I and the excised 150 bp DNA fragment was subsequently cloned into pUC19 plasmid DNA restricted with *Sma* I and *Xba* I. The presence of the cloned fragments was verified by restriction enzyme digestion of the recombinant plasmid DNA with *Kpn* I and *Xba* I. In addition, the integrity of the cloned DNA fragments was verified by automated nucleic acid sequencing using the ABI PRISM™ Big Dye™ Terminator Ready Reaction kit (Perkin Elmer), as described previously (Section 2.2.7), except that the universal M13 forward (-21) sequencing primer was used.

3.2.7   Construction of recombinant pALacZsd(DSM1707)498A and pALacZsd(DSM1707)498B reporter vectors

3.2.7.1 Cloning of the 498A and 498B DNA fragments into pALacZsd

The DNA fragments were recovered from the constructed pUC498A and pUC498B subclones by *Kpn* I and *Xba* I restriction enzyme digestion. Following gel purification of the respective DNA fragments, they were ligated into similarly prepared pALacZsd vector DNA at 15°C for 16 h. The ligation reaction mixtures (10 μl) contained 1 μl of a 10 × DNA ligation buffer (660 mM Tris.HCl; 10 mM DTT; 50 mM MgCl₂; 10 mM ATP; pH 7.5), 1 U T4 DNA ligase (Roche; 1 U/μl) and the ratio of insert to vector was typically 6:1. Following transformation of competent

E. coli JM109 cells, the cells were plated onto LB-agar plates supplemented with 20 µg/ml tetracycline. Recombinant transformants were then randomly selected for further characterization and grown overnight at 37°C in 10 ml of LB-broth supplemented with 20 µg/ml tetracycline.

3.2.7.2 PCR screening of putative recombinant transformants

To verify the presence of cloned insert DNA in the constructed reporter vectors, plasmid DNA was extracted by the alkaline lysis method and used as template DNA in PCR reactions with oligonucleotide primers JB-1 and JB-2. The PCR reaction mixture (50 µl) contained $1 \times$ Taq polymerase buffer (50 mM KCl; 10 mM Tris-HCl (pH 9.0); 0.1% Triton® X-100), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 25 ng of template DNA, 50 pmol of each primer and 1 U of Taq DNA polymerase (Promega). Following an initial denaturation of 95°C for 2 min, the samples were subjected to 30 cycles of amplification at 94°C, 1 min; 52°C, 30 s and 72°C, 1 min followed by a final extension step at 72°C for 5 min. Control reaction mixtures containing distilled water and all other reagents but no template DNA as well as reaction mixtures containing plasmids pALacZsd and pALacZsd$_{(DSM1707)}$498 (clone 498) were included. Following PCR amplification, aliquots of the reaction mixtures were analyzed by 2% (w/v) agarose gel electrophoresis. Recombinant clones containing the cloned insert DNA was designated pALacZsd$_{(DSM1707)}$498A and pALacZsd$_{(DSM1707)}$498B, respectively, and used in all subsequent assays.

3.2.8 Determination of β-galactosidase activity of the recombinant pALacZsd reporter vectors

3.2.8.1 Preparation of competent *P. aeruginosa* cells

Competent *P. aeruginosa* cells were prepared and transformed essentially by the procedures described by Olsen *et al*. (1982). A single colony of *P. aeruginosa* DSM1707, grown overnight at 37°C on a TN-agar plate (0.5% tryptone; 0.1% dextrose; 0.25% yeast extract; 0.4% sodium nitrate; 1.2% agar) (Olsen and Shipley, 1973), was inoculated into 100 ml TN-broth and grown at 37°C to an OD$_{550}$ of between 0.3 and 0.5. The cells from 30 ml of the culture were collected

in Corex tubes by centrifugation at 4000 rpm for 10 min at 4°C in a pre-cooled Sorvall HB 4 rotor. The pellet was suspended in 15 ml ice-cold filter-sterilized 0.15 M $MgCl_2$, incubated on ice for 5 min, pelleted as before and gently resuspended in 7.5 ml of the ice-cold $MgCl_2$ solution. After incubation on ice for 20 min, the cells were again collected by centrifugation and the pellet finally resuspended in 750 µl of ice-cold $MgCl_2$.

3.2.8.2 Transformation of competent *P. aeruginosa* cells

The competent cells were transformed by addition of 500 ng plasmid DNA (pALacZsd, pALacZsdIP, pALacZsd$_{(DSM1707)}$498, pALacZsd$_{(DSM1707)}$498A or pALacZsd$_{(DSM1707)}$498B) to 200 µl of competent cells in a sterile glass tube. Following incubation on ice for 1 h, the cells were incubated at 37°C for 3 min and chilled on ice for 5 min. After addition of 500 µl of LB-broth, the transformation mixtures were incubated at 37°C for 3 h with shaking. The cells were plated in aliquots of 100-200 µl onto LB-agar plates supplemented with 40 µg/ml tetracycline and incubated overnight at 37°C.

3.2.8.3 Culturing conditions

A single colony of freshly-streaked cultures of each transformant as well as *P. aeruginosa* DSM1707 was inoculated into 50 ml modified FABG medium. After incubation at 37°C for 7 h, the exponentially growing cultures were inoculated to an OD_{540} of 0.05 into 2 ml FABG medium in 28-ml McCartney bottles with or without glass wool (0.05 g; mean diameter 15 µm, Merck). All cultures, except *P. aeruginosa* DSM1707, were supplemented with 40 µg/ml tetracycline. The cultures were incubated for 16 h at 37°C with agitation. Planktonic cells 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 cells. The supernatant of these cultures was carefully aspirated, transferred to a new microfuge tube and referred to as planktonic cells grown in the presence of glass wool (PGW). Following addition of 2 ml of FABG medium to the glass wool, the samples were vortexed for 1 min to remove the biofilm biomass from the glass wool substrate, and the recovered supernatants were transferred to new tubes to be used for β-galactosidase assays and protein concentration determinations, respectively. Efficient removal of the biofilm cells from the glass wool substratum was verified by light microscopy.

3.2.8.4 β-galactosidase assay

β-galactosidase activity was assayed using ONPG (Roche) as a chromogenic substrate according to the methods described by Miller (1972). Samples of each growth phase (500 µl) were incubated for 20 min at 4°C to stop further growth of the cells. For activity assays, 500 µl Z-buffer (0.06 M Na_2HPO_4; 0.04 M NaH_2PO_4; 0.01 M KCl; 0.001 M $MgSO_4$; 0.5 M β-mercaptoethanol) was added to each tube followed by the addition of 10 µl 0.1% SDS and 20 µl chloroform to lyse the bacterial cells. After incubation at 28°C for 10 min, 200 µl ONPG substrate (4 mg/ml in Z-buffer) was added to each tube and mixed to initiate the colour reaction. The reactions were terminated by the addition of 500 µl of 1 M Na_2CO_3 after 10 min and the tubes were then briefly centrifuged to collect the cellular debris and glass wool. Hydrolysis of ONPG was quantified by transferring 200 µl of each supernatant to wells in a microtitre plate and measuring the absorbance at 414 nm using a Titretek multiscan MCC/340 apparatus. All assays were performed in triplicate and on at least two separate occasions.

3.2.8.5 Protein concentration determination

The protein concentration of the planktonic, PGW and attached (biofilm) samples, prepared as described above, was determined by the method of Bradford (1976) using a commercial kit (Bio-Rad protein assay) and bovine serum albumin (BSA) as standard. The bacterial cells from samples of each growth phase (500 µl) were collected by centrifugation at 15 000 rpm for 5 min and the cell pellets were resuspended in 500 µl UHQ water prior to being heated to 95°C for 10 min. The suspensions were subsequently incubated on ice for 10 min and sonicated by 4 pulses of 15 s each using a 4710 Series Ultrasonic Homogenizer at an output of 40%, after which 50 µl of each sample was added to 1.5 ml protein assay reagent (Bradford reagent), mixed well and the absorbance at 595 nm determined. UHQ water was used to zero the absorbancy readings and the protein concentration (µg/ml) was then determined from the prepared standard curve. The β-galactosidase activity per total cellular biomass was determined by dividing the β-galactosidase values by the protein concentration to ensure that the difference in β-galactosidase activity was due to a difference in promoter activity, and not due to variations in the yield of biomass.

3.2.9   Generation of *P. aeruginosa* pMiniCTX transconjugants

3.2.9.1 Construction of recombinant and control pMiniCTX-GFP reporter plasmids

To allow investigation of the promoter activity at single cell level, promoter-*gfp* reporter vector constructs were prepared which could be stably integrated into the genome of *P. aeruginosa* using the pMiniCTX vector (Hoang *et al.*, 2000). The strategy used for the construction of plasmid pMiniCTX-498-GFP is indicated in Fig. 3.2. The insert DNA was recovered from the recombinant pGEM®-T-Easy-498 vector by excision with *Kpn* I and *Xba* I, gel-purified and then cloned into similarly prepared pJBA27 vector DNA to yield the intermediate vector pJBA27-498, containing the *gfp* gene under transcriptional control of the putative σ^{54} promoter. The 498-*gfp* DNA fragment was subsequently ligated into pMiniCTX as a 2.0 kbp *Kpn* I - *Not* I fragment and the ligation mixture was transformed into competent *E. coli* JM109 cells, prepared as described previously (Section 2.2.2). The transformation mixtures were plated onto selective LS-LB-agar plates (1% bacto-tryptone; 0.5% bacto-yeast extract; 0.4% NaCl; 1.2% agar; pH 7.4) supplemented with 20 µg/ml tetracycline. Putative recombinant transformants were selected and characterized by colony PCR analysis as described below. One of the recombinants was selected for further use and designated as pMiniCTX-498-GFP. For the construction of a control promoterless pMiniCTX-GFP reporter plasmid, plasmid pJBA27 was digested with *Xba* I and *Not* I to yield a 1.7 kb DNA fragment containing the promoterless *gfp* gene which was then cloned into pMiniCTX digested with *Not* I and *Spe* I. Following transformation of competent *E. coli* JM109 cells, a recombinant plasmid, pMiniCTX-GFP, was selected based on colony PCR analysis. As a positive control in the subsequent investigations, plasmid pMiniCTX-IP-GFP was kindly provided by A. van Schalkwyk (Department of Microbiology and Plant Pathology, University of Pretoria) and contains the *gfp* reporter gene cloned under the transcriptional control of the constitutive *lac* promoter.

3.2.9.2 Colony PCR

Selected colonies were transferred to tubes containing 500 µl UHQ water, mixed and boiled for 5 min at 95°C. The cellular debris was pelleted by centrifugation for 2 min at 15 000 rpm and 5 µl of the supernatant, containing the DNA, was used as template in the PCR reactions. In
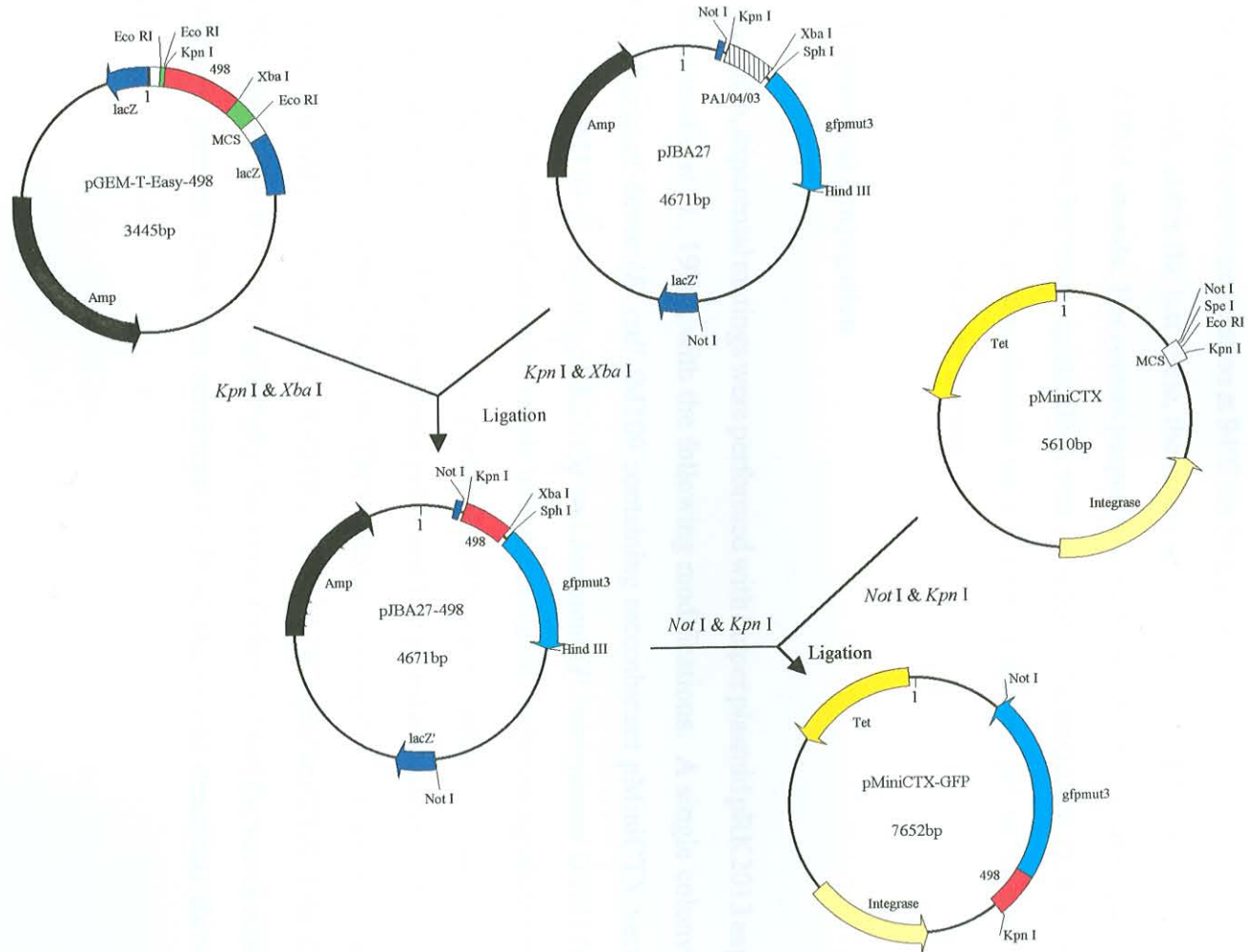
Fig. 3.2   Construction of the recombinant pMiniCTX-498-GFP reporter plasmid.

addition, the PCR reaction mixtures contained $1 \times$ PCR buffer, 1.5 mM $MgCl_2$, 50 pmol each of the EBF and EBR oligonucleotide primers (Table 3.1), each dNTP at a concentration of 0.2 mM and 1 U of Taq DNA polymerase (Promega) in a final reaction volume of 50 µl. The reaction tubes were placed in a GeneAmp 9600 thermal cycler (Perkin Elmer) and following an initial denaturation of 95°C for 2 min, the reactions were subjected to 25 cycles of amplification using the following conditions: denaturation at 94°C for 30 s, annealing at 57°C for 30 s and elongation at 72°C for 1 min. After the last cycle, the reactions were kept at 72°C for 5 min to complete synthesis of all DNA strands. For control purposes, a reaction mixture containing distilled water and all other reagents but no template DNA was included. The amplified PCR products were subsequently analyzed by electrophoresis on a 1% (w/v) agarose gel in the presence of an appropriate DNA molecular weight marker.

3.2.9.3 Triparental conjugation

For conjugation, triparental matings were performed with helper plasmid pRK2013 as previously described (Deretic *et al.*, 1986), with the following modifications. A single colony of freshly streaked cultures of donor (*E. coli* JM109 containing recombinant pMiniCTX vector DNA), helper (*E. coli* JM109 containing pRK2013) and recipient (*P. aeruginosa* DSM1707) strains were mixed on a LB-agar plate with a sterile inoculation needle and then incubated overnight at 37°C. Following incubation, the mixed growth was streaked on selective medium consisting of LB-agar supplemented with 300 µg/ml tetracycline and 100 µg/ml ampicillin, and incubation was continued at 37°C for a further 24 to 48 h. The resulting *P. aeruginosa* recombinant strains were designated DSM1707::pMiniCTX-GFP, DSM1707::pMiniCTX-498-GFP and DSM1707::pMinCTX-IP-GFP, respectively. Successful integration of the vector constructs into *P. aeruginosa* genomic DNA was confirmed by PCR analysis as described above (Section 3.2.9.2).

3.2.10 Microscopic analysis of recombinant and control pMiniCTX-GFP reporter plasmids

3.2.10.1 Preparation of glass slides

Glass slides were prepared by soaking the slides for 1 h in a 10% KOH solution prepared in ethanol after which they were thoroughly washed in distilled water and ethanol. The slides were rinsed in distilled water, air-dried and then coated with gelatin by soaking the slides for 30 min at 70°C in a solution containing 0.1% gelatin and 0.01% $KCr(SO_4)_2$. The slides were used once they were air dry.

3.2.10.2 Fluorescent microscopy

A single colony of freshly-streaked wild-type *P. aeruginosa* DSM1707, DSM1707::pMiniCTX-GFP, DSM1707::pMiniCTX-498-GFP and DSM1707::pMiniCTX-IP-GFP were each inoculated into 100 ml FABG medium. After incubation at 37°C for 7 h, the exponentially growing cultures were inoculated to an OD_{540} of 0.05 into 2 ml FABG medium with or without glass wool (0.05 g; mean diameter 15 μm, Merck) in 28-ml McCartney bottles. The cultures were incubated for 16 h at 37°C with agitation and then prepared for microscopy. For microscopic analysis of planktonic and PGW cells, 5 μl of the cultures were transferred to separate gelatin-coated slides and spread evenly over the surface of the slides. For microscopic analysis of attached (biofilm) cells, sections of the glass wool were carefully transferred to microscope slides using sterile tweezers and pryed apart, taking care not to disrupt the biofilm. Coverslips were mounted onto the glass slides and sealed with Cutex to prevent the samples from drying out. The cells were examined by epifluorescence microscopy using a Zeiss Axiovert 200 fluorescent microscope fitted with a 63x/1.4 Zeiss Neofluor objective and the no. 10 Zeiss filter set (excitation, 470 to 490 nm; emission, 515 to 565 nm; dichroic, 510 nm). The images were captured using a Nikon charge-coupled device (CCD) camera. All samples were analyzed in triplicate and images were captured for at least three separate microscope fields.

3.2.11 Flow chamber experiments

Biofilms were grown at 37°C in flow cells with individual dimensions of 2 × 2 × 15 mm, supplied with FABG medium as described by Heydorn *et al.* (2002). The substratum consisted of a microscope glass coverslip. Exponentially growing cultures of DSM1707::pMiniCTX-498-GFP and DSM1707::pMiniCTX-GFP (200 µl of each) were injected into separate channels, using a 2 ml syringe with a 0.6 × 30 mm gauge needle. After inoculation, the flow channels were left for 1 h at 37°C and the flow was then switched on at 0.44 mm/s using a Watson Marlow 205S peristaltic pump. Random images were acquired at various time intervals after inoculation using an inverted Zeiss Axiovert 200 fluorescent microscope fitted with a 63x/1.4 Zeiss Neofluor objective. The images were captured with a CCD camera (Nikon).

3.3   RESULTS

3.3.1   Construction of pUC19 subclones containing putative IHF binding and σ^{54} promoter sequences

Results of the sequence analysis performed in Chapter 2 indicated that several of the clones displaying up-expression of a *lacZ* reporter gene following attachment to a glass wool substratum contained putative σ^{54} promoter sequences. However, only one of these, clone 498, contained a sequence corresponding to an IHF binding site, in addition to the σ^{54} promoter sequence. Furthermore, the DNA fragment mapped 62 bp upstream of a gene encoding a flagellar hook protein (FlgE). Since structural components, such as flagella, pili and adhesins, have been shown to play an important role in facilitating the adherence of *P. aeruginosa* to surfaces (O'Toole and Kolter, 1998a), clone 498 was selected for further characterization. To investigate, the DNA fragment contained in clone 498 was first cloned into pUC19 which would facilitate subsequent construction of subclones containing the IHF and σ^{54} promoter regions, respectively. The cloning strategy is indicated in Fig. 3.1.

Due to the pALacZsd reporter plasmid being present in low copy numbers in *P. aeruginosa*, the yield of insert DNA following plasmid DNA extraction, restriction enzyme digestion and purification of insert DNA was too low to allow further DNA manipulation steps. Consequently,

a PCR-based approach was adopted whereby a sufficient amount of the insert DNA could be obtained. Using pALacZsd$_{(DSM1707)}$498 (clone 498) as template DNA and oligonucleotide primers JB-1 and JB-2, PCR was carried out as described under Materials and Methods (Section 3.2.2). An aliquot of the reaction mixture was analyzed by agarose gel electrophoresis and a single discreet DNA fragment of approximately 430 bp was observed (Fig. 3.3). This is slightly larger than the expected size of 313 bp due to primer JB-1 annealing in the multiple cloning site (MCS) at the 5' end of the insert DNA, and primer JB-2 annealing 97 bp downstream of the 3' end of the MCS in the *lacZ* gene.

The agarose gel-purified amplicon was subsequently cloned into the pGEM®-T-Easy vector and restriction of the derived recombinant plasmids with *Eco* RI resulted in the excision of a 430 bp DNA fragment, indicating that the amplicon was successfully cloned into the pGEM®-T-Easy vector. Since the recombinant pGEM®-T-Easy vector lacked the appropriate restriction enzyme recognition sites which would allow for the construction of the desired subclones, the insert DNA was recovered by digestion with *Eco* RI and *Xba* I (Fig. 3.4, lane 3) and recloned into similarly prepared pUC19 vector DNA. A number of colonies resulting from the transformation of competent *E. coli* JM109 cells were selected, plasmid DNA extracted and characterized by restriction enzyme digestion. A recombinant plasmid from which an insert of the expected size was excised by *Eco* RI and *Xba* I digestion (Fig. 3.4, lane 5) was selected, designated as pUC498 and used in the construction of the desired subclones.

A single *Bsr* BI site located 170 nt from the 5' end of the full-length 498 insert DNA separates the putative IHF and σ^{54} consensus sequences from each other (Fig. 3.1). However, pUC19 contains three such sites, located at nucleotide positions 496, 737 and 2538. Thus, digestion of pUC498 with *Bsr* BI and *Eco* RI resulted in 5 restriction fragments corresponding in length to 170, 226, 241, 541 and 1801 bp. The 170 bp restriction fragment corresponded to the expected length of the DNA fragment containing the putative IHF binding sequence. Restriction of pUC498 with *Bsr* BI and *Xba* I also resulted in the generation of 5 restriction fragments corresponding in length to 74, 150, 241, 714 and 1801 bp. The DNA fragment of 150 bp corresponded to the expected length of the DNA fragment containing the putative σ^{54} promoter sequence. The respective DNA fragments were therefore excised from the agarose gel, purified by the geneclean method and then ligated into *Sma* I and *Eco* RI and *Sma* I and *Xba* I digested

			1500
			1000
			800
			600
430			500
			400
			300
			200
			100
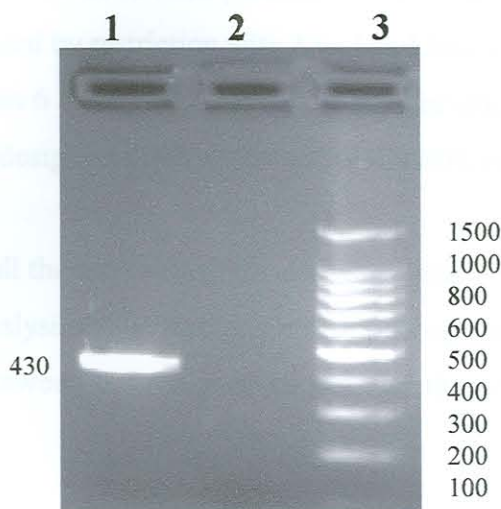
Fig. 3.3   Agarose gel electrophoretic analysis of the DNA product obtained by PCR amplification of the cloned DNA fragment of pALacZsd$_{(DSM1707)}$498. Lane 1, sample of the reaction mixture following the PCR; Lane 2, negative control reaction lacking template DNA. The size of the PCR product (in bp) is indicated to the left of the figure, while the sizes of the 100 bp molecular weight marker (Promega) are indicated to the right of the figure (lane 3).

Fig. 3.4   Agarose gel electrophoretic analysis of recombinant pGEM®-T-Easy and pUC19 plasmids. Lane 1, Molecular weight marker; Lane 2, linear pGEM®-T-Easy vector DNA; Lane 3, plasmid pGEM®-T-Easy-498 restricted with *Eco* RI and *Xba* I; Lane 4, *Eco* RI-linearized pUC19; Lane 5, plasmid pUC498 restricted with *Eco* RI and *Xba* I; Lane 6, plasmid pUC498A restricted with *Kpn* I and *Xba* I; Lane 7, plasmid pUC498B restricted with *Kpn* I and *Xba* I. The sizes of the 100 bp molecular weight marker (Promega) in base pairs are indicated to the left of the figure.

pUC19, respectively. Since both *Bsr* BI and *Sma* I generate blunt ends, the termini are compatible. After transformation, recombinant plasmids were selected from which insert DNA of the expected size were excised by restriction with *Kpn* I and *Xba* I which flanked the cloned DNA fragments (Fig. 3.4, lanes 6 and 7, respectively). The subclones containing the IHF and σ^{54}consensus sequences were designated pUC498A and pUC498B, respectively.

The nucleic acid sequence of all the recombinant clones were determined to verify the integrity of the cloned insert DNA. Analysis of the resulting electropherograms indicated no alterations in the nucleotide sequence between these cloned copies and the original copy of the DNA fragment.

3.3.2 Construction of recombinant pALacZsd$_{(DSM1707)}$498A and pALacZsd$_{(DSM1707)}$498B reporter vectors

To assay the activity of the putative σ^{54} promoter as well as the contribution of the putative IHF binding site towards the promoter activity, the insert DNA from subclones pUC498A and pUC498B was recloned into the pALacZsd reporter vector. The pALacZsd vector (Weyers, 1999) contains a Tet^{r} gene, a MCS upstream of a promoterless *lacZ* reporter gene and transcriptional and translational terminators upstream of the reporter gene to reduce basal levels of reporter gene expression. The broad host range of the vector permits replication of the plasmid in a wide variety of Gram-negative bacteria, including *E. coli* and *P. aeruginosa*. A ribosomal binding site (RBS) was also inserted upstream of the *lacZ* gene to facilitate gene expression in *E. coli* and *P. aeruginosa*. A plasmid map of the vector is indicated in Fig. 3.5a.

To prepare the vector DNA, pALacZsdIP, containing the *lac* promoter from the pBluescript vector, was digested with *Kpn* I and *Xba* I. This resulted in two DNA restriction fragments of approximately 1.0 and 13.3 kbp. The size of these fragments corresponded to the size of the *lac* promoter fragment and the pALacZsd vector, respectively. As the *Kpn* I and *Xba* I sites are located in close proximity to each other in the multiple cloning site of pALacZsd, the use of pALacZsdIP served as a control to verify that the vector DNA had indeed been digested by both restriction enzymes. The DNA fragments containing the putative IHF and σ^{54} promoter sequences were recovered from pUC498A and pUC498B, respectively, using *Kpn* I and *Xba* I

and the appropriate vector and insert DNA fragments were subsequently gel-purified, ligated and then transformed into competent *E. coli* JM109 cells.

Due to the small size of the insert DNA (<200 bp) and the large size of the vector DNA (13.3 kbp), coupled with the fact that pALacZsd is a low copy number plasmid, screening of putative recombinants by means of restriction enzyme digestion proved to be inefficient. Thus, as the DNA fragments were cloned directionally into the reporter plasmid, thereby obviating the need for transcriptional orientation determination by restriction enzyme mapping, recombinant transformants were rather identified by colony PCR analysis. The size of the amplified PCR products (Fig. 3.5b) was 120 bp larger than the size of the cloned DNA fragments due to the annealing sites of the JB-1 and JB-2 primers being located within the vector DNA. As expected, when pALacZsd, containing no insert DNA, was used as a template in a PCR reaction, a 120 bp fragment was amplified (Fig. 3.5b, lane 5). No amplification occurred in the control reaction lacking template DNA.

3.3.3   β-galactosidase activity assays

For β-galactosidase activity assays, the recombinant and control pALacZsd reporter vector constructs were transformed into competent *P. aeruginosa* DSM1707 cells and β-galactosidase expression was assayed by using ONPG as a chromogenic substrate. *P. aeruginosa* DSM1707 was included in these assays as a control to determine the residual β-galactosidase activity in the cells, while *P. aeruginosa* DSM1707 cells transformed with pALacZsdIP, containing the *lac* promoter from pBluescript, served as a positive control. Following overnight incubation of the cultures, planktonic cells 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 attached (biofilm) cells. The planktonic cells from these latter cultures were also recovered and referred to as PGW cells. The planktonic cells grown in the presence of glass wool (PGW) have been reported to differ from planktonic cells grown in the absence of glass wool as well as biofilm cells (Steyn *et al.*, 2001; Oosthuizen *et al.*, 2002). Based on their different proteomic profiles, PGW cells have thus been proposed to represent a phenotypically unique population of cells. Consequently, the activity of the IHF binding site and the σ^{54} promoter element was also assayed, for comparative purposes, in PGW cells. All assays were performed in triplicate and on at least two separate
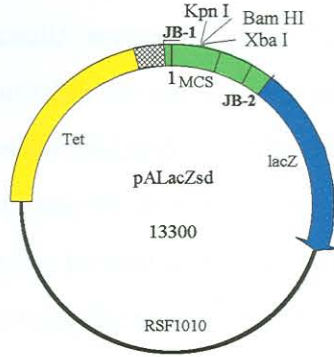
Fig. 3.5a   Plasmid map of pALacZsd, indicating the annealing sites of primers JB-1 and JB-2.
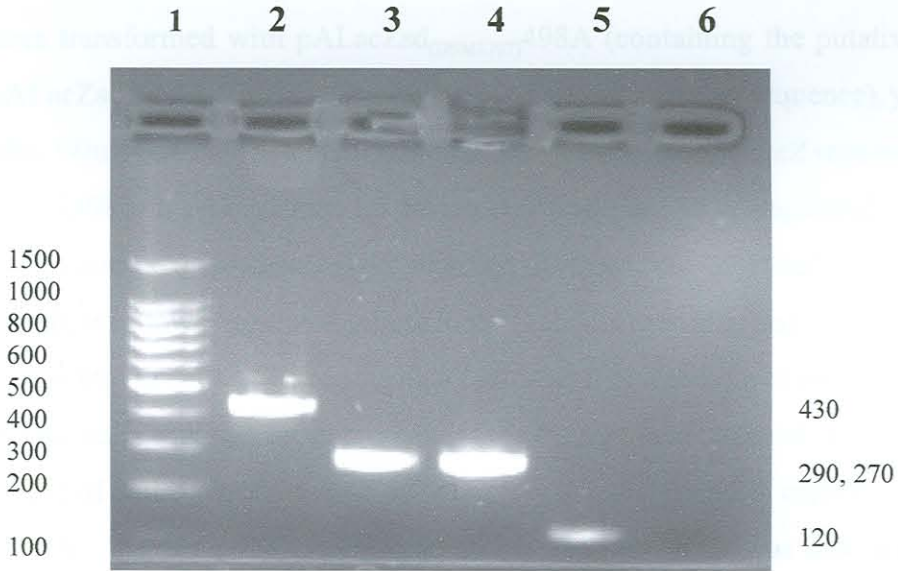
Fig. 3.5b   Agarose gel electrophoresis indicating amplification of the cloned DNA fragments in the recombinant pALacZsd reporter vector constructs. Lane 1, Molecular weight marker; Lane 2, amplified insert DNA from pALacZsd$_{(DSM1707)}$498; Lane 3, amplified insert DNA from pALacZsd$_{(DSM1707)}$498A; Lane 4, amplified insert DNA from pALacZsd$_{(DSM1707)}$498B; Lane 5, amplified product from pALacZsd. The control reaction (Lane 6) contained no DNA template. The sizes of a 100-bp molecular weight marker (Promega) are indicated to the left of the figure, while the sizes of the PCR amplicons (in bp) are indicated to the right of the figure.

occasions. The results were analyzed as described in Materials and Methods (Section 3.2.8.5) and are presented in Fig. 3.6.

Analysis of the results obtained for *P. aeruginosa* DSM1707 cells indicated a lack of β-galactosidase expression. These results served to confirm that *P. aeruginosa* is a β-galactosidase-negative host and thus suitable for use with the pALacZsd reporter vector. Cells containing the promoterless control vector pALacZsd displayed a low level of β-galactosidase activity. This low basal level of activity may be the result of slight transcriptional read-through from the tetracycline resistance (Tet^r) gene located at the 5' end of the cloned promoter fragment (Fig. 3.5a). As expected, the cells containing the positive control vector, pALacZsdIP, displayed high levels of β-galactosidase activity under all the growth conditions investigated.

Comparison of the results obtained for cultures transformed with the pALacZsd$_{(DSM1707)}$498 reporter construct (containing both the putative IHF binding and σ^{54} promoter sequences) to those obtained for cultures transformed with pALacZsd$_{(DSM1707)}$498A (containing the putative IHF binding site) and pALacZsd$_{(DSM1707)}$498B (containing the putative σ^{54} promoter sequence), yielded the following results. When compared to planktonic cells, expression of the *lacZ* reporter gene from pALacZsd$_{(DSM1707)}$498 was up-regulated 1.5-fold in biofilm cells, and up-regulated 1.2-fold in PGW cells. By contrast, reporter gene expression from the IHF-deficient clone, pALacZsd$_{(DSM1707)}$498B, was down-regulated in both PGW (1.2-fold) and attached cells (5.3-fold) when compared to planktonic cells. The results also indicated that although expression of the *lacZ* gene in planktonic cells was similar, expression of the *lacZ* gene in attached cells was 7.5-fold higher in the case of cells containing pALacZsd$_{(DSM1707)}$498 compared to cells containing pALacZsd$_{(DSM1707)}$498B. As expected, expression of the reporter gene was low for cells containing pALacZsd$_{(DSM1707)}$498A.

Based on the above results, it was concluded that the putative σ^{54} promoter displays up-expression of the *lacZ* reporter gene following attachment of *P. aeruginosa* cells to a surface and that the IHF binding site is critical for optimal expression from this promoter.

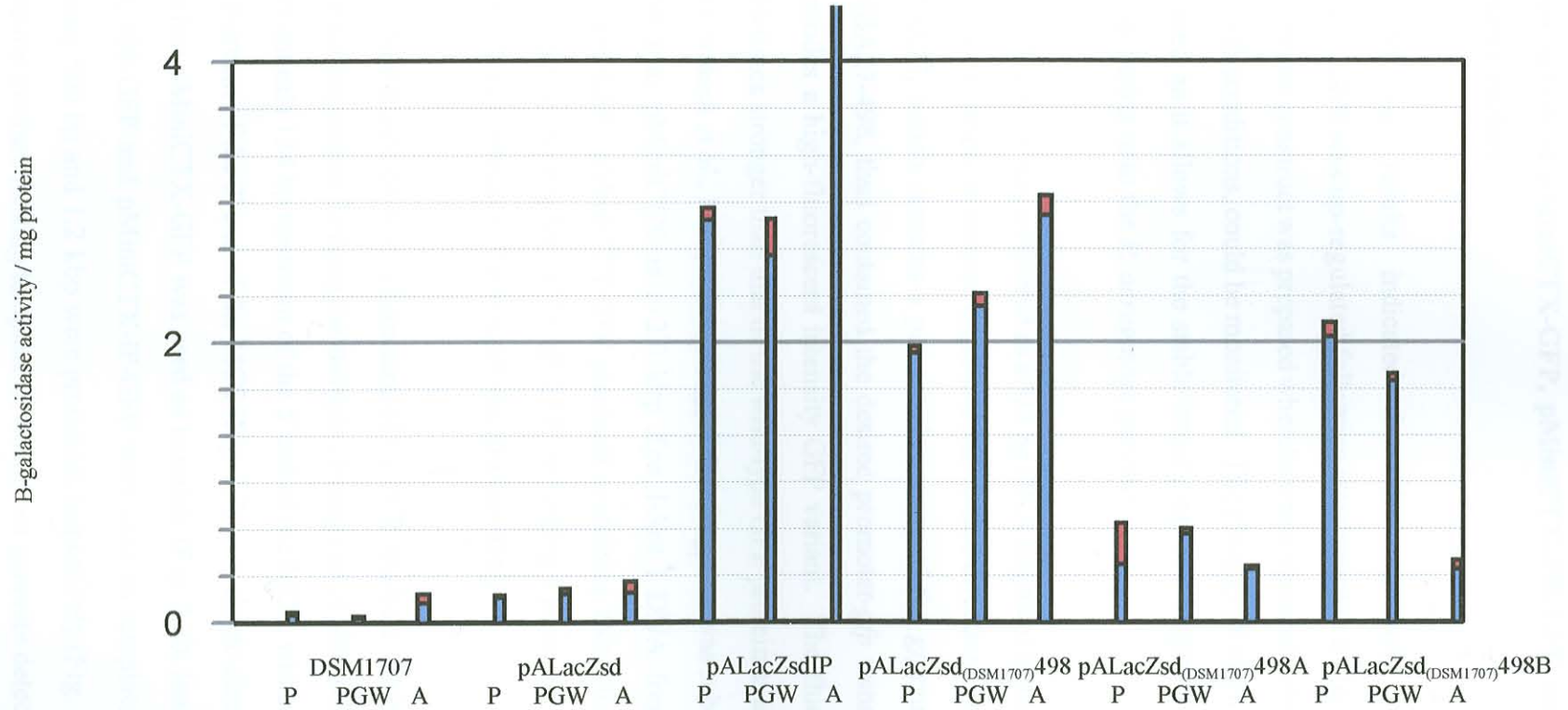Fig. 3.6   Graph displaying the β-galactosidase activity per total cellular biomass (in Miller units) of the different promoter-*lacZ* constructs in planktonic (P) cells, planktonic sells grown in the presence of glass wool (PGW) and attached (A) cells. pALacZsdIP was used as a positive control vector, whereas pALacZsd was used as a negative control vector. Standard deviations are indicated by the red bars.

3.3.4 Construction of pMiniCTX-GFP, pMiniCTX-498-GFP and pMiniCTX-IP-GFP reporter vectors

Since the previous results indicated that the regulatory element contained in pALacZsd$_{(DSM1707)}$498 was up-regulated following attachment to a glass wool substratum (Fig. 3.6), a GFP reporter construct was prepared whereby transcriptional activity in single cells, under different growth conditions, could be monitored. The pMiniCTX cloning system (Hoang *et al.*, 2000) was used as it allows for the stable integration of a single copy of the promoter-*gfp* transcriptional fusion onto the *P. aeruginosa* genome.

The 498 DNA fragment was recovered as a 313 bp DNA fragment from pGEM®-T-Easy-498 by *Kpn* I and *Xba* I restriction enzyme digestion and cloned directionally into similarly prepared plasmid pJBA27, which contains a promoterless copy of the *gfp*mut3 gene. The resultant plasmid, pJBA27-498, thus contained the desired promoter-*gfp* transcriptional fusion. The *gfp*mut3 encodes a high-fluorescent intensity GFP variant. The fluorescence signal of this mutant is 20-times stronger than that of the wild-type GFP protein at an excitation wavelength of 488 nm (Cormack *et al.*, 1996). The 498-*gfp* DNA fragment was subsequently recloned from pJBA27-498 into pMiniCTX as a 2.0 kbp *Kpn* I-*Not* I DNA fragment to yield plasmid pMiniCTX-498-GFP. pMiniCTX-GFP plasmids containing either the *lac* promoter obtained from plasmid pBluescript (pMiniCTX-IP-GFP) or lacking a promoter (pMiniCTX-GFP) were used as controls in subsequent fluorescent microscopy analysis.

The recombinant plasmids were characterized by PCR analysis. Primers EBF and EBR that anneal to vector sequences flanking the multiple cloning site of pMiniCTX-GFP were used. The EBF primer anneals 134 bp upstream of the 5' end of the MCS, while the EBR primer anneals 37 bp downstream of the 3' end of the MCS (Fig. 3.7a). A single discreet band of 250 bp was observed when pMiniCTX-GFP was used as template (Fig. 3.7b, lane 2). In the cases where pMiniCTX-498-GFP and pMiniCTX-IP-GFP were used as templates, expected products of approximately 500 bp and 1.2 kbp were produced, respectively (Fig. 3.7, lanes 3 and 4). The control reaction mixture lacking template DNA did not generate detectable product.
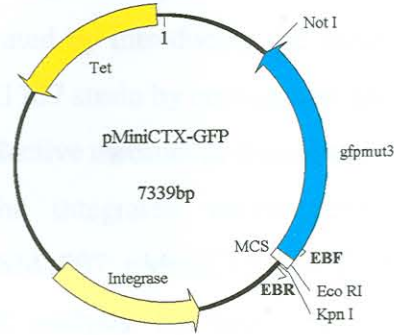
Fig. 3.7a   Plasmid map of pMiniCTX-GFP, indicating the annealing sites of primers EBF and EBR.

E. coli   *P. aeruginosa*
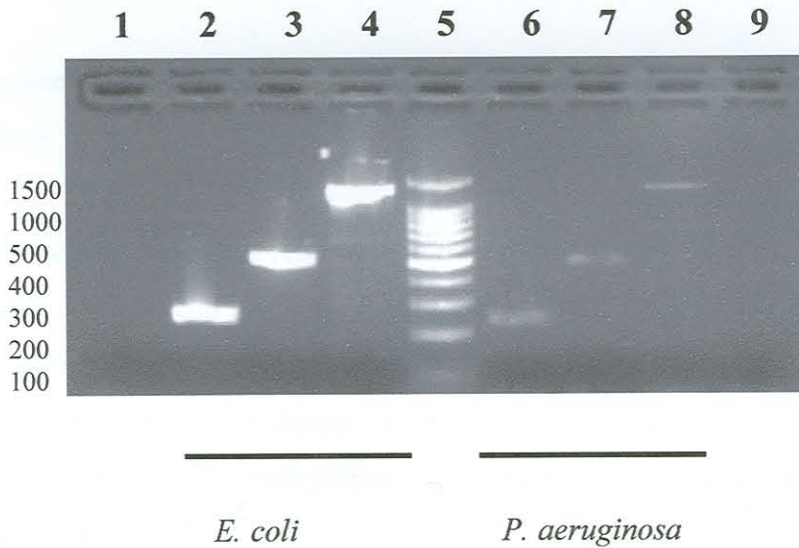
Fig. 3.7b   Agarose gel electrophoresis indicating amplification of the cloned DNA fragments in the recombinant pMiniCTX-GFP reporter vector constructs in *E. coli* (Lanes 2, 3 and 4), and following integration of the recombinant vectors onto the *P. aeruginosa* genome (Lanes 6, 7 and 8). Lanes 1 and 9, control reactions lacking template DNA; Lanes 2 and 6, amplified insert DNA from pMiniCTX-GFP in *E. coli* and DSM1707::pMiniCTX-GFP, respectively; Lanes 3 and 7, amplified insert DNA from pMiniCTX-498-GFP in *E. coli* and DSM1707::pMiniCTX-498-GFP, respectively; Lanes 4 and 8, amplified insert DNA from pMiniCTX-IP-GFP in *E. coli* and DSM1707::pMiniCTX-IP-GFP, respectively; Lane 5; Molecular weight marker. The sizes of a 100-bp molecular weight marker (Promega) are indicated to the left of the figure.

3.3.5 Generation and characterization of *P. aeruginosa* DSM1707 strains containing integrated copies of pMiniCTX-GFP, pMiniCTX-498-GFP and pMiniCTX-IP-GFP

*P. aeruginosa* strains harboring integrated copies of pMiniCTX-GFP, pMiniCTX-498-GFP and pMiniCTX-IP-GFP were generated by introducing the recombinant and control pMiniCTX vectors into the wild-type DSM1707 strain by conjugation and selecting for subsequent single recombination events using a selective medium as described in Materials and Methods (Section 3.3.4). The presence of the integrated vector constructs in the resultant strains DSM1707::pMiniCTX-GFP, DSM1707::pMiniCTX-498-GFP and DSM1707::pMiniCTX-IP-GFP, were confirmed by PCR analysis. By making use of primers EBF and EBR and chromosomal DNA as template to amplify the region spanning the MCS, amplicons of the expected sizes were obtained (Fig. 3.7b; lanes 6 to 8). No similar bands were observed in control reactions containing chromosomal DNA of wild-type *P. aeruginosa* DSM1707 or reactions lacking template DNA (Fig. 3.7b, lane 9).

3.3.6 Fluorescence microscopy of *P. aeruginosa* strains grown in the presence or absence of a surface

To investigate attachment-dependent regulation of the *flgE* regulatory element, contained in the 498 DNA fragment, at single cell level, the *P. aeruginosa* DSM1707, DSM1707::pMiniCTX-GFP, DSM1707::pMiniCTX-498-GFP and DSM1707::pMiniCTX-IP-GFP strains were cultured in FABG medium in the presence or absence of glass wool. Following incubation, samples of planktonic, PGW and biofilm cells were prepared for fluorescent microscopy and the results are indicated in Figures 3.8 to 3.11.

No fluorescence was observed for *P. aeruginosa* DSM1707, DSM1707::pMiniCTX-GFP and DSM1707::pMiniCTX-498-GFP when planktonic cells grown in the absence or presence of glass wool (PGW) were analyzed. This was despite numerous cells being visible under light microscopy (Fig. 3.8 [B,D]; Fig. 3.9 [B,D]; Fig. 3.10 [B,D], respectively). Although biofilms could be clearly seen 16 h after incubation, *P. aeruginosa* DSM1707 and DSM1707::pMiniCTX-GFP displayed a very low level of fluorescence in attached cells (Fig. 3.8 [E]; Fig. 3.9 [E], respectively). This may be due to autofluorescence being higher in biofilm cells than their
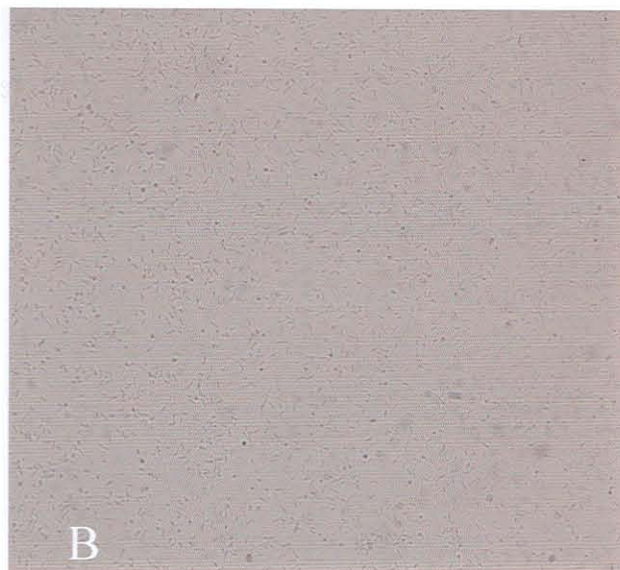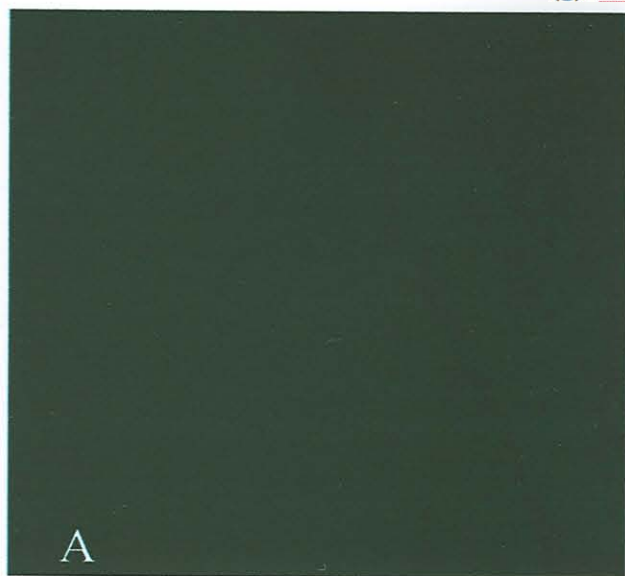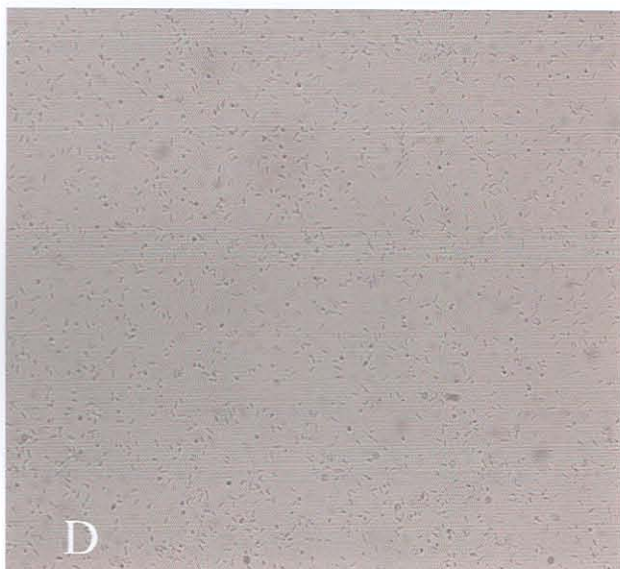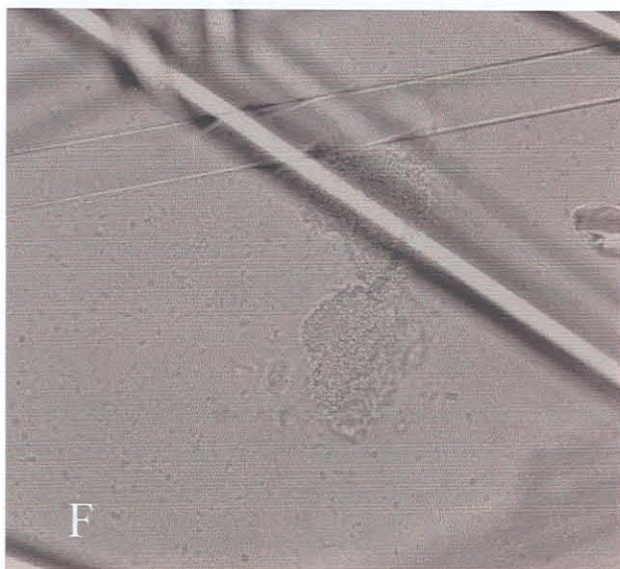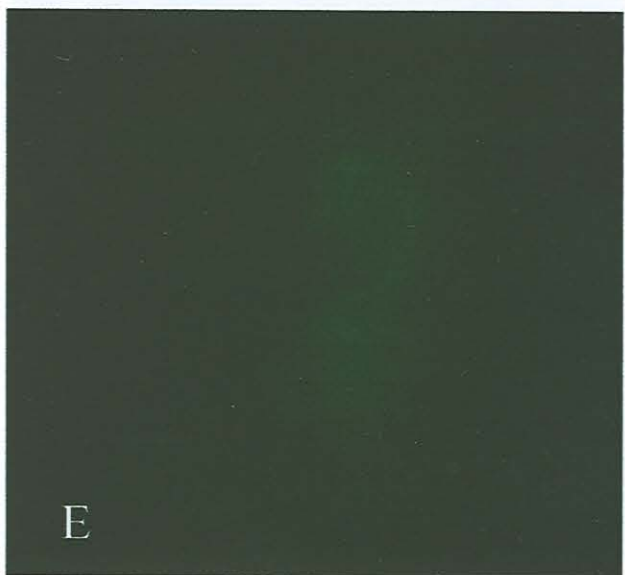
Fig. 3.8   Fluorescent (A, C, E) and light (B, D, F) photomicrographs of a 16 h *P. aeruginosa* DSM1707 culture. Magnification X630.

Fig. 3.9   Fluorescent (A, C, E) and light (B, D, F) photomicrographs of a 16 h
            DSM1707::pMiniCTX-GFP culture. Magnification X630.

Fig. 3.10   Fluorescent (A, C, E) and light (B, D, F) photomicrographs of a 16 h DSM1707::pMiniCTX-498-GFP culture. Magnification X630.

Fig. 3.11   Fluorescent (A, C, E) and light (B, D, F) photomicrographs of a 16 h DSM1707::pMiniCTX-IP-GFP culture. Magnification X630.

planktonic counterparts due to local depletion of the added iron. By contrast, DSM1707::pMiniCTX-IP-GFP fluoresced brightly in planktonic, PGW and attached cells (Fig. 3.11 [A,C,E]). This was not unexpected, since the *lac* promoter is a strong constitutive promoter. Expression of GFP fluorescence in single cells of DSM1707::pMiniCTX-498-GFP in the attached state (Fig. 3.10 [E]) was similar to that of DSM1707::pMiniCTX-IP-GFP. This result is in agreement with the results obtained by ONPG analysis, indicating that the promoter in the 498 DNA fragment is up-regulated by attachment to a surface. However, not all cells fluoresced intensely, suggesting that the *flgE* gene may be heterogeneously expressed in biofilms.

3.3.7 Flow chamber experiment

In order to study transcriptional activity of the *flgE* promoter during biofilm development in a flow through system on a glass coverslip as substratum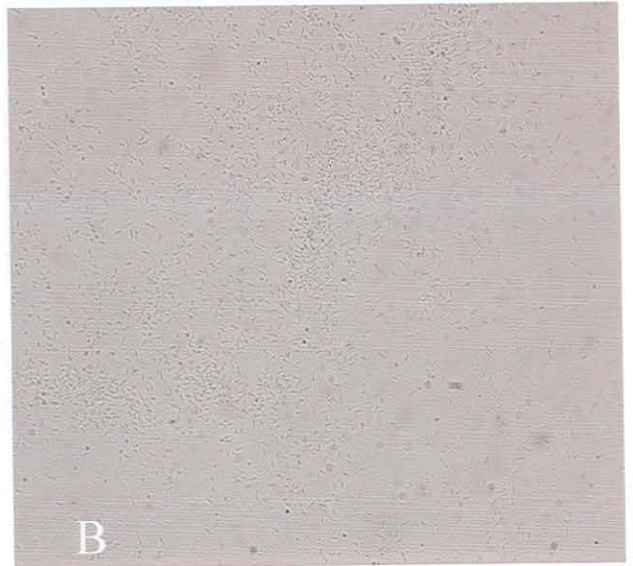, DSM1707::pMiniCTX-498-GFP was inoculated into a flow chamber and *gfp* expression monitored by epifluorescence microscopy over time. Two hours after inoculation, single cells could be seen attaching to the surface (Fig. 3.12 [B]). Not all of the cells fluoresced, but those that did, fluoresced brightly. These cells were either attached or planktonic in the presence of glass. After 4 h more cells fluoresced, though at a lower level, while at 6 h after inoculation, microcolony formation started and a pattern of fluorescence similar to 4 h was observed (results not shown). After 16 h, microcolonies with water channels were visible (Fig. 3.12 [D]) and fluorescence were mainly due to single, bright cells being present around the microcolonies (Fig. 3.12 [C]). Thicker microcolonies with water channels were observed 24 and 48 h after inoculation and these showed similar fluorescence patterns as those observed at 16 h (Fig. 3.12 [E,F,G,H]). Structured biofilms formed within 72 h, became more dense after 96 h, and these were thick, complex biofilm structures after 144 h (Fig. 3.12 [I to P]). With respect to the fluorescence pattern, although an increase in fluorescence was observed after 72 h, the fluorescence was heterogeneous with some cells and cell clusters in the biofilms fluorescing brighter than the rest of the cells.

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Fig. 3.12   Fluorescent (A, C, E, G) and light (B, D, F, H) photomicrographs of biofilm development of *P. aeruginosa* DSM1707::pMiniCTX-498-GFP on glass in a flow chamber at 2 h (A,B), 16 h (C, D), 24 h (E, F) and 48 h (G, H). Size bar depicts 10 µm.
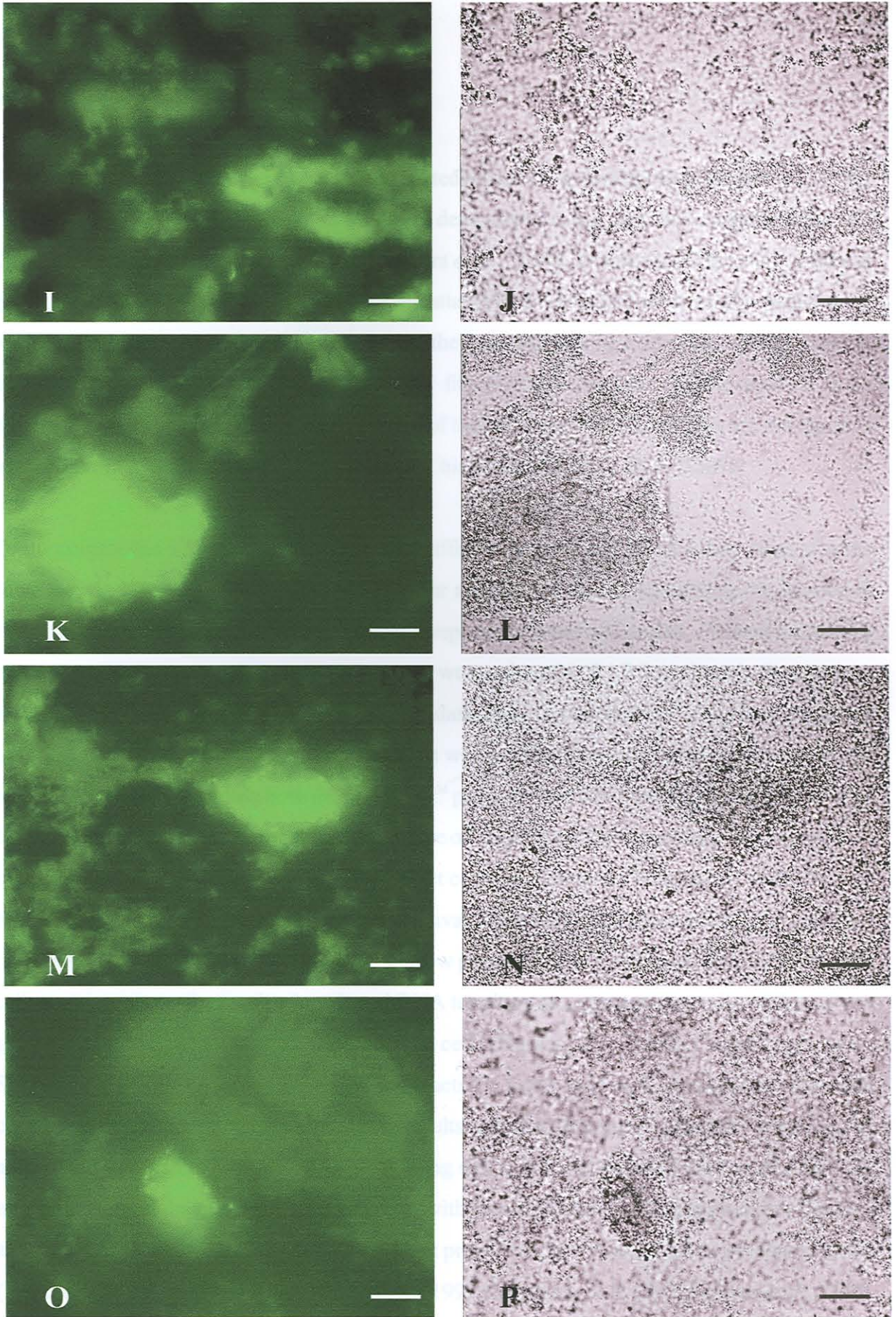
Fig. 3.12 (continued) Fluorescent (I, K, M, O) and light (J, L, N, P) photomicrographs of biofilm development of *P. aeruginosa* DSM1707::pMiniCTX-498-GFP on glass in a flow chamber at 72 h (I, J), 96 h (K, L), 120 h (M, N) and 144 h (O, P). Size bar depict 10 μm.

3.4   DISCUSSION

Transcription of several genes that are up-regulated in biofilm-growing cells (Davies *et al.*, 1993; O'Toole *et al.*, 1998) has been reported to be dependent on the alternative sigma factor, σ^{54} (Zielinski *et al.*, 1992; Boucher *et al.*, 2000; Arora *et al.*, 1997). In this part of the study, reporter gene technology was used to characterize the attachment-dependent activity of a putative σ^{54} promoter which mapped to a region upstream of the *P. aeruginosa flgE* gene, encoding a flagellar hook protein (Chapter 2). Flagella and other fimbrial structures have been reported to be required during the initial stages of attachment of the cells to a surface, but they are not reported to be required in well-developed *P. aeruginosa* biofilms (Whiteley *et al.*, 2001).

To investigate the σ^{54} promoter activity under different growth conditions and to determine the contribution of an IHF binding site to promoter activity, subclones containing the respective elements were prepared in the broad-host-range reporter plasmid pALacZsd. *P. aeruginosa* cells transformed with the respective reporter vectors were cultured in FABG medium in the presence and absence of glass wool. Using a β-galactosidase assay, up-expression of the *lacZ* gene was observed upon attachment of the cells to a glass wool substratum, but the up-regulation for the reporter construct containing both the putative σ^{54} promoter and IHF binding site was higher than that for the construct containing only the putative σ^{54} promoter. The observed promoter activity, in planktonic and PGW cells, from the construct containing only the putative σ^{54} promoter may have been due to non-specific binding of the activator proteins to the supercoiled DNA (Brahms *et al.*, 1995; Revet *et al.*, 1995). However, 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). Low β-galactosidase activity was observed for the reporter construct containing the IHF binding site only. These results indicated that the σ^{54} promoter is induced by attachment to a surface and that the IHF binding site is in some way involved in this biofilm-associated up-regulation. This is in agreement with previous studies which have shown that IHF aids in transcription from several σ^{54}-dependent promoters, but does not act as a promoter itself (Bertoni *et al.*, 1998; Carmona and Magasanik, 1996; Carmona *et al.*, 1997; Hoover *et al.*, 1990).

Although the data obtained from the *in vitro* β-galactosidase assays yielded useful results, it does,

however, not allow for detailed studies regarding promoter activity at a single cell level. Given the stability of the β-galactosidase enzyme, the observed up-regulation may have been due to up-expression of the reporter molecule at any given stage(s) of biofilm development. Consequently, recombinant pMiniCTX-GFP reporter vectors were constructed which contained DNA fragments containing the IHF binding site and/or the σ⁵⁴ promoter sequence as well as the *lac* promoter as a positive control and promoterless *gfp* gene as a negative control. The use of *gfp* as a reporter of promoter activity allowed for studying single cells in a nondestructive manner and without the addition of exogenous substrates or energy sources (Bloemberg *et al.*, 1997). In addition, GFP-marked cells could be visualized by standard epifluorescence microscopy.

P. aeruginosa strains containing integrated copies of the respective *gfp* fusion constructs were subsequently cultured in medium in the presence and absence of glass wool. Samples of the planktonic, PGW and biofilm cells were analyzed by fluorescence microscopy. Initial culturing of the strains in LB-broth resulted in high background fluorescence for *P. aeruginosa* DSM1707 (data not shown). This may have been due to *P. aeruginosa* DSM1707 producing siderophores under iron-limiting conditions (Reimann *et al.*, 2001). Addition of ferrous sulphate to the growth medium resulted in decreased autofluorescence, however, it did not reduce autofluorescence to such a level that clear differences between the different strains and growth conditions could be observed. However, growth of the cultures in FABG medium (Heydorn *et al.*, 2002) reduced the autofluorescence of *P. aeruginosa* and clear differences could be observed for the up-regulation of the reporter molecule upon attachment. Up-expression of the reporter molecule was observed for 498-*gfp* fusions in biofilm cells compared to planktonic and PGW cells, indicating that the promoter is up-regulated upon attachment to a glass wool substratum at a single cell level (Fig. 3.10).

It is well known that biofilms are heterogeneous, which consequently limits the study of transcriptional regulation of a specific gene. Thus, assaying the whole population for reporter activity may not reveal the heterogeneity in a subset of that population. To overcome this limitation, a flow cell system with GFP as a reporter molecule was used to investigate the activity of the regulatory element that mapped upstream of the *flgE* gene on the *Pseudomonas* genome. Biofilm formation is a dynamic process consisting of cells attaching to a surface, forming microcolonies by growth and division and the addition of more single cells, which eventually

differentiates into well-developed three-dimensional pillar-like structures (biofilms) interspersed with water channels (Costerton *et al.*, 1995). Cells that detach from these biofilms can attach at another location and initiate the formation of a new biofilm. The fluorescence pattern observed at various time intervals of biofilm development (Fig. 3.12) indicated that some single cells fluoresced as early as 2 h. This indicates a role for flagella in the early attachment or transport of these cells to the surface. The GFP variant used in this study was the stable version with a long half-life (Tombolini *et al.*, 1997), thereby resulting in cells fluorescing long after initiation and termination of transcription of *flgE*. Consequently, the lower fluorescence observed in cell clusters at later time intervals (6 h to 48 h) could have been due to division of the cells. However, the increase in fluorescence in cell clusters after three days of incubation may be due to a signal for detachment of the cluster from the biofilm, a process that would require flagella.

Based on the results obtained during the course of this study, the following model for the role of *flgE* in biofilm development is proposed. FlgE has been reported to be part of the hook-basal body structure (HBB) of the flagellum, of which the completion is a control checkpoint in the synthesis of flagella. An incomplete HBB results in complete inhibition of expression of the late flagellar genes (Karlinsey *et al.*, 2000; Mullin *et al.*, 2001). Since FlgE forms an integral part of the HBB, and a complete HBB is needed for the synthesis of flagella, up-expression of *flgE* will lead to up-expression of the late flagellar genes and therefore complete synthesis of flagella. Flagella have previously been shown to be important in the initial attachment of bacteria to a surface, presumably due to transport of the cells to the surface (O'Toole and Kolter, 1998a; 1998b; Watnick *et al.*, 1999; Pratt and Kolter, 1998). However, they seem to be dispensable in microcolonies and fluorescence in cells visualized in this phase may be due to newly attached cells. Once the biofilm is well-developed, or the localized environmental conditions become unfavourable for growth in the biofilm, expression of *flgE* in pockets/cell clusters in the biofilm may once again favour the formation of flagella and the cells or cell clusters may swim to the planktonic phase or to a new niche for attachment. The signals responsible for this response are still unclear, but may be related to quorum sensing. Thus, the results obtained in this part of the study indicate a role for flagella not only in the initial attachment of the bacteria to the substrate, but also in the detachment of bacteria from the biofilm.

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.

CONGRESS CONTRIBUTIONS DURING THE COURSE OF THIS STUDY

1. Local conference presentations

1.1   Meiring, M.S., J.B. Weyers, V.S. Brözel and J. Theron. Comparative sequence analysis of biofilm-specific regulatory elements in *P. aeruginosa* PAO1. BIO-Y2K Combined Millenium Meeting, Grahamstown, 23-28 January 2000.

1.2   Meiring, M.S., J.B. Weyers, V.S. Brözel and J. Theron. Genetic characterisation of biofilm-specific regulatory elements in *P. aeruginosa* PAO1. Seventeenth Congress of the South African Genetics Society, Pretoria, 27-30 June 2000.

2. International conference presentations

2.1   Meiring, M.S., V.S. Brözel and J. Theron. Genetic characterisation of biofilm-specific regulatory elements in *P. aeruginosa* PAO1. Biofilms 2000, Big Sky, Montana, 16-20 July 2000.

2.2   Mac Donald, R., M.S. Meiring, J. Theron and V.S. Brözel. The role of the alternative sigma factors RpoS and RpoN on the formation of *P. aeruginosa* biofilms. Biofilms 2000, Big Sky, Montana, 16-20 July 2000.

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1    GATCTCGCGT TCGCGATGGC TCAGGCAGAC CGGGTTGGAC ATCAGCATCG GATGCTCCAG
61   GTCGGTCAGC TTCTGGGTCA GCAACTCGAT CATGCAACGC AGCCGCAGGC GGATTTCCTC
121  GCGCTCGAAG CTGGAGATGT TCTGCTGGTC GCGCGCCACG GAAAGCACGC TGAGCAAATT
181  GTTCGGCGCG CGGATCACCG CGGTATAGCC GGCGGCCTTG GCCATCTGGA TGGCCTCCAG
241  GGTCTCGGTG AGCGAACCGA TC
```
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61   GATGTGCACG CTGAAGATCA CCATGTAGTC CACCAAGCCG TGGTTGGACT CGGTGAAATA
121  CTTCTGTACC TCGTTGACCT TCTGGCTCTC GTAGTTCGAC CAGGTGTTCT CGGTGCCATT
181  CACCACGCCG GTCTGCAAGC CCTGGTAGAC CTCGGCGAAG GACATCTTCC GGAGATC
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Nucleotide sequence of pALacZsd(DSM1707)267

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1    GATCTCGTCC ATGCTCACGC CAAGGGTCTC CGCCTTGCGC CGGTCGATGT CCAGGCGGAT
61   C
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Nucleotide sequence of pALacZsd(DSM1707)278

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61   GGTGAAGGCC GCCATCGAAG GGCGCGAGAT GCAGGTGGAG GACGTGGTGC GGATC
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Nucleotide sequence of pALacZsd(DSM1707)457

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121  AGCATTGTCG GAACAGCCTC TGGAGACGGG AAGGTGTGGG GGCCGGCCGG GCCGGCCCGC
181  AGAGGCCGTG CATTCTACCG TAAAGCACCG TCCGGCGGCA CCCGCGCCGA TGGCTATCGC
241  ATAGCCCTCC ATCGGATCGC CGCGCGCAAT CGCGCGTTGC GTTCCTTCAT CGGCCAGGGC
301  TACTACAACT GCCACACCCC GGCACCGATC
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Nucleotide sequence of pALacZsd(DSM1707)461

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1    GATCATTTGG CATCTTTGGC TTGATTCGTC AAAAGCAATG AAAAATCGGC TAATACGACG
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121  TGACCGCCAG GCATCTCCTA TACTCAGGCT CCGCTCCCTG CGCAGCCTCT CTGGCAAGGT
181  CACTTCATGA ACAAGAAAAA CCGCCACCCC GCCGACGGCA AGCAGCCCAT CACCATCTTT
241  GGCCCGGACT TCCCCTTCGC CTTCGACGAC TGGCTGGAAC ACCCCGCAGG CCTGGGCAGC
301  ATCCCAGCAG ATC
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Nucleotide sequence of pALacZsd(DSM1707)498

```
1    GATCAGGCGC GGCATGACCA GCTCGGCGGT CTCGAAGGTG GTGAAGCGTT CGCCGCAGGC
61   CAGGCACTCG CGGCGCCGGC GCACCTGATC
```
Nucleotide sequence of pALacZsd(DSM1707)560

```
1    GATCTGCTCC ACTTCCAGCA GCAGTTCGCC CAGCGGCGGA TAGTTGAAGT CCCGCTCGAG
61   CAGGGTCGGG CGCACGCCGA AGCGACGATA GGCGGCTCCG AGCAAGGCCC AGACGCCGGG
121  CTTCACGGCG GCACCATGGG TGTCGATC
```
Nucleotide sequence of pALacZsd(DSM1707)561

```
1     GATCCTGTTC TCGCTGTGCT CGGCGCTGTC CGGGCTGGTC GGCGGCTTCC TCAGCCTGCT
61    GCTGTTCCGC GGCATCATGG GCCTGGCCGA GGGGCCGATC TGCGCCTTGC CTTGCAGGCC
121   GGTGGCCAGG CCGACGTCGA CGTTGTTGTT CTCGTAGGCG CCGATC
```
Nucleotide sequence of pALacZsd(DSM1707)569

```
1     GATCATCGTC GACGAAGCCC ACCGGCGCGG CATCGAGGTG CGCGTCGACG ACGCCGAGGG
61    CGGCCTGTTC ACCCTGACCC AGGGCGGCCG GCAGATC
```
Nucleotide sequence of pALacZsd(DSM1707)570

```
1     GATCGCACCG CAGTTCGTAA AACCCTACGT CAAGGGTGAC AAGCACGATG CACACGACGC
61    CGAGGCGATC
```
Nucleotide sequence of pALacZsd(DSM1707)614

```
1     GAGCGTTTCG CCCGCTACAC CCGCATCAGC ATGTTCAACC TGCTGCGCCG TTCGGCCGAC
61    GTCGCGGTCG GCGGCGTGCA GGTGATGAAG TTCGGGGAGT ACGTGCACTA GCTGTACGTT
121   CCCACCAGCC TCAACCTGGT GAAGATGAAG CCGCTGCACT GGCGCCGGGG GGGTCGATC
```
Nucleotide sequence of pALacZsd(DSM1707)633

```
1     CCGGCCTGCA AGGCCGGCCG GCCATTGGCG TAGCTCAAGC CACGGCGCGC GACCTGCTCG
61    GCCTGGGCCG CATCGCCCTG GGCCAGGCGC ACCTGGGCCA GGCGATAGAG CACCTGCGGC
121   TCGCGCGGAG CGATGCGCTG GGCTCGCTCG AGGCTGGCGG CGGCGCTGTT CAGGTCGCCG
181   CTGCCCTGCT GTTGCTGGGC GGTTGTGAGC ATCGCCAGGA CCGGCCCGTC CAACTGCTCG
241   TCGGCCGCCA GGCTGCCGGC CGAGCCGGAG GCCGGAATGC CGGTGGGCGC GGAACGACTG
301   GCGCCGCTGG GCGGCATGTT GTAGCTGCCA CCCAGCGGTG GCGTGGAGGC GCTGCTGGGC
361   GGAGCCTGGT ACTGGGTGCC GGTGCCGAGC GGAGCGCTGC TGATCGGCGC GGCGCCGGAC
421   TGCGCCGGGA AAGTCTGGAT C
```
Nucleotide sequence of pALacZsd(DSM1707)699

```
1     CTCAGCGAGG AGTGCGGACG CCAGGTGCTG GCGGTGGTCG GCGATAGCGA CAGCCTGGTG
61    GAGCGCCTGG CCGACCTGCC GCCGGAAAGC ATCCTGATCA CCGAGCTGGG CTTGCCTGGC
121   CAGCGCAGCC GCGACGGTAT CCACCTGGTG GAATGGCTGA CCCGGCACTG CCCGCAAATG
181   AAGGTGATGG TCTACTCGGT GTTCAGCGCG CCGTTGCTGG CCAAGGCGGT CCTGCGCAGC
241   GGCGCCTCGG CCTACATCAG CAAGCGCAGC CCGCTGGAGA CGTTGAAGGC GGCGCTGGAG
301   TGCATGGCGC TCGGACAGAC CTTCCTCGAC CCCGGCCTGC ACCCGCAGCG GCATACCGGC
361   AAGCCGCTGA GCCCCACCGA GGTGGACATC CTCCGGCGCC TGGCGCGTGG CGACACGGTC
421   AGCGAGATC
```
Nucleotide sequence of pALacZsd(DSM1707)703

```
1     GATCCGTGGC GCCGCCGAGT TGCTCGACGG CGAGATGCCG GCCGAGCAGC GGGCGCGCTT
61    CGTCGGCAAC ATCGCCGGCG AGAGCGAGCG CCTGCAACAA CTGATC
```
Nucleotide sequence of pALacZsd(DSM1707)707

```
1     GATCGTCGAT ATCGTTGCCG GCGACTATGT GACGCGGAAC TACCAGGCGG CGGCGATGAA
61    CGGACGGATC GTGCAGATCG GCGTGATCAA GGGCAAGGCC GCCGAAGTCG ACCTGTTCCC
121   GATGCTGAGC AAGCGCCTGG TCCATCTTGG CTCGACCCTG CGCTCGCGGA GCCACGACGA
181   GAAAGGCGCG ATC
```
Nucleotide sequence of pALacZsd(DSM1707)877

```
1      TGCTGGCGGC TCATGGCCTG GCGCCGCATC AGCGTGTAGG CCTCCTCCTC GCCGCAATGC
61     TTCATCTTCA TCAGCAGGCC CTTGGCCAGC TCGATGCGCT TGCGCTCGGC CAGCTGCGCC
121    TCGCGCGCCT GCAGTTGAGC GCGCAGCGCC TGGTCGCTCT CGAAGCGCGC CATGGCGACG
181    TCGAGGATCG GCTCGGCGCG AGGGTCCTGG GCGCGCTCGA CAAGGGTGCG GAAGGCATTC
241    TCCACCACCC AGCGATCGAT CACCCGGTCG AAGCCCAGGC GTGCGGCGAA CTCCACCATC
301    TCGCGGCAGA CCGCCTCGGT GGTAGCGGCC TTGCGCAGCC GGGCGACGCT GTCGAAGAGA
361    AGGCTGGCGT AGTCGGTCAT GGCGCGGGCA TTCCTGGCTT TTTCCGCATG ATCC
```
Nucleotide sequence of pALacZsd$_{(DSM1707)}$889

```
1      ATGCGACCGG CCAGGGCGCT GGCCAGGGTG CAGCCGGAGC CATGGTAGCT GCCGGGCAGG
61     CGCTGGCAGG TGAAGGTGCG GCGGCTGCCG TCGCGGCTGT ACAGGCGATT GTGCACTTCG
121    CTTTCGTCGC CGTGTCCGCC GGTGATC
```
Nucleotide sequence of pALacZsd$_{(DSM1707)}$921

```
1      TCGTCCCGCT TCGGGCTGAA AGTGCGACCG CCCGGGCGGG CGTCAGTCCC CGACGGCAGC
61     GATCAGGACG CCACAACCCA CGCCGCAGTA GCAGCAGGTC AAAGCGGTGG TGCGCAGCGG
121    GGAATCTGTC GACATGCTCG GCTCCTGTTA TCGGCGAGAG ATCAACTGGA CCGTGATGAT
181    CAGCGAGGGC ACCCTGTTCG TCACCGACGA GAACGGCCAG CACGAGTTGC AGCTGGAGTG
241    GCTGCAGGGC GAGCGTTGAT C
```
Nucleotide sequence of pALacZsd$_{(DSM1707)}$930