CHAPTER 3

EXTRACELLULAR ALKALINE PROTEASE SYNTHESIS BY DIFFERENT RECOMBINANT *Pseudomonas aeruginosa* STRAINS

3.1 INTRODUCTION

It has long been recognized that bacteria can switch from free-living unicellular organisms to sessile multicellular communities known as biofilms (Costerton *et al.*, 1995; Costerton *et al.*, 1987). This transition to surface-attached growth is known to result in diverse changes in gene expression, which causes the attaching cells to become phenotypically and metabolically distinct from their planktonic counterparts (Davey and O'Toole, 2000; Costerton *et al.*, 1995; Davies and Geesey, 1995). *P. aeruginosa* is one of the most studied biofilm-forming organisms and has emerged as a model organism in the study of surface- and biofilm-induced gene expression. In recent years, a number of different approaches have been used to identify genes involved in the formation, development and maintenance of *P. aeruginosa* biofilms. These approaches have included reporter gene technology (Weyers, 1999), random insertion transposon mutagenesis (O'Toole and Kolter, 1998a), proteomic analyses (Steyn *et al.*, 2001) and DNA microarray analyses (Whiteley *et al.*, 2001). The results of these investigations have led to the identification of numerous genes of which the expression is up-regulated in biofilm-growing cells. These include, amongst many others, the alginate biosynthetic genes *algC* (Davies and Geesy, 1995; Davies *et al.*, 1993) and *algD* (Hoyle *et al.*, 1993; Rice *et al.*, 1995), the type IV pilin subunit-encoding gene *pilA* (O'Toole *et al.*, 2000) and genes encoding an outer membrane protein (OmlA) and translocation proteins (TatA and TatB) (Whiteley *et al.*, 2001).

Since biofilms immobilized to a surface are responsible for much of the microbial activity found in natural environments, the potential for altered gene expression by up-regulation at surfaces and in biofilms might have enormous implications in biotechnology. The rates of biodegradation of pollutant chemicals are markedly influenced when bacteria adhere to surfaces (Larsson *et al.*, 1993; Holm *et al.*, 1992), and consequently increasing use is being made of reactors utilizing microorganisms immobilized in biofilms for the degradation of pollutant liquid and gaseous chemicals (Rothenburger and Atlas, 1993; van Loosdrecht and Heijnen, 1993). In addition, the immobilized cells are capable of continuous growth by shedding of the old cells and thus retaining a metabolically active cell population. These properties of immobilized (biofilm) cells, together with the use of promoters that are actively up-regulated in cells growing as a biofilm, may enable the development of a biofilm-specific expression system whereby heterologous enzymes and/or other products can be expressed in a cost-effective manner.

There is, however, no single strategy for achieving maximal expression of every cloned gene and the high-level production of homologous and heterologous proteins in a host organism often also depends on many other factors. These may include the unique structural features of the gene sequence, the stability and translational efficiency of mRNA, intracellular or extracellular expression of the protein, degradation of the protein by host cell proteases and major differences in codon usage between the heterologous gene and native host organism (Old and Primrose, 1994; Balbas and Bolivar, 1990). In response to the need for high expression levels, many different approaches and strategies have therefore been developed whereby gene expression can be modulated (Old and Primrose, 1994; Labes *et al.*, 1990). The molecular biological features that have been manipulated include, amongst other, the nature of the transcriptional promoter, the strength of the ribosome binding site, the number of copies of the cloned gene and whether the gene is plasmid-borne or integrated into the genome of the host cell, and the efficiency of translation in the host organism.

Recent advances in the understanding of the formation and development of mature biofilms, together with the potential biotechnological applications associated with this mode of bacterial growth, are making biofilm-forming bacteria attractive hosts for the development of biofilm-specific expression systems whereby heterologous proteins can be produced. In this study, *P. aeruginosa* and alkaline protease (AprA), which is secreted by a dedicated type I secretion system, were used as models for developing such a biofilm-specific expression system. Consequently, this part of the investigation was directed at evaluating different strategies whereby extracellular alkaline protease expression could be increased. Since alkaline protease is naturally expressed and secreted by *P. aeruginosa*, many of the above-mentioned features whereby gene expression can be modulated, could be excluded from this study. Thus, the aims of this second part of the study were to evaluate the use of different (i) promoters (constitutive vs. attachment-inducible promoters), (ii) gene dosages (a single integrated copy of the expression cassette vs. multiple extrachromosomal copies thereof) and (iii) growth modes (planktonic vs. biofilm growth) as means whereby the level of extracellular alkaline protease could be increased.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial strains, plasmids and culture media

The bacterial strains and plasmids used in this part of the study are listed in Table 3.1. The *E. coli* and *P. aeruginosa* strains were routinely cultured at 37°C in LB-broth or on LB-agar plates. The following antibiotics were used in order to maintain the plasmid DNA in *E. coli*: tetracycline at 20 $\mu g.ml^{-1}$, ampicillin at 100 $\mu g.ml^{-1}$ and gentamicin at 7 $\mu g.ml^{-1}$. To maintain chromosomal insertions in *P. aeruginosa* DSM1707, gentamicin was used at a concentration of 50 $\mu g.ml^{-1}$ and tetracycline at 100 $\mu g.ml^{-1}$. All antibiotics were purchased from Sigma-Aldrich. Plasmids pJB3Tc20 and mini-CTX were kindly supplied by S. Valla, Norwegian University of Science and Technology, Trondheim, Norway, and H.P. Schweizer (Department of Microbiology, Colorado State University), respectively. The *E. coli* – *Bacillus* shutle vector pSVB1 was provided by K-D. Wittchen (Institute for Microbiology, Westfälische Wilhelms-Universität, Münster, Germany).

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

Strains, plasmids or primers	Relevant properties	Source or reference
Strains:		
E. coli DH5α	*recA endA1 hsd*R17 *sup*E4 *gyr*A96 *rel*A1 Δ(*lac*ZYA-*arg*F)U169 (ϕ80d*lac*ZΔM15)	Promega
P. aeruginosa DSM1707	Prototroph (PAO1)	#DSM
DSMap0	Alkaline protease null mutant of *P. aeruginosa* DSM1707	This study
DSM1707CTXIPAPR	pCTX-IPAPR integrated onto DSM1707 genome	This study
DSM1707CTX703APR	pCTX-703APR integrated onto DSM1707 genome	This study
DSM1707CTXalgDAPR	pCTX-algDAPR integrated onto DSM1707 genome	This study
DSMap0CTXIPAPR	pCTX-IPAPR integrated onto DSMap0 genome	This study
DSMap0CTX703APR	pCTX-703APR integrated onto DSMap0 genome	This study
DSMap0CTXalgDAPR	pCTX-algDAPR integrated onto DSMap0 genome	This study
DSM1707pJBIPAPR	DSM1707 transformed with pJB-IPAPR	This study
DSM1707pJB703APR	DSM1707 transformed with pJB-703APR	This study
DSM1707pJBalgDAPR	DSM1707 transformed with pJB-algDAPR	This study
DSMap0pJBIPAPR	DSMap0 transformed with pJB-IPAPR	This study
DSMap0pJB703APR	DSMap0 transformed with pJB-703APR	This study
DSMap0pJBalgDAPR	DSMap0 transformed with pJB-algDAPR	This study

Table 3.1 (continued)

Strains, plasmids or primers	Relevant properties	Source or reference
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
pALacZsd703	pALacZsd containing a DNA fragment up-regulated by attachment	Weyers, 1999
pUC18	ColE1, ϕ80d*lacZ*, *lacI*, Amp^R, cloning vector	Stratagene
pUC18-algD	pUC18 containing the *algD* promoter	This study
pUC18-703	pUC18 containing a DNA fragment (703) up-regulated by attachment	A. van Schalkwyk
pGEM® – T Easy	Amp^R, cloning vector for PCR products	Promega
pGEM-lP	pGEM® – T Easy containing PCR-amplified *lac* promoter	This study
pGEM-AlgD	pGEM® – T Easy containing PCR-amplified *algD* promoter	This study
pAPR	pUC18 containing cloned *aprA* and *aprI* genes	This study
plPAPR	pAPR containing the *lac* promoter upstream of the *aprAI* genes	This study
p703APR	pAPR containing the 703 DNA fragment upstream of the *aprAI* genes	This study
palgDAPR	pAPR containing the *algD* promoter upstream of the *aprAI* genes	This study
pSVB1	Tet^R, Amp^R, *LacZ*, *E.coli-Bacillus* shuttle vector	K-D. Wittchen
pSVBalgD	pSVB1 containing the *algD-aprAI* expression cassette	This study
pSVB703	pSVB1 containing the 703-*aprAI* expression cassette	This study
mini-CTX	Tet^R, self-proficient integration vector with *tet*, *FRT-attP-MCS*, *ori*, *int*, *oriT*	Hoang *et al.*, 2000
pCTX-lPAPR	mini-CTX containing the *lac-aprAI* expression cassette	This study
pCTX-703APR	mini-CTX containing the 703-*aprAI* expression cassette	This study
pCTX-algDAPR	mini-CTX containing the *algD-aprAI* expression cassette	This study
pJB3Tc20	Amp^R, Tet^R, *P. aeruginosa* and *E. coli* shuttle plasmid	S. Valla
pJB-lPAPR	pJB3Tc20 containing the *lac-aprAI* expression cassette	This study
pJB-algDAPR	pJB3Tc20 containing the *algD-aprAI* expression cassette	This study
pJB-703APR	pJB3Tc20 containing the 703-*aprAI* expression cassette	This study

Table 3.1 (continued)

Strains, plasmids or primers	Relevant properties	Source or reference
*Primers:		
AN1	5' – GAAGGTACCTGGCGCTACCGT TCGT – 3'	C.J. Cooper
AN2	5' – GGGGTCTAGACGGTGATAGGATGTTTTCTCT – 3'	C.J. Cooper
JT1	5' – GAATTCGAGGATCCTACCCGGGGATC – 3'	A. van Schalkwyk
JT2	5' – GGTCGAGCTCTAGAGGATC – 3'	A. van Schalkwyk
703F	5' – CAGGACGTTGTAAAACGACCGG – 3'	This study
pUC/M13 Forward	5' – GTTTCCCAGTCACGAC – 3'	Roche
pUC/M13 Reverse	5' – GTAAAACGACGGCCAGT – 3'	Roche

DSM - Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany

* Underlined are the restriction endonuclease sites for *Kpn*I in AN1, *Xba*I in AN2, *Eco*RI in JT1 and *Sac*I in JT2

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

3.2.2.1 Culturing conditions

A single colony of freshly-streaked cultures of *P. aeruginosa* DSM1707 as well as *P. aeruginosa* DSM1707 containing pALacZsd, pALacZsdIP and pALacZsd703 was inoculated into 10 ml LB-broth. The respective cultures were incubated at 37°C until an OD_{540} of 0.1, after which an aliquot (500 µl) of the exponentially growing cultures were inoculated into 2 ml LB-broth in 2-ml Eppendorf tubes with or without glass wool (0.025 g; mean diameter 15 µm, Merck). All cultures, except *P. aeruginosa* DSM1707, were supplemented with 100 $\mu g.ml^{-1}$ 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 latter consisted of both the surrounding suspended and glass wool-attached cells. To dislodge the attached cells from the glass wool substrate, each of the samples was vortexed for 1 min. Efficient removal of the attached cells from the glass wool substratum was verified by light microscopy. Aliquots of the different planktonic and biofilm-microcosm samples were then transferred to new tubes to be used for β-galactosidase assays and protein concentration determinations, respectively.

3.2.2.2 β-galactosidase activity 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 $mg.ml^{-1}$ 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 7 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 microtitre reader. All assays were performed in triplicate.

3.2.2.3 Protein concentration determination

The protein concentration of planktonic and biofilm-microcosm samples was determined using a commertial kit (Pierce Coomassie Plus) and bovine serum albumin (BSA) as standard. To determine the protein concentration, bacterial cells from the respective samples (500 µl) were collected by centrifugation at 12 000 × *g* for 5 min and the cell pellets were suspended 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 three 15-s pulses using an ultrasonic homogenizer (Cole-Palmer Series 4710 Ultrasonic Homogenizer), after which 50 µl of each sample was added to 1.5 ml Coomassie plus Protein Assay Reagent (Pierce), mixed well and the absorbance at 495 nm determined. UHQ water containing Coomassie plus Protein Assay Reagent was used to zero the absorbency readings. 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.

3.2.3 PCR amplification of different promoters

The constitutive *lac* promoter and the attachment-inducible *algD* promoter were obtained by PCR amplification using pALacZsdIP vector and *P. aeruginosa* chromosomal DNA as template DNA, respectively. Each of the reaction mixtures (50 µl) contained 10 ng of plasmid DNA or 100 ng of chromosomal DNA as template DNA, 1 × polymerase buffer (50 mM KCl, 10 mM Tris·HCl (pH 9.0), 0.1% [v/v] Triton X-100), 0.2 mM of each dNTP and 1 U of *Taq* DNA polymerase (Promega). For amplification of the *lac* promoter, 25 pmol of the JT1 and JT2 primers were used, while 25 pmol of the AN1 and AN2 primers were used to amplify the *algD* promoter (Table 3.1). The reaction tubes were placed in a Perkin-Elmer GeneAmp 2400 thermocycler. Following initial denaturation for 3 min at 95°C, the reactions were subjected to 25 cycles of denaturation for 30 s at 95°C, annealing for 40 s at 54°C (primers JT1 and JT2) or 30 s at 72°C (primers AN1 and AN2) and elongation for 1 min at 72°C. For control purposes, reaction mixtures containing UHQ water and all other reagents but no template DNA were included. The amplicons were resolved on a 1% (w/v) agarose gel in 1 × TAE buffer and visualised by UV-induced fluorescence after staining with ethidium bromide as described in Section 2.2.3.

3.2.4 Cloning of the amplicons into pGEM® – T Easy

Both the *lac* and *algD* promoter amplicons were purified from agarose gels using a silica suspension and then ligated into pGEM® – T Easy vector DNA (Promega) as described in Section 2.2.6. Following transformation of competent *E. coli* DH5α cells, a number of putative recombinant transformants were selected by blue/white colour selection from LB-agar plates supplemented with 100 $\mu g.ml^{-1}$ ampicillin. The putative recombinant transformants were cultured overnight at 37°C in LB-broth and the extracted plasmid DNA was then characterized by agarose gel electrophoresis and by restriction endonuclease digestion with *Eco*RI. Recombinant plasmids yielding DNA inserts of the expected size were designated pGEM-IP and pGEM-algD, respectively. In addition, the integrity of the cloned amplicons was verified by automated nucleic acid sequencing using the ABI PRISM™ BigDye™ Terminator Ready Reaction kit (Perkin-Elmer) and universal pUC/M13 forward and reverse sequencing primers as described previously (Section 2.2.12).

3.2.5 Construction of recombinant pAPR plasmids containing different promoters

The recombinant plasmid pAPR had previously been constructed (Chapter 2) and contains a cloned copy of the promoterless *aprAI* genes. To allow for the transcription of these genes by making use of various different promoters, several promoter-*aprAI* constructs were prepared as indicated in Fig. 3.1 to Fig. 3.3. The promoters used in these analyses consisted of the constitutive *lac* promoter, the attachment-inducible *algD* promoter and a *P. aeruginosa*-specific regulatory element, which will be referred to as 703. All molecular cloning techniques used in this part of the study are in accordance with those described in Chapter 2.

3.2.5.1 Construction of pIPAPR

The *lac* promoter DNA insert was recovered from the recombinant pGEM-IP clone by *Eco*RI and *Sac*I restriction enzyme digestion, purified from an agarose gel and then ligated into similarly prepared pAPR vector DNA. Following transformation of competent *E. coli* DH5α cells, the cells were plated onto LB-agar plates supplemented with 100 $\mu g.ml^{-1}$ ampicillin. Transformants were then randomly selected and characterized by restriction enzyme digestion following plasmid DNA extraction. A recombinant clone containing the cloned *lac* promoter was designated pIPAPR and used in subsequent DNA manipulations.

3.2.5.2 Construction of palgDAPR

The *algD* promoter DNA insert was recovered from the recombinant pGEM-algD vector by excision with both *Kpn*I and *Xba*I, gel-purified and then cloned into similarly prepared pUC18 vector DNA to yield pUC18-algD. The multiple cloning site (MCS) of pUC18, however, lacked the appropriate restriction endonuclease recognition sites to allow cloning of the promoter in the correct transcriptional orientation into pAPR. Thus, the *algD* promoter was cloned as a *Kpn*I - *Sal*I fragment into the *E. coli-Bacillus* shuttle vector, pSVB1, to create vector pSVBalgD. The *algD* promoter was then recovered from pSVBalgD by *Eco*RI and *Sac*I restriction enzyme digestion and subsequently ligated into similarly prepared pAPR vector DNA. The ligation mixture was transformed into competent *E. coli* DH5α cells and putative recombinant transformants were randomly selected and characterized by restriction enzyme digestion following plasmid DNA extraction. One of the recombinant clones containing the cloned *algD* promoter was selected for further use and designated palgDAPR.

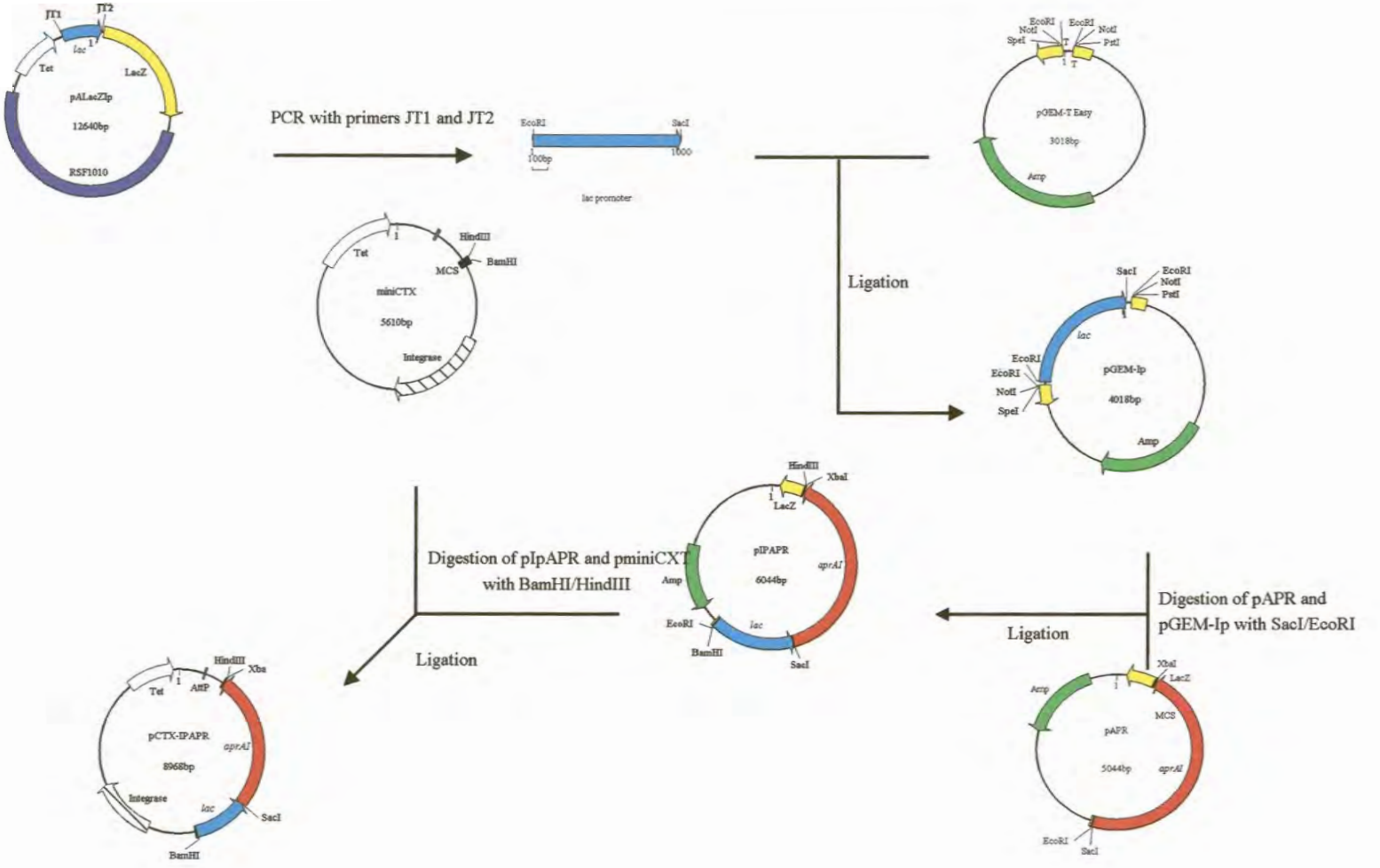

Fig. 3.1 Diagrammatic representation of the construction of plasmid pCTX-IPAPR.

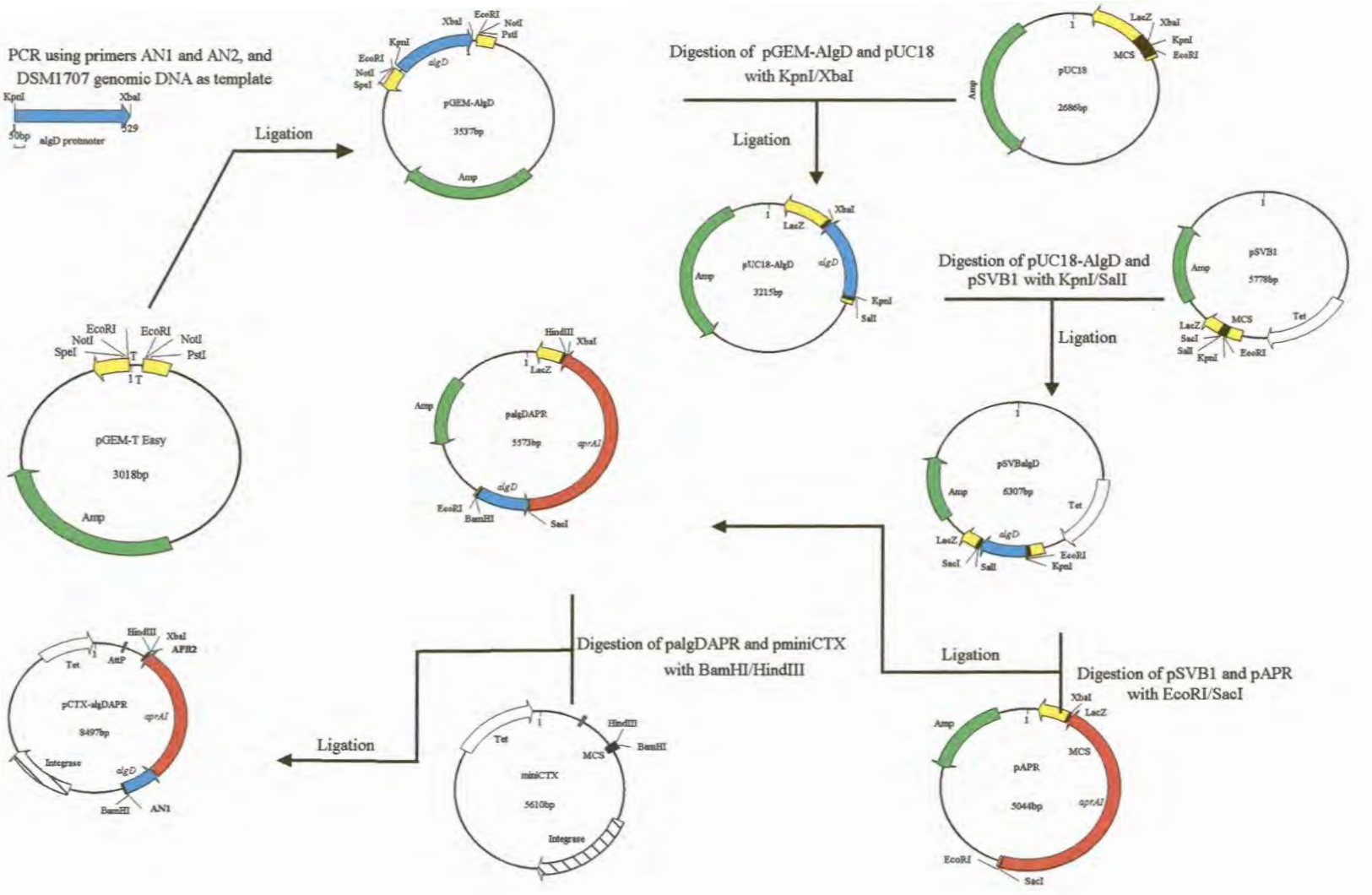

Fig. 3.2 Diagrammatic representation of the construction of plasmid pCTX-algDAPR.

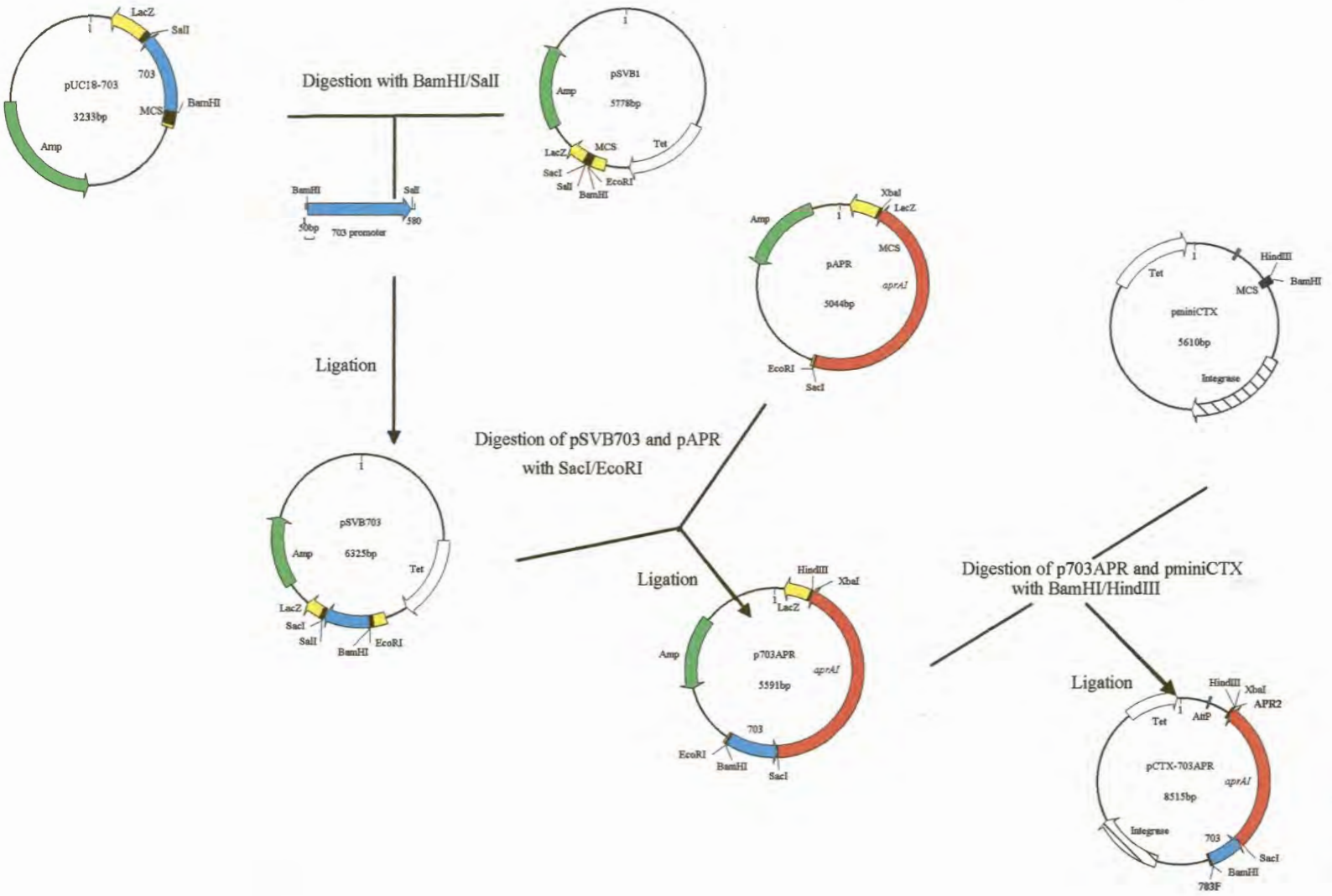

Fig. 3.3 Diagrammatic representation of the construction of plasmid pCTX-703APR.

3.2.5.3 Construction of p703APR

Plasmid pUC18-703, containing a cloned copy of the putative 703 promoter, was used as the source for construction of the recombinant p703APR plasmid DNA. The recombinant pUC18-703 plasmid DNA was digested with *Bam*HI and *Sal*I and the excised 703 DNA fragment was subsequently cloned into similarly digested pSVB1 vector DNA to yield vector pSVB703. To enable cloning of the 703 DNA fragment into pAPR, both the recombinant pSVB703 and pAPR plasmid DNA were digested with *Eco*RI and *Sac*I. The promoter-containing DNA fragment and restricted pAPR vector DNA were purified from an agarose gel, ligated overnight and then transformed into competent *E. coli* DH5α cells. The presence of the cloned DNA fragment was verified by restriction enzyme digestion of the isolated recombinant plasmid DNA and a recombinant clone, designated p703APR, was selected and used in all subsequent DNA manipulations.

3.2.6 Generation of *P. aeruginosa* mini-CTX transconjugants

3.2.6.1 Construction of recombinant mini-CTX plasmids containing different promoter-*aprAI* constructs

The mini-CTX cloning system (Hoang *et al.*, 2000) was used in order to stably integrate a single copy of the different promoter-*aprAI* expression cassettes onto the *P. aeruginosa* genome. To enable cloning of the *lac*/*algD*/703 promoter-*aprAI* cassettes into the mini-CTX vector, plasmids pIPAPR, palgDAPR and p703APR as well as mini-CTX vector DNA were digested with both *Bam*HI and *Hind*III. Each of the different promoter-*aprAI* fragments and restricted mini-CTX vector DNA were purified from agarose gels and ligated overnight before being transformed into competent *E. coli* DH5α cells. Recombinant transformants were selected from LB-agar plates supplemented with 20 $\mu g.ml^{-1}$ tetracycline. After overnight incubation at 37°C, plasmid DNA was extracted and further characterized by restriction enzyme digestion. The recombinant plasmids were designated pCTX-IPAPR, pCTX-algDAPR and pCTX-703APR, respectively.

3.2.6.2 Preparation and transformation of competent *P. aeruginosa* cells

Competent cells of both the parental *P. aeruginosa* DSM1707 as well as mutant DSMap0 strains were prepared and transformed essentially by the procedures previously described (Section 2.2.13). However, the protocol was adapted as follows to accommodate large-scale preparation of the competent cells. After inoculating 100 ml of TN-broth with a single colony of *P. aeruginosa* DSM1707 or DSMap0, the cultures were grown at 37°C to an OD_{540} of between 0.3 and 0.5. The cells from 30 ml of each culture were collected in polypropolene tubes by centrifugation at 4000 × *g* for 10 min at 4°C in a Sorval SS-34 rotor. The pellets were 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 $MgCl_2$ solution. After incubation on ice for 20 min, the cells were again collected by centrifugation and the pellets finally resuspended in 750 µl of ice-cold $MgCl_2$.

The prepared competent DSM1707 and DSMap0 cells were transformed by the addition of approximately 500 ng plasmid DNA (pCTX-IPAPR, pCTX-algDAPR and pCTX-703APR) to 200 µl of competent cells in a sterile Eppendorf 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 the addition of 500 µl of LB-broth, the transformation mixtures were incubated at 37°C for 3 h with shaking and then plated in aliquots of 100-200 µl onto LB-agar plates supplemented with 100 $\mu g.ml^{-1}$ tetracycline and incubated overnight.

3.2.6.3 Screening of putative transconjugants

Following incubation of the agar plates, colonies resulting from the transformation of both the parental DSM1707 and mutant DSMap0 strains were selected and analyzed for the presence of integrated copies of the different promoter-*aprAI* cassettes by PCR analyses. Whereas oligonucleotide primers JT1 and APR2 were used to amplify a 3.3-kb amplicon consisting of the 5' end of the *lac* promoter and the 3' end of the *aprI* gene, oligonucleotide primers AN1 and APR2 was used to amplify a 2.9-kb amplicon consisting of the 5' end of the *algD* promoter and the 3' end of the *aprI* gene. Oligonucleotide primers 703F and APR2 was used to amplify a 2.9-kb amplicon consisting of the 5' end of the putative 703 promoter and the 3' end of the *aprI* gene. The PCR reactions were performed as described in Section 3.2.3,

except that primer annealing was performed at 53°C for 45 s (primers APR2 and JT1), 58°C for 45 s (primers APR2 and AN1) or 55°C for 45 s (primers APR2 and 703F). For these analyses, UHQ water served as a negative control, while chromosomal DNA extracted from the parental and respective isogenic mutant strains provided sample template DNA. Following PCR amplification, aliquots of the various reaction mixtures were analyzed by gel electrophoresis on a 1% (w/v) agarose gel.

3.2.7 Generation of *P. aeruginosa* strains harbouring recombinant pJB3Tc20 plasmids

3.2.7.1 Construction of recombinant pJB3Tc20 plasmids containing different promoter-*aprAI* expression cassettes

By contrast to the mini-CTX system which allows for the stable integration of a single copy of the promoter-*aprAI* vector constructs onto the genome of *P. aeruginosa*, plasmid pJB3Tc20 is a high-copy-number plasmid that permits extrachromosomal replication of the recombinant DNA in a wide variety of Gram-negative bacteria, amongst other *E. coli* and *P. aeruginosa* (Blatney *et al.*, 1997). Thus, recombinant pJB3Tc20 plasmids were constructed in order to increase the number of cloned copies of the different promoter-*aprAI* expression cassettes within transformed cells. The recombinant pCTX-lPAPR, pCTX-algDAPR and pCTX-703APR plasmids served as sources for the construction of recombinant pJB3Tc20 plasmids. Both the recombinant pCTX-lPAPR and pCTX-703APR as well as pJB3Tc20 plasmid DNA was restricted with *Eco*RI and *Xba*I. The *lac-aprAI* and 703-*aprAI* DNA inserts as well as restricted pJB3Tc20 vector DNA were purified from agarose gels, ligated overnight and then transformed into competent *E. coli* DH5α cells. Similarly, the *algD-aprAI* DNA insert was recovered from the recombinant pCTX-algDAPR plasmid following digestion with *Eco*RI and *Hind*III and then cloned into identically digested pJB3Tc20 vector DNA. Recombinant transformants were selected from LB-agar plates supplemented with 20 $\mu g.ml^{-1}$ tetracycline after incubation overnight at 37°C, and further characterized by restriction enzyme digestion following plasmid DNA extraction. The selected recombinant plasmids were designated pJB-lPAPR, pJB-703APR and pJB-algDAPR, respectively.

3.2.7.2 Tri-parental conjugation

As an alternative to the transformation of competent *P. aeruginosa* cells, the different recombinant pJB3Tc20 plasmid constructs were introduced into *P. aeruginosa* by tri-parental conjugation (Greener *et al.*, 1992). Using a sterile inoculation needle, a loop-full of growth of donor (*E. coli* DH5α containing the recombinant pJB3Tc20 vector DNA), helper (*E. coli* DH5α containing the helper plasmid pRK600) and recipient (*P. aeruginosa* DSM1707 or DSMap0) strains were mixed on a LB-agar plate lacking antibiotics and incubated overnight at 37°C. The mixed growth was then streaked onto selective LB-agar medium to select for successfully transformed cells. In the case of the parental DSM1707 strains, the LB-agar was supplemented with 100 $\mu g.ml^{-1}$ tetracycline and 150 $\mu g.ml^{-1}$ ampicillin. In the case of the mutant DSMap0 strains, the LB-agar was supplemented with 100 $\mu g.ml^{-1}$ tetracycline, 150 $\mu g.ml^{-1}$ ampicillin and 50 $\mu g.ml^{-1}$ gentamicin. The agar plates were subsequently incubated at 37°C until colonies became visible.

3.2.7.3 Colony PCR

Selected colonies were transferred to Eppendorf tubes containing 500 µl UHQ water, vortexed and boiled for 5 min at 95°C. The cellular debris was pelleted by centrifugation for 2 min at 12 000 × *g* and 5 µl of the supernatant, containing the DNA, was used as template in the PCR reactions. In addition, the PCR reaction mixtures contained 1 × polymerase buffer, 1.5 $MgCl_2$, 25 pmol each of the appropriate oligonucleotide primers (Section 3.2.6.3), 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 2400 thermocycler (Perkin-Elmer) and subjected to 25 cycles of amplification using conditions identical to those described in Section 3.2.6.3. For control purposes, a reaction mixture containing UHQ 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.

3.2.8 Extracellular proteolytic assays

The extracellular proteolytic activity of planktonic and biofilm-microcosm populations of the different wild-type *P. aeruginosa* DSM1707 and isogenic mutant DSMap0 strains were determined as follows. Briefly, single colonies of the various recombinant DSM1707 and DSMap0 strains were inoculated into 20 ml TSB broth, supplemented with the appropriate antibiotics, in 100-ml Erlenmeyer flasks with or without glass wool (4 g; mean diameter 15 µm, Merck). Following overnight incubation at 30°C with agitation, the cells of each culture were pelleted by centrifugation at 5000 × *g* for 10 min. The cell-free culture supernatants (20 ml) were dialysed against 30 mM Tris buffer (pH 7.5) for 30 h before being concentrated by lyophilization. The proteolytic activity of the supernatants was subsequently investigated spectrophotometrically using azocasein as substrate as described in Section 2.2.16. Total culturable counts were obtained by serially diluting an aliquot (100 µl) of each of the planktonic and biofilm-microcosm samples in ddH_2O and plating in triplicate onto LB-agar plates whereafter the colonies were counted after incubation of the plates at 37°C for 18 h. The azocasein hydrolysis activity per total cellular biomass was determined by dividing the azocasein hydrolysis values by the total cuturable count to ensure that the difference in extracellular proteolytic activity was due to a difference in promoter activity, and not due to variations in the yield of biomass. All of the above assays were performed on three separate samples.

3.3 RESULTS

This part of the study was aimed at evaluating different approaches whereby the level of extracellular alkaline protease of *P. aeruginosa* might be increased. The recombinant plasmid pAPR had previously been constructed (Chapter 2) and contains a cloned DNA fragment with the promoterless *aprA* and *aprI* genes. Both these cloned genes contain a Shine-Dalgarno sequence to allow for efficient translation in *P. aeruginosa* and a transcriptional terminator is situated at the 3' end of the *aprI* gene (Duong *et al.*, 1992). To allow for transcription of these genes, several different promoter-*aprAI* constructs were prepared. The promoters used in these analyses consisted of the constitutive *lac* promoter and the attachment-inducible promoters, *algD* and 703 (see below). Amongst the various approaches

commonly used to increase the level of heterologous protein expression in the host organism, the use of the above promoters as well as the effect of differences in gene dosage and culturing conditions, were specifically investigated in this part of the study.

3.3.1 β-galactosidase activity assays

In an earlier study undertaken by Weyers (1999), several genetic elements, which were thought to be important in regulating the expression of genes required for the attachment of *P. aeruginosa* to a surface, were isolated. One of these elements, 703, was subsequently chosen for use in this study. BLAST homology searches against the *P. aeruginosa* PAO1 genome sequence (Stover *et al.*, 2000; www.pseudomonas.com) indicated that the genetic element mapped 808 bp upstream from the *P. aeruginosa* PA2897 gene, which encodes for a putative transcription regulatory protein.

To assay the promoter activity of the 703 regulatory element, the β-galactosidase expression of *P. aeruginosa* DSM1707 containing recombinant and control pALacZsd reporter vector constructs (Table 3.1) was assayed 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 containing pALacZsdIP, which contains the strong constitutive *lac* promoter from the pBluescript vector, 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 biofilm-microcosm cells. All assays were performed in triplicate and the results were analyzed as described in Materials and Methods (Section 3.2.2.3), and are presented in Fig. 3.4.

The results obtained for *P. aeruginosa* DSM1707 cells indicated a lack of β-galactosidase expression and served to confirm that *P. aeruginosa* is a β-galactosidase-negative host and thus suitable for use with the pALacZsd reporter vector. As expected, cells containing the promoterless control vector pALacZsd displayed a low level of β-galactosidase activity, while cells containing the positive control vector pALacZsdIP displayed high levels of β-galactosidase activity under all the growth conditions investigated. The results furthermore

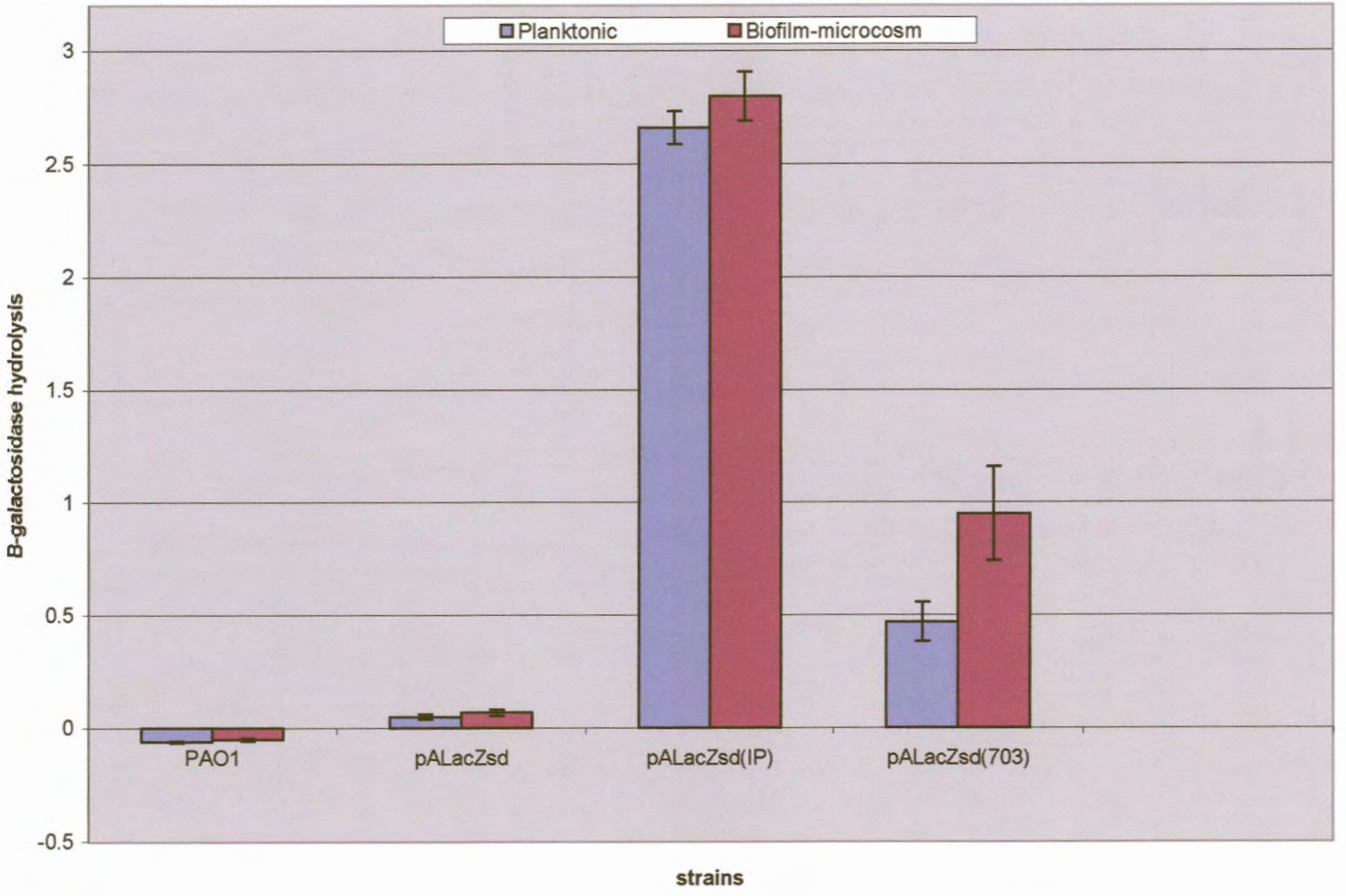

Fig. 3.4 Graph displaying the β-galactosidase activity per total cellular biomass (A_{414}/protein) of the 703 promoter-*lacZ* construct in planktonic (P) cells and biofilm-microcosm (A) cells. pALacZsdIP was used as a positive control vector, whereas pALacZsd was used as a negative control vector. Error bars denote one standard deviation of the mean.

indicated that the reporter gene expression from pALacZsd703 was 5.6-fold lower in the planktonic population and 2.9-fold lower in the biofilm-microcosm compared to the results obtained for cultures containing pALacZsdIP. However, the *lacZ* reporter gene from pALacZsd703 was up-regulated 2-fold in the biofilm-microcosm compared to the planktonic population. Based on these results, it was concluded that the cloned DNA insert of pALacZsd703 displayed promoter activity and that this activity was up-regulated by attachment of the *P. aeruginosa* cells to a substratum. Consequently, the putative attachment-inducible 703 promoter was included for further use in this study.

3.3.2 Construction of recombinant plasmids pCTX-IPAPR, pCTX-algDAPR and pCTX-703APR

3.3.2.1 Construction of pCTX-IPAPR

The reporter vector pALacZsdIP contains a constitutive *lac* promoter and was shown in the preceding section to display a high level of promoter activity in both the planktonic population and biofilm-microcosm populations of *P. aeruginosa* (Fig. 3.4). To amplify the *lac* promoter, a PCR-based approach was adopted by making use of oligonucleotide primers that anneal to vector sequences flanking the multiple cloning site (MCS) of pALacZsd into which the *lac* promoter had been cloned (Weyers, 1999). The strategy for constructing plasmid pCTX-IPAPR is indicted in Fig. 3.1.

Using pALacZsdIP as template DNA and oligonucleotide primers JT1 (containing a *Eco*RI site) and JT2 (containing a *Sac*I site), PCR was performed as described under Materials and Methods (Section 3.2.3). Following agarose gel electrophoresis of the reaction mixture, a single amplicon of the expected size (1 kb) was obtained. The agarose gel-purified amplicon was subsequently cloned into the pGEM® – T Easy vector. Restriction of the derived recombinant plasmid DNA with both *Eco*RI and *Sac*I resulted in the excision of a 1-kb product, indicating that the amplicon was successfully cloned into the vector. A recombinant clone, pGEM-IP, was selected and the integrity of the cloned insert DNA was verified by automated nucleic acid sequencing analysis prior to it being used in the construction of the desired clones.

To construct the intermediate vector pIPAPR, the *lac* promoter DNA insert was recovered from pGEM-IP by digestion with both *Eco*RI and *Sac*I and recloned into similarly prepared pAPR vector DNA. A number of colonies resulting from the transformation of competent *E. coli* DH5α cells were selected, plasmid DNA extracted and characterized by agarose gel electrophoresis and by restriction enzyme digestion. A recombinant plasmid from which an insert of the expected size (1 kb) was excised by *Eco*RI and *Sac*I digestion was selected and designated pIPAPR. The *lac* promoter was thus cloned in the correct transcriptional orientation upstream from the previously cloned *aprAI* genes. The *lac* promoter-*aprAI* expression cassette was finally cloned into mini-CTX as a *Bam*HI - *Hind*III DNA fragment to yield plasmid pCTX-IPAPR. Characterization of the recombinant plasmid by restriction with *Bam*HI and *Hind*III (Fig. 3.5, lane 3) resulted in two DNA fragments corresponding in size to the *lac* promoter-*aprAI* expression cassette (3.3 kb) and linearized mini-CTX vector DNA (5.6 kb).

3.3.2.2 Construction of pCTX-algDAPR

The product encoded by the *algD* gene is involved in the biosynthesis of the exopolysaccharide alginate (Núñez *et al.*, 2000; Tavares *et al.*, 1999; Gacesa, 1998) and expression of the *algD* operon has been reported to be up-regulated following attachment of *P. aeruginosa* cells to a surface (Hoyle *et al.*, 1993). Recently, the *algD* promoter was also shown to up-regulate the expression of a *lacZ* reporter gene following attachment of the *P. aeruginosa* cells to a glass wool substratum (Cooper, 2003). Oligonucleotide primers were thus designed to amplify a region of the *P. aeruginosa* genome comprising nucleotides 3961887 to 3962464, which contains the attachment-inducible *algD* promoter sequence together with upstream regulatory sequences. The strategy used for constructing plasmid pCTX-algDAPR is indicated in Fig. 3.2.

Using genomic DNA isolated from *P. aeruginosa* DSM1707 as template, PCR was carried out with primers AN1 (containing a *Kpn*I site) and AN2 (containing a *Xba*I site). An aliquot of the PCR reaction mixture was analysed by agarose gel electrophoresis and a single major DNA band of approximately 500 bp was observed. The amplicon was purified from the agarose gel and subsequently cloned into the pGEM® – T Easy vector. The putative recombinant plasmids were analysed for *algD* promoter-specific DNA inserts by digesting

the plasmid DNA with both *Kpn*I and *Xba*I. This resulted in excision of the expected 500 bp DNA fragment, indicating that the amplicon was successfully cloned. The integrity of the cloned *algD* promoter in a selected clone, pGEM-algD, was verified by automated nucleic acid sequencing and used in further DNA cloning procedures.

Since the recombinant pGEM-algD vector lacked appropriate enzyme recognition sites within the multiple cloning site (MCS), which would facilitate construction of the desired clones, the DNA insert was recovered by digestion with *Kpn*I and *Xba*I and first cloned into similarly prepared pUC18 vector DNA to yield pUC18-algD, before being cloned as a *Sal*I-*Kpn*I DNA fragment into pSVB1 vector DNA. The derived recombinant plasmid was designated pSVBalgD and used in the construction of the intermediate vector palgDAPR. To construct palgDAPR, the *algD* promoter DNA fragment was excised from pSVBalgD by digestion with both *Eco*RI and *Sac*I to allow for cloning of the promoter sequence in the correct transcriptional orientation into similarly digested pAPR vector DNA, upstream from the previously cloned *aprAI* genes. The *algD* promoter-*aprAI* expression cassette was then cloned into mini-CTX vector DNA as a *Bam*HI-*Hind*III DNA fragment to complete construction of the pCTX-algDAPR plasmid. Digestion of the recombinant pCTX-algDAPR plasmid DNA with *Bam*HI and *Hind*III resulted in excision of the cloned 2.9-kb DNA insert, indicating that the expression cassette was successfully cloned into the vector (Fig. 3.5, lane 5).

3.3.2.3 Construction of pCTX-703APR

Earlier in this study, the putative 703 promoter was shown to be responsible for up-regulated expression of the *lacZ* reporter gene upon attachment of *P. aeruginosa* cells to a glass wool substratum and was subsequently included in this investigation as a second attachment-inducible *P. aeruginosa*-specific promoter. The strategy used for the construction of plasmid pCTX-703APR is indicated in Fig. 3.3.

The putative attachment-inducible 703 promoter had been previously cloned into pUC18 vector DNA. Consequently, the 547-bp DNA fragment was recovered from the recombinant pUC18-703 plasmid by digestion with both *Bam*HI and *Sal*I, purified from an agarose gel and then cloned into pSVB1, which had also been digested by these enzymes to generate plasmid pSVB703. This cloning step was required, as discussed above, to allow for cloning of the 703

promoter sequence in the correct transcriptional orientation relative to the *aprAI* genes in the recombinant pAPR plasmid DNA. The 703 promoter DNA insert was thus recovered from pSVB703 by restriction with *Eco*RI and *Sac*I, and cloned into similarly prepared pAPR vector DNA to yield the intermediate vector p703APR. The 703 promoter-*aprAI* expression cassette was subsequently recloned into the *Bam*HI and *Hind*III sites of the mini-CTX vector DNA to generate the pCTX-703APR plasmid. Digestion of the recombinant pCTX-703APR plasmid DNA with both *Bam*HI and *Hind*III resulted in digestion products of approximately 5.6 kb and 2.9 kb (Fig. 3.5, lane 7). The sizes of these fragments corresponded with the expected size of the vector DNA and the size of the cloned 703 promoter-*aprAI* cassette, respectively.

3.3.3 Generation and characterization of parental *P. aeruginosa* DSM1707 and isogenic mutant DSMap0 strains containing integrated copies of the respective recombinant mini-CTX plasmids

Competent cells of both the parental *P. aeruginosa* DSM1707 and isogenic alkaline protease-deficient DSMap0 strains were prepared and then transformed with each of the newly constructed recombinant mini-CTX plasmids. Parental and mutant *P. aeruginosa* strains harbouring integrated copies of pCTX-IPAPR, pCTX-algDAPR and pCTX-703APR were selected for single recombination events using a selective medium as described in Materials and Methods (Section 3.2.6.2). Whereas the parental *P. aeruginosa* strains containing integrated copies of the different vector constructs were designated DSM1707CTXIPAPR, DSM1707CTXalgDAPR and DSM1707CTX703APR, the strains generated from the alkaline protease-deficient DSMap0 strain were designated as DSMap0CTXIPAPR, DSMap0CTXalgDAPR and DSMap0CTX703APR.

The presence of the integrated vector constructs in the different strains was confirmed by colony PCR analyses. In each case, the region spanning the promoter-*aprAI* cassette was amplified by making use of primer APR2 in conjunction with primers JT1, AN1 or 703F. Whereas primer APR2 anneals 133 nt downstream from the 3' end of the *aprI* gene, the promoter-specific primers anneal to the 5' ends of the respective *lac*, *algD* and 703 promoter sequences. The use of primers JT1 and APR2 together with chromosomal DNA from strains

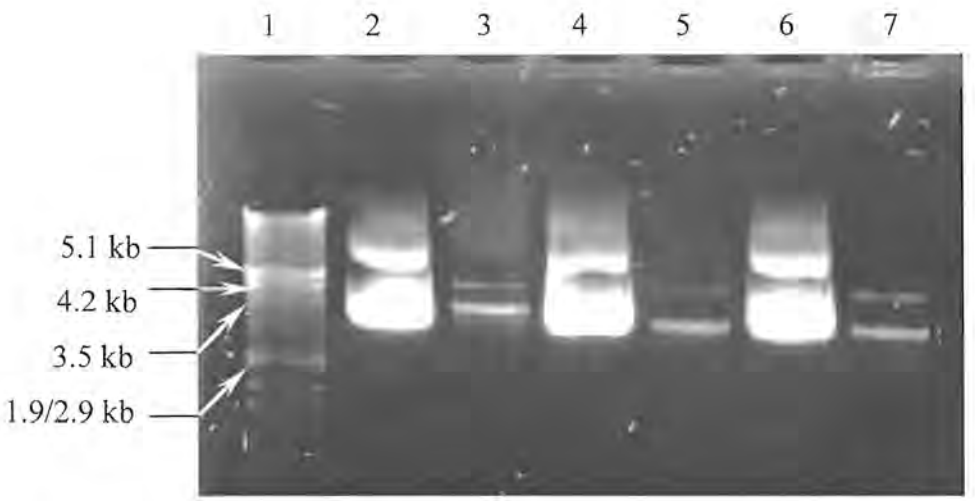

Fig. 3.5 Agarose gel electrophoretic analysis of the recombinant plasmids pCTX-IPAPR, pCTX-algDAPR, and pCTX-703APR. Lane 1, DNA molecular weight marker; Lane 2, uncut plasmid pCTX-IPAPR; Lane 3, plasmid pCTX-IPAPR digested with *Bam*HI and *Hind*III; Lane 4, uncut plasmid pCTX-algDAPR; Lane 5, plasmid pCTX-algDARP digested with *Bam*HI and *Hind*III; Lane 6, uncut pCTX-703APR; Lane 7, plasmid pCTX-703APR digested with *Bam*HI and *Hind*III. The sizes of the molecular weight marker, phage lambda DNA restricted with *EcoRI* and *Hind*III, are indicated to the left of the figure.

DSM1707CTXIPAPR and DSMap0CTXIPAPR, each yielded an expected 3.3-kb *lac* promoter-*aprAI* product following PCR amplification (Figs. 3.6 and 3.7, lanes 3). Primers AN1 and APR2 were used to successfully amplify a 2.9-kb *algD* promoter-*aprAI* product when DSM1707CTXalgDAPR and DSMap0CTXalgDAPR chromosomal DNA were used as template in the respective PCR reactions (Figs. 3.6 and 3.7, lanes 4). By making use of DSM1707CTX703APR and DSMap0CTX703APR chromosomal DNA as template in the respective PCR reactions and primers 703F and APR2, an expected 2.9-kb 703 promoter-*aprAI* product was amplified (Figs. 3.6 and 3.7, lanes 5). No similar bands to those obtained above were observed in control reactions lacking template DNA (Figs. 3.6 and 3.7, lanes 6).

Thus, the strains derived from the parental *P. aeruginosa* DSM1707 strain each contained, in addition to the *aprAI* genes located in the *apr* operon, an additional copy of the *aprAI* genes but under transcriptional control of different promoters. Each of the strains derived from the mutant DSMap0 strain, however, contained only a single copy of the constructed promoter-*aprAI* expression cassettes. The respective strains were subsequently used in all further analyses.

3.3.4 Construction of recombinant plasmids pJB-IPAPR, pJB-algDAPR and pJB-703APR

To increase the dosage of the different promoter-*aprAI* expression cassettes within transformed cells, DNA inserts from the above pCTX clones were recloned into the high-copy-number pJB3Tc20 vector. The *lac* promoter- and 703 promoter-*aprAI* expression cassettes were recovered from pCTX-IPAPR and pCTX-703APR, respectively, using *Eco*RI and *Xba*I and then cloned into pJB3Tc20 vector DNA, which had been digested in the same manner. After transformation, recombinant plasmid DNA was selected from which insert DNA of the expected size was excised by digestion with *Eco*RI and *Xba*I (Fig. 3.8, lanes 3 and 7). The size of the observed restriction DNA fragments corresponded to the size of the pJB3Tc20 vector DNA (7 kb) and the size of either the cloned *lac* promoter-*aprAI* DNA insert (3.3 kb) or the cloned 703 promoter-*aprAI* DNA insert (2.9 kb). The recombinant clones were designated pJB-IPAPR and pJB-703APR, respectively.

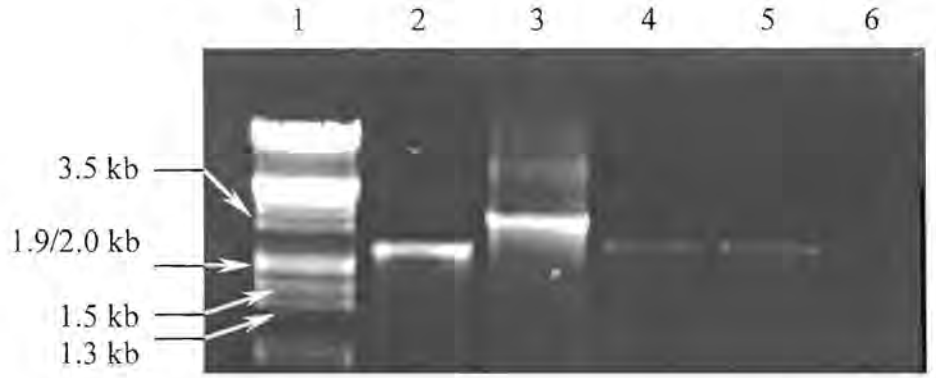

Fig. 3.6 Agarose gel electrophoretic analysis of PCR products generated from DSM1707 cell lysates containing integrated copies of different promoter-*aprAI* constructs. Lane 1, DNA molecular weight marker; Lane 2, PCR amplification of DSM1707 using primers APR1 and APR2; Lane 3, PCR amplification of DSM1707CTXIPAPR using primers JT1 and APR2; Lane 4, PCR amplification of DSM1707CTXalgDAPR using primers AN1 and APR2; Lane 5, PCR amplification of DSM1707CTX703APR using primers 703F and APR2; Lane 6, negative control reaction lacking template DNA. Sizes of the molecular weight marker, phage lambda DNA restricted with *Eco*RI and *Hind*III, are indicated to the left of the figure.

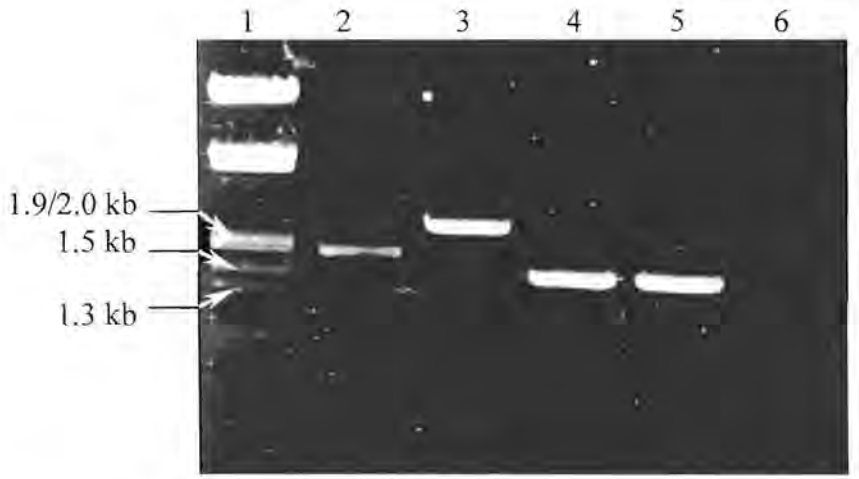

Fig. 3.7 Agarose gel electrophoretic analysis of PCR products generated from DSMap0 cell lysates containing integrated copies of different promoter-*aprAI* constructs. Lane 1, Molecular weight marker; Lane 2, PCR amplification of DSMap0 template using primers APR1 and APR2; Lane 3, PCR amplification of DSMap0CTXIPAPR using primers JT1 and APR1; Lane 4, PCR amplification of DSMap0CTXalgDAPR using primers AN1 and APR2; Lane 5, PCR amplification of DSMap0CTX703APR using primers 703F and APR2 with as template; Lane 6, negative control reaction lacking template DNA. Sizes of the molecular weight marker, phage lambda restricted with *Eco*RI and *Hind*III, are indicated to the left of the figure.

To construct the pJB-algDAPR plasmid, the *algD* promoter-*aprAI* expression cassette was recovered from pCTX-algAPR and cloned as an *Eco*RI - *Hind*III DNA fragment into the pJB3Tc20 vector DNA. The resultant recombinant plasmid DNA was characterized by restriction enzyme digestion. Restriction of the recombinant plasmid DNA with *Eco*RI and *Hind*III resulted in two DNA fragments corresponding in size to the 7-kb pJB3Tc20 vector DNA and the 2.9-kb *algD* promoter-*aprAI* DNA insert (Fig. 3.8, lane 5).

Each of the recombinant pJB3Tc20 plasmid constructs were subsequently introduced into both the wild-type *P. aeruginosa* DSM1707 as well as mutant DSMap0 strains by conjugation. The presence of the recombinant vector constructs in the resultant DSM1707 and DSMap0 strains was then confirmed by colony PCR analyses. Cell lysates were thus prepared from both the transformed and untransformed control strains, and by making use of the appropriate primers and cell lysates (Section 3.2.7.3) regions corresponding to the promoter-*aprAI* cassettes were amplified. Amplicons of the expected sizes were obtained, and no similar bands were obtained in control reactions prepared from untransformed parental DSM1707 and mutant DSMap0 strains, or in reactions lacking template DNA (results not shown).

3.3.5 Extracellular proteolytic activity of *P. aeruginosa* DSM1707 and DSMap0 cells containing integrated copies of the recombinant mini-CTX vector constructs

The extracellular proteolytic activity of planktonic and biofilm-microcosm populations of the different recombinant *P. aeruginosa* DSM1707 strains (DSM1707CTXIPAPR, DSM1707CTXalgDAPR and DSM1707CTX703APR) and different recombinant DSMap0 strains (DSMap0CTXIPAPR, DSMap0CTXalgDAPR and DSMap0CTX703APR) was assayed using azocasein as substrate. *P. aeruginosa* strains DSM1707 and DSMap0 were included in these assays as controls whereby the wild-type and residual extracellular proteolytic activities could be determined, respectively. All assays were performed in triplicate and the results were analysed as described in Materials and Methods (Section 3.2.8), and are presented in Fig. 3.9.

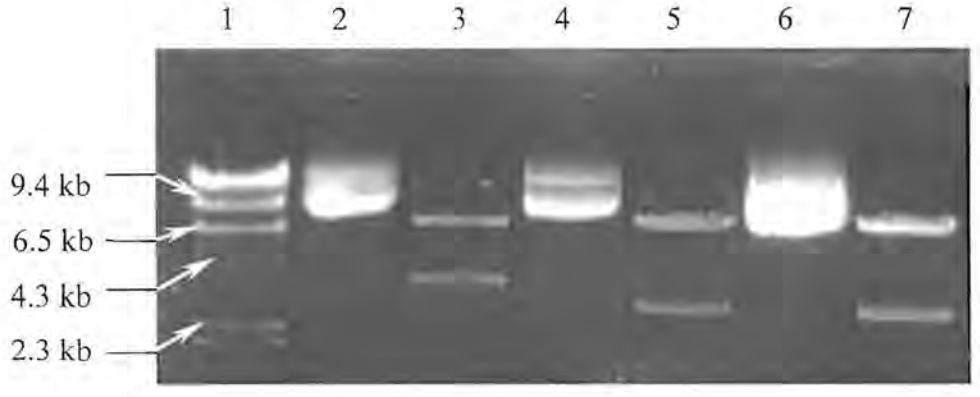

Fig. 3.8 Agarose gel electrophoretic analysis of the recombinant pJB3Tc20 vector constructs containing different promoter-*aprAI* expression cassettes. Lane 1, DNA molecular weight marker; Lane 2, uncut plasmid pJB-IPAPR; Lane 3, plasmid pJB-IPAPR digested with *Eco*RI and *Xba*I; Lane 4, uncut plasmid pJB-algDAPR; Lane 5 plasmid pJB-algDAPR digested with *Eco*RI and *Hind*III; Lane 6, uncut plasmid pJB-703APR; Lane 7, plasmid pJB-703APR digested with *Eco*RI and *Xba*I. The sizes of the molecular weight marker, phage lambda DNA restricted with *Hind*III, are indicated on the left of the figure.

3.3.5.1 Extracellular proteolytic activity of planktonic cultures

Analysis of the results obtained for the strains derived from *P. aeruginosa* DSM1707 (Fig. 3.9a) indicated that both the DSM1707CTXIPAPR and DSM1707CTXalgDAPR strains yielded similar proteolytic activities as the parental *P. aeruginosa* DSM1707 strain, while slightly less proteolytic acitivity was observed for the DSM1707CTX703APR strain. The level of activity displayed by these aforementioned two strains was approximately 0.9-fold lower than that of the parental strain, and 1.2-fold higher than that of the DSM1707CTX703APR strain. By contrast, the planktonic cultures of the strains derived from DSMap0 displayed low levels of extracellular proteolytic activity. Although the level of activity was almost half of the activity observed for the corresponding DSM1707-derived strains, it was nevertheless slightly higher (1.3- to 1.5-fold) than the residual extracellular proteolytic activity displayed by the parental DSMap0 strain (Fig. 3.9a).

3.3.5.2 Extracellular proteolytic activity of biofilm-microcosms

The extracellular proteolytic activity of biofilm-microcosms from the strains derived from *P. aeruginosa* DSM1707 (Fig. 3.9b) resembled the results previously obtained for the planktonic cultures (Fig. 3.9a). The respective strains yielded similar proteolytic activities, which was only slightly lower (1.0- to 1.3-fold) than the parental DSM1707 strain, but were at least twice as high as the level of proteolytic activity displayed by the corresponding strains derived from DSMap0. The levels of extracellular proteolytic activity observed for biofilm-microcosms of strains derived from DSMap0 (Fig. 3.9b) were also similar to each other, but were, however, slightly higher than the values of the corresponding planktonic cultures (Fig. 3.9a).

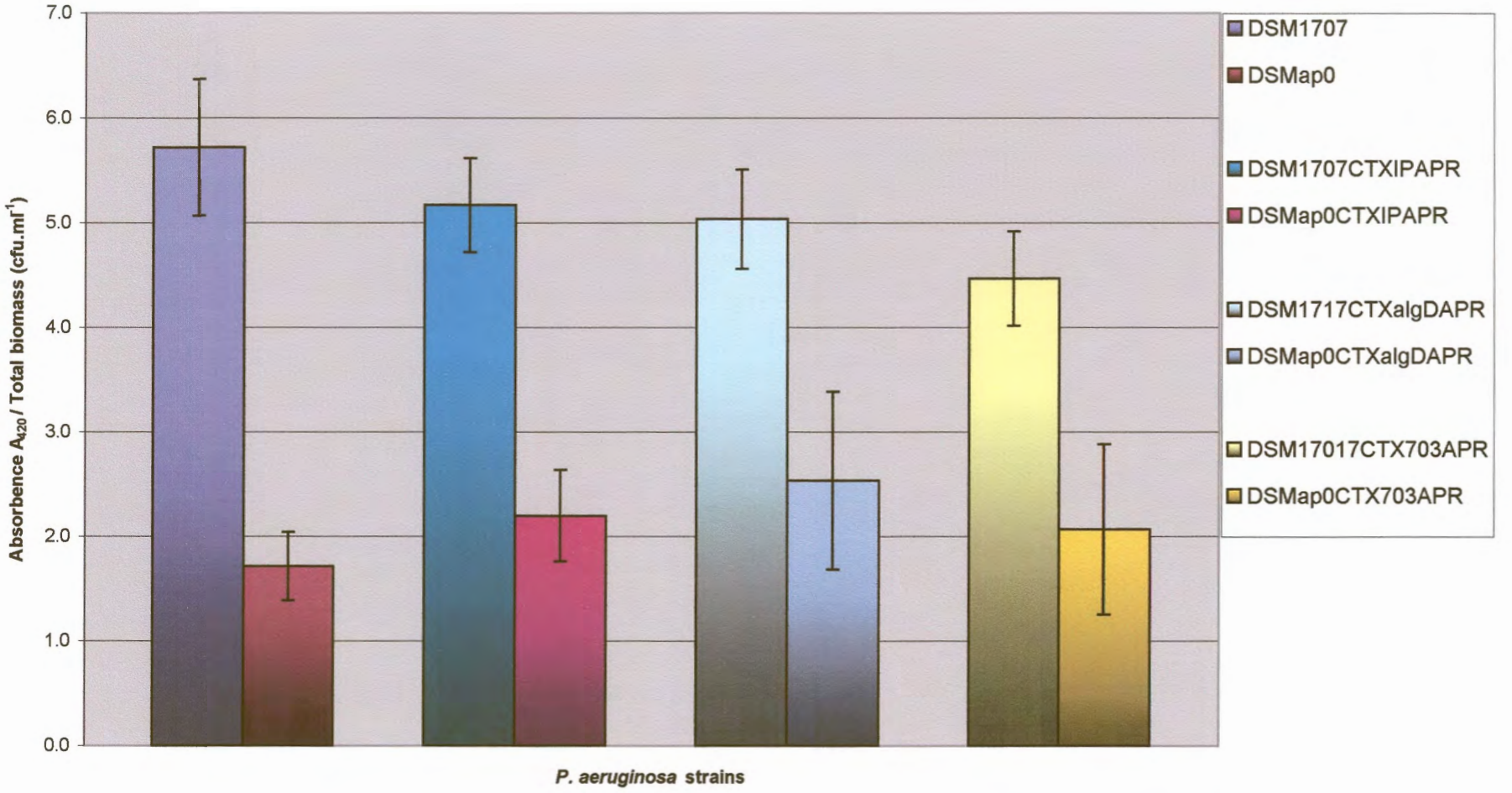

Fig. 3.9a Extracellular proteolytic activity per total biomass (in cfu.ml^{-1}) of planktonic cultures of strains derived from the *P. aeruginosa* DSM1707 and alkaline-deficient mutant DSMap0 strains. The strains each contain an integrated copy of the respective promoter-*aprAI* expression cassettes. Error bars denote one standard deviation of the mean.

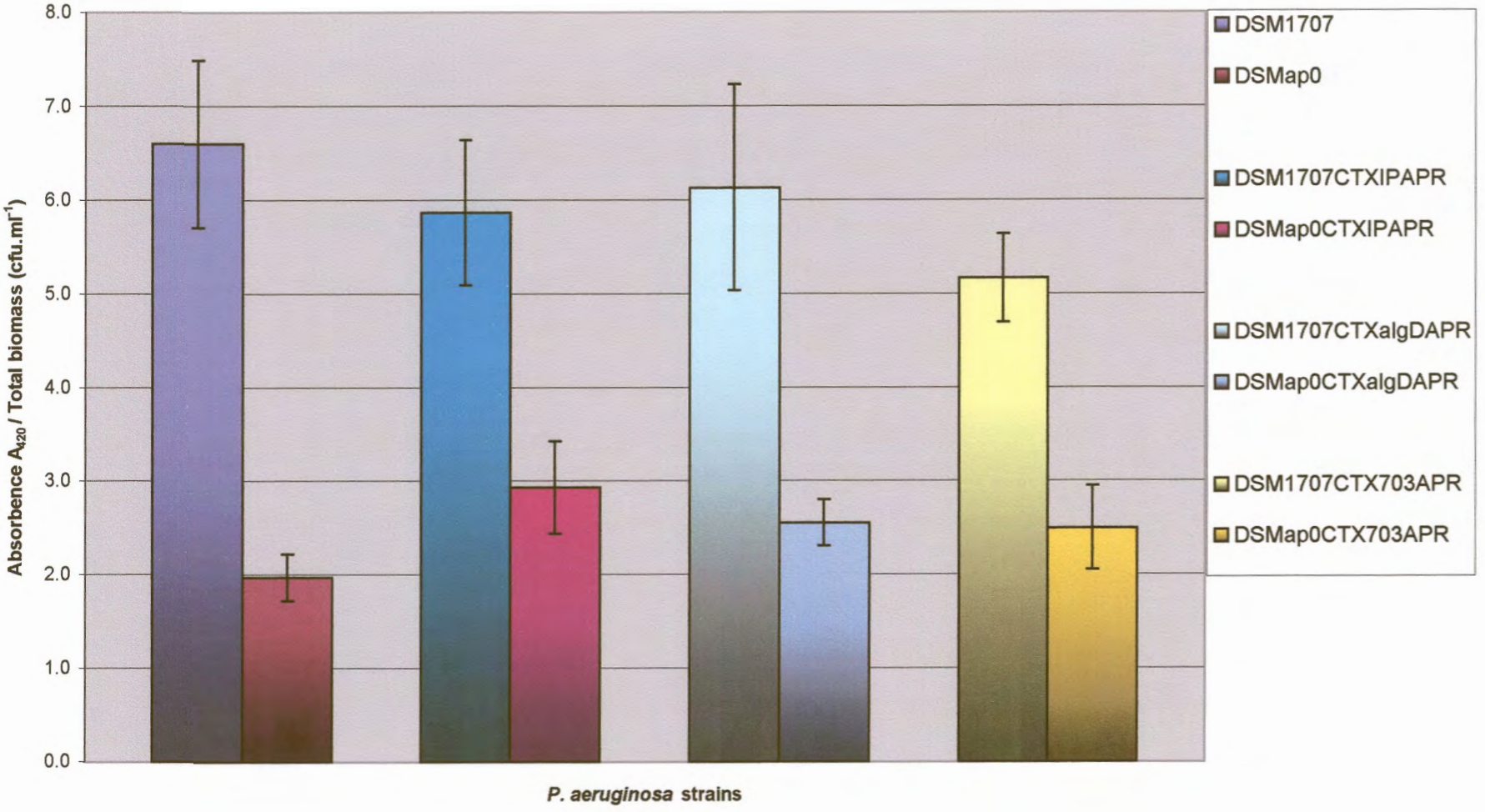

Fig. 3.9b Extracellular proteolytic activity per total biomass (in cfu.ml^{-1}) of biofilm-microcosms of strains derived from the *P. aeruginosa* DSM1707 and alkaline-deficient mutant DSMap0 strains. The strains each contain an integrated copy of the respective promoter-*aprAI* expression cassettes. Error bars denote one standard deviation of the mean.

3.3.6 Extracellular proteolytic activity of *P. aeruginosa* DSM1707 and DSMap0 strains transformed with different recombinant pJB3Tc20 vector constructs

To investigate what the effect would be on the extracellular proteolytic activity of *P. aeruginosa* if the respective promoter-*aprAI* expression cassettes were supplied in higher dosages, the expression cassettes were cloned into the high-copy-number pJB3Tc20 plasmid, and the constructed recombinant vectors (Table 3.1) were then introduced into the parental DSM1707 and isogenic mutant DSMap0 strains by tri-parental matings. The extracellular proteolytic activity of planktonic populations and biofilm-microcosms of the transformed DSM1707 and DSMap0 strains, as well as untransformed strains, were assayed as above using azocasein as substrate. The results of these analyses are indicated in Fig. 3.10.

3.3.6.1 Extracellular proteolytic activity of planktonic cultures

Analysis of the results obtained for planktonic cultures of *P. aeruginosa* DSM1707 transformed with the different recombinant pJB3Tc20 vector constructs (Fig. 3.10a) indicated that the respective recombinant strains each displayed similar levels of extracellular proteolytic activity. The level of extracellular proteolytic activity was, however, on average, 2.3-fold lower than that observed for the parental *P. aeruginosa* DSM1707 strain. These levels of activity were notably lower (at least 2-fold) than those previously observed for the planktonic cultures of *P. aeruginosa* DSM1707 harboring an integrated copy of the different promoter-*aprAI* expression cassettes (Fig. 3.9b). Moreover, the levels of extracellular proteolytic activity were comparable to those observed for the *P. aeruginosa* DSMap0 cultures transformed with the corresponding recombinant vector constructs. The extracellular proteolytic activity of planktonic cultures of DSMap0 cells transformed with the different recombinant pJB3Tc20 vector constructs (Fig. 3.10a) were identical to each other, and was 1.4-fold higher than the residual extracellular proteolytic activity observed for the untransformed *P. aeruginosa* DSMap0 strain.

3.3.6.2 Extracellular proteolytic activity of biofilm-microcosms

The results that were obtained for biofilm-microcosms of the *P. aeruginosa* DSM1707 and DSMap0 strains transformed with the various different recombinant pJB3Tc20 vector constructs (Fig. 3.10b) displayed the same trend as previously observed for the planktonic

cultures (Fig. 3.10a). The level of extracellular proteolytic activity displayed by the biofilm-microcosms of the respective recombinant DSM1707 strains was similar to each other, but was nevertheless higher than the activity displayed by the corresponding planktonic cultures. Similarly, the extracellular proteolytic activities displayed by biofilm-microcosms of the various recombinant DSMap0 strains were also similar to each other, and the activities displayed by these strains were marginally higher (1.5- to 1.8-fold) compared to the corresponding planktonic values. The biofilm-microcosm of nonrecombinant *P. aeruginosa* DSM1707 displayed the same high level of extracellular proteolytic activity observed throughout these analyses (Fig. 3.10b).

3.4 DISCUSSION

The minimal requirement of an effective gene expression system is the presence of a promoter sequence upstream from a cloned gene. The expression of the cloned gene is generally enhanced by placing the gene under control of a constitutively transcribed strong promoter. However, a high level of continual expression of a cloned gene is often detrimental to the host cell, because it may create an energy drain, thereby impairing essential host functions. Thus, it may be preferable to rather control transcription in such a way that the cloned gene is expressed only at a specific stage in the host cell growth cycle and only for a specified duration (Goldstein and Doi, 1995). This objective was attempted by making use of two attachment-inducible promoters, namely *algD* and 703. Whereas the *algD* promoter has been reported to be up-regulated during attachment of *P. aeruginosa* cells to a surface (Hoyle *et al.*, 1993), the uncharacterized 703 promoter was shown by β-galactosidase assays to be up-regulated in the *P. aeruginosa* biofilm-microcosm compared to the corresponding planktonic population (Fig. 3.4). For comparative purposes, the strong constitutive *lacI* promoter was also included in these investigations. Differences in gene dosage were investigated as an alternative strategy whereby extracellular alkaline protease expression might be increased. Generally, the level of gene expression is proportional to the number of transcribed gene copies in the host cell (Summers, 1998). Thus, it can be expected that by increasing the plasmid copy number, there will be a concomitant increase in the amount of protein that is being synthesized. Consequently, two fundamentally different cloning vectors were used in this investigation.

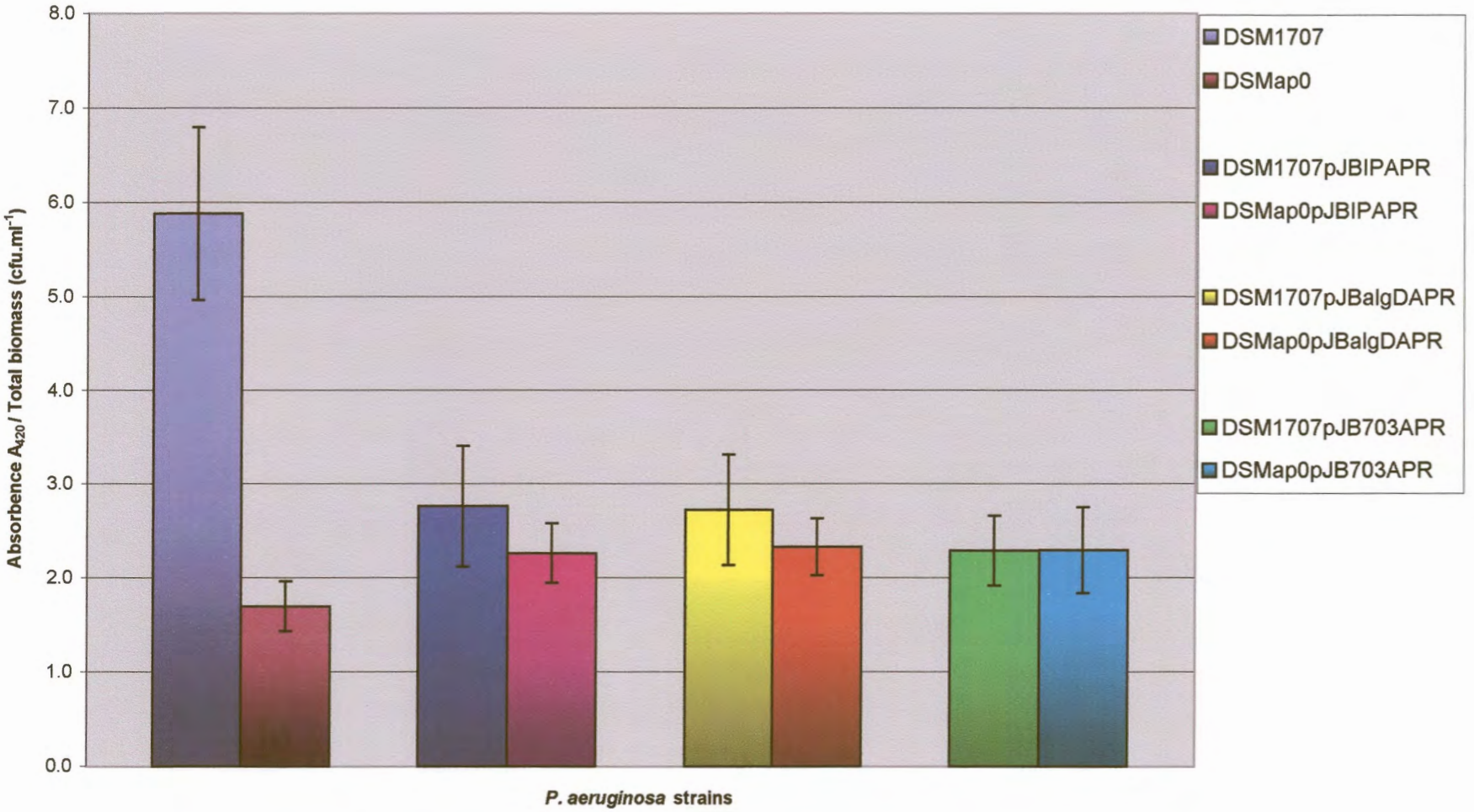

Fig. 3.10a Extracellular proteolytic activity per total biomass (in cfu.ml^{-1}) of planktonic cultures of strains derived from the *P. aeruginosa* DSM1707 and alkaline-deficient mutant DSMap0 strains. The strains have each been transformed with the high-copy-number recombinant pJB3Tc20 vector constructs harbouring the respective promoter-*aprAI* expression cassettes. Error bars denote one standard deviation of the mean.

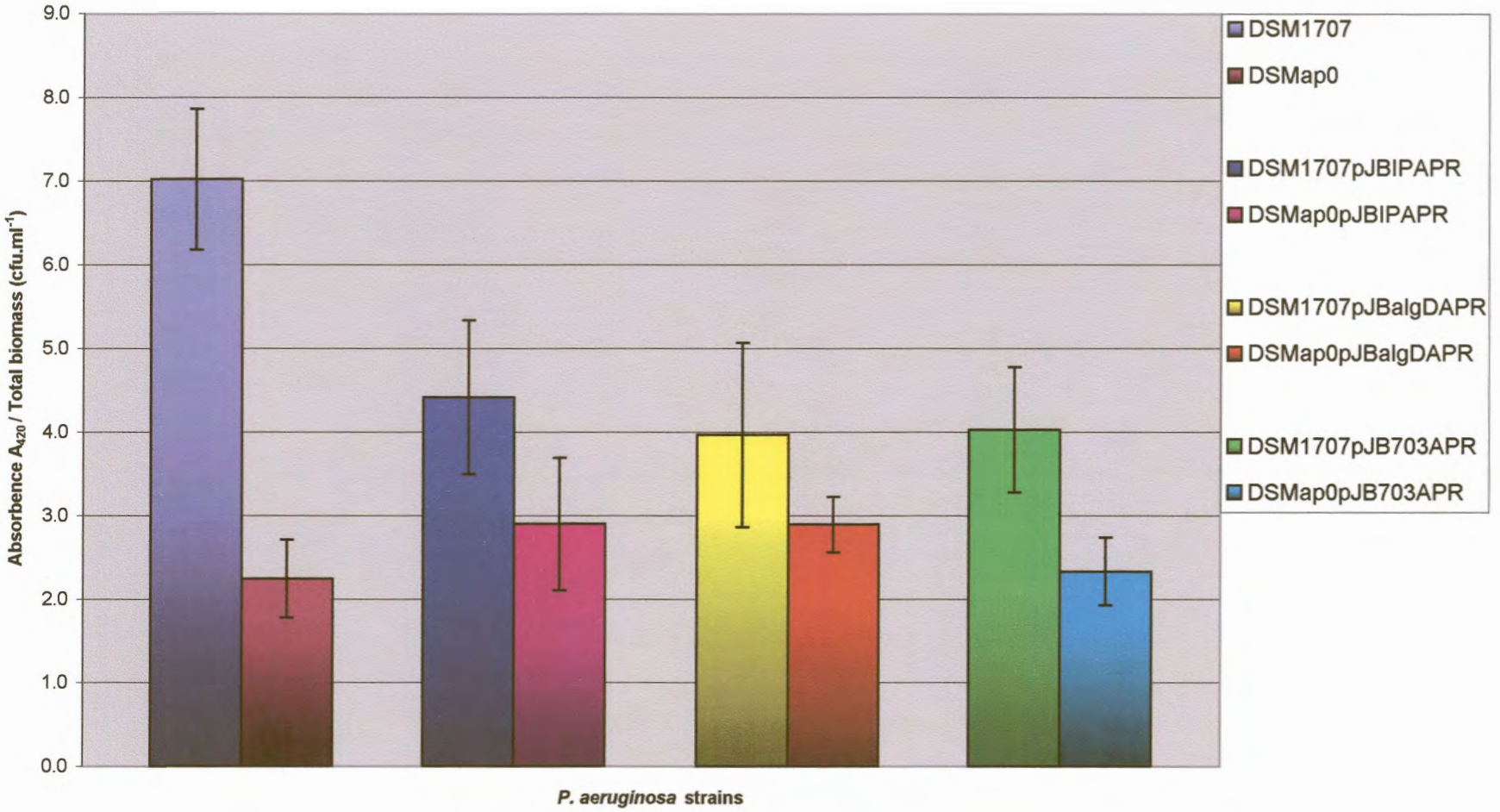

Fig. 3.10b Extracellular proteolytic activity per total biomass (in cfu.ml^{-1}) of biofilm-microcosms of strains derived from the *P. aeruginosa* DSM1707 and alkaline-deficient mutant DSMap0 strains. The strains have each been transformed with the high-copy-number recombinant pJB3Tc20 vector constructs harbouring the respective promoter-*aprAI* expression cassettes. Error bars denote one standard deviation of the mean.

The mini-CTX cloning system consists of a *Pseudomonas*-specific integration plasmid that allows integration of a single copy of the recombinant plasmid into the genome of pseudomonads (Hoang *et al.*, 2000). By contrast, the pJB3Tc20 vector is a high-copy-number plasmid and capable of replicating extrachromosomally in a wide variety of different Gram-negative bacteria (Blatney *et al.*, 1997). Thus, by making use of recombinant plasmids derived from these different vectors, the effect of a single integrated copy of the different promoter-*aprAI* expression cassettes on the level of extracellular alkaline protease could be investigated and compared to the effect of multiple extrachromosomal copies of these expression cassettes.

To investigate, expression cassettes that consisted either of the *lacI*, *algD* or 703 promoters cloned upstream of the *aprAI* genes were constructed. *P. aeruginosa* strains containing a genomically integrated copy of the different promoter-*aprAI* expression cassettes were subsequently derived from the parental DSM1707 and alkaline-deficient DSMap0 strains by making use of the mini-CTX cloning system. In an effort to increase the dosage of the different promoter-*aprAI* expression cassettes, each of the constructed expression cassettes was also cloned into pJB3Tc20 and introduced into the DSM1707 and DSMap0 strains by tri-parental conjugations. The extracellular proteolytic activity of planktonic and biofilm cultures of all the respective recombinant strains was subsequently determined by azocasein hydrolysis assays (Smibert and Krieg, 1994).

The results that were obtained during the course of these investigations can be summarized as follows. (i) Recombinant strains that have been derived from the parental DSM1707 strain and harboured a genomically integrated copy of the respective promoter-*aprAI* expression cassettes yielded similar (but never higher) levels of extracellular proteolytic activity as the parental strain, irrespective of the promoter or culturing conditions used. By contrast, recombinant DSM1707-derived strains harbouring multiple extrachromosomal copies of the different promoter-*aprAI* expression cassettes displayed reduced levels of extracellular proteolytic activity, although higher levels of activity were observed in the biofilm-microcosms compared to the planktonic cultures. (ii) The recombinant strains that have been derived from DSMap0 each displayed similar low levels of extracellular proteolytic activity, irrespective of the promoter used, dosage of the different expression cassettes or culturing conditions. (iii) The level of extracellular proteolytic activity displayed by the non-recombinant parental DSM1707 strain remained high in both the planktonic population and

biofilm-microcosm. Thus, it could be concluded that none of the strategies employed in this study resulted in an increase in the level of extracellular alkaline protease expression.

Notably, all the DSM1707-derived strains contained an intact *apr* operon that could enable secretion of the alkaline protease through the intact alkaline protease secretion machinery. Yet, unlike the parental DSM1707 strain, which displayed a high level of extracellular proteolytic activity throughout this investigation, the level of proteolytic activities displayed by strains derived from DSM1707 decreased with a concomitant increase in the number of promoter-*aprAI* expression cassettes. Since it is reasonable to expect that the levels of proteolytic activity should be the same or higher in all DSM1707-derived strains, the obtained results may therefore imply that the alkaline protease was, in some way, inactivated or neutralized through an increase in the number of promoter-*aprAI* expression cassettes. A possible explanation for this might be that AprI, which is homologous to the *Erwinia chrysanthemi* alkaline protease inhibitor (Létoffé, *et al.*, 1989), indeed acts as an inhibitor, and not as a chaperone, of AprA activity. Since the *aprAI* genes are assumed to be coexpressed in all of the recombinant *aprAI*-promoter constructs, an increased level of AprA would result in a corresponding increase in the level of AprI. These increased levels of AprI may subsequently lead to inhibition of AprA, as is evidenced by the lower levels of proteolytic activity displayed by strains containing increasing copies of the promoter *aprAI* constructs.

In this study, the use of the attachment-inducible promoters *algD* and 703 did not result in a significant up-regulation of the extracellular proteolytic activity of biofilm-microcosms when compared to planktonic cultures. The lack of up-regulation in extracellular proteolytic activity in biofilm-microcosms may not be entirely surprising. Reporter gene studies have shown that although *P. aeruginosa* cells initiated up-regulation of *algC* synthesis 15 min after attachment of the cells to a glass surface, the level of *algC* synthesis decreased after 2 h of incubation (Davies and Geesey, 1995; Davies *et al.*, 1993). In addition, not all cells exhibited induction of *algC* expression upon attachment and the cells that did not undergo *algC* up-regulation were less able to remain attached. It is neither known when actual up-regulation of the attachment-inducible *algD* and 703 promoters occur during the biofilm developmental phase nor is it known for how long these promoters are actually transcriptionally active. Thus, the low levels of up-regulated extracellular proteolytic activity observed in instances

where these promoters have been used, could have been due to limited transcriptional activity of the respective promoters. With regard to the *algD* promoter, various environmental factors have been reported to affect its transcriptional activity. These include high osmolarity, high oxygen tension, ethanol exposure and nitrogen limitation (Gacesa, 1998; De Vault *et al.*, 1990; Zielinski *et al.*, 1992), while growth under anaerobic conditions was shown to induce *algD* transcription only marginally (De Vault *et al.*, 1990). In the case of the 703 promoter, it is not known at present which other factor(s) may be important in regulating its transcriptional activity.

In conclusion, the results obtained in this part of the investigation indicated that the modulation of alkaline protease gene expression through the use of different promoters or an increased gene dosage in planktonic cultures and biofilm-microcosms of *P. aeruginosa* did not result in increased levels of extracellular proteolytic activity. Several factors may have accounted for this lack of observed up-regulation in extracellular proteolytic activity. These include the imposing of a metabolic load on the host organism by high plasmid DNA copy numbers, intracellular inhibition of alkaline protease by AprI, and regulation of the transcriptional activity of the respective attachment-inducible promoters by environmental factors. These insights may prove valuable in facilitating the design of alternative strategies whereby the limitations, as pointed out by this study, can be effectively addressed and overcome.

CHAPTER 4

CONCLUDING REMARKS

THE MORE WE STUDY BACTERIA, THE MORE WE SEEM TO UNDERSTAND THEM AND THE MORE THE QUESTIONS, BUT ABOVE ALL, THE MORE FRIGHTNING REALIZATION: THEY SEEM MORE EVOLVED, MORE STRUCTURED AND MORE ORGANIZED THAN WE

J.J.Smith

Most microorganisms, amongst them, *Pseudomonas aeruginosa*, possess the ability to adhere to surfaces in both natural and industrial habitats. Biofilms, the almost inevitable consequence of microbial adhesion to surfaces, consist of communities of microorganisms immobilized onto surfaces and are responsible for much of the microbial activity found in natural and biotechnological situations. Numerous reports have indicated that certain physiological characteristics of bacteria are altered when the organisms adhere to a surface (Davey and O'Toole, 2000; Davies and Geesy, 1995) and reporter gene technology has demonstrated that the expression of some genes is altered in bacteria at surfaces when compared to their planktonic counterparts (Heydorn *et al.*, 2000; Davies *et al.*, 1993). The up-regulation of gene expression at surfaces and in biofilms may find specific application in the development of a self-driving biofilm-specific expression system whereby enzymes and/or other products can be expressed. In this investigation, *P. aeruginosa* and alkaline protease (AprA) served as models towards the development of such a biofilm-specific expression system. This investigation was thus primarily aimed at evaluating the usefulness of different strategies whereby the level of extracellular alkaline protease expression may be increased. The information that has evolved during the course of this study is briefly summarized and some suggestions regarding future research in this field will be made.

In the first part of the study, an alkaline protease-deficient *P. aeruginosa* strain, DSMap0, was generated of which the *aprAI* genes were disrupted by insertion of a gentamicin resistance cassette. Whereas the *aprA* gene encodes the alkaline protease (AprA), the *aprI* gene encodes a protein of unknown function (AprI), but is thought to play a role as intracellular inhibitor or chaperone of the alkaline protease (Duong *et al.*, 1992). Due to the possibility that AprI may act as a chaperone for alkaline protease activity, both the *aprA* and *aprI* genes were consequently targeted for directed insertional inactivation. To prevent inactivation of the secretion mechanism through disruption of the upstream secretory proteins AprDEF, the gentamicin gene used in this study was flanked at both termini by transcriptional and translational terminators (Luckow *et al.*, 1993). Various different PCR assays were performed to confirm that only the *aprAI* genes were disrupted through the insertion of the gentamicin resistance cassette. Furthermore, proteolytic assays indicated a lack of intracellular proteolytic activity in both the mutant and parental *P. aeruginosa* strains, but the mutant DSMap0 strain displayed a six-fold reduction in extracellular proteolytic activity compared to the parental DSM1707 strain. Although these results provided indirect evidence that the *aprAI* genes were indeed inactivated, further research would be required to

verify whether or not the secretory genes are intact and indeed successfully expressed. Transcription of the *aprDEF* genes may be investigated by Northern blot analysis, while correct assembly of the secretory proteins may be investigated by Western blot analysis using anti-AprX serum. AprX, a protein of unknown function, is also secreted by the alkaline protease secretion machinery (Duong *et al.*, 2001) and extracellular detection of this protein may therefore indicate that the export machinery is intact and fully functional.

In the second part of the study, different strategies were investigated as a means whereby the level of extracellular alkaline protease expression could be increased. The strategies were based on the use of different promoters (constitutive vs. attachment-inducible), gene dosages (a single integrated copy of the expression cassettes vs. multiple extrachromosomal copies thereof) and growth modes of the cultures (planktonic vs. biofilm growth). The results that were obtained during the course of these investigations indicated that none of the strategies employed in this study appears to have resulted in increased levels of extracellular proteolytic activity when compared to the wild-type parental *P. aeruginosa* DSM1707 strain. However, a number of conclusions drawn from these results, as well as factors to consider in future investigations, will be highlighted in the following sections.

I. The extracellular proteolytic activities of cultures from *P. aeruginosa* DSM1707 strains containing multiple extrachromosomal copies of the promoter-*aprAI* expression were at no stage higher than the activities observed for cultures from strains containing an integrated copy thereof, and none of these recombinant strains displayed proteolytic activities higher than the parental DSM1707 strain. This may have been due to inhibition of the intracellular alkaline protease by AprI.

Several alternative approaches may be investigated as means whereby alkaline protease secretion from *P. aeruginosa* could be improved. These may include the deletion of the gene encoding AprX, which is secreted by the same secretion apparatus used for alkaline protease secretion, or deletion of the gene encoding AprI, which may serve as an inhibitor of the alkaline protease. Alternatively, components of the alkaline protease secretion machinery may be expressed at higher levels. This might be achieved by either overexpressing the *aprDEF* genes *in trans* on a plasmid or by, preferably, placing the entire *apr* operon (with and without the *aprX* and *aprI* genes to allow for comparative analysis) under transcriptional control of a strong promoter. Both approaches may aid in increased or more efficient

secretion of overexpressed alkaline protease, but the effect(s) that these high levels of secretory protein expression may have on the host cells need to be properly evaluated. A very different approach may involve fusing the structural gene encoding the alkaline protease to a DNA fragment encoding a signal peptide, thereby enabling its secretion via a different secretion pathway. However, the presence of a signal peptide sequence does not necessarily guarantee a high rate of secretion and may result in increased competition for secretion (Cheah *et al.*, 1994; Gottesman, 1996; Wilcox and Studnicka, 1988). The alkaline protease-encoding gene may also be fused to a gene encoding a protein, which is very efficiently secreted, with DNA encoding a protease recognition site separating the two genes. Functional alkaline protease may then be released from the fusion protein by digestion with the protease. Finally, the use of a different export pathway might be considered. The type V (autotransporter) secretion pathway appears to be most appropriate (Section 1.4.4). A typical autotransporter contains three domains: an amino-terminal sequence for secretion across the inner membrane by the *sec* system, an integral passenger or functional domain, and a carboxy-terminal β-domain (Henderson *et al.*, 1998). The β-domain inserts into the outer membrane to form a pore structure through which the passenger domain passes to the cell surface. The nature of the passenger domain does not appear to be important, as foreign proteins can be substituted for this domain and still be successfully secreted (Suzuki *et al.*, 1995). Thus, by combining recombinant DNA technology to construct such an autotransporter consisting of the alkaline protease as passenger domain and placing this unit under transcriptional control of different promoters, its usefulness may be assessed.

II. The intracellular proteolytic activities of cultures from the *P. aeruginosa* DSMap0 strains, which contained an *apr* operon of which the *aprAI* genes had been disrupted, were always lower than in the corresponding recombinant DSM1707 strains and resembled the activity displayed by the parental DSMap0 strain. Since the structural gene of the secreted protein in type I secretion systems is usually clustered with the genes encoding the secretory proteins (Binet *et al.*, 1997; Fath and Kolter, 1993), the obtained results may indicate that the secretion process is inefficient when these genes do not form part of the same operon structure.

Closer inspection of the *P. aeruginosa apr* operon reveals that the secretory genes *aprDEF*, located upstream of the *aprA* structural gene encoding alkaline protease, is separated from the *aprA* by an intergenic region of 359 bp (Fig.1.8). Since no intergenic regions separate the

aprD, *E* and *F* genes, it may indicate that the secretory proteins are synthesized first, followed by a slight pause before the alkaline protease is synthesized. Such a delay may ensure assembly of the secretion machinery prior to alkaline protease synthesis and secretion. This may be required, as the alkaline protease has a short intracellular half-life (160 s) and is rapidly degraded if not immediately secreted (Duong *et al.*, 1991). Similar results have been reported for *E. coli* α-hemolysin (Oropeza-Wekerle *et al.*, 1989). Thus, expression of the *aprAI* genes from a location different to that of the *apr* operon may result in expression of the alkaline protease prior to assembly of the secretion apparatus, thereby resulting in increased levels of intracellular alkaline protease, which is subsequently rapidly degraded prior to secretion. In addition, no information is available on the factors regulating expression of the *apr* operon in *P. aeruginosa* cells. Thus, it may be worthwhile cloning the entire operon under transcriptional control of the attachment-inducible promoters used in this study or, alternatively, a single strong promoter such as the *lac* promoter.

III. No significant up-regulation of extracellular proteolytic activity was observed in biofilm-microcosms of the different *P. aeruginosa* DSM1707 and DSMap0 strains compared to their corresponding planktonic cultures. This, however, may have been related to the nature of the promoters used. In addition, several factors relating to biofilm architecture and development would also need to be considered during the development of a biofilm-specific expression system.

The attachment-inducible promoters used in this study, *algD* and 703, may be subject to transcriptional control from environmental factors. This has been reported to be the case for the *algD* promoter, and high osmolarity, oxygen tension and growth under anaerobic conditions have all been reported to influence its transcriptional activity (Gacesa, 1998; Zielinski *et al.*, 1992; De Vault *et al.*, 1990). Thus, further detailed characterization of these promoters, especially of the 703 promoter, may be required. Although β-galactosidase activity assays have shown up-regulation of the 703 promoter activity following attachment of the *P. aeruginosa* cells to glass wool (Fig. 3.4), it should be kept in mind that these assays may only yield an average value for the whole population. Towards characterizing these promoters, future studies may entail the construction of promoter fusions with unstable variants of a reporter gene, such as *gfp* encoding the green fluorescence protein (GFP), and the expression monitored by time-lapse video microscopy. This should yield information

regarding when actual up-regulation of the promoter occurs and also as to the duration of transcriptional activity. Such information may prove valuable, as the *algC* promoter is also attachment-inducible, but the level of *algC* synthesis has been reported to decrease 2 h after attachment and not all cells induced *algC* expression upon attachment (Davies and Geesey, 1995).

With regards to biofilm architecture, several studies have indicated that biofilms are very heterogenous, containing microcolonies of bacterial cells encased in an EPS matrix and is separated from other microcolonies of bacterial cells by interstitial voids or water channels (Tolker-Nielsen *et al.*, 2000; Costerton *et al.*, 1995; Lawrence *et al.*, 1991). This structure of the biofilms may therefore have implications for mass transport of nutrients into, and CO_2 and metabolic by-products out of biofilms. Furthermore, the maximum growth potential of a bacterial biofilm is not only limited by the availablility of nutrients in the immediate environment, but also by the perfusion of those nutrients to cells within the biofilm, the removal of metabolic by-products and the expression of quorum-sensing molecules released in response to nutrient limitation. Other factors that also control biofilm development and maturation include internal pH, oxygen perfusion, carbon source and osmolarity (Chandy and Angels, 2001; Davies *et al.*, 1998; Allison *et al.*, 1998; McLean *et al.*, 1997; Carpentier and Cerf, 1993; La Tourette Prosser *et al.*, 1987). All of the above factors are especially important in terms of optimizing expression system development based on biofilm activity, as the establisment of concentration gradients of the above factors within the biofilm structure may result in heterogenous gene expression For example, in the case of *P. aeruginosa*, expression of alkaline phosphatase was located only in a thin layer at the biofilm-bulk fluid interface and the cells in the bulk of the biofilm were found to be physiologically inactive (Xu *et al.*, 1998; Huang *et al.*, 1998). Furthermore, reporter gene studies by De Kievit *et al.* (2001) indicated that *rhlI* is expressed by only 5 to 15% of the biofilm cells and these were concentrated around the base of the biofilm.

In conclusion, attempts to use *P. aeruginosa* for the development of a robust biofilm-specific expression system have not yet fulfilled any expectations. Notwithstanding the possibility that polar effects may have resulted in the low levels of extracellular proteolytic activity observed for the DSMap0-derived strains, the use of DSM1707-derived strain allowed the identification of a number of "bottlenecks". The detailed characterization of these bottlenecks is a prerequisite for the improvement of *P. aeruginosa* as host for the secretory production of

industrially relevant heterologous proteins. It should, however, be kept in mind that *P. aeruginosa* is an opportunistic human pathogen. Consequently, should proof of the concept be obtained, the knowledge generated from such studies should be applied to an avirulent *P. aeruginosa* strain or the use of other Gram-negative expression host organisms should be explored.