

## 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 gelatinous 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,  $\sigma^{54}$  (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  $\sigma^{54}$ - or  $\sigma^{22}$  (AlgU/AlgT)-dependent (Boucher *et al.*, 2000). Also, transcription of the *pilA* gene of *P. aeruginosa* requires, in addition to  $\sigma^{54}$ , 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  $\sigma^{54}$ -dependent. Thus,  $\sigma^{54}$  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  $\beta$ -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,  $\sigma^{54}$ . 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  $\sigma^{54}$  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.  $\beta$ -galactosidase assays and fluorescent microscopy were performed in FABG medium (0.2% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; 0.6% Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O; 0.3% KH<sub>2</sub>PO<sub>4</sub>; 0.3% NaCl; 0.0001% MgCl<sub>2</sub>; 0.00001% CaCl<sub>2</sub>; 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
<b>Strains</b>		
<i>E. coli</i> JM109	F' <i>traD36 lacI<sup>q</sup> Δ(lacZ)M15 proA<sup>+</sup>B<sup>+</sup>/e14-(McrA<sup>-</sup>) Δ(lac<sup>+</sup>proAB) thi gyrA96 (NaI<sup>r</sup>) endA1 hsdR17 (r<sub>k</sub><sup>-</sup>m<sub>k</sub><sup>+</sup>) relA1 supE44 recA1</i>	Promega
<i>P. aeruginosa</i> DSM1707	Prototroph (PAO)	DSM <sup>a</sup>
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
<b>Plasmids</b>		
pALacZsd	Tet <sup>r</sup> , IncQ/RSF1010, t, T, promoterless <i>lacZ</i> gene	Weyers, 1999
pALacZsdIP	pALacZsd containing the <i>lac</i> promoter from Weyers, 1999 pBluescript (co-ordinates 719-1719)	
pALacZsd <sub>(DSM1707)</sub> 498	pALacZsd containing a fragment up-regulated by attachment (contains putative IHF and $\sigma^{54}$ sites)	Weyers, 1999
pALacZsd <sub>(DSM1707)</sub> 498A	subclone A from pALacZsd498 (putative IHF site)	This study
pALacZsd <sub>(DSM1707)</sub> 498B	subclone B from pALacZsd498 (putative $\sigma^{54}$ site)	This study
pGEM <sup>®</sup> -T-Easy	Amp <sup>r</sup> , cloning vector for PCR products	Promega
pGEM <sup>®</sup> -T-Easy-498	pGEM <sup>®</sup> -T-Easy containing a fragment up-regulated by attachment (contains putative IHF and $\sigma^{54}$ sites)	This study
pJBA27( <i>gfpmut3</i> )	Amp <sup>r</sup> , pUC18Not <sub>-PA1/04/03</sub> -RBSII- <i>gfpmut3*</i> -T <sub>0</sub> -T <sub>1</sub>	Andersen <i>et al.</i> , 1998
pMiniCTX	Tet <sup>r</sup> , self-proficient integration vector with <i>tet</i> , <i>FRT-attP-MCS</i> , <i>ori</i> , <i>int</i> , and <i>oriT</i>	Hoang <i>et al.</i> , 2000
pMiniCTX-GFP	pMiniCTX containing promoterless <i>gfp</i> gene from pJBA27	This study
pMiniCTX-498-GFP	pMiniCTX containing cloned 498- <i>gfp</i> fusion	This study
pMiniCTX-IP-GFP	pMiniCTX-GFP containing the <i>lac</i> promoter from pBluescript (co-cordinates 719-1719)	Van Schalkwyk, 2001
pRK2013	ColE1 <i>mob</i> <sup>+</sup> <i>tra</i> <sup>+</sup> (RK2)Kan <sup>r</sup>	Figurski & Helinski, 1979
pUC19	Amp <sup>r</sup> , 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
<b>Primers</b>		
EBF	5'-GCATCACCTTCACCCTCTCC-3'	This study
EBR	5'-CCGGCGCGCGTAATACG-3'	This study
JB-1	5'-GAATTCGAGCTGGGTAC-3'	Weyers, 1999
JB-2	5'-GTTTTCCAGTCACGAC-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<sup>®</sup> X-100), MgCl<sub>2</sub> 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<sup>™</sup> 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<sup>®</sup> 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<sup>®</sup>-T-Easy vector

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

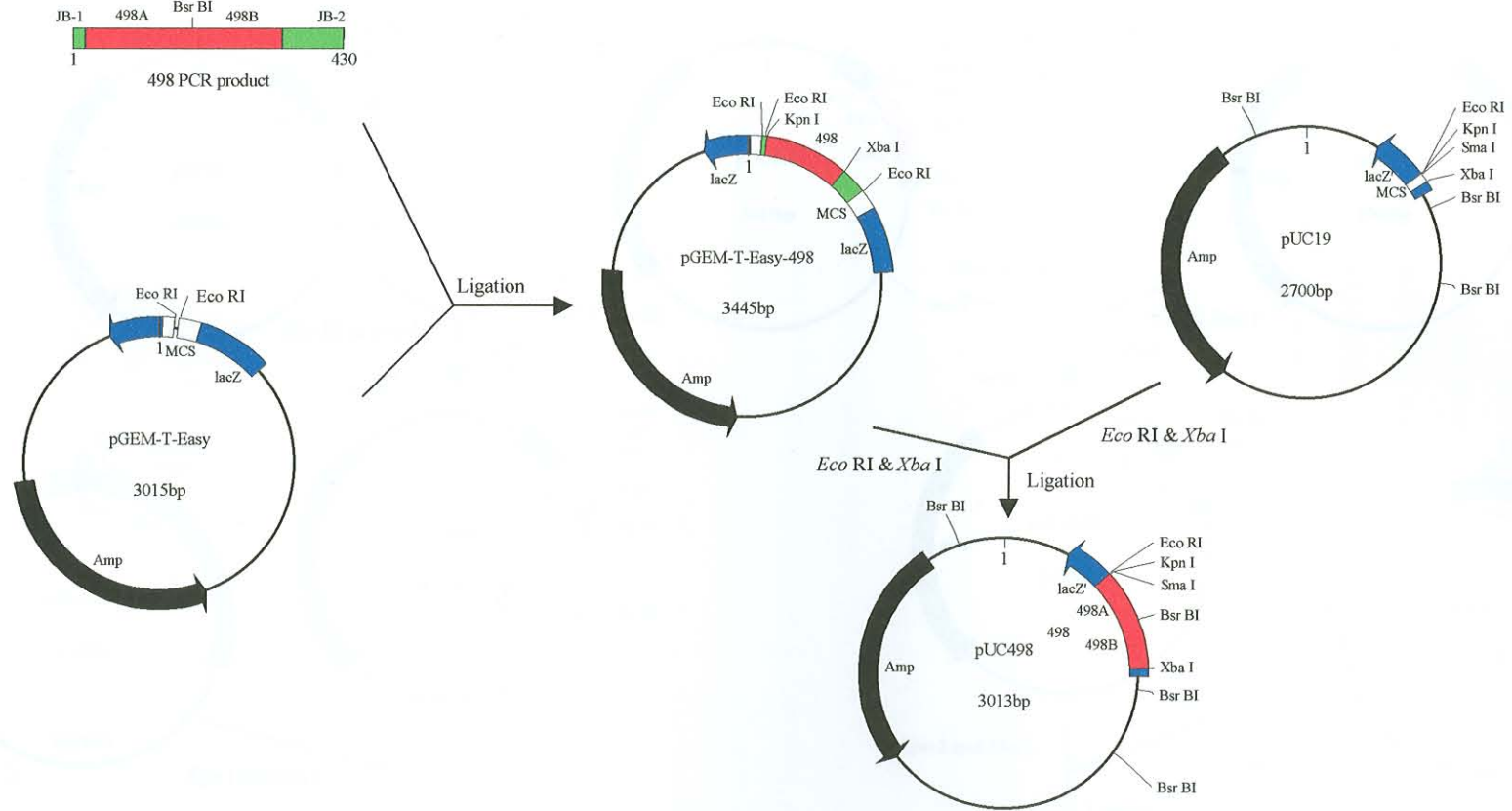
Ligation of the purified amplicons and the linear pGEM<sup>®</sup>-T-Easy vector was performed for 16 h at 4°C in a total volume of 10 µl. The reaction mixture contained 1 µl of 10 × DNA ligase buffer (660 mM Tris.HCl; 10 mM DTT; 50 mM MgCl<sub>2</sub>; 10 mM ATP; pH 7.5), 50 ng of pGEM<sup>®</sup>-T-Easy vector, 300 ng insert DNA, 3 U T4 DNA ligase (Promega, 3U/µl), and UHQ water. Competent *E. coli* JM109 cells, prepared as described in Section 2.2.2, were transformed following addition of the ligation reaction mixture (5 µl), using the heat shock-method (Section 2.2.3). After overnight incubation, recombinant transformants with a Gal<sup>-</sup> phenotype were selected, cultured and subjected to restriction enzyme digestion. A recombinant plasmid, containing the cloned amplicon was designated pGEM<sup>®</sup>-T-Easy-498.

### 3.2.5 Restriction endonuclease digestions

All restriction enzyme digestions were performed in sterile Eppendorf tubes and contained the appropriate concentration of salt (using the 10 × buffer supplied by the manufacturer) for the specific enzyme and 5-10 U of enzyme per µg of plasmid DNA. Reaction volumes were small (15-20 µl) and incubation was for 1-1.5 h at 37°C for all restriction enzymes, except for *Sma* I which was incubated at 25°C. When digestion entailed the use of two enzymes requiring different salt concentrations for optimal activity, the plasmid DNA was first digested with the enzyme requiring a lower salt concentration, after which the salt concentration was adjusted and the second enzyme added. All restriction enzymes were supplied by Roche, Promega or New England Biolabs. The digestion products were analyzed by agarose gel electrophoresis and sized according to their migration in the gel as compared to that of a 100 bp molecular weight marker (Promega).

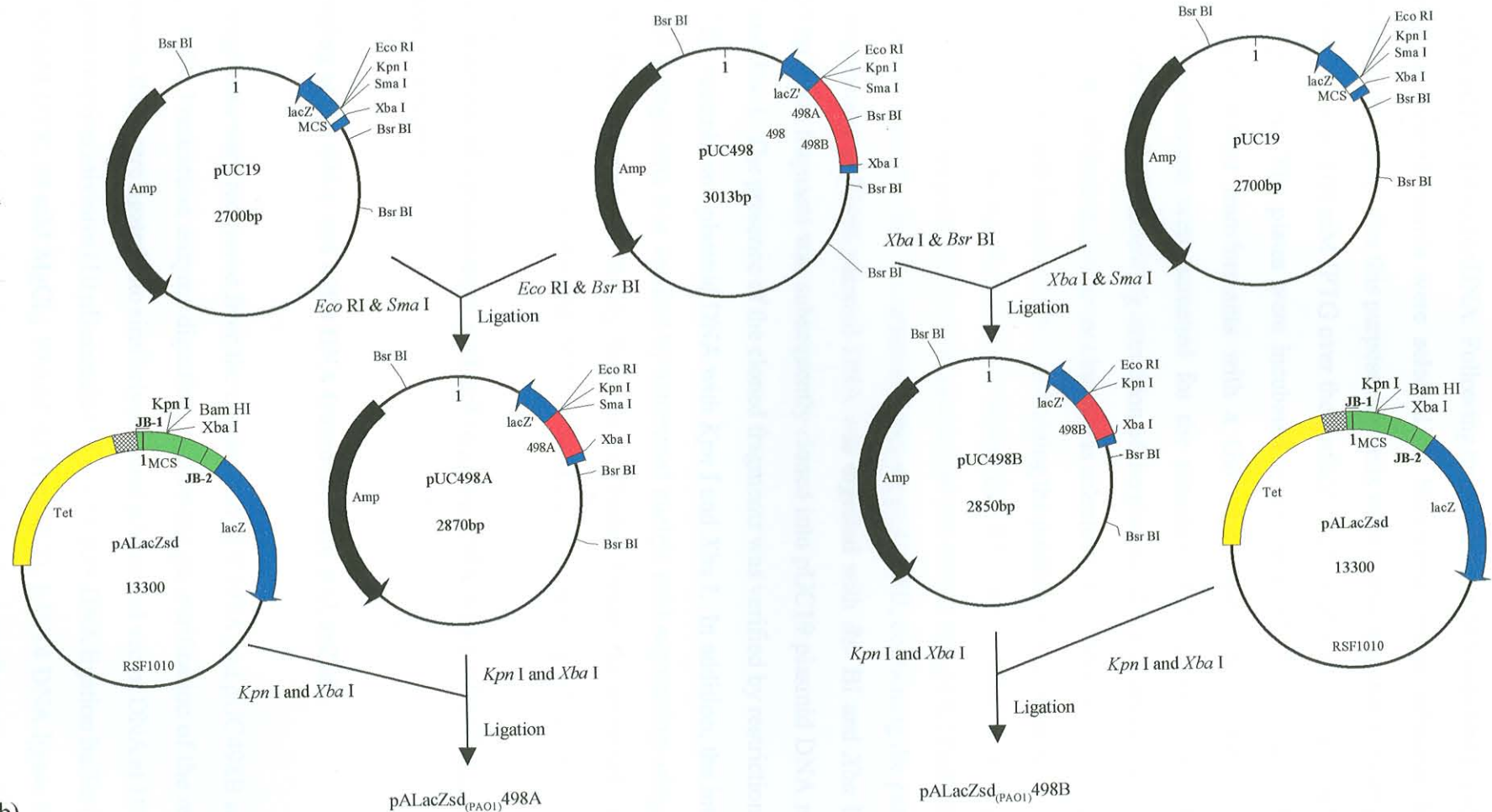
### 3.2.6 Construction of recombinant pUC498A and pUC498B plasmids

To separately assay the activity of the putative  $\sigma^{54}$  promoter and to determine the importance of the putative integration host factor (IHF) sequence in promoter activity, the insert DNA was first cloned into pUC19 prior to constructing subclones containing the putative  $\sigma^{54}$  and IHF elements. The strategy for the construction of the respective subclones is shown in Fig. 3.1. The cloned 320 bp PCR-amplified DNA fragment was recovered by *Eco* RI and *Xba* I restriction of the recombinant pGEM<sup>®</sup>-T-Easy-498 plasmid DNA, agarose gel-purified and then ligated into



(a)  
Fig. 3.1

Construction of the recombinant reporter vectors pALacZsd<sub>(DSM1707)</sub>498A and pALacZsd<sub>(DSM1707)</sub>498B containing putative IHF and  $\sigma^{54}$  consensus sites, respectively. (a) Cloning of the 498 DNA fragment into pUC19.



(b)

Fig. 3.1

Construction of the recombinant reporter vectors pALacZsd<sub>(DSM1707)</sub>498A and pALacZsd<sub>(DSM1707)</sub>498B containing putative IHF and  $\sigma^{54}$  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  $\mu$ l of 2% (w/v) X-gal and 10  $\mu$ l of 100 mM IPTG over the surface of LB-agar plates supplemented with 100  $\mu$ g/ml ampicillin. The plates were incubated overnight at 37°C and observed for the presence of recombinant transformants with a Gal<sup>-</sup> 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  $\sigma^{54}$  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<sub>(DSM1707)</sub>498A and pALacZsd<sub>(DSM1707)</sub>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  $\mu$ l) contained 1  $\mu$ l of a 10  $\times$  DNA ligation buffer (660 mM Tris.HCl; 10 mM DTT; 50 mM MgCl<sub>2</sub>; 10 mM ATP; pH 7.5), 1 U T4 DNA ligase (Roche; 1 U/ $\mu$ l) 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 × Taq polymerase buffer (50 mM KCl; 10 mM Tris-HCl (pH 9.0); 0.1% Triton® X-100), 1.5 mM MgCl<sub>2</sub>, 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<sub>(DSM1707)</sub>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<sub>(DSM1707)</sub>498A and pALacZsd<sub>(DSM1707)</sub>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<sub>550</sub> 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<sub>2</sub>, incubated on ice for 5 min, pelleted as before and gently resuspended in 7.5 ml of the ice-cold MgCl<sub>2</sub> 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<sub>2</sub>.

### 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<sub>(DSM1707)</sub>498, pALacZsd<sub>(DSM1707)</sub>498A or pALacZsd<sub>(DSM1707)</sub>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<sub>540</sub> 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 $\beta$ -galactosidase assay

$\beta$ -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  $\mu$ l) were incubated for 20 min at 4°C to stop further growth of the cells. For activity assays, 500  $\mu$ l Z-buffer (0.06 M Na<sub>2</sub>HPO<sub>4</sub>; 0.04 M NaH<sub>2</sub>PO<sub>4</sub>; 0.01 M KCl; 0.001 M MgSO<sub>4</sub>; 0.5 M  $\beta$ -mercaptoethanol) was added to each tube followed by the addition of 10  $\mu$ l 0.1% SDS and 20  $\mu$ l chloroform to lyse the bacterial cells. After incubation at 28°C for 10 min, 200  $\mu$ l 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  $\mu$ l of 1 M Na<sub>2</sub>CO<sub>3</sub> 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  $\mu$ l 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  $\mu$ l) were collected by centrifugation at 15 000 rpm for 5 min and the cell pellets were resuspended in 500  $\mu$ l 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  $\mu$ l 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 ( $\mu$ g/ml) was then determined from the prepared standard curve. The  $\beta$ -galactosidase activity per total cellular biomass was determined by dividing the  $\beta$ -galactosidase values by the protein concentration to ensure that the difference in  $\beta$ -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<sup>®</sup>-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  $\sigma^{54}$  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  $\mu$ g/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  $\mu$ l 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  $\mu$ l of the supernatant, containing the DNA, was used as template in the PCR reactions. In

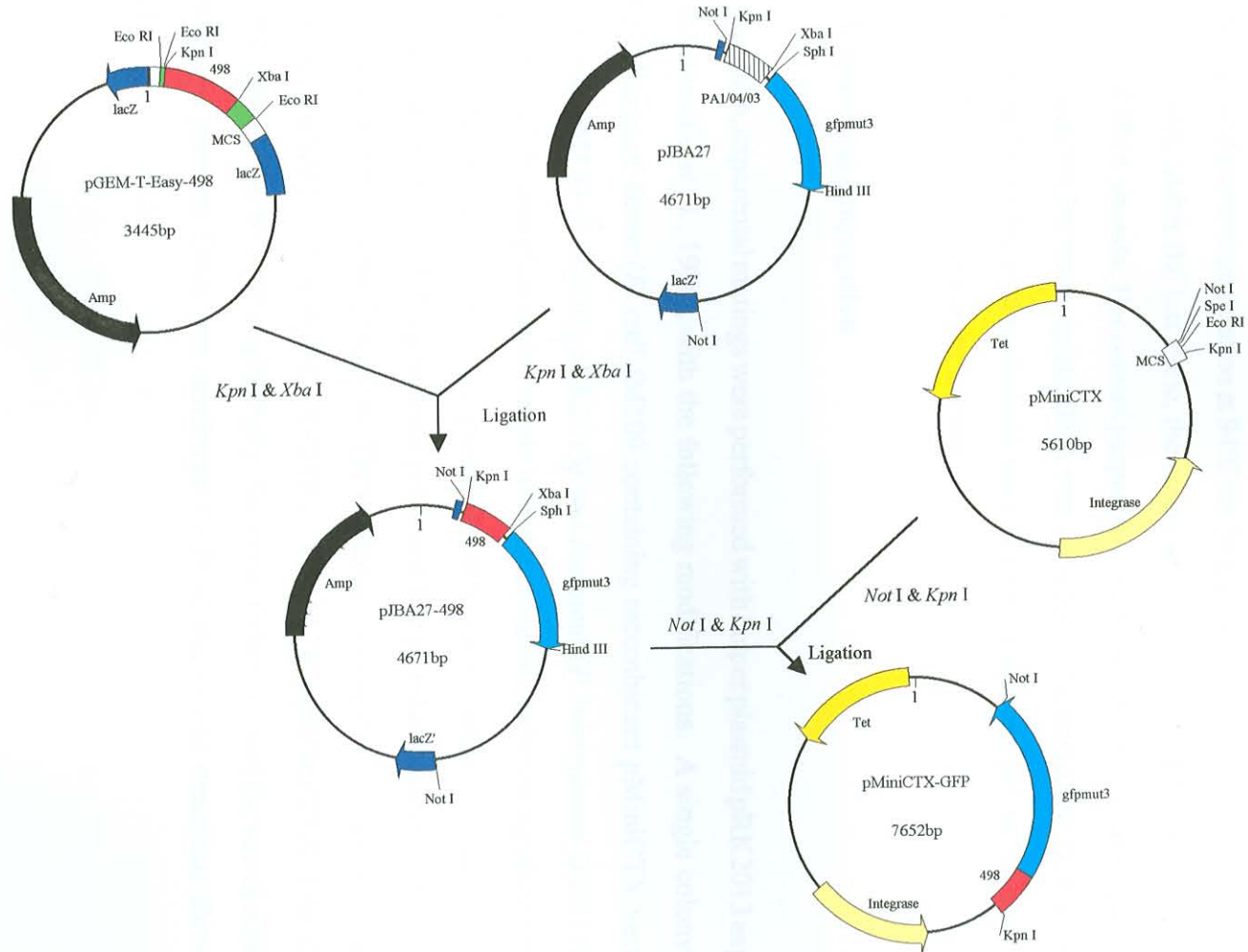


Fig. 3.2

Construction of the recombinant pMiniCTX-498-GFP reporter plasmid.

addition, the PCR reaction mixtures contained 1 × PCR buffer, 1.5 mM MgCl<sub>2</sub>, 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::pMiniCTX-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%  $\text{KCr}(\text{SO}_4)_2$ . 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  $\text{OD}_{540}$  of 0.05 into 2 ml FABG medium with or without glass wool (0.05 g; mean diameter 15  $\mu\text{m}$ , 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  $\mu\text{l}$  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 $\sigma^{54}$ 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  $\sigma^{54}$  promoter sequences. However, only one of these, clone 498, contained a sequence corresponding to an IHF binding site, in addition to the  $\sigma^{54}$  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  $\sigma^{54}$  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<sub>(DSM1707)</sub>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 discrete 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<sup>®</sup>-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<sup>®</sup>-T-Easy vector. Since the recombinant pGEM<sup>®</sup>-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  $\sigma^{54}$  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  $\sigma^{54}$  promoter sequence. The respective DNA fragments were therefore excised from the agarose gel, purified by the gene clean method and then ligated into *Sma* I and *Eco* RI and *Sma* I and *Xba* I digested

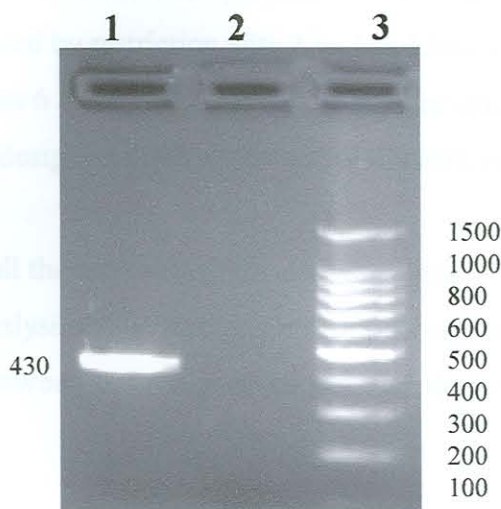


Fig. 3.3

Agarose gel electrophoretic analysis of the DNA product obtained by PCR amplification of the cloned DNA fragment of pALacZsd<sub>(DSM1707)</sub><sup>498</sup>. 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<sup>®</sup>-T-Easy and pUC19 plasmids. Lane 1, Molecular weight marker; Lane 2, linear pGEM<sup>®</sup>-T-Easy vector DNA; Lane 3, plasmid pGEM<sup>®</sup>-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  $\sigma^{54}$  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<sub>(DSM1707)</sub>498A and pALacZsd<sub>(DSM1707)</sub>498B reporter vectors

To assay the activity of the putative  $\sigma^{54}$  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<sup>r</sup> 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  $\sigma^{54}$  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 $\beta$ -galactosidase activity assays

For  $\beta$ -galactosidase activity assays, the recombinant and control pALacZsd reporter vector constructs were transformed into competent *P. aeruginosa* DSM1707 cells and  $\beta$ -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  $\beta$ -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  $\sigma^{54}$  promoter element was also assayed, for comparative purposes, in PGW cells. All assays were performed in triplicate and on at least two separate

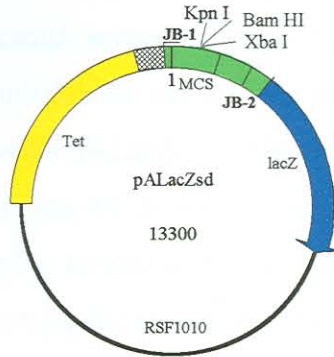


Fig. 3.5a Plasmid map of pALacZsd, indicating the annealing sites of primers JB-1 and JB-2.

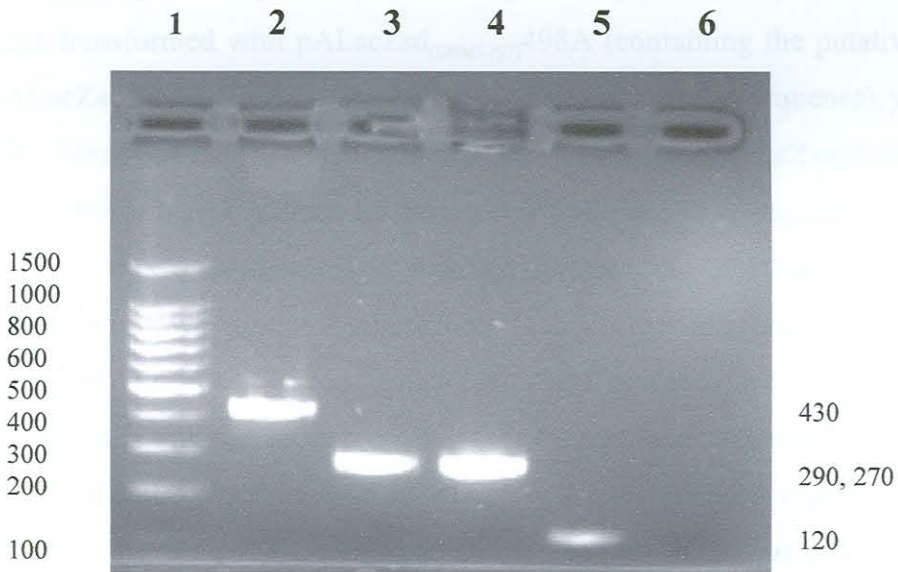


Fig. 3.5b Agarose gel electrophoresis indicating amplification of the cloned DNA fragments in the recombinant pALacZsd reporter vector constructs. Lane 1, Molecular weight marker; Lane 2, amplified insert DNA from pALacZsd<sub>(DSM1707)</sub>498; Lane 3, amplified insert DNA from pALacZsd<sub>(DSM1707)</sub>498A; Lane 4, amplified insert DNA from pALacZsd<sub>(DSM1707)</sub>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  $\beta$ -galactosidase expression. These results served to confirm that *P. aeruginosa* is a  $\beta$ -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  $\beta$ -galactosidase activity. This low basal level of activity may be the result of slight transcriptional read-through from the tetracycline resistance (Tet<sup>r</sup>) 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  $\beta$ -galactosidase activity under all the growth conditions investigated.

Comparison of the results obtained for cultures transformed with the pALacZsd<sub>(DSM1707)</sub>498 reporter construct (containing both the putative IHF binding and  $\sigma^{54}$  promoter sequences) to those obtained for cultures transformed with pALacZsd<sub>(DSM1707)</sub>498A (containing the putative IHF binding site) and pALacZsd<sub>(DSM1707)</sub>498B (containing the putative  $\sigma^{54}$  promoter sequence), yielded the following results. When compared to planktonic cells, expression of the *lacZ* reporter gene from pALacZsd<sub>(DSM1707)</sub>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<sub>(DSM1707)</sub>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<sub>(DSM1707)</sub>498 compared to cells containing pALacZsd<sub>(DSM1707)</sub>498B. As expected, expression of the reporter gene was low for cells containing pALacZsd<sub>(DSM1707)</sub>498A.

Based on the above results, it was concluded that the putative  $\sigma^{54}$  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.

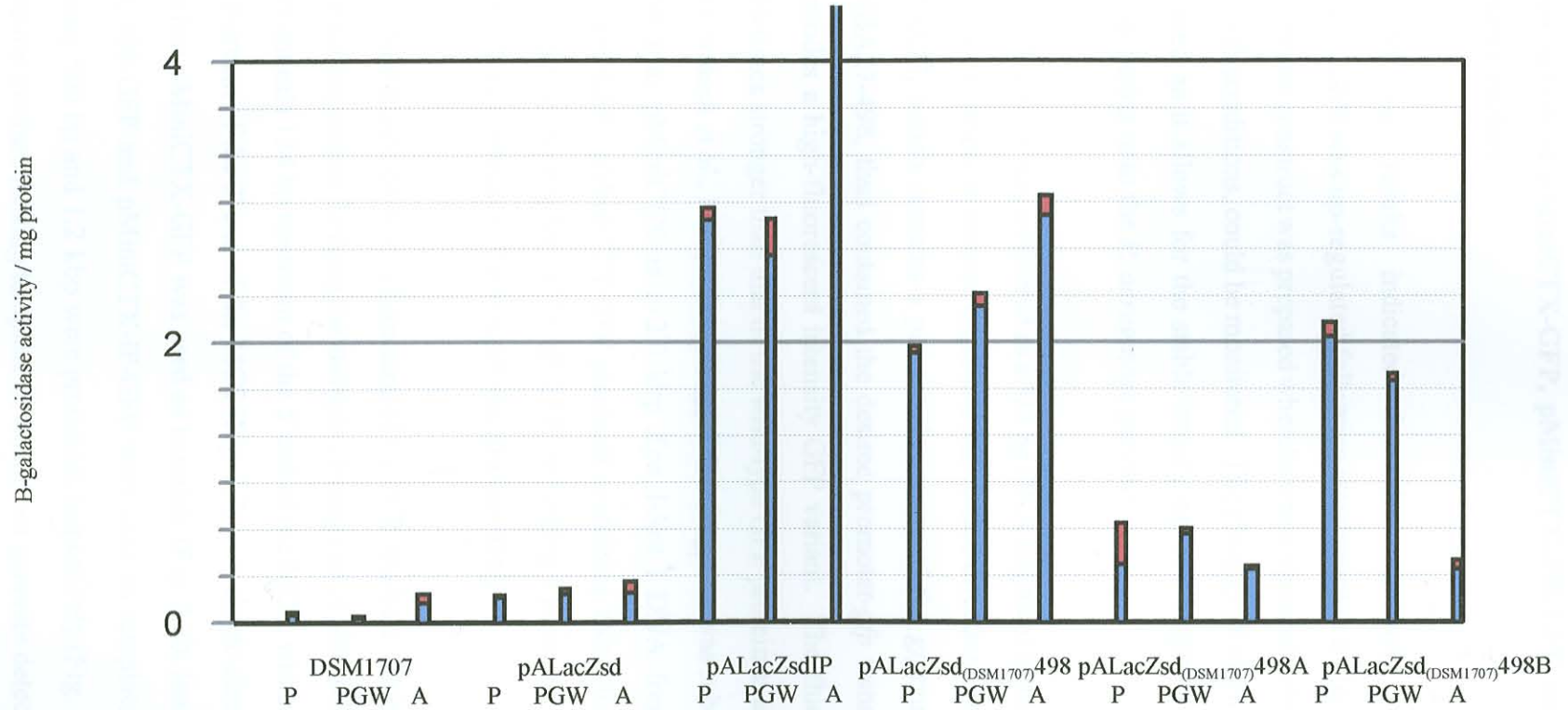


Fig. 3.6

Graph displaying the  $\beta$ -galactosidase activity per total cellular biomass (in Miller units) of the different promoter-*lacZ* constructs in planktonic (P) cells, planktonic cells 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<sub>(DSM1707)</sub>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<sup>®</sup>-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 *gfpmut3* gene. The resultant plasmid, pJBA27-498, thus contained the desired promoter-*gfp* transcriptional fusion. The *gfpmut3* 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.

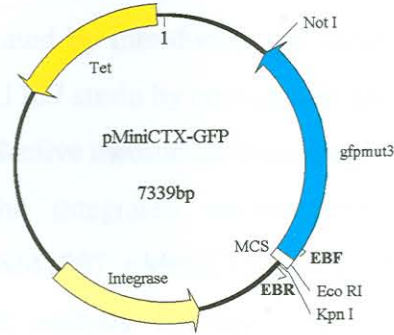


Fig. 3.7a Plasmid map of pMiniCTX-GFP, indicating the annealing sites of primers EBF and EBR.

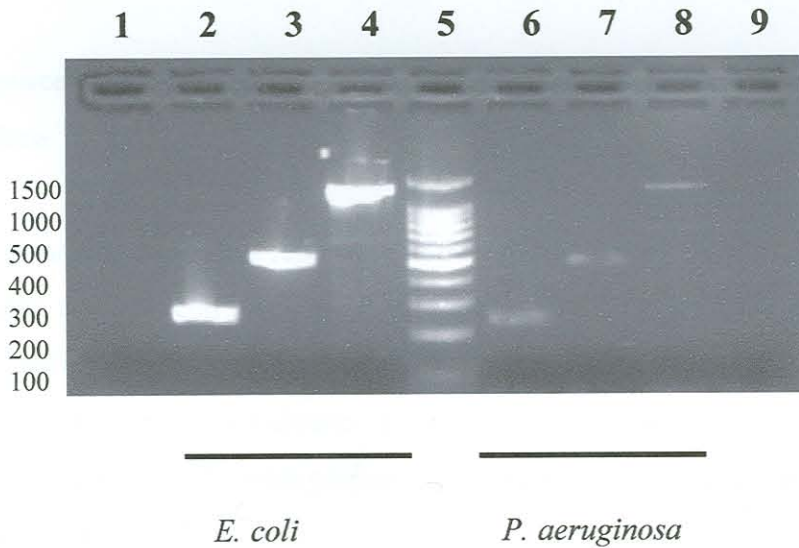


Fig. 3.7b Agarose gel electrophoresis indicating amplification of the cloned DNA fragments in the recombinant pMiniCTX-GFP reporter vector constructs in *E. coli* (Lanes 2, 3 and 4), and following integration of the recombinant vectors onto the *P. aeruginosa* genome (Lanes 6, 7 and 8). Lanes 1 and 9, 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

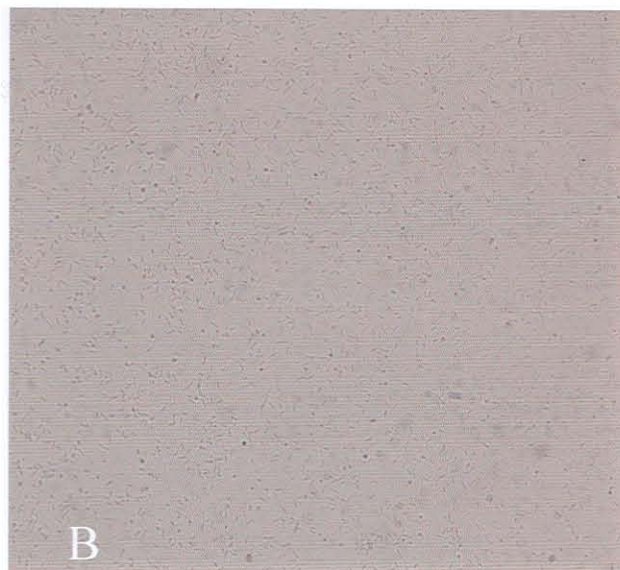
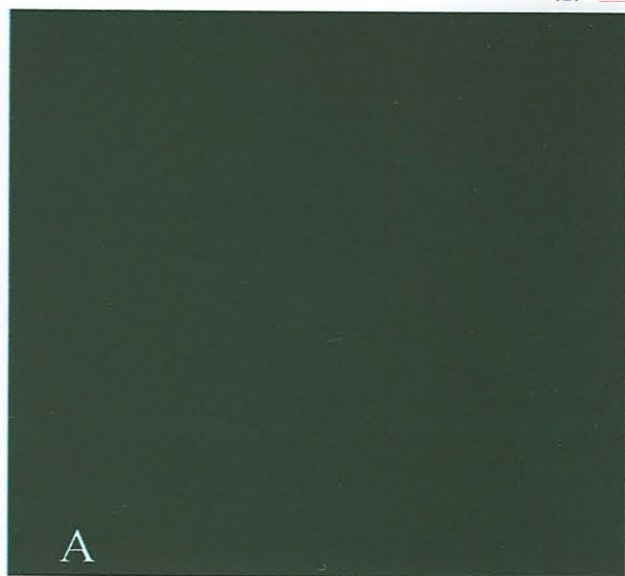
*P. aeruginosa* strains harboring integrated copies of pMiniCTX-GFP, pMiniCTX-498-GFP and pMiniCTX-IP-GFP were generated by introducing the recombinant and control pMiniCTX vectors into the wild-type DSM1707 strain by conjugation and selecting for subsequent single recombination events using a selective 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

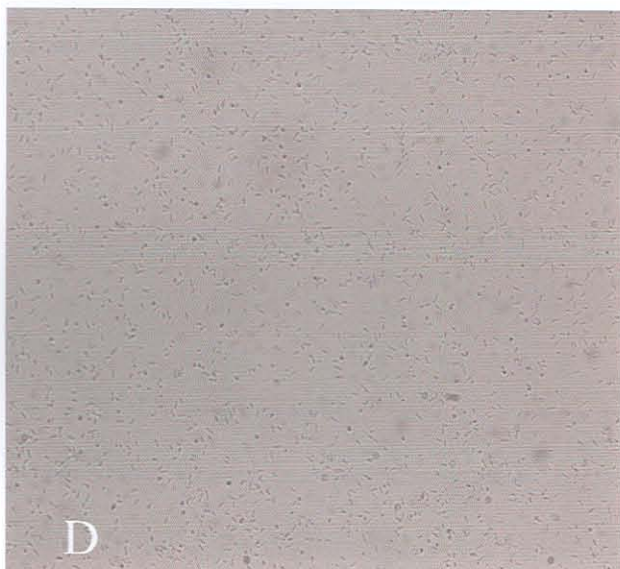
To investigate attachment-dependent regulation of the *flgE* regulatory element, contained in the 498 DNA fragment, at single cell level, the *P. aeruginosa* DSM1707, DSM1707::pMiniCTX-GFP, DSM1707::pMiniCTX-498-GFP and DSM1707::pMiniCTX-IP-GFP strains were cultured in FABG medium in the presence or 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

Planktonic



PGW



Attached

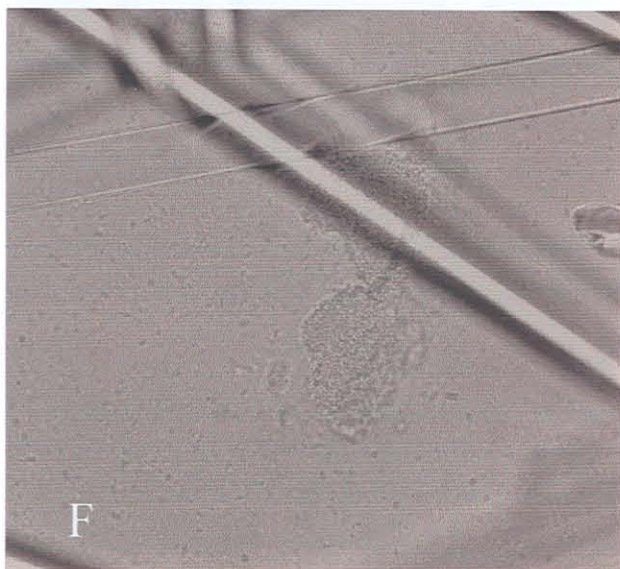
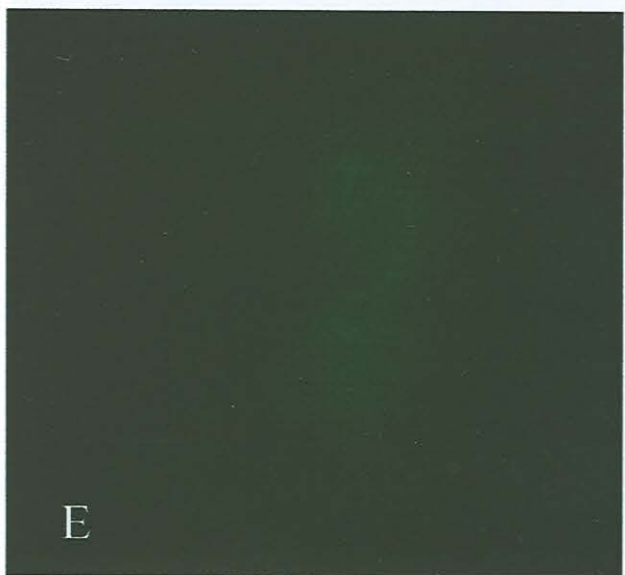
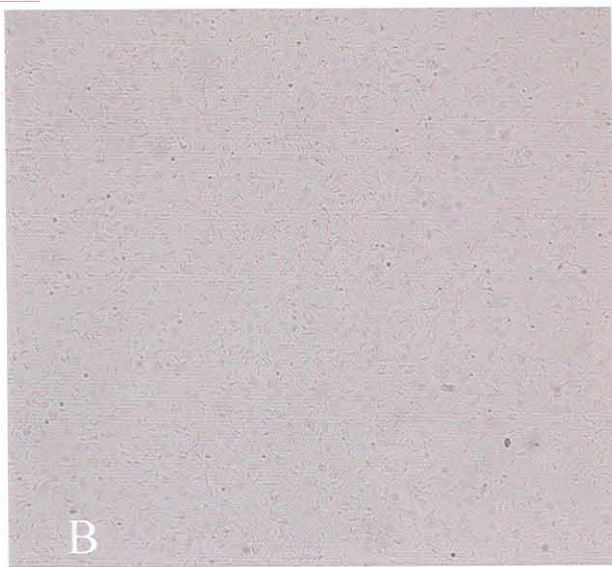


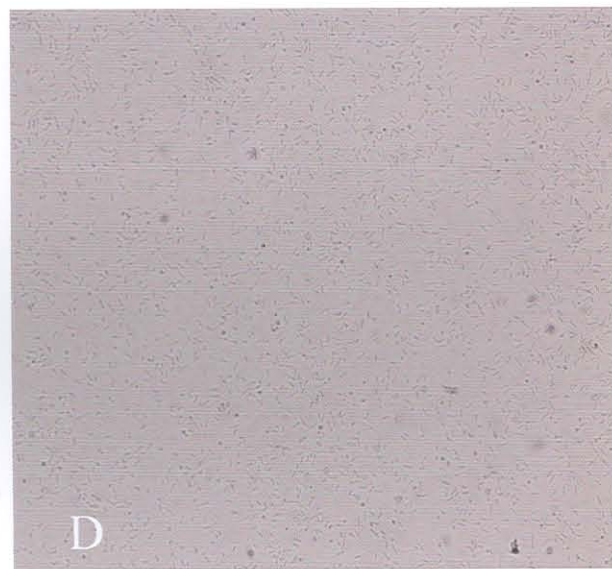
Fig. 3.8

Fluorescent (A, C, E) and light (B, D, F) photomicrographs of a 16 h *P. aeruginosa* DSM1707 culture. Magnification X630.

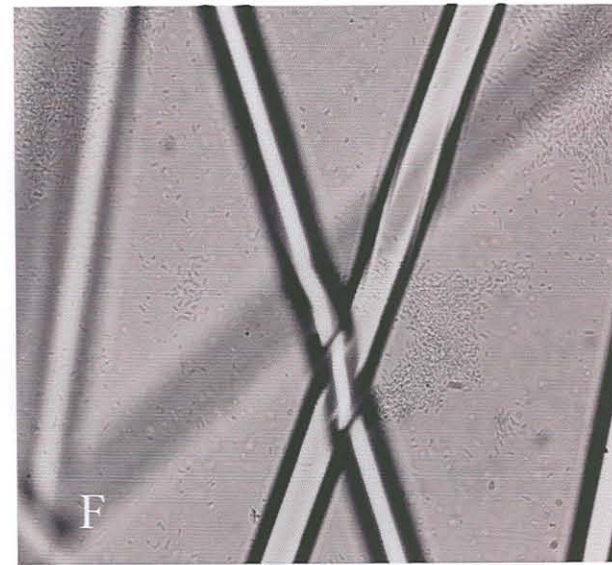
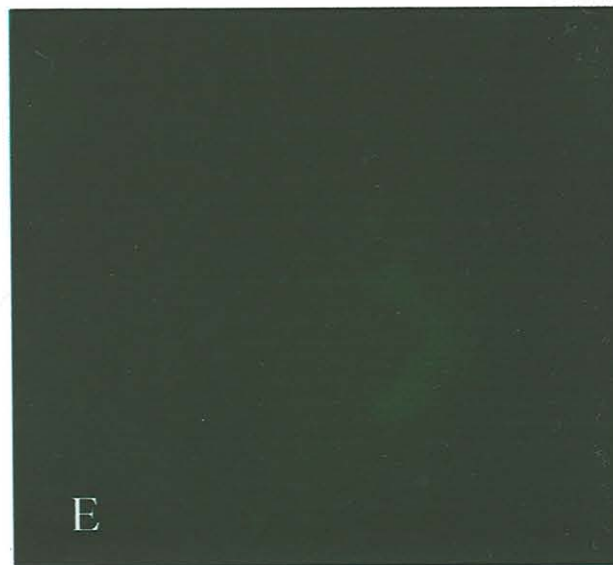
Planktonic



PGW

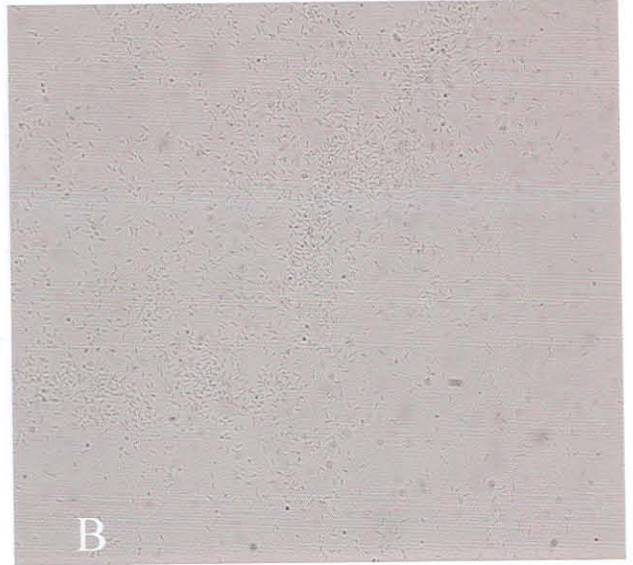
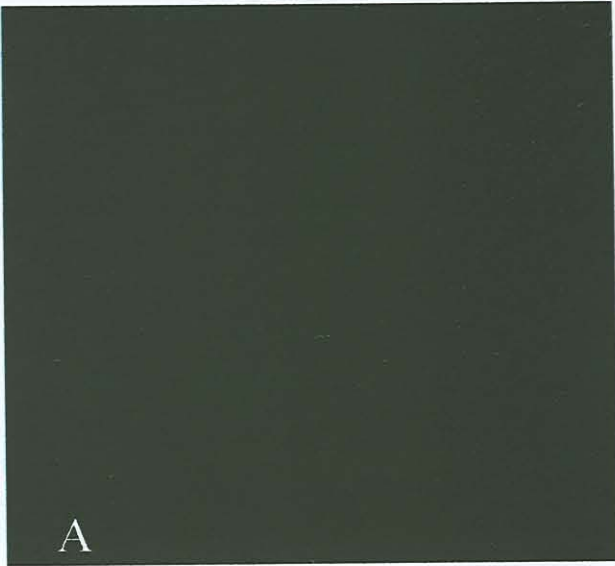


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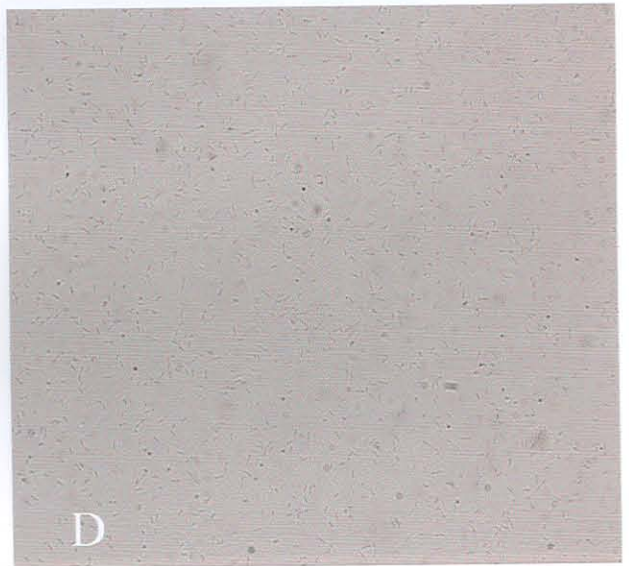
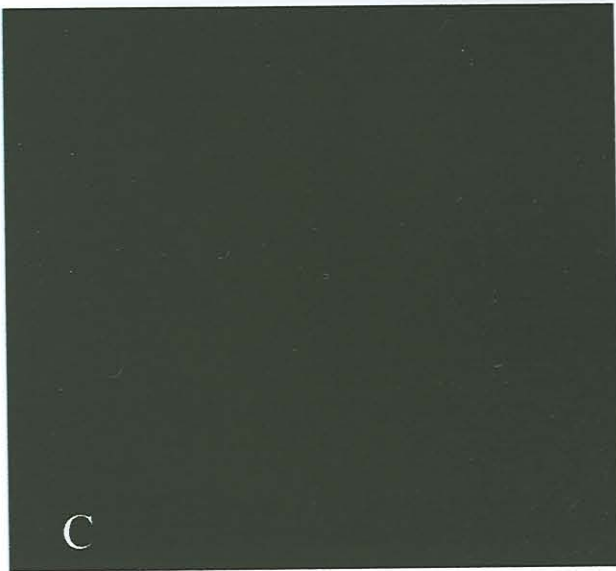


**Fig. 3.9** Fluorescent (A, C, E) and light (B, D, F) photomicrographs of a 16 h DSM1707::pMiniCTX-GFP culture. Magnification X630.

Planktonic



PGW



Attached

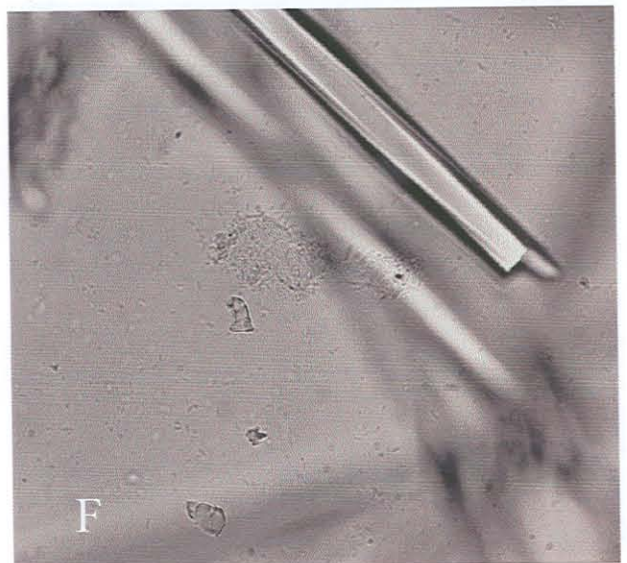
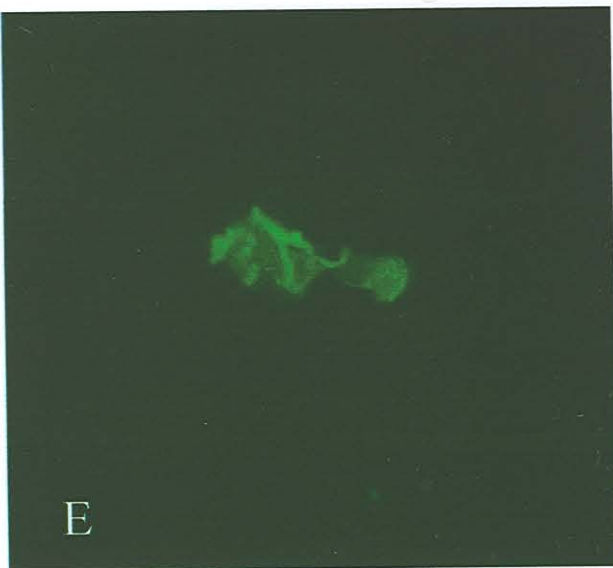
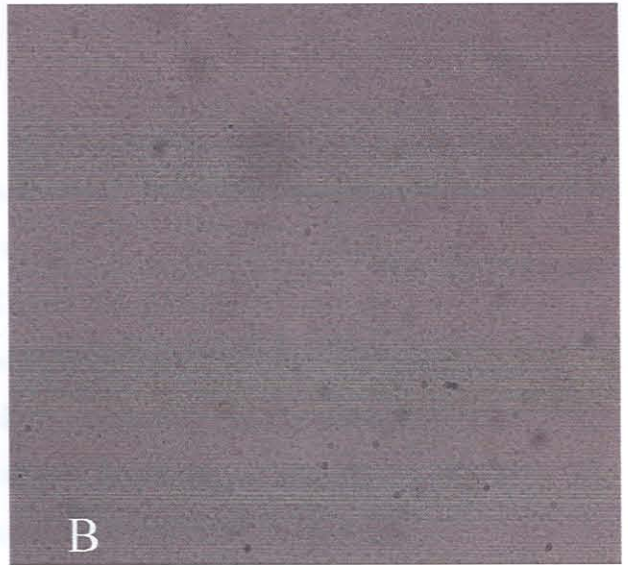
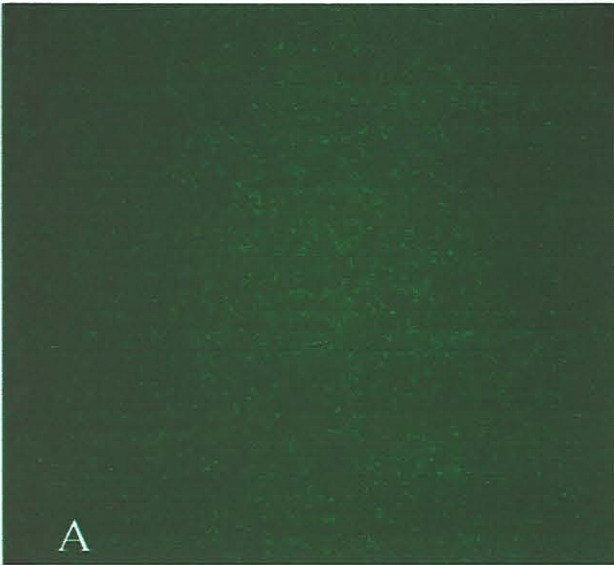
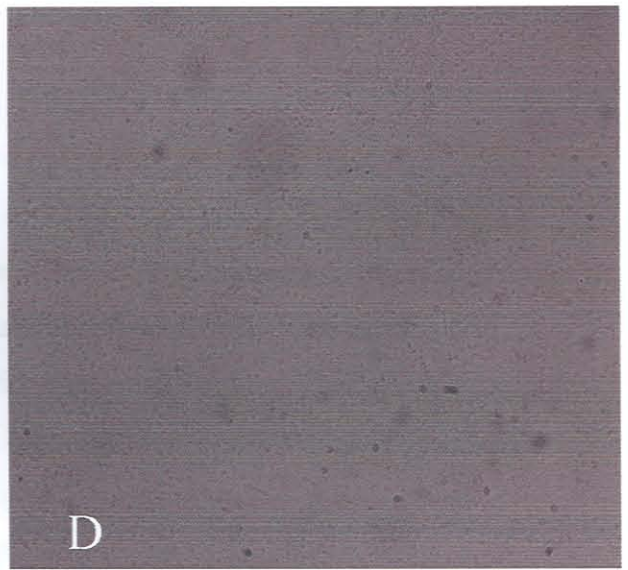
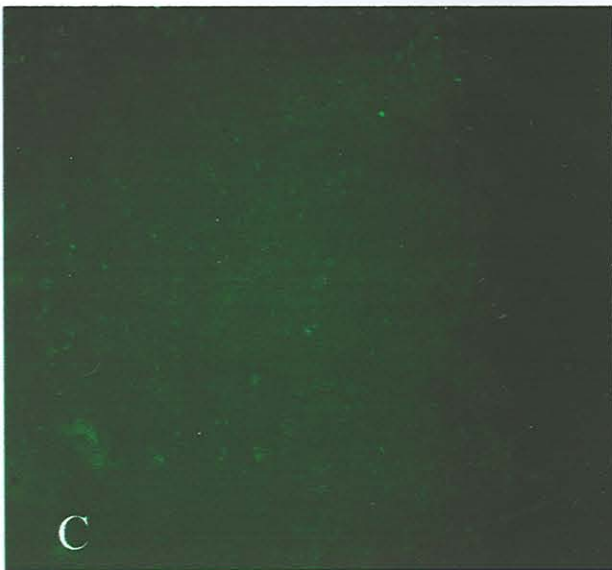


Fig. 3.10 Fluorescent (A, C, E) and light (B, D, F) photomicrographs of a 16 h DSM1707::pMiniCTX-498-GFP culture. Magnification X630.

Planktonic



PGW



Attached

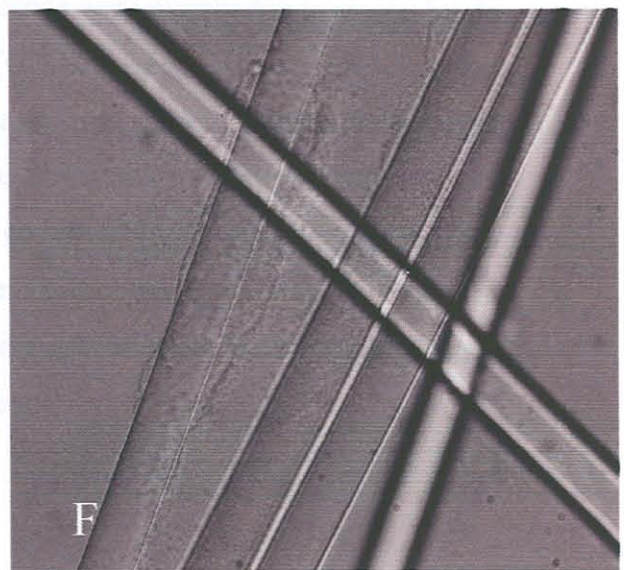
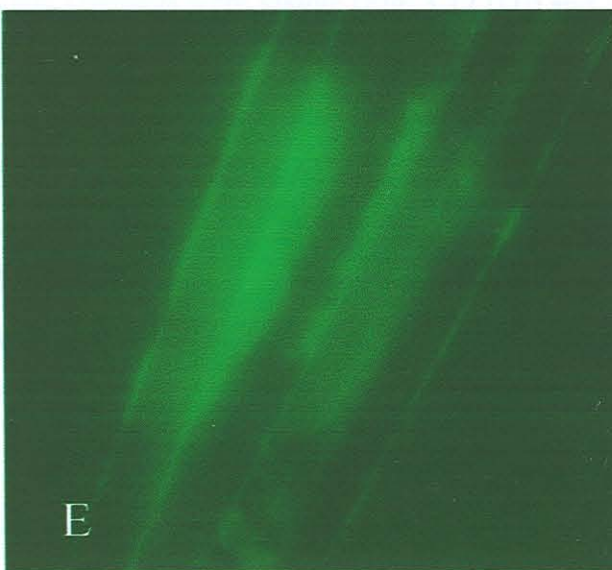


Fig. 3.11 Fluorescent (A, C, E) and light (B, D, F) photomicrographs of a 16 h DSM1707::pMiniCTX-IP-GFP culture. Magnification X630.

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.

Fig. 3.12 Fluorescent (A, C, E, G) and light (B, D, F, H) phase micrographs of biofilms developed by 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). Scale bar depicts 10 µm.



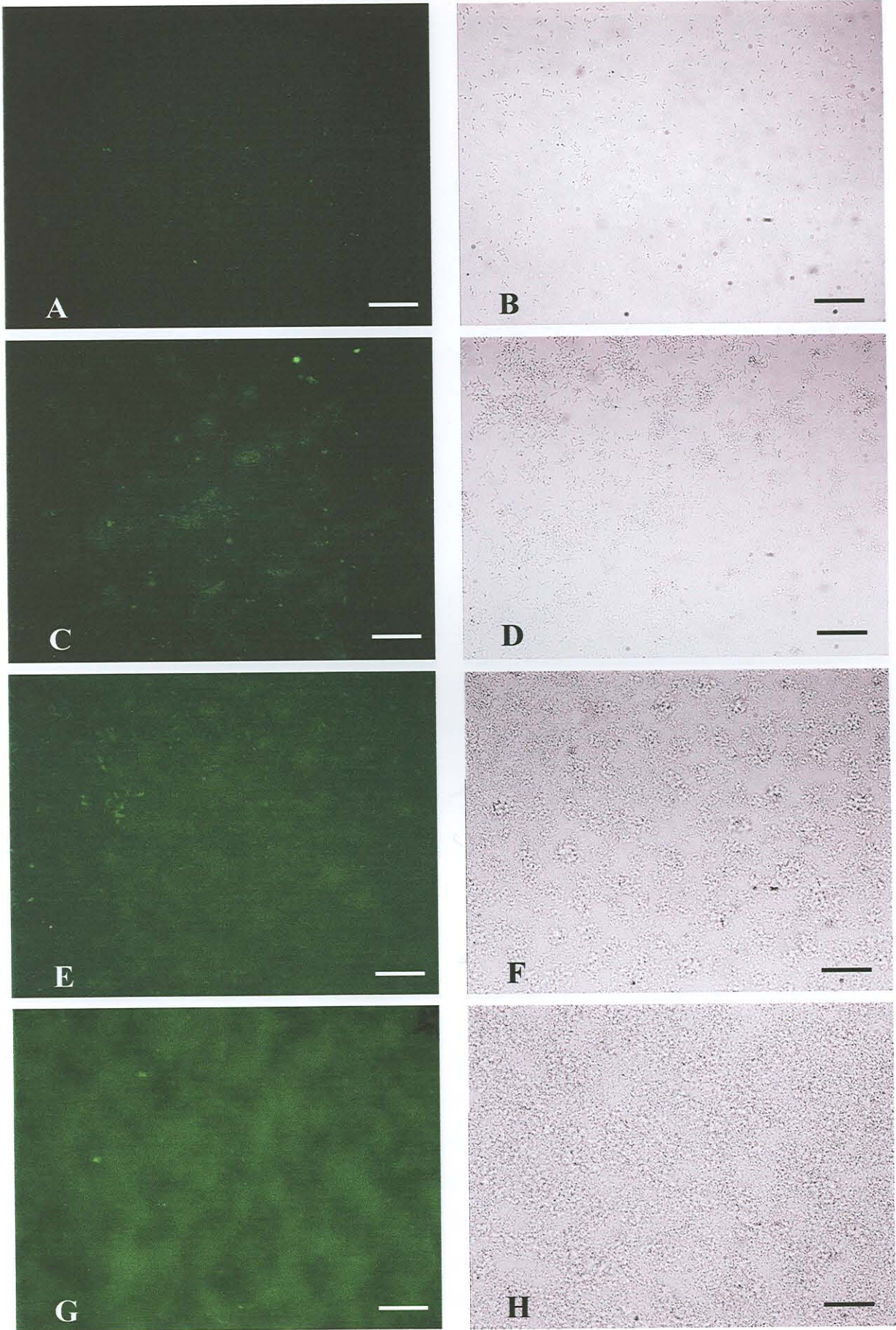


Fig. 3.12

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  $\mu\text{m}$ .

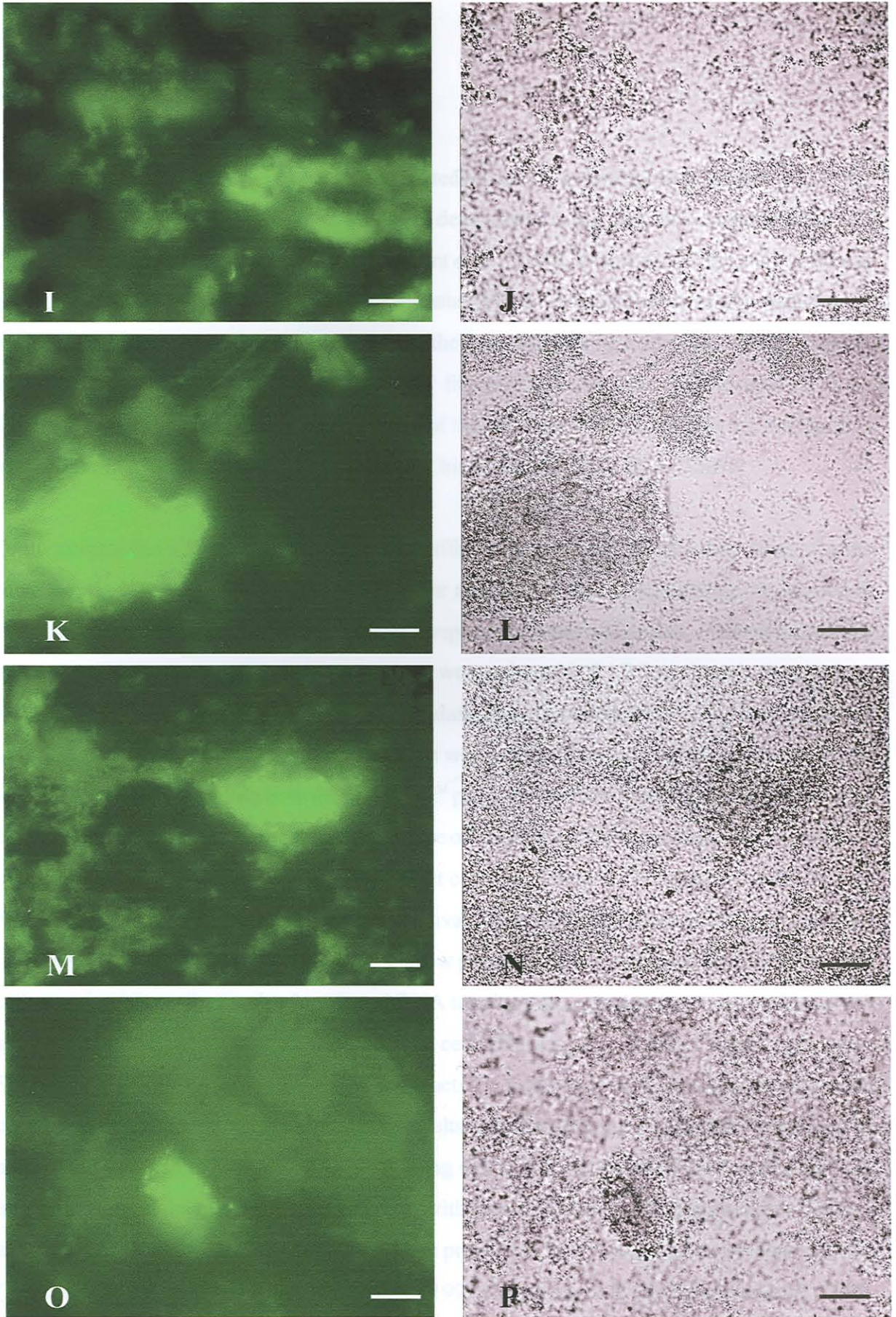


Fig. 3.12 (continued) Fluorescent (I, K, M, O) and light (J, L, N, P) photomicrographs of biofilm development of *P. aeruginosa* DSM1707::pMiniCTX-498-GFP on glass in a flow chamber at 72 h (I, J), 96 h (K, L), 120 h (M, N) and 144 h (O, P). Size bar depict 10 μm.

### 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,  $\sigma^{54}$  (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  $\sigma^{54}$  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  $\sigma^{54}$  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  $\beta$ -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  $\sigma^{54}$  promoter and IHF binding site was higher than that for the construct containing only the putative  $\sigma^{54}$  promoter. The observed promoter activity, in planktonic and PGW cells, from the construct containing only the putative  $\sigma^{54}$  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  $\beta$ -galactosidase activity was observed for the reporter construct containing the IHF binding site only. These results indicated that the  $\sigma^{54}$  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  $\sigma^{54}$ -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*  $\beta$ -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  $\beta$ -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  $\sigma^{54}$  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 (Karlinsky *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.