CHAPTER 4

THE EFFECT OF A REPORTER VECTOR CONSTRUCT ON THE BIOFILM PHYSIOLOGY OF *Pseudomonas aeruginosa*

(The style of this chapter is in accordance with that of the Journal *Microbiology*)

4.1 SUMMARY

The phenotypic effects that a *lacZ*-based reporter vector construct, pALacZsd, has on the planktonic, SIP and biofilm populations of *P. aeruginosa* was investigated. The data obtained indicate that *P. aeruginosa* cells containing the pALacZsd vector are phenotypically different to untransformed *P. aeruginosa* cells. *P. aeruginosa* cells transformed with pALacZsd were found to have more protein biomass per cellular volume than untransformed cells and plasmid DNA concentrations were found to differ in total attached cultures when compared to planktonic cultures. Since the pALacZsd vector contains a tetracycline resistance gene upstream from the multiple cloning site, the effect of transcriptional read-through from this gene on β-galactosidase expression was assayed in the presence of various concentrations of tetracycline. The results indicated a linear correlation between tetracycline concentration and basal β-galactosidase activity for planktonic cultures, but no similar correlation could be observed for biofilm and associated cultures. Cloning of a promoterless DNA fragment into the pALacZsd vector had no significant effect on the basal levels of β-galactosidase activity. Thus, the pALacZsd reporter vector is appropriate for use in studies relating to the characterisation of biofilm-specific promoters.

4.2 INTRODUCTION

Plasmids have been found to be ubiquitous in the *Pseudomonas* group; however, their frequency of occurrence varies greatly in a particular species, or group of species, and in different microbial habitats (Boronin, 1992). Two decades of research into *P. aeruginosa* plasmids have illustrated the wide occurrence of plasmids carrying antibiotic resistance in bacteria obtained from surgical patients, patients with urinary tract infections and patients suffering severe burn wounds (Boronin, 1992). More often than not, a large number of these *P. aeruginosa* isolates have been biofilm organisms as is generally observed in *P. aeruginosa* isolates of a medical origin.

Studies of specific gene expression in bacteria have been greatly facilitated by the use of reporter genes. The construction of the relevant fusions between the promoters of interest and appropriate reporter genes, coupled with the simple quantitative assays for enzymes like β-galactosidase and luciferase, has allowed detailed investigations of gene regulation. Such investigations have been performed successfully in planktonic cultures of many bacterial species, for which the addition and spatial distribution of enzymatic substrates do not represent any problems (Andersen *et al.*, 1998), unlike the analysis of heterogeneous and complex biofilm populations.

In growing cells, the rate of transcription is not the same as the level of expression (Warner & Lolkema, 2002). The latter is determined by both the rate of transcription and the growth rate. Two important points, therefore, that must be considered when interpreting data obtained for promoter-*lacZ* fusions are: (1) the expression level must have reached a steady state value; and (2) when comparing β-galactosidase activities, the growth rates in the two situations must be the same (Warner and Lolkema, 2002). To date, most efforts aimed at providing a reporter vector for use in biofilms, have paid very little attention to the effect that the extrachromosomal or integrated vector construct, has on the physiology of the organism of interest.

A reporter vector has recently been constructed by Weyers (1999) in order to allow screening of a *P. aeruginosa* a genomic library for putative attachment-inducible regulatory elements. The vector construct is based on the pAL4000 vector (Greener *et al.*, 1992). The *luc* gene of pAL4000, situated downstream from the multiple cloning site, was removed and replaced with a *lacZ* gene obtained from a yeast episomal plasmid, YEp62 (Broach *et al.*, 1983). The

reporter vector was designated pALacZsd (Fig. 4.1). A 1 000 bp *Sau*3AI DNA fragment containing the constitutive *lacI* promoter from the pBluescript plasmid was cloned into the pALacZsd vector, yielding plasmid pALacZsdIP (Fig. 4.1). This vector construct served as a positive control for the analysis of promoter activity in the pALacZsd vector system (Weyers, 1999). The pALacZsd *tetA* gene codes for an energy-dependant membrane-associated protein, which exports tetracycline from the bacterial cell.

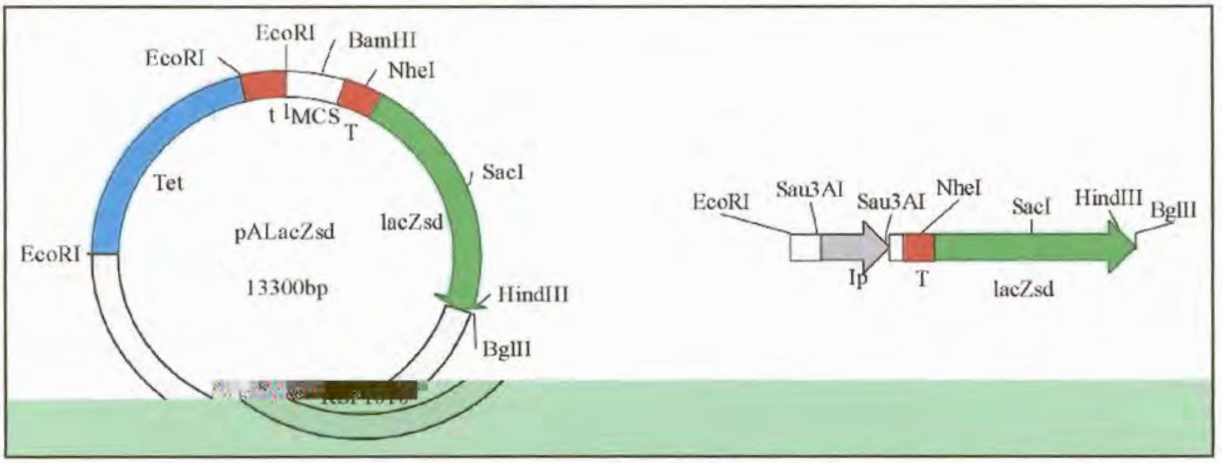

Figure 4.1: Schematic representation of the pALacZsd vector (left) and the insert used to create the pALacZsdIP vector (right). The pALacZsd vector contains a multiple cloning site (MCS), a *lacZ* gene (lacZsd), part of the RSF1010 replicon (RSF1010), a *tetA* tetracycline resistance gene (Tet), a transcriptional terminator (t), a translation termination fragment (T) and a ribosome-binding site downstream of T.

Using the recently constructed pALacZsd reporter vector, the aim of this study was to investigate the phenotypic effect that the extrachromosomal reporter vector may have on *P. aeruginosa* planktonic, SIP and biofilm populations.

4.3 METHODS

4.3.1 Bacterial strains, media and growth conditions

4.3.1.1 General growth conditions

The bacterial strains and plasmids used in this study are listed in Table 4.1. *E. coli* JM105, which was used for the maintenance and amplification of plasmid DNA, was grown at 37°C in LB-broth supplemented with 10 $\mu g.ml^{-1}$ tetracycline. *P. aeruginosa* PAO (DSM 1707) cultures were grown as previously described in Section 3.3.1. When required, the medium was supplemented with 40 $\mu g.ml^{-1}$ tetracycline (Roche Diagnostics, Randburg, South Africa), prepared in a 50% ethanol solution. Where indicated, NaCl was added to a final concentration of 0.7 M (LB + NaCl) and ethanol to a concentration of 2.5% (v/v) (LB + EtOH). Due to the addition of tetracycline, which had been prepared in ethanol, the final ethanol concentration was slightly higher (2.9% [v/v]). The *P. aeruginosa* cultures were incubated at 37°C for 16 h and the total attached biomass was then separated into SIP and biofilm biomass as previously described (Section 3.3.1).

4.3.1.2 Growth conditions used when assaying for β-galactosidase activity

P. aeruginosa PAO (DSM 1707) was grown as described above from stock cultures (frozen at -70°C), after which aliquots (500 µl) were transferred to 2-ml microfuge tubes with or without 0.025 g of glass wool (Merck, Darmstadt, Germany; mean standard diameter = 10 µm). After culturing at 37°C with agitation for 16 h, the total attached biomass was separated into SIP and biofilm biomass and fresh broth (500 µl) was added to the glass wool-attached biomass matrix. For investigations aimed at determining the effect of different tetracycline concentrations on basal levels of β-galactosidase activity, only the planktonic and total attached (SIP + biofilm) samples were assayed.

4.3.2 Analytical Methods

The biomass yield, culturable count, total cellular protein concentration and cellular volume were determined as previously described (Section 3.3.2).

Table 4.1: Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant properties	Reference or source
Strains:		
Escherichia coli JM105		J. B. Weyers*
Pseudomonas aeruginosa DSM 1707	Prototroph	DSM†
Plasmids:		
pALacZsd	pAL4000 (Greener *et al.*, 1992) containing a promoterless *lacZ* in place of the *luc* gene	J. B. Weyers
pALacZsdIP	pALacZsd containing the *lacI* promoter from pBluescript® SK (+) (co-ordinates 719-1719)	J. B. Weyers
pALacJK	pALacZsd containing 437 bp of the open reading frame of the *P. aeruginosa aprA* gene	This study

*: Mr J. B. Weyers, Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria, South Africa
†: Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany

4.3.3 Molecular Techniques

4.3.3.1 Preparation and transformation of competent cells

Competent *E. coli* JM105 cells were prepared and transformed according to the procedures described by Rees (1986). For *P. aeruginosa*, cultures were grown on TN media (0.5% [w/v] tryptone; 0.1% [w/v] dextrose; 0.25% [w/v] yeast extract; 0.4% [w/v] $NaNO_3$) (Olsen & Shipley, 1973) prior to the preparation of competent cells. Competent *P. aeruginosa* cells were then prepared and transformed as described by Olsen *et al.* (1982). Transformed cultures were grown to an OD_{540} = *ca.* 0.5, aliquotted and stored in 50% glycerol at –70°C. Fresh stock cultures were used for each assay.

4.3.3.2 Determination of plasmid DNA concentrations from P. aeruginosa

Cultures grown in the presence of glass wool (total attached biomass) were incubated at 37°C with agitation for 16 h and then treated as follows. Using a 1 ml micropipette, the SIP cells were transferred from the test tubes into 2-ml microfuge tubes and collected by centrifugation at 16 000 × g for 5 min. The glass wool-biofilm matrix was subsequently added to the SIP cells collected in the microfuge tube. The cells from planktonic cultures grown in the absence of glass wool were collected by centrifugation as described above. Plasmid DNA was then extracted by an alkaline lysis procedure (Sambrook *et al.*, 1989) and the DNA pellet dissolved in 1 ml of ddH_2O. Following analysis on a 1% agarose gel (Promega, Madison, USA), the plasmid DNA concentration was quantified by ultraviolet spectrometry. Both the A_{260} and A_{260}/A_{280} readings were measured using a Cary 1E UV visible spectrophotometer

(Varian, Mulgrave, Victoria, Australia).

4.3.3.3 Construction of the pALacJK vector

The pALacJK vector was constructed to determine the effect, if any, of a promoterless DNA fragment on the basal levels of β-galactosidase activity in the transformed cells. For this purpose, a portion of the coding region of the *aprA* gene, corresponding to co-ordinates 1357441 to 1357878 of the *P. aeruginosa* genome, was cloned into the multiple cloning site of the pALacZsd vector. Briefly, *P. aeruginosa* genomic DNA was isolated using the method of Jansen (1995) and then used as template DNA for the polymerase chain reaction (PCR) amplification of the 437 bp portion of the *aprA* gene. The reaction mixture consisted of 1 µl of template DNA, 1.5 mM $MgCl_2$, 100 pmol of each primer (APR1: 5'–GCGACGTCGAGCTGTGTCAGTTTGGAC–3'; APR2: 5'–CGCTCTAGAGAGGTCGATC ACAACCTG–3'), 250 µM of each dNTP, 1.5 U *Taq* DNA polymerase (Southern Cross Biotechnology, Johannesburg, South Africa) and 10% (v/v) dimethyl sulphoxide (DMSO). The PCR product was purified by precipitation with 10% 3 M NaOAc (pH 7.6) and 2 volumes 100% ethanol.

Following digestion of the *aprA* amplicon and pALacZsd vector DNA with both *Pst*I and *Xba*I, the DNA fragments were purified from the agarose gel using the freeze-squeeze protocol (Benson, 1984) and then ligated prior to transformation of competent *E. coli* JM105 cells. Putative recombinant clones were screened by restriction enzyme digestion with *Pst*I and *Xba*I, and by PCR analysis, as described above. The nucleic acid sequence of the cloned insert DNA was determined using an ABI PRISM™ Big Dye™ Terminator Cycle Sequencing Kit (Perkin Elmer, Foster City, CA, USA) and primers APR1 and APR2 according to the manufacturer's instructions. A recombinant clone was designated pALacJK and used in all subsequent assays.

4.3.4 β-galactosidase activity assay

β-galactosidase activity was assayed in liquid cultures using 2-nitrophenyl-β-D-galactopyranoside (ONPG) as a chromogenic substrate according to the methods described by Miller (1972). Briefly, after incubation of the cultures at 4°C for 20 min, an equal volume (500 µl) of ice-cold 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 the respective cultures. The cells were

vortexed and lysed by addition of 20 µl chloroform and 10 µl 0.1% SDS. After incubation at 28°C for 10 min, 200 µl ONPG (4 $mg.ml^{-1}$ in Z-buffer) was added. After incubation for 15 min at room temperature, the reaction was terminated by the addition of 500 µl of 1 M Na_2CO_3. The cell debris and glass wool were pelleted by centrifugation at 16 000 × g for 5 min. Hydrolysis of ONPG was quantified by transferring 200 µl of each reaction mixture to a microtitre plate and measuring the absorbance at 414 nm using a Titretek multiscan MCC/340 microtitre plate reader. β-galactosidase activity was expressed as either the A_{414} (amount of ONPG hydrolysed in 15 min) or A_{414} / µg total cellular protein.

4.4 RESULTS

4.4.1 Variation in biomass

As seen by the standard deviation in the culturable count (Fig. 4.2), optical density (Fig. 4.3) and total cellular protein concentration (Fig. 4.4), there was a substantial variation in the results of triplicate samples grown from the same inoculum. This variation was inherent to all the experimental protocols. The standard deviations shown throughout are therefore a function of the differences in the final biomass of cultures, and should not be taken as an indication of the variation of the above measurements on a cellular level.

4.4.2 Culturable counts and total biomass (A_{540})

P. aeruginosa transformed with pALacZsd plasmid DNA and grown in LB-S in the presence of glass wool (total attached biomass) yielded double the culturable count compared to planktonic cultures (4.63E+09 compared to 2.73E+09) (Fig. 4.2). There were 1.6- and 15-fold more cells in the SIP population compared to the planktonic and biofilm populations, respectively. However, when compared to untransformed *P. aeruginosa* cultures (Fig. 3.5), the culturable counts were 1.7-fold lower for the planktonic population, 2.6-fold lower for the biofilm and 1.9-fold lower for the SIP population. Nevertheless, the biofilm population of *P. aeruginosa* transformed with pALacZsd corresponded to *ca.* 17% of the planktonic population, or 8.5% of the total culturable count of cultures grown in the presence of glass wool (total attached biomass). These results were in agreement with those obtained for untransformed *P. aeruginosa* cultures (Section 3.4.3). This correlated with the observed optical density values (Fig. 4.3) when differences in cell volume under the different growth conditions are taken into account. The decrease in the culturable count of the biofilm population of *P. aeruginosa* transformed with pALacZsd may be due to a decrease in

culturability of the cells rather than being the "true" culturable count. This may be due to the observed decrease in cellular volume (refer to Section 4.4.4) that is thought to be a direct consequence of the tetracycline concentrations at the glass wool – growth medium interface.

Approximately 56% of the *P. aeruginosa* cells transformed with pALacZsd grown in the presence of a glass wool surface and 0.7 M NaCl (LB + NaCl) were attached to the surface (Fig. 4.2), significantly higher than those grown in either LB-S or LB + EtOH. The SIP population displayed a lower culturable count (3.3-fold) than the planktonic population. This was in contrast to the transformed *P. aeruginosa* cultures grown in LB-S and to untransformed *P. aeruginosa* cultures grown in LB + NaCl (Fig. 3.5). The biofilm population had 2.6-fold less cells than the planktonic population, but 1.3-fold more cells than the SIP population. These differences correlated with the observed decreases in optical density (Fig. 4.3) and protein biomass (Fig. 4.4).

Approximately 30% of the *P. aeruginosa* cells transformed with pALacZsd grown in the presence of a glass wool surface and 2.9% ethanol (LB + EtOH) were attached to the surface (Fig. 4.2), significantly higher than those grown in LB-S but not those grown in LB + NaCl. The SIP population again displayed a lower culturable count (1.3-fold) than the planktonic population. The biofilm population had 3-fold less cells than the planktonic population and 2.3-fold less cells than the SIP population. These differences correlated with the observed differences in optical density (Fig. 4.3) and protein biomass (Fig. 4.4).

The small amounts of ethanol present in the culture media as well as the presence of tetracycline influence the culturability issues seen previously [high culture broth osmolarity was previously seen to influence the culturability of both planktonic and biofilm cells (Section 3.4.3)]. In the presence of plasmid DNA, tetracycline and NaCl or ethanol, culturability of all the respective populations (including the SIP population) was affected when compared to untransformed *P. aeruginosa* cells grown under identical conditions (Fig. 3.5).

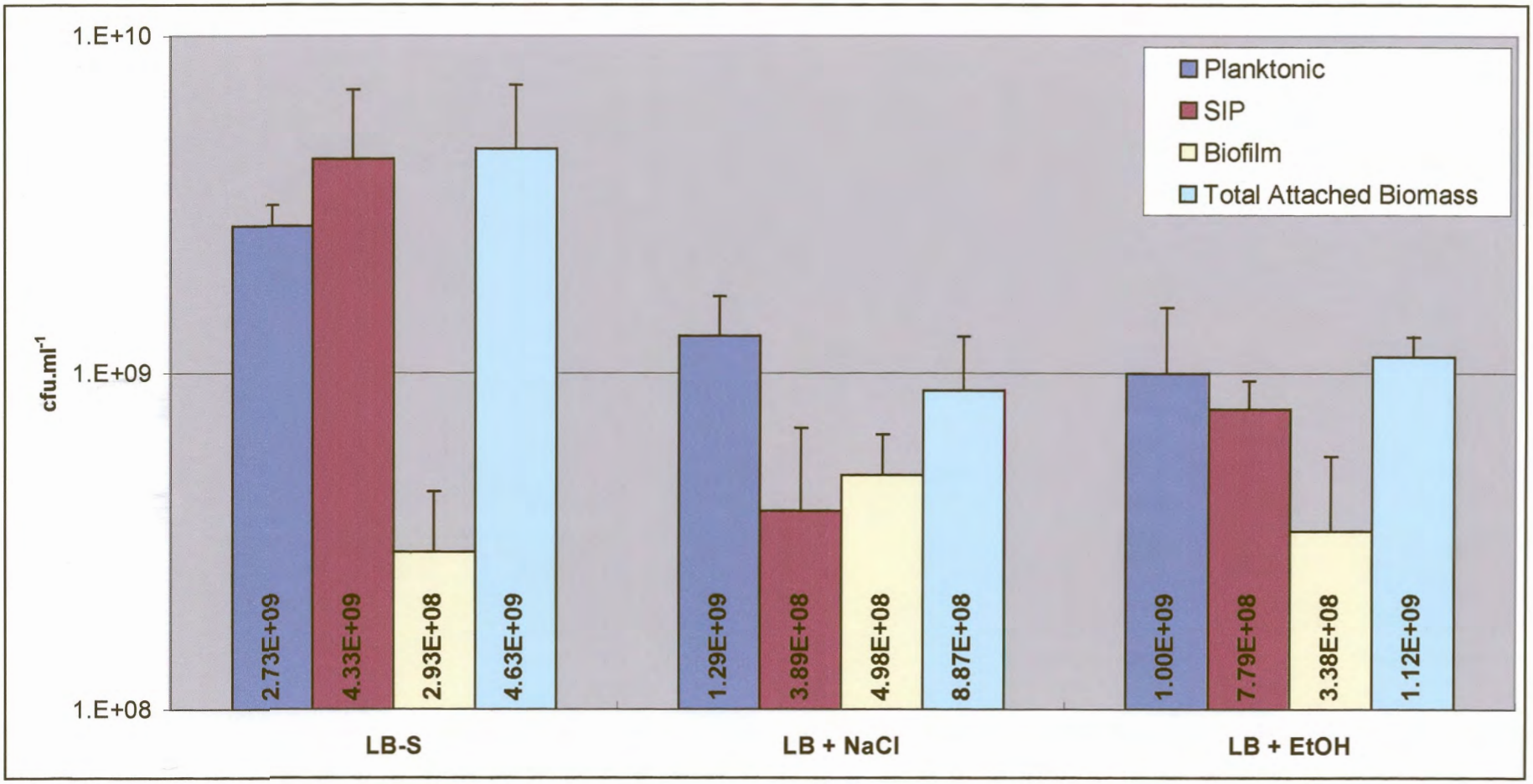

Fig. 4.2: Culturable count of *P. aeruginosa* transformed with pALacZsd cultured for 16 h in LB broth without salt (LB-S), with 0.7 M NaCl (LB + NaCl) and 2.9% (v/v) ethanol (LB + EtOH). Error bars denote one standard deviation of the mean.

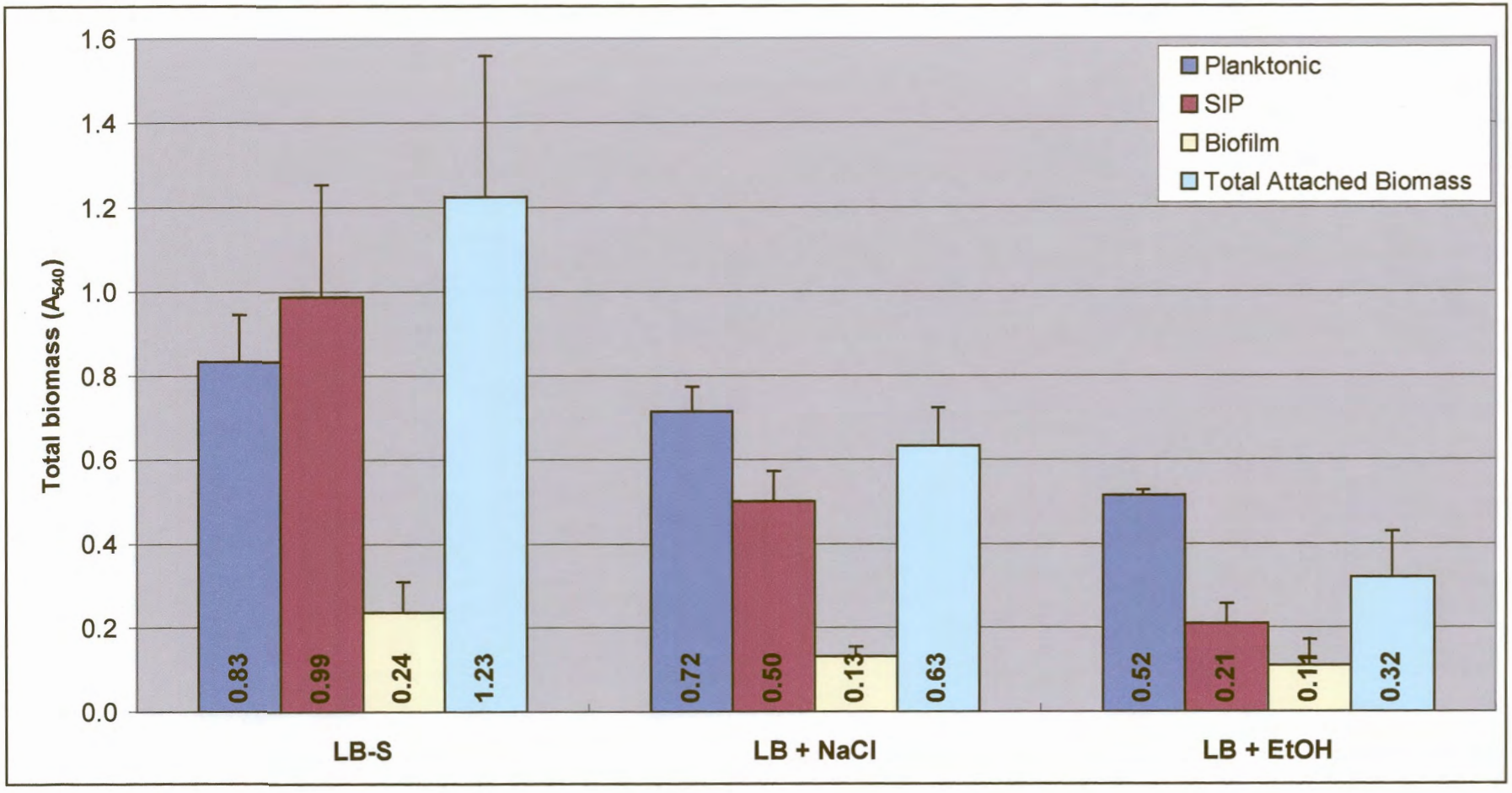

Fig. 4.3: Optical density (A_{540}) of *P. aeruginosa* transformed with pALacZsd cultured for 16 h in LB broth without salt (LB-S), with 0.7 M NaCl (LB + NaCl) and 2.9% (v/v) ethanol (LB + EtOH). Error bars denote one standard deviation of the mean.

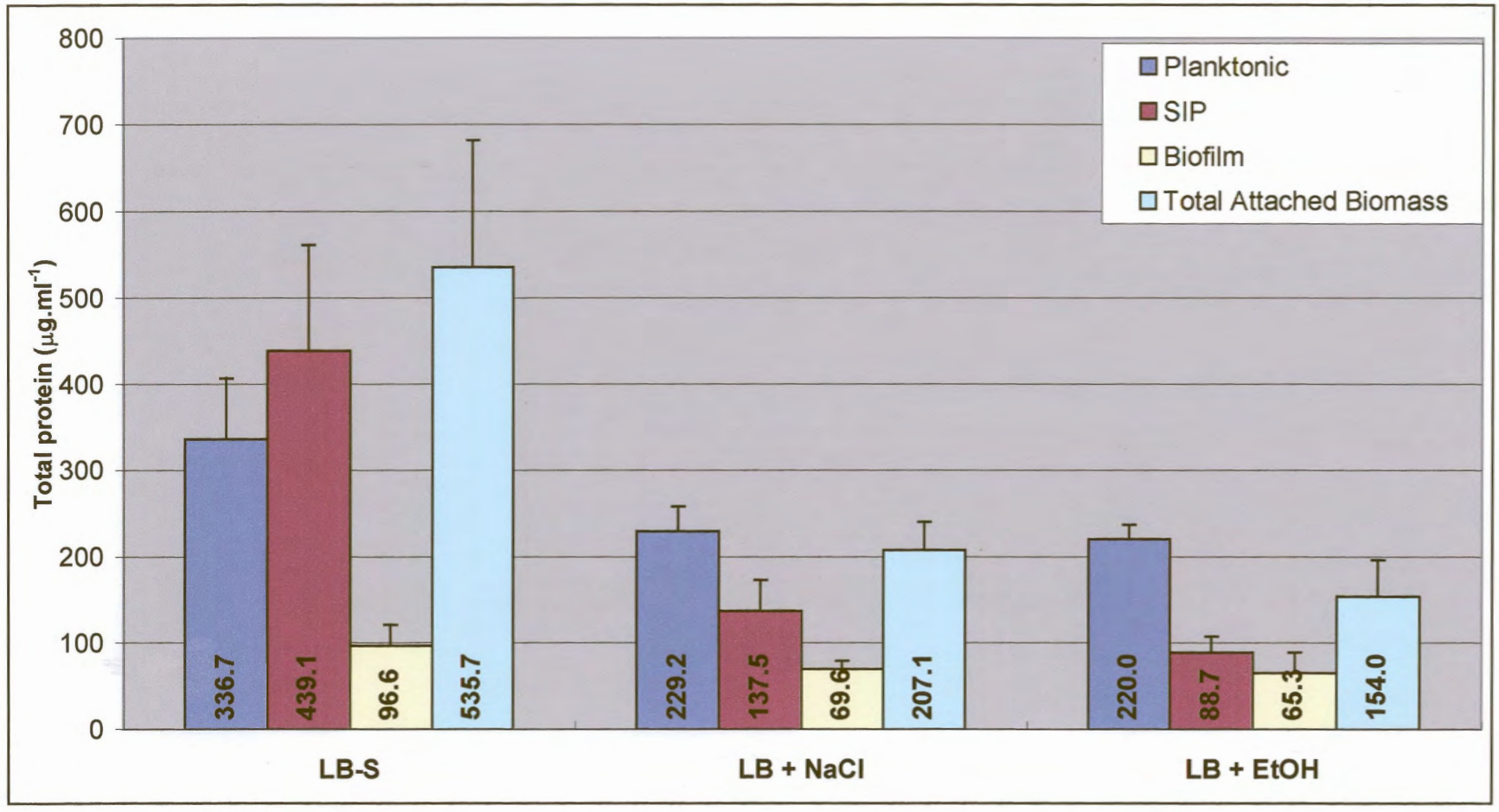

Fig. 4.4: Total cellular protein of *P. aeruginosa* transformed with pALacZsd cultured for 16 h in LB broth without salt (LB-S), with 0.7 M NaCl (LB + NaCl) and 2.9% (v/v) ethanol (LB + EtOH). Error bars denote one standard deviation of the mean.

4.4.3 Total cellular protein

The total protein concentration of cultures of *P. aeruginosa* transformed with pALacZsd and grown in LB-S in the presence of glass wool (total attached biomass) was 1.6-fold higher than that of planktonic cultures. The biofilm population had a protein biomass *ca.* equivalent to 18% of the total attached biomass (Fig. 4.4). Compared to the planktonic population, the biofilm population exhibited a 3.5-fold decrease in total protein concentration, while the SIP population exhibited a 1.3-fold increase in total protein concentration (Fig. 4.4). When compared to untransformed *P. aeruginosa* cultures, the biofilm population of *P. aeruginosa* transformed with pALacZsd grown in LB-S with glass wool increased to slightly higher (0.5%) than that observed for untransformed *P. aeruginosa* in LB-S.

For cultures of *P. aeruginosa* transformed with pALacZsd and grown in LB + NaCl, the SIP population had 1.7-fold less protein biomass and the biofilm population 3.3-fold less protein biomass than the planktonic populations (Fig. 4.4). The biofilm population had a protein biomass *ca.* equivalent to 33.6% of the total protein biomass of the population grown in the presence of glass wool. This represents 16% more protein biomass in the biofilm population than that of untransformed *P. aeruginosa* cultured under identical conditions (Fig. 3.7).

The SIP population of *P. aeruginosa* transformed with pALacZsd and grown in LB + EtOH, had 2.5-fold less protein biomass than the planktonic population, while the biofilm population had 3.4-fold less protein biomass compared to the planktonic population (Fig. 4.4). The biofilm population had a protein biomass *ca.* equivalent to 42% of the total attached protein biomass of the population grown in the presence of a glass wool surface. This is similar to the biofilm population of untransformed *P. aeruginosa* grown in LB + EtOH (48%) (Fig. 3.7).

4.4.4 Cellular volume

Planktonic cultures of *P. aeruginosa* transformed with pALacZsd grown to an OD_{540} of 0.41 had a cell volume of $2.707 \pm 1.624\ \mu m^3$. After 16 h of growth, the SIP cells had a similar cell volume compared to planktonic cells, but biofilm cells had 1.4-fold less volume (Fig 4.5). By contrast, both SIP and biofilm populations of untransformed *P. aeruginosa* cultures showed an increase in cellular volume compared to the planktonic population (Fig. 3.8).

Planktonic cultures of *P. aeruginosa* transformed with pALacZsd grown in LB + NaCl had 1.5-fold less cell volume than those grown in LB-S. Although the cell volume of biofilm cells was *ca.* the same as planktonic cells, the cells of the SIP population showed a 1.7-fold

increase in cell volume (Fig. 4.5). Whereas the planktonic and SIP cells of untransformed *P. aeruginosa* (Fig. 3.8) were found statistically (P > 95%) to differ from the biofilm cells, cell volume of planktonic and biofilm cells of the transformed *P. aeruginosa* cultures were statistically different from the SIP cells (P > 95%).

Transformed *P. aeruginosa* cultures grown in LB + EtOH medium were found to exhibit no statistically significant (P > 95%) difference in cell volume between either planktonic, SIP or biofilm populations (Fig 4.5) This is in contrast to untransformed *P. aeruginosa* cells (Fig. 3.8), where planktonic and biofilm populations were shown to be significantly (P > 95%) different to the SIP population.

4.4.5 The effect of tetracycline concentration on basal β-galactosidase levels in the pALacZsd vector system under different growth conditions

In all β-galactosidase activity assays, basal levels of β-galactosidase expression could be observed in *P. aeruginosa* cells transformed with the promoterless pALacZsd reporter vector. The source of these basal levels of expression was thought to be due to transcriptional read-through from the *tetA* gene located upstream from the 5' end of the multiple cloning site. To investigate, *P. aeruginosa* cells transformed with pALacZsd were cultured in LB-S, LB + NaCl and LB + EtOH media containing different concentrations of tetracyline (0 – 80 $\mu g.ml^{-1}$) and the β-galactosidase activity of planktonic (Fig. 4.6) and total attached (Fig. 4.7) samples was subsequently assayed.

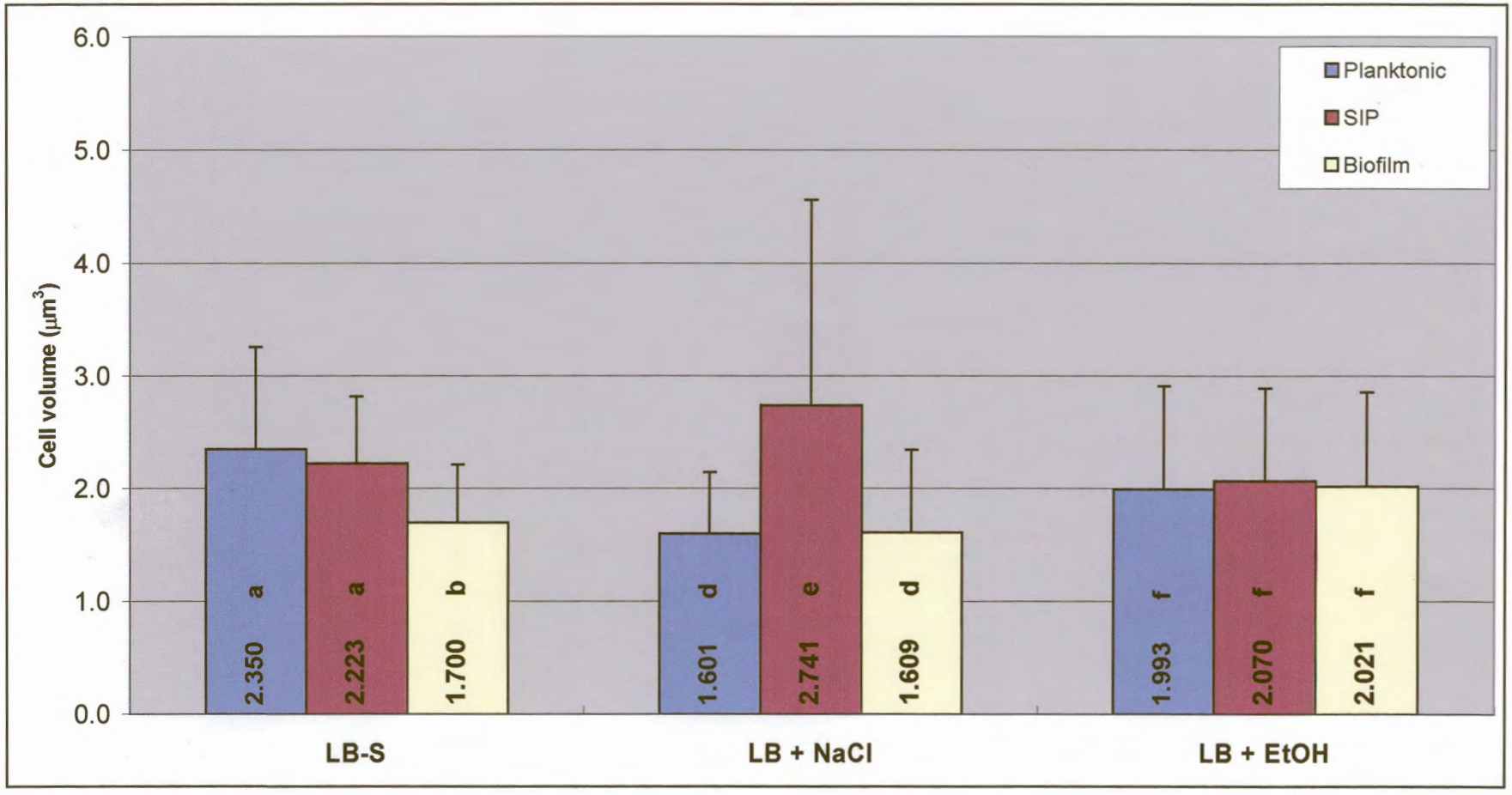

Fig. 4.5: Cell volumes of planktonic, surface influenced planktonic (SIP) and biofilm cells of *P. aeruginosa* transformed with pALacZsd grown for 16 h in LB broth without salt (LB-S), with 0.7 M NaCl (LB + NaCl) and 2.9% (v/v) ethanol (LB + EtOH). Means with different superscripts indicate statistically significant differences ($P<0.05$). Error bars denote one standard deviation of the mean.

A linear relationship between tetracycline concentration and β-galactosidase activity, which differed in the planktonic populations grown in LB-S, LB + NaCl, and LB + EtOH, was obtained (Fig 4.6). These results indicated that the *tetA* gene was upregulated in response to increased levels of tetracycline, which inadvertently caused an increase in basal β-galactosidase levels. The basal β-galactosidase level at a tetracycline concentration of 0 $\mu g.ml^{-1}$ was found to differ for each of the three different growth conditions and increased proportionally as the tetracycline concentration was increased (Fig. 4.6). The influence of tetracycline concentration on total attached cultures (SIP + biofilm) was markedly different to that obtained for planktonic cultures. For example, basal β-galactosidase activity at 0 $\mu g.ml^{-1}$ tetracycline for total attached cultures (SIP + biofilm) was higher than that of their planktonic counterparts. By contrast to planktonic cultures, a linear relationship between the tetracycline concentration and β-galactosidase activity was not observed (Fig. 4.7). This may indicate that cellular behaviour was markedly different either due to the SIP or the biofilm population or both for each growth medium used.

4.4.6 Hydrolysis of ONPG (A_{414}) and plasmid DNA concentrations in planktonic and total attached cultures of *P. aeruginosa* pALacZsd

Splitting of ONPG per total protein concentration (Table 4.2) indicates that the amount of enzyme per biomass ($A_{414}.\mu g^{-1}$) was similar in planktonic and total attached cultures, but markedly different in total attached cultures grown in LB + EtOH. The amount of plasmid DNA per biomass was found to be lower in the total attached environment. The amount of plasmid DNA per ONPG hydrolysis was 1.5-fold higher in planktonic populations grown in LB-S than in total attached cultures. Total attached cultures grown in LB + EtOH proved to have more plasmid $DNA.A_{414}^{-1}$ than the LB-S biofilm, *ca.* equivalent to LB-S planktonic populations. Similar ratios were obtained for plasmid DNA concentrations using the pJB3Tc20 vector system (Blatny *et al.*, 1997) as those obtained using the pALacZsd vector (data not shown).

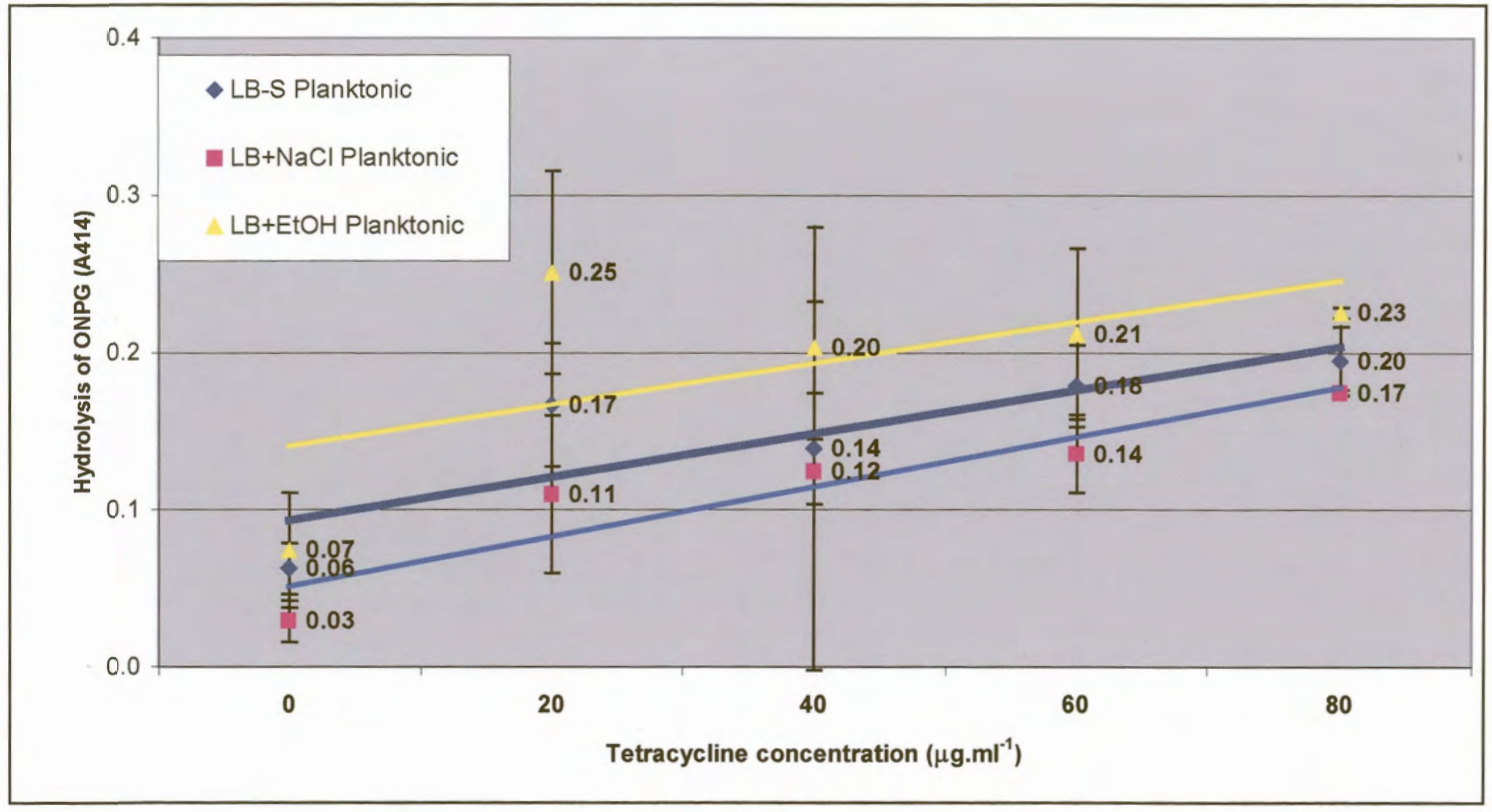

Fig. 4.6: The effect of tetracycline concentration (in $\mu g.ml^{-1}$) and growth conditions on basal β-galactosidase levels in planktonic cells. Error bars denote one standard deviation of the mean.

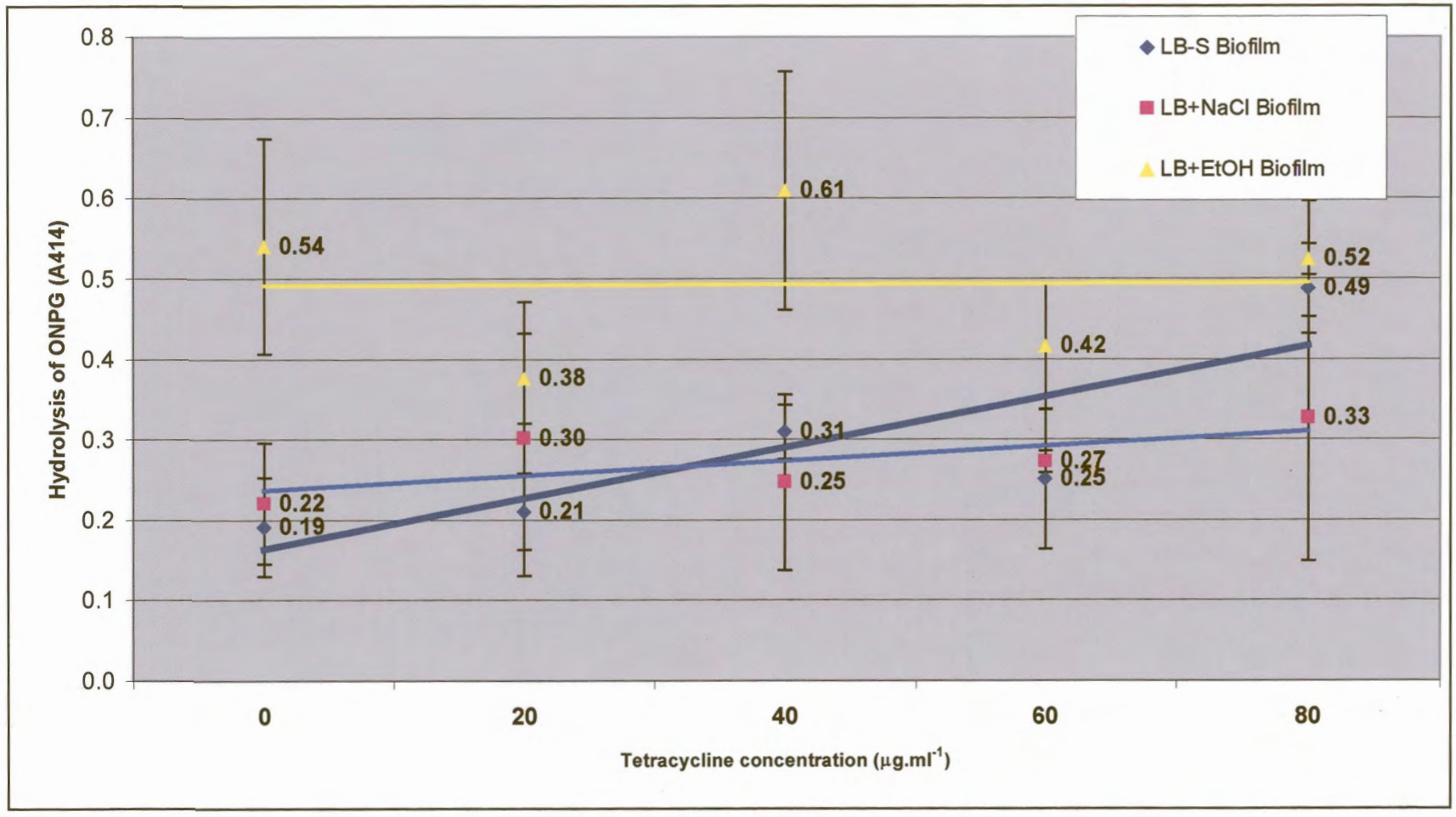

Fig. 4.7: The effect of tetracycline concentration (in $\mu g.ml^{-1}$) and growth conditions on basal β-galactosidase levels in total attached (attached + SIP) cultures. Error bars denote one standard deviation of the mean.

Table 4.3: Hydrolysis of ONPG (A_{414}), total protein biomass and plasmid DNA concentrations in planktonic and total attached cultures of *P. aeruginosa* pALacZsd

	LB-S (Planktonic)	**LB-S (Total Attached)**	**LB + EtOH (Total Attached)**
Tetracycline Concentration ($\mu g.ml^{-1}$)	40	40	40
A_{414}* / Biomass ($A_{414}.\mu g\ protein^{-1}$) × 100	8.72	6.61	4.25
pALacZsd DNA / Biomass ($ng.\mu g\ protein^{-1}$) × 100	3.91	1.99	1.78
pALacZsd DNA / A_{414} ($ng.A_{414}^{-1}$) × 100	4.48	3.00	4.19

*: Hydrolysis of ONPG

4.4.7 β-galactosidase activity in the pALacJK vector

To determine whether cloned insert DNA would have any effect on the basal β-galactosidase levels, a promoterless portion of the *P. aeruginosa aprA* gene was cloned into the pALacZsd vector. Comparison of *P. aeruginosa* cells transformed with the constructed pALacJK vector to cells transformed with the promoterless pALacZsd vector, indicated that the presence of an insert had no effect on the basal levels of β-galactosidase expression in either planktonic, SIP or biofilm populations grown in LB-S (Fig. 4.8). By contrast, *P. aeruginosa* cells transformed with pALacZsdIP indicated high levels of β-galactosidase in the planktonic, SIP and biofilm populations (Fig. 4.8). This was expected, since the *lacI* promoter is a strong constitutive promoter. These results indicate that the pALacZsd reporter vector was suitable for use in the assaying of biofilm-specific promoter activity. In addition, *P. aeruginosa* PAO (DSM1707) cells indicated a lack of endogenous β-galactosidase expression, thereby confirming its suitability as a host for use with the pALacZsd reporter vector.

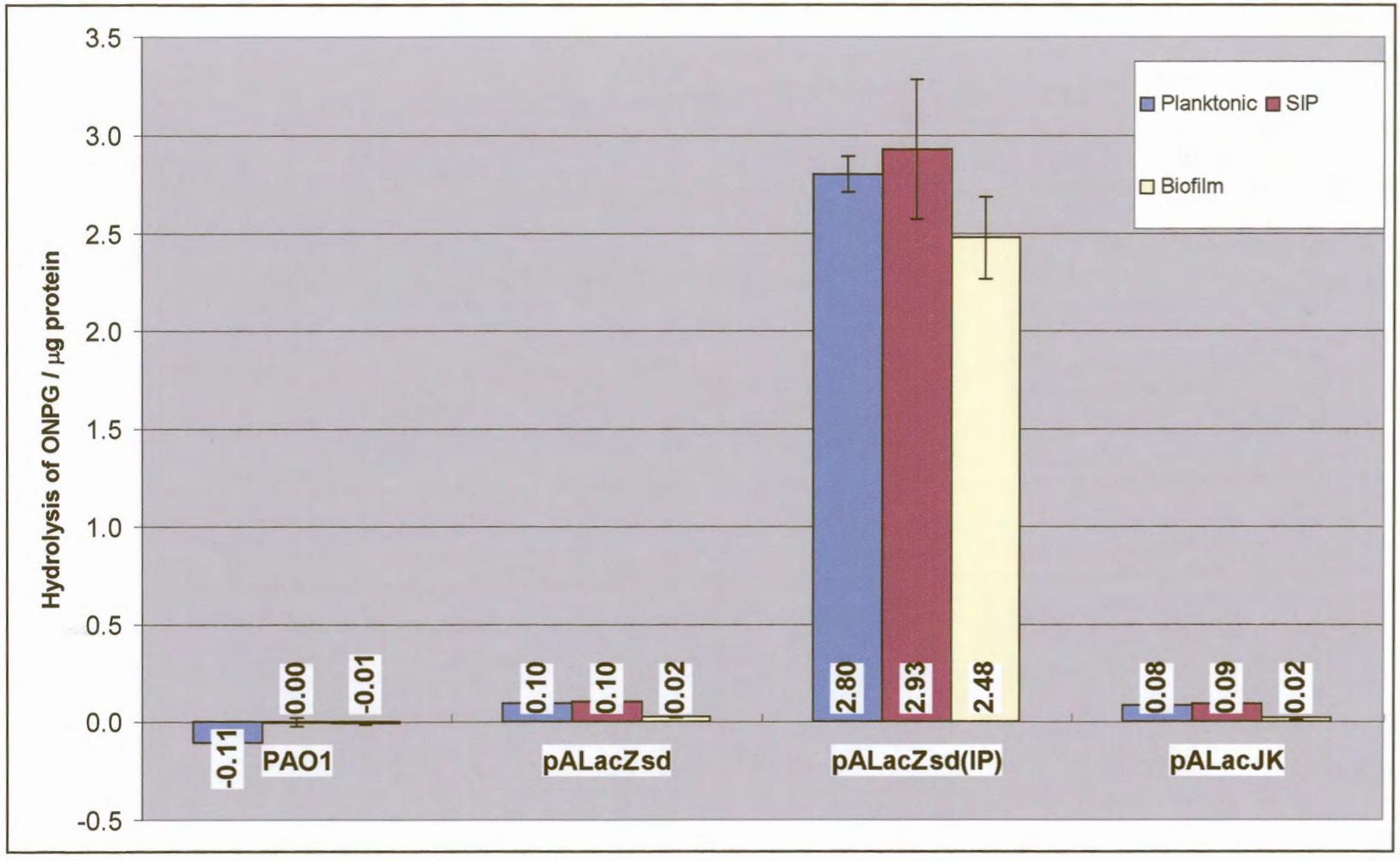

Fig. 4.8: ONPG hydrolysis per µg protein for *P. aeruginosa* pALacJK cultured for 16 h in LB broth without salt (LB-S). Error bars denote one standard deviation of the mean.

4.5 DISCUSSION

In this study the effect of a plasmid reporter construct, pALacZsd, on the physiology of *P. aeruginosa* planktonic, SIP and biofilm populations was investigated and compared to the corresponding populations of untransformed *P. aeruginosa* PAO (DSM1707). The presence of vector DNA and tetracycline had very little effect on biomass measurements in either planktonic, SIP or biofilm cultures grown in LB-S as the total protein concentrations of transformed *P. aeruginosa* cells were found to be similar to those obtained for untransformed *P. aeruginosa*. The data presented does, however, suggest an increase in the compactness (unit protein biomass per cellular volume) of cells of transformed planktonic and SIP populations, when compared to untransformed populations grown in LB-S.

P. aeruginosa biofilm-grown cells transformed with pALacZsd were found to be phenotypically different to planktonic cells grown in the same medium as discussed in Chapter 3. Moreover, they were found to be phenotypically different to untransformed *P. aeruginosa* cells. Previously, untransformed *P. aeruginosa* biofilm cells grown in LB-S were reported to exhibit an increase in cellular volume, and therefore perhaps a higher growth rate, than planktonic cells (Chapter 3). *P. aeruginosa* cultures transformed with the pALacZsd vector system, however, no longer exhibit this phenomenon, in fact the situation seems to be reversed in that biofilm cells are seen to have a smaller volume than both the planktonic and SIP populations.

In untransformed *P. aeruginosa* cultures, the yield of SIP cells was clearly greater than the yield of planktonic cells in broth with or without NaCl. Upon transformation with the pALacZsd vector, the yield of SIP cells grown in LB + NaCl was reduced to a level below that of planktonic populations, indicating that substantially less cells detached from the biofilm into the liquid phase. This may be as a consequence of up-regulation of alginate biosynthesis, due to the additional 0.4% ethanol present in these cultures, as demonstrated in untransformed *P. aeruginosa* populations grown in LB + EtOH (Chapter 3). Cultures grown in LB + EtOH are seen to be physiologically different to those grown in LB-S, however, there is more similarity between cultures grown in LB + NaCl and those grown in LB + EtOH than was previously seen in untransformed *P. aeruginosa* cultures. This phenomenon can similarly be attributed to the additional 0.4% ethanol present in LB + NaCl grown populations. Small additional amounts of ethanol seem to be able to change the dynamics of LB + NaCl grown populations as well as LB + EtOH grown cultures when compared to untransformed *P.*

aeruginosa populations cultured in the same medium.

The effect of various tetracycline concentrations on basal β-galactosidase activity in total attached cultures, which may be due to the physiological differences observed between untransformed biofilm and untransformed SIP populations (Chapter 3), showed no correlation for cultures grown in LB-S, LB + NaCl or LB + EtOH. 0.7 M NaCl was found to depress basal β-galactosidase expression levels, whereas the addition of ethanol increases the basal expression levels. Basal levels of β-galactosidase activity were found to be markedly different for planktonic and total attached (SIP + biofilm) cultures as well as between the different biofilms formed in the different growth media. The differences between planktonic and total attached cultures as well as between total attached cultures themselves supports the hypothesis that the physiology of both SIP and biofilm bacteria in the different growth media is unique to each growth state (Chapter 3). The high basal β-galactosidase levels with the increase in tetracycline concentrations may be due to transcriptional readthrough from the hyperinduced *tetA* gene, though the promoter is 1200 bp upstream of *lacZ*.

Concentrations of plasmid DNA per ONPG hydrolysis indicate that in total attached cultures grown in LB-S and LB + EtOH only half the amount of plasmid DNA is needed to obtain the equivalent amount of enzyme as planktonic cultures. Any up-regulation of promoter activity observed in total attached cultures (combined SIP and biofilm activity) therefore, should be at least twice as high as planktonic cultures in order to be considered "true" up-regulation. It is suspected, at least under these conditions, that the "SIP-biofilm" mode of growth induces an increase in plasmid DNA copy number in the "SIP-biofilm" mode of growth.

The physiological data presented here shows that *P. aeruginosa* containing an R-type plasmid is physiologically different to *P. aeruginosa* PAO (DSM 1707). The addition of tetracycline coupled with conferring resistance of *P. aeruginosa* to tetracycline do not have an "equal and opposite effect" on cellular physiology.

CHAPTER 5

EXPRESSION OF THE *Pseudomonas aeruginosa* PAO (DSM 1707) *algD* PROMOTER IS AFFECTED BY ENVIRONMENTAL STIMULI AND WHEN GROWN AS A BIOFILM

(The style of this chapter is in accordance with that of the Journal *Microbiology*)

5.1 SUMMARY

The attachment of *Pseudomonas aeruginosa* to a surface with the subsequent formation of a biofilm as well as environmental stimuli cause either expression or up-regulation of genes involved in the production of alginate, a bacterial exopolysaccharide produced in large quantities. The physicochemical conditions affecting up-regulation of the *P. aeruginosa* PAO (DSM1707) *algD* promoter were investigated using an *algD-lacZ* transcriptional fusion. The effects on promoter activity of osmolarity, water and nutrient availability as well as the presence of a surface for biofilm formation were studied. Promoter activity was assayed in liquid cultures using 2-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate for the β-galactosidase enzyme. The presence of 2% (w/v) glucose in combination with minimal M63 salts medium (MMG) upregulated *algD* promoter activity in biofilm populations and down-regulated in planktonic populations when compared to levels obtained in LB medium lacking NaCl (LB-S). *algD* promoter activity in SIP populations was downregulated in both MMG and LB-S. The presence of 0.7 M NaCl (LB + NaCl) enhanced *algD* promoter activity in biofilm populations only, while promoter activity was repressed in the planktonic and SIP population. The addition of 0.7 M NaCl to cultures grown in MMG (MMG + NaCl) was deleterious to promoter activity in the biofilm population. The effect of ethanol on *algD* promoter activity differed greatly from that observed for NaCl. Whereas the *algD* promoter was upregulated in planktonic populations grown in LB + EtOH, it was found to be upregulated in both planktonic and biofilm populations grown in MMG + EtOH medium. The data presented indicates that at least five separate factors, *i.e.* osmolarity, water availability, nutrient availability, growth as a biofilm and growth in the presence of a biofilm, influence the regulation of *algD*, either individually or in combination.

5.2 INTRODUCTION

The biofilm mode of growth has long been considered to provide optimal habitat conditions for many species of bacteria. The formation of *P. aeruginosa* biofilms is accompanied by the production of a mucoid exopolysaccharide (Costerton *et al.*, 1987) similar to that of the alginates of marine algae (Drummond *et al.*, 1962) and *Azotobacter vinelandii* (Gorin & Spencer, 1966). Cystic Fibrosis (CF) is an autosomal recessive disease that is characterised by disturbances in electrolyte transport and mucus secretion from exocrine glands and secretory epithelia (Berry *et al.*, 1989). The abnormalities in exocrine secretions of CF patients include altered electrolyte levels and atypical glycoproteins. The accumulation of abnormal fluids in the respiratory tract of CF patients appears to predispose the CF patient to chronic lung infection with *P. aeruginosa*. With prolonged infection, *P. aeruginosa* shifts to a mucoid phenotype that produces large amounts of alginate. The presence of this extracellular mucus compounds problems associated with the already viscous lung environment of CF patients (Berry *et al.*, 1989).

In *P. aeruginosa*, the synthesis and export of alginate, a co-polymer of N-acetyl guluronic and N-acetyl mannuronic acid (Rehm & Valla, 1997), is catalysed by a number of enzymes, most of which are located in a 12-member operon controlled from the promoter upstream of the *algD* gene (Gacesa, 1998). At least 24 genes have been directly implicated in alginate biosynthesis, showing complex regulation involving both specific gene products and some global regulators (Gacesa, 1998). The committal step, however, remains the conversion of GDP-mannose to GDP-mannuronate by the *algD* gene product (Gacesa, 1998).

The *algD* promoter is controlled by the *algR* (an environmentally responsive regulatory gene) and *algU* gene products. *algU*, which encodes the alternative sigma factor σ^{22}, undergoes a strong transcriptional activation in mucoid *P. aeruginosa* cells (Deretic *et al.*, 1987b). Published data indicates that *algU* is the first open reading frame of a five-member operon containing *mucA-D* (Martin *et al.*, 1993a; Martin *et al.*, 1993b; Boucher *et al.*, 1996). The products of the *mucA* and *mucB* (anti-σ factor) genes repress the activity of the AlgU protein. Mucoid CF isolates frequently have a defective *mucA* allele and inactivation of *mucA* or *mucB* results in the mucoid phenotype (Martin *et al.*, 1993b). Spontaneous reversion to the non-mucoid phenotype is often due to a suppressor mutation in *algU* (DeVries & Ohman, 1994). The periplasmic protein MucB (AlgN) was proposed to function by facilitating the interaction of the transmembrane protein MucA with cytoplasmic σ^{22} (Mathee *et al.*, 1997).

According to this model, MucA transduces a signal that leads to rapid turnover of σ^{22} and therefore affects the control of σ^{22}-dependant promoters like *algD* and, thus, the expression of all the other genes in the alginate biosynthetic cluster. *mucD* is orthologous to the *Escherichia coli* periplasmic protease and chaperone DegP. DegP homologues are known virulence factors that play a protective role in stress response in various species (Yorgey *et al.*, 2001). *mucD* mutants have been shown to be impaired in their ability to cause disease (Yorgey *et al.*, 2001) as well as up-regulating *algD* activity, thereby resulting in alteration to the mucoid phenotype. The exact function of MucC is unknown, but is thought to be a regulator of some sort (Gacesa, 1998). Boucher *et al.* (1997) have shown that the frequency of *mucA* mutations causing mucoidy in *P. aeruginosa* isolates taken from CF patients is as high as 84%. The most common mutation (observed in 25% of the strains containing *mucA* mutations) was a deletion of a G within the homopolymeric stretch of 5 residues located between positions 429 and 433 of the *mucA* coding sequence (Boucher *et al.*, 1997). The mechanism by which these mutations occur is unknown at present.

The chemical environment has been found to have a considerable affect on transcription from *algD*. A two-component signal transduction pathway comprising the putative sensor proteins AlgQ (kinase) and AlgZ, interacting with regulatory proteins like AlgR and AlgB, has been identified. The positive regulators bind, after phosphorylation, upstream of the *algD* promoter leading to the formation of a superhelical structure with the aid of the histone-like protein, AlgP, causing activation of transcription (Rehm & Valla, 1997). Transcriptional activation of *algD* increases due to increases in osmolarity [with the exception of cells grown in the presence of a silicone rubber surface (Hoyle *et al.*, 1993)], which is thought to be due to an increase in DNA supercoiling (Berry *et al.*, 1989). Ethanol (a commonly-used dehydrating agent) as well as isopropanol has been found to be capable of inducing concentration-dependent transcription from *algD* (DeVault *et al.*, 1990). Changes in growth temperature (Leitão *et al.*, 1992), pH, nitrogen or phosphate limitation and oxygen availability (Leitão & Sá-Correira, 1997; Gacesa, 1998) all have an affect on *algD* promoter activation, while growth under anaerobic conditions induces *algD* transcription marginally (DeVault *et al.*, 1990). Glucose has been reported to stimulate *algD* transcription and alginate production (Ma *et al.*, 1997), but this contradicts earlier work, which proposed that glucose repression of *algD* occurs (DeVault *et al.*, 1991).

The objective of this part of the study was thus to determine the effect of *P. aeruginosa* PAO

(DSM1707) growing as a biofilm as well as in various chemical environments on *algD* promoter activity.

5.3 MATERIALS AND METHODS

5.3.1 Bacterial strains, media and growth conditions

The bacterial strains and plasmid DNA used in this study are listed in Table 5.1. *E. coli* JM105 and *E. coli* XL1-Blue, which were used for the maintenance and amplification of plasmid DNA, were grown at 37°C in Luria-Bertani (LB) broth supplemented with 10 μg.ml^{-1} tetracycline. *P. aeruginosa* was grown either in MMG broth (minimal M63 salts supplemented with 0.2% [w/v] glucose, 1 mM $MgSO_4$, 0.5% [w/v] Casamino Acids) (O'Toole & Kolter, 1998), or in LB-S broth with the addition of 40 μg.ml^{-1} tetracycline (Roche Diagnostics, Randburg, South Africa). When required, NaCl was added to a final concentration of 0.7 M (LB + NaCl) and ethanol to a concentration of 2.5% (v/v) (LB + EtOH). However, the final ethanol concentration was slightly higher at 2.9% (v/v) due to the 0.4% ethanol added in combination with the tetracycline (Section 4.3.1.1). The *P. aeruginosa* cultures were incubated at 37°C with agitation at 200 rpm until mid-exponential phase (OD_{540} = *ca.* 0.5), diluted to OD_{540} = 0.1 in pre-warmed (37°C) LB-S and aliquots (500 μl) were then transferred to 2-ml microfuge tubes with or without 0.025 g of glass wool (Merck, Darmstadt, Germany; mean standard diameter = 10 μm). After growth at 37°C for 16 h, the total attached biomass was separated into SIP and biofilm biomass as described in Section 3.3.1, cooled to 4°C and β-galactosidase activity assays were performed (Section 5.3.2). The optimal NaCl and ethanol concentrations required for maximal *algD* promoter activity was determined by assaying the β-galactosidase activity of planktonic cultures grown at various NaCl (0 – 1 M) and ethanol (0 – 5% [v/v]) concentrations.

5.3.2 Assay for β-galactosidase activity

β-galactosidase activity was assayed in liquid cultures using 2-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate as previously described (Section 4.3.4). β-galactosidase activity was expressed as either the A_{414} (amount of ONPG hydrolysed in 15 min) or A_{414} / μg total cellular protein.

Table 5.1: Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant properties	Reference or source
Strains:		
Escherichia coli XL1-Blue	Δ(lacZ)M15	Stratagene*
Escherichia coli JM105	Δ(lacZ)M15	J. B. Weyers†
Pseudomonas aeruginosa DSM 1707	Prototroph	DSM‡
Plasmids:		
pCR-Script™-Amp SK(+)	pBluescript® SK (+) with a *Srf*I cloning site	Stratagene
PALacZsd	pAL4000 (Greener *et al.*, 1992) containing a promoter-less *lacZ* in place of the *luc* gene	J. B. Weyers
PALacZsdIP	pALacZsd containing the *lac* promoter from pBluescript (co-ordinates 719-1719)	J. B. Weyers
PALacZsdAg	pALacZsd containing the *algD* promoter from the *P. aeruginosa* genome (co-ordinates 3961979 to 3962457)	This study

*: Stratagene®, La Jolla, CA, USA
†: Mr J. B. Weyers, Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria, South Africa
‡: Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany

5.3.3 Analytical Methods

Total cellular protein concentrations of the above-mentioned cultures were determined using the Bradford assay, as described in Section 3.3.2, and used to standardise the results obtained for the β-galactosidase activity assay (Section 5.3.2).

5.3.4 DNA Manipulations

5.3.4.1 Construction of the algD reporter vector pALacZsdAg

The pALacZsdAg vector was constructed to study the activity of the *algD* promoter in *P. aeruginosa* under various culture conditions. The *algD* promoter sequence, inclusive of the upstream regulatory elements, corresponding to co-ordinates 3961979 to 3962457 of the *P. aeruginosa* genome, was inserted into the *Kpn*I and *Xba*I sites of the pALacZsd vector. Briefly, *P. aeruginosa* genomic DNA was isolated using the method of Jansen (1995) and used as template DNA for the PCR amplification of the *algD* promoter region. The reaction mixture consisted of 1 µl of a 1:100 dilution of template DNA, 4 mM $MgCl_2$, 50 pmol of each of the primers AN1 (5' – GAAGGTACCTGGCGCTACCGTTCGT – 3') and AN2 (5' – GGGGTCTAGACGGTGATAGGATGTTTTCTCT – 3'), 250 µM of each dNTP and 1.5 U *Taq* DNA polymerase (Promega, Madison, USA). The 478-bp amplicon was concentrated by precipitation with 10% 3 M NaOAc (pH 7.6) and 2 volumes 100% ethanol and then cloned into the PCR-Script™-Amp SK(+) vector. The nucleic acid sequence of the cloned insert

DNA was determined using an ABI PRISM™ Big Dye™ Terminator Cycle Sequencing Kit (Perkin Elmer, Foster City, CA, USA) and primers AN1 and AN2, in accordance with the manufacturer's instructions.

The PCR-Script™-Amp SK(+) - *algD* recombinant plasmid as well as the pALacZsd vector were extracted from *E. coli* XL1-Blue and *E. coli* JM105, respectively, by an alkaline lysis method (Sambrook *et al.*, 1989) and digested (Sambrook *et al.*, 1989) with both *Kpn*I and *Xba*I. The 478-bp *algD* promoter DNA fragment as well as the linearized pALacZsd vector DNA was gel-purified using the freeze-squeeze protocol (Benson, 1984), ligated and transformed into competent *E. coli* JM105 cells (Section 4.3.3.1). Putative recombinant clones were screened both by digestion with *Kpn*I and *Xba*I, and by using PCR as described above. A recombinant clone was designated pALacZsdAg and used in all subsequent assays.

The pALacZsd, pALacZsdIP and pALacZsdAg reporter vectors were subsequently transformed into competent *P. aeruginosa* DSM 1707 cells (Section 4.3.3). The transformed cultures were grown to an OD_{540} = *ca.* 0.5, aliquotted and stored in 50% glycerol at –70°C. Fresh culture stocks were used for each series of β-galactosidase activity assays.

5.4 RESULTS

5.4.1 Construction of the pALacZsdAg vector

The 478-bp *P. aeruginosa algD* promoter, inclusive of the upstream regulatory elements, was isolated by PCR amplification and cloned into the *Kpn*I and *Xba*I sites of the pALacZsd vector. Putative recombinant clones were screened both by digestion with *Kpn*I and *Xba*I and using PCR (Fig. 5.1). A recombinant clone was designated pALacZsdAg and used in all subsequent assays.

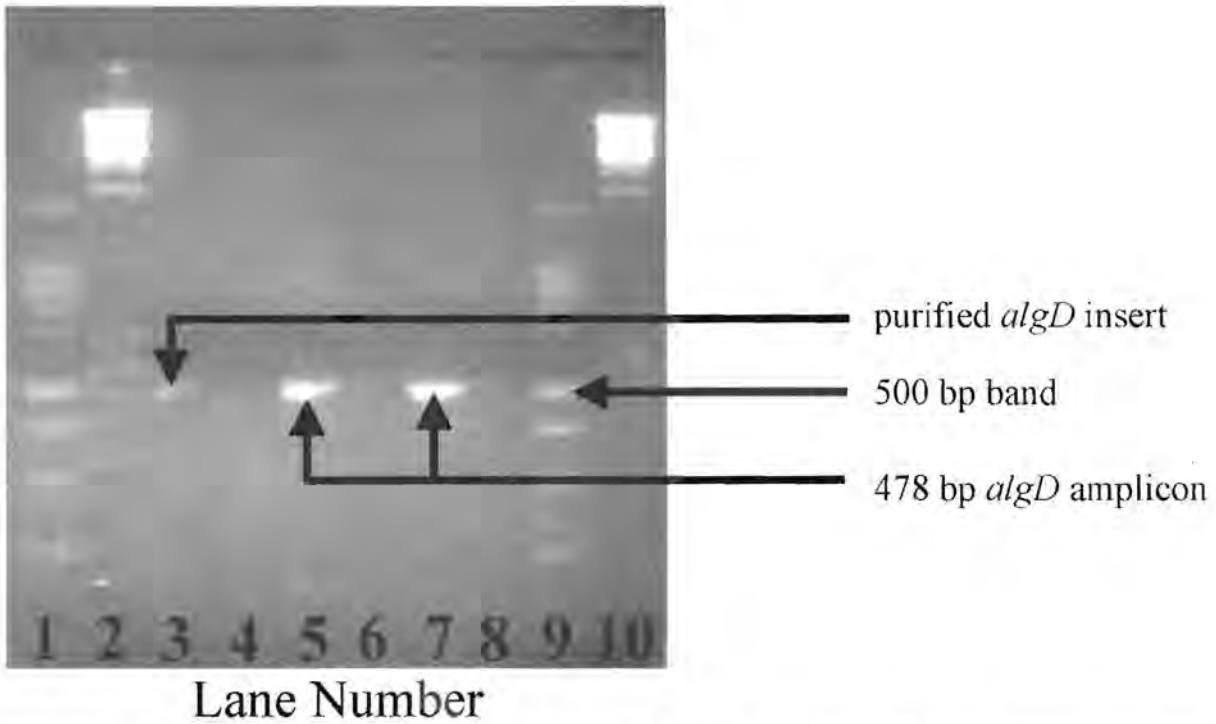

Fig. 5.1: A purified *algD* promoter (lane 3) was ligated into the pALacZsd reporter vector and recombinant clones were identified following PCR amplification of a 478-bp *algD* amplicon (lanes 5 and 7). Lanes 1 and 9, 100 bp DNA Ladder (Promega, Madison, USA) and lanes 2 and 10, phage λ DNA digested with *Hind*III.

5.4.2 Determination of optimal NaCl and EtOH concentrations for *algD* promoter activity in planktonic cultures

The data obtained (Fig. 5.2) shows that the *algD* promoter activity increased as the osmolarity of the LB growth medium increased, but beyond 0.8 M NaCl the level of promoter activity decreased. The NaCl concentration for maximal *algD* promoter activity was found to range between 0.5 M and 0.8 M. This is in agreement with data published by Deretic *et al.* (1989) and Berry *et al.* (1989) who found that high osmolarity resulted in increased expression of the *algD* gene.

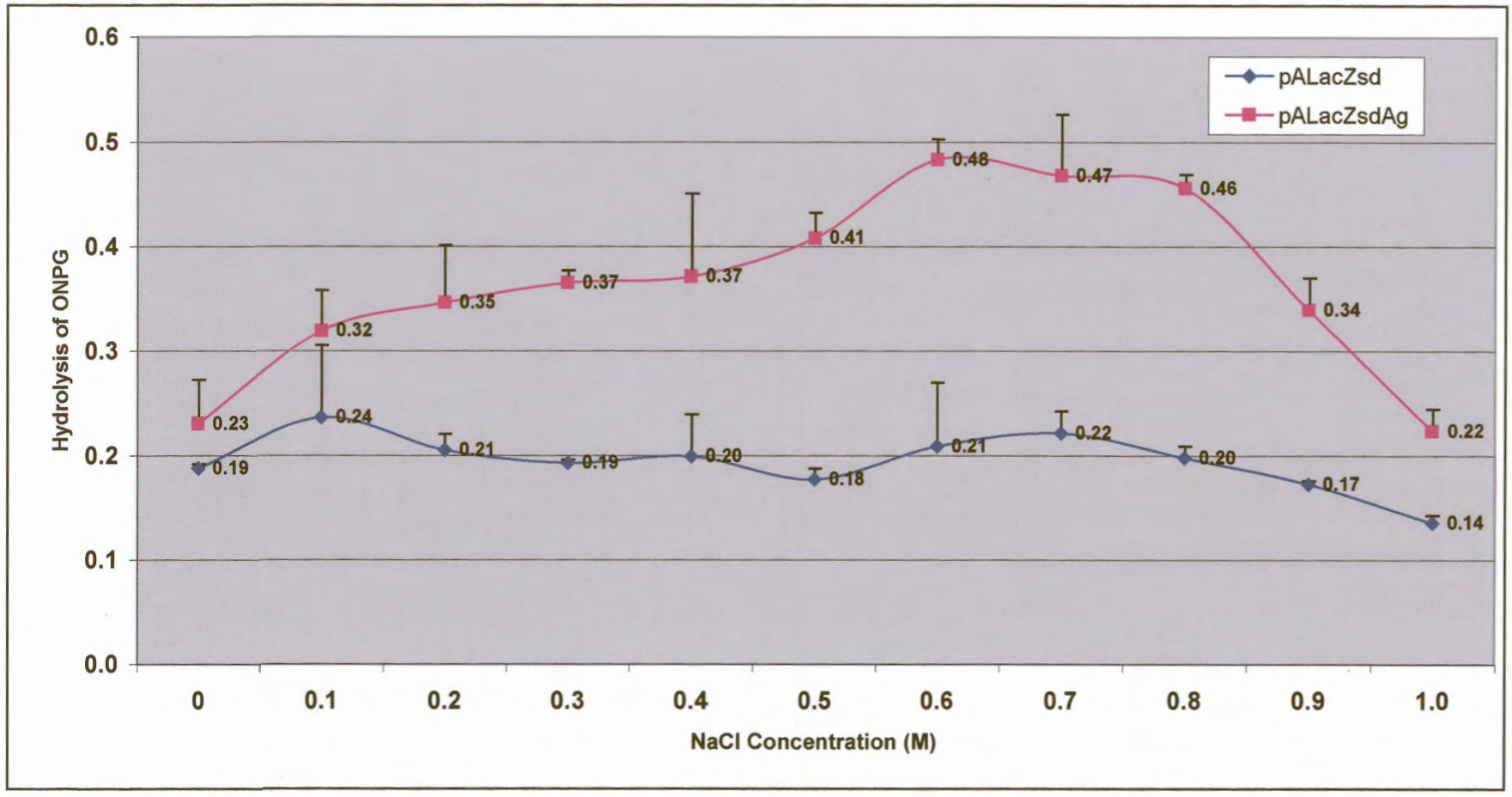

Fig. 5.2: *algD* promoter activity as measured by β-galactosidase activity assays on 16 h planktonic cultures of *P. aeruginosa* (pALacZsdAg) grown at various NaCl concentrations in LB, using ONPG as substrate. Error bars denote one standard deviation of the mean.

The EtOH concentration for maximal *algD* promoter activity in planktonic *P. aeruginosa* cultures was determined to be 2.9% (Fig. 5.3). Unlike the rather weak affect of 0.7 M NaCl, which caused a 2-fold up-regulation of *algD* promoter activity, ethanol strongly upregulated expression from the *algD* promoter 8-fold. The results were in agreement with those obtained by DeVault *et al.* (1990). Consequently, all media used in subsequent analysis contained 0.7 M NaCl or 2.9% (v/v) ethanol.

5.4.3 *algD* promoter activity

5.4.3.1 algD promoter activity in low salt broth

The *algD* promoter displayed high activity in planktonic cultures grown in LB-S (Fig. 5.4). By contrast, *algD* promoter activity was 2.5-fold lower in the biofilm population, and 4-fold lower in the SIP population compared to that in the planktonic population. However, the *algD* promoter activity was 1.6-fold higher in the biofilm population compared to the SIP population. These results indicate that both direct association of cells with the surface, as well as growth of suspended cells in close proximity to a surface-grown (biofilm) culture, lead to down-regulation of transcription from *algD*. The presence of 2% (w/v) glucose in combination with minimal M63 salts (MMG) upregulated *algD* promoter activity in biofilm populations when compared to the levels obtained in the corresponding populations of cultures grown in LB-S. For cultures grown in MMG, *algD* promoter activity was abolished in the SIP population, while the promoter activity in the biofilm population was 2.7-fold higher than in the planktonic population (Fig. 5.4).

5.4.3.2 algD promoter activity in high salt broth

The *algD* promoter activity was enhanced by 0.7 M NaCl (LB + NaCl) in biofilm populations when compared to the corresponding population of cultures grown in LB-S. By contrast, promoter activity was lower in planktonic populations and totally abolished in the SIP population (Fig. 5.4). Interestingly, up-regulation of the *algD* promoter in the presence of a glass wool surface and 0.7 M NaCl (LB + NaCl) was 3-fold higher than with a glass wool surface alone (LB-S). The *algD* promoter activity in cultures grown in MMG + NaCl was similar for planktonic and SIP populations grown in LB + NaCl, but it was, however, abolished in the biofilm population (Fig. 5.4). These results indicate that the metabolic flux, dictated by the different nutrient make-up of MMG medium, influenced regulation of the *algD* promoter in biofilm cells differently than in planktonic and SIP cells.

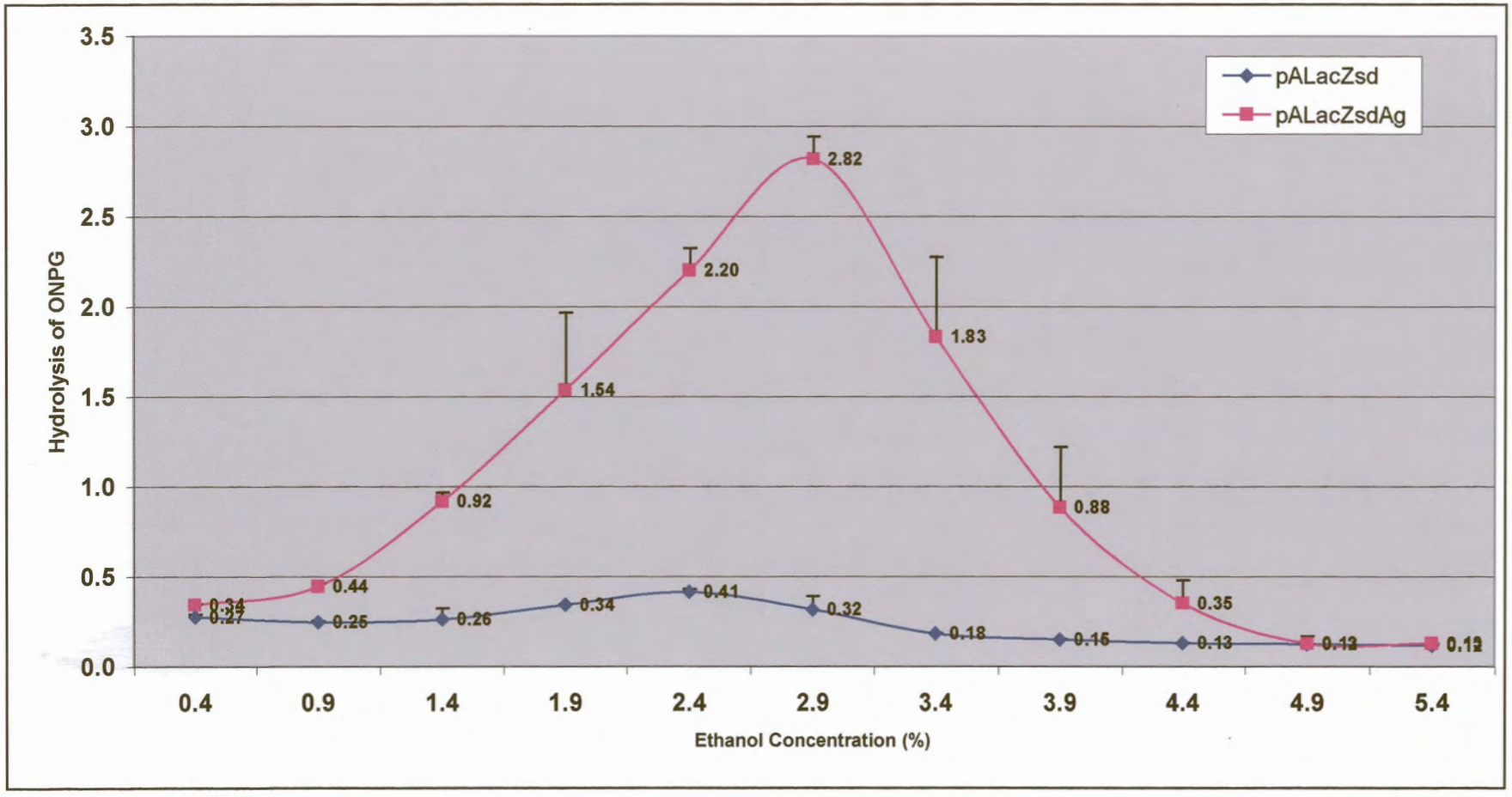

Fig. 5.3: *algD* promoter activity as measured by β-galactosidase activity assays on 16 h planktonic cultures of *P. aeruginosa* (pALacZsdAg) grown at various ethanol concentrations in LB, using ONPG as substrate. Error bars denote one standard deviation of the mean.

5.4.3.3 algD *promoter activity in broth containing 2.9% (v/v) ethanol*

The planktonic population of cultures grown in LB + EtOH showed extreme up-regulation of the *algD* promoter, as has been reported previously (DeVault *et al.*, 1990). The same was true for the planktonic population grown in MMG + EtOH, albeit to a lesser degree (Fig. 5.4). Unlike NaCl, ethanol did not exert an additive effect with surface-associated growth in MMG media, since the levels of activity were similar to those in biofilm populations grown in MMG medium lacking ethanol. The *algD* promoter activity in the SIP populations was again very low.

5.5 DISCUSSION

The *algD* promoter activity was shown to be influenced not only by salinity (Fig. 5.2) and ethanol (Fig. 5.3), as has been established by Berry *et al.* (1989) and DeVault *et al.* (1990), respectively, but also by growth as a biofilm, growth in the vicinity of a biofilm (SIP) and nutrient availability (Fig. 5.4). Conflicting reports regarding the activation of the *algD* promoter by glucose have been noted in the literature. DeVault *et al.* (1991) demonstrated that the *algD* promoter in planktonic populations was sensitive to glucose repression when grown in nutrient broth supplemented with up to 5% glucose, while Ma *et al.* (1997) demonstrated an increase in *algD* transcription in planktonic populations when *P. aeruginosa* was grown in LB-broth supplemented with 5% glucose. From the data presented here, glucose (MMG) was found to be capable of up-regulating the *algD* promoter in biofilm populations by 3-fold when compared to the cultures grown without glucose (LB-S). *algD* activity in the biofilm population grown in MMG is up-regulated compared to planktonic cultures while biofilm cultures grown in LB-S are down-regulated when compared to the corresponding planktonic population. The transcriptional inactivation seen in the SIP population of cultures grown in MMG, as well as the data presented above, may be evidence of the role of catabolic repression in alginate synthesis in SIP populations of cells, as hypothesized by DeVault *et al.* (1991) and points out the possibility of alternative mechanisms for alginate synthesis. Alternatively upregulation in the biofilm population and due to salt in MMG medium may be δ^{54} dependent but overridden in LB-S due to the presence of nitrogen in the medium.

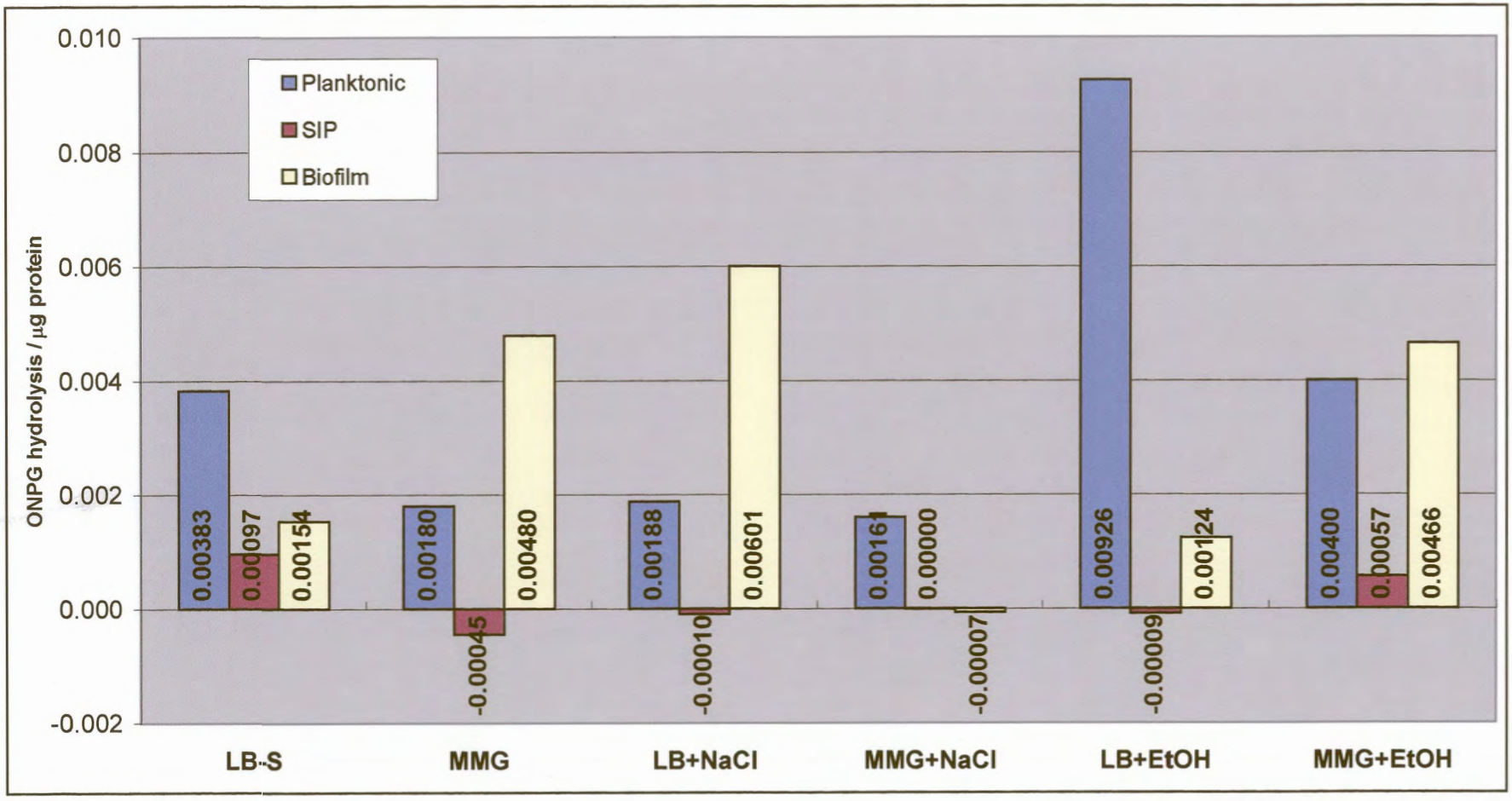

Fig. 5.4: *algD* promoter activity after 16 h of growth as influenced by LB-S and MMG media with and without 0.7 M NaCl or 2.9% (v/v) ethanol.

Attachment to a surface plays a major role in the expression of *algD* and therefore in control of alginate synthesis. This was demonstrated in MMG by cultures, in the presence of a surface, having substantially higher *algD* promoter activity than planktonic cultures. This same phenomenon was observed for cultures grown in the presence of NaCl (LB + NaCl). However, a cumulative affect for activity caused by NaCl and activity caused by the surface is only seen in LB + NaCl. It appears that the surface itself is being sensed by the bacterium. A combination of NaCl and glucose abolished both activity due to the surface and activity due to NaCl as evidenced in MMG + NaCl media. This is further evidenced by the fact that the accumulation and subsequent increase in NaCl concentration at the surface would tend to be inhibitory to *algD* transcription, as shown in the concentration gradient for this substance (Fig. 5.2). It can be postulated that NaCl merely provides optimal environmental conditions for signalling between the surface and the bacterium-itself. The previously reported down-regulation of the *algD* promoter in the presence of NaCl and a silicone rubber surface (Hoyle *et al.*, 1993) correlates well with the data obtained for cultures grown in MMG + NaCl. This is understandable, as Hoyle *et al.* (1993) grew their cultures in a basal salts medium containing L-glutamate. NaCl, therefore, under conditions where catabolite repression occurs, is capable of inactivating alginate biosynthesis. Under environmental conditions where the TCA cycle is utilized for metabolism, the reverse occurs. NaCl is then able to cause substantially elevated levels of *algD* transcription in biofilm populations.

Ethanol does not exert its effect on the *algD* promoter in the same manner as NaCl. As also reported in previous studies, ethanol up-regulates the *algD* promoter in planktonic populations (DeVault *et al.*, 1990; Edwards & Saunders, 2001). However, biofilm populations do not show an increase in *algD* promoter activity when compared to media lacking ethanol (Fig. 5.4). Ethanol is thought to exert its effect on the cell by causing membrane perturbation (Sissons *et al.*, 1996); however, the mechanism by which alginate biosynthesis is up-regulated in planktonic populations remains to be elucidated. Alginate concentrations (Table 3.1) as well as visual images of biofilm cells embedded in large amounts of EPS (Fig. 3.4g) do not correlate with the observed low levels of *algD* promoter activity. The data may indicate that translational control of *algD* *via* increased mRNA stability in the LB + EtOH environment is taking place. Alginate concentrations in the SIP population of cultures grown in LB + EtOH indicated that this may be the case as transcription of *algD* has ceased, however, alginate concentrations remained similar to concentrations in the biofilm population (Table 3.1). A similar scenario has been shown for

Bacillus subtilis when exposed to ethanol whereby the half-life of the mRNA of the general stress protein, GsiB, is increased to *ca.* 20 min (Jürgen *et al.*, 1998).

The metabolic flux dictated by the different nutrient make-up in MMG medium may influence *algD* promoter regulation in biofilm-cultured and planktonic cells differently than SIP cells, as demonstrated by the abolished *algD* promoter activity in SIP cells grown in MMG. Catabolite repression control is known to be involved in the regulation of virulence factors in many Gram-negative bacteria (Sage & Vasil, 1997) and is also involved in biofilm development (O'Toole *et al.*, 2000). It should therefore be expected that cultures either primed for attachment (planktonic cells) or attached to a surface should show up-regulation of alginate when grown in MMG. SIP cells are hypothesised to show down-regulation of *algD* in MMG due to the "detachment phenotype" (Section 3.5). Activity of the *algD* promoter was abolished in the biofilm population of cultures grown in MMG + NaCl, showing the fine intricacies at work behind up-regulation of the alginate biosynthetic operon. NaCl (high osmolarity) causes production of osmoprotectants, which in turn, enable catabolite repression control of the *plcH* gene (Sage & Vasil, 1997). The metabolic pathways of *plcH* catabolite repression and glucose catabolite repression have been shown to differ (Sage & Vasil, 1997). It is therefore probable that the induction of both the glucose and *plcH* metabolic pathways may in consort be responsible for inactivating alginate production in the biofilm phenotype. The presence of a glass wool surface, however, was shown to contribute to the observed phenomenon, as planktonic populations remained unaffected. Ethanol in combination with MMG showed drastically different effects on *algD* promoter activity. Cultures grown in MMG + EtOH behaved as described above for cultures grown in LB + NaCl. The mechanism by which ethanol exerts its effect on *algD* promoter activity in MMG media seems to be identical to that described for LB-NaCl.

In conclusion, it is apparent that at least five separate factors, *i.e.* osmolarity, water availability, nutrient availability, growth as a biofilm and growth in the presence of a biofilm, influence the regulation of *algD* and therefore alginate biosynthesis. The data presented shows that *P. aeruginosa* is able to alter levels of alginate gene expression according to the environmental conditions in which it finds itself. Furthermore, attachment to a surface, in conjunction with other environmental conditions, plays an important role in both the up-regulation as well as the down-regulation of *algD*.

CHAPTER 6

CHARACTERISATION OF A NOVEL *PSEUDOMONAS AERUGINOSA* PAO (DSM1707) REGULATORY ELEMENT

(The style of this chapter is in accordance with that of the Journal *Microbiology*)

6.1 SUMMARY

The attachment of bacteria to surfaces and the concomitant formation of biofilms results in various physiological changes due to the up- and down-regulation of a number of genes, including those involved in exopolysaccharide production. In a previous study, putative attachment-inducible regulatory elements of *Pseudomonas aeruginosa* PAO (DSM 1707) were identified using the pALacZsd vector system. The aim of this study was to further characterise one of these regulatory elements. Nucleic acid sequence analysis of the selected regulatory element, pALacZsd65, indicated that it contained two cloned DNA fragments. The chimera 1 element mapped on the reverse strand of PA2464, but lacked significant homology to any known open reading frames in other bacteria. By contrast, the second chimera mapped 483 bp upstream of the recently described *pepA (phpA)* gene, which has been reported to affect expression of the *P. aeruginosa* alginate operon. The effect of different physicochemical conditions, previously found to up-regulate *algD* promoter activity in planktonic and biofilm cultures (Chapter 5), was also investigated in this study. Promoter activity of clone pALacZsd65 was assayed in liquid cultures using 2-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate for the β-galactosidase enzyme. Differences in promoter activity were observed across various NaCl and ethanol concentrations with maximal expression of the reporter gene observed at concentrations of 0.2 M NaCl and 2.4% (v/v) ethanol in planktonic cultures. The data presented indicates that the pALacZsd65 construct may contain a promoter sequence, or part thereof, that is influenced by detachment of *P. aeruginosa* from a surface.

6.2 INTRODUCTION

The attachment of *P. aeruginosa* to surfaces and the concomitant formation of biofilms are accompanied by various physiological changes (Fletcher, 1991; Dagostino *et al.*, 1991), which are the result of altered regulation of a number of genes. These include genes involved in the production of alginate (Davies & Geesey, 1995), the most noteworthy being the *algC* (Davies *et al.*, 1993) and *algD* (Hoyle *et al.*, 1993; Rice *et al.*, 1995) genes. Recent studies have shown the up-regulation of a number of other genetic elements that may be necessary for biofilm formation and maintenance, including *crc* (O'Toole *et al.*, 2000), *lasI* and *rhlI* (Davies *et al.*, 1998; De Kievit *et al.*, 2001), *flgK*, *lasB* and *rhlA* (Sauer *et al.*, 2002) as well as a large number of other genes reported by Whiteley *et al.* (2001).

Gene regulation has been found to be altered in biofilms cells on a transcriptional (Whiteley *et al.*, 2001; Weyers, 1999) as well as a translational (Sauer *et al.*, 2002) level, thus resulting in major differences in the phenotypes of planktonic and biofilm populations. Sauer *et al.* (2002) have characterised five stages of biofilm development, namely (1) reversible attachment; (2) irreversible attachment; (3) maturation-1; (4) maturation-2; and (5) dispersion. Using two-dimensional gel electrophoresis, they were able to demonstrate significant differences between the proteomes of the various stages of biofilm development as well as a change in expression levels of more than 50% of the proteome when maturation-2 stage biofilms were compared to planktonic cells. Weyers (1999) isolated a number of regulatory elements which upregulated expression of a LacZ reporter gene at least 3-fold in biofilm populations compared to planktonic populations, and Whiteley *et al.* (2001) recently reported differential expression of 73 different genes following a comparison of the gene expression profiles in mature biofilms and planktonic populations using DNA microarrays.

Published texts to date imply that the opportunistic pathogen *P. aeruginosa* "prefers the biofilm mode of growth" due to biofilm cells being protected from agents that would otherwise be lethal under normal growth conditions. The bacterial biofilm is thought to be more resistant to antimicrobial agents (Costerton *et al.*, 1987; Allison & Gilbert, 1995; Brown *et al.*, 1995), ultra-violet light (Degiorgi *et al.*, 1996), heat (Deog-Hwan & Marshall, 1995), and a host of other environmental factors. Findings presented in this dissertation have, however, questioned the widely held belief that *P. aeruginosa* occurs predominantly as a biofilm. The data presented thus far have shown that there is many more suspended (SIP) than biofilm cells (Chapters 3 and 4). Furthermore, when observing the formation and

development of biofilms in flow cells, many suspended cells (SIP) pass the surface without showing any inclination to attach to the surface (personal observation). It can therefore not be argued that biofilm cells are more capable of dealing with environmental pressures such as those mentioned above. The question thus arises as to why the majority (*ca.* 80%) of cells in a *P. aeruginosa* population, grown in the presence of sufficient surface area for attachment, "voluntarily" remain in suspension and not attached to the surface. [This phenomenon can be observed in Figs. 3.2, 3.3 and 3.4 where much of the glass wool has no biofilm or attached cells]. A possible explanation may be that a large number of cells detach from the surface (Section 3.5), since the SIP population constantly has a greater biomass than the biofilm population under conditions where the theoretical growth rate of the biofilm population is 1.3X greater than that of the SIP population (Section 3.4.6).

Little is known about the many genes that may be involved in the formation and regulation of *P. aeruginosa* biofilms, let alone those involved in the detachment process. A number of suspected attachment-inducible regulatory elements have been isolated by Weyers (1999). These are thought to control the expression of genes that may aid or play roles in the attachment of *P. aeruginosa* to a surface, subsequent biofilm formation and detachment. The objective of this study was to examine one of these regulatory elements, namely pALacZsd65, in greater detail.

6.3 MATERIALS AND METHODS

6.3.1 Bacterial strains, media and growth conditions

The bacterial strains and plasmid DNA used in this study are listed in Table 6.1. *E. coli* JM105, used for the maintenance and amplification of plasmid DNA, was cultured as previously described (Section 5.3.1). *P. aeruginosa* PAO (DSM 1707) was grown either in MMG (Section 5.3.1) or in LB-S (Section 3.3.1) with the addition of 40 $\mu g.ml^{-1}$ tetracycline (Section 4.3.1.1). When required, NaCl was added to a final concentration of 0.2 M (LB + NaCl) and ethanol to a final concentration of 2.4% (v/v) (LB + EtOH). The *P. aeruginosa* cultures were incubated at 37°C with agitation at 200 rpm until mid-exponential phase (OD_{540} = *ca.* 0.5), diluted to OD_{540} = 0.1 in pre-warmed (37°C) LB-S and aliquots (500 µl) were then transferred to 2-ml microfuge tubes with or without 0.025 g of glass wool (Merck, Darmstadt, Germany; mean standard diameter = 10 µm). After growth at 37°C for 16 h, total attached biomass was separated into SIP and biofilm biomass, as described in Section 3.3.1, cooled to 4°C and β-galactosidase activity assays were performed. The optimal NaCl and ethanol

concentration required for maximal promoter activity of clone pALacZsd65 was determined by assaying the β-galactosidase activity of planktonic cultures grown at various NaCl (0 – 1 M) and ethanol (0 – 5% [v/v]) concentrations.

6.3.2 Assay for β-galactosidase activity

β-galactosidase activity was assayed in liquid cultures using 2-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate for the β-galactosidase enzyme as previously described (Section 4.3.4). β-galactosidase activity was expressed as either the A_{414} (amount of ONPG hydrolysed in 15 min) or A_{414} / μg total cellular protein.

6.3.3 Analytical Methods

Total protein concentrations of the above-mentioned cultures were determined using the Bradford assay as described in Section 3.3.2, and used to standardise the results obtained for the β-galactosidase activity assay.

6.3.4 DNA Manipulations

6.3.4.1 Construction of the pALacZsd65 clone

The pALacZsd65 clone was obtained by screening a previously constructed library aimed at isolating *P. aeruginosa* biofilm regulatory elements (Weyers, 1999). Briefly, *P. aeruginosa* genomic DNA was isolated using the method of Jansen (1995), digested with the *Sau*3AI restriction endonuclease and the restriction fragments cloned into the pALacZsd vector using the *Bam*H1 restriction endonuclease site within the multiple cloning site of the vector. The clones were then screened for β-galactosidase activity in the presence of glass wool (total attached cultures), which was compared to the activity obtained from planktonic cultures (Weyers, 1999). An arbitrary ratio of at least 3:1 for attached: planktonic activity of the reporter vector was considered as significant transcriptional up-regulation of the various regulatory elements isolated. One of these attachment-induced regulatory elements, pALacZsd65, was selected and used in all of the subsequent investigations.

The pALacZsd65 construct as well as the control pALacZsd and pALacZsdIP reporter vectors was transformed into competent *P. aeruginosa* DSM 1707 cells (Section 4.3.3). The transformed cultures were grown to an OD_{540} = *ca.* 0.5, aliquotted and stored in 50% glycerol at –70°C. Fresh culture stocks were used for each series of β-galactosidase activity assays.

Table 6.1: Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant properties	Reference or source
Strains:		
Escherichia coli JM105	Δ(lacZ)M15	Stratagene*
Pseudomonas aeruginosa DSM 1707	Prototroph	DSM†
Plasmids:		
pALacZsd	pAL4000 (Greener *et al.*, 1992) containing a promoterless *lacZ* in place of the *luc* gene	J. B. Weyers‡
pALacZsdIP	pALacZsd containing the *lac* promoter from pBluescript (co-ordinates 719-1719)	J. B. Weyers
pALacZsd65	pALacZsd containing a biofilm regulatory element and a second chimera from the *P. aeruginosa* genome (co-ordinates 4288237 to 4288459 and 2781439 to 2781214)	J. B. Weyers

*: Stratagene®, La Jolla, CA, USA
†: Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany
‡ Mr J. B. Weyers, Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria, South Africa

6.3.4.2 Nucleic acid sequence analysis of the regulatory element contained in pALacZsd65

The 445-bp insert DNA in the pALacZsd vector was sequenced using an ABI PRISM™ Big Dye™ Terminator Cycle Sequencing Kit (Perkin Elmer, Foster City, CA, USA) and primers JB1 (5' – GAATTCGAGCTCGGTAC – 3') and JB2 (5' – GTTTTCCCAGTCACGAC – 3') according to the manufacturers instructions. Sequence analysis was performed using the *Pseudomonas* Genome Project's database (http://www.pseudomonas.com) and BLAST2 (http://www.ncbi.nlm.nih.gov).

6.4 RESULTS

6.4.1 Nucleic acid sequence analysis of the regulatory element contained in pALacZsd65

Nucleic acid sequence analysis indicated that the cloned insert DNA in pALacZsd65 consisted of two distinct DNA fragments mapping on different regions of the *P. aeruginosa* genome (Fig. 6.1). These were designated chimera 1 and chimera 2, respectively. The 226-bp chimera 1 element mapped on the reverse strand of PA2464, 6 571 bp upstream of the *gtdA* gene, but lacked significant homology to any other known genes when compared to nucleic acid sequences deposited in the GenBank database. The second chimera (223 bp) mapped 483 bp upstream of the recently described *pepA* (*phpA*) gene (Woolwine & Wozniak, 1999; Woolwine *et al.*, 2001), which is reported to have an effect on expression of the *P.*

aeruginosa alginate operon. The *pepA* (*phpA*) gene appears to form the first gene in a putative operon consisting of three genes, namely *pepA* (*phpA*), *holC* and PA3833, a gene with an as yet unknown function (Fig. 6.2).

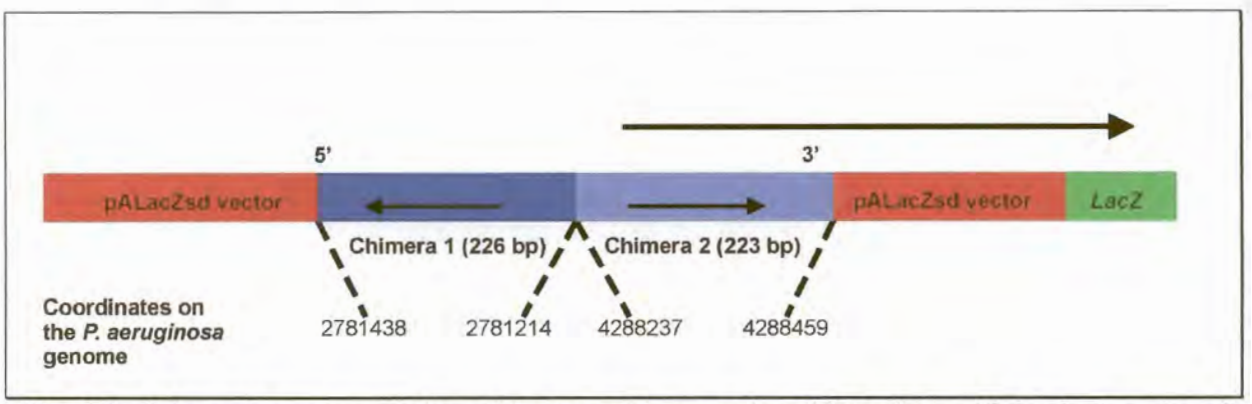

Fig. 6.1: Schematic representation of the two chimeras cloned into the multiple cloning site of the pALacZsd vector. Arrows indicate the putative transcriptional direction in which the insert DNA was cloned into the vector.

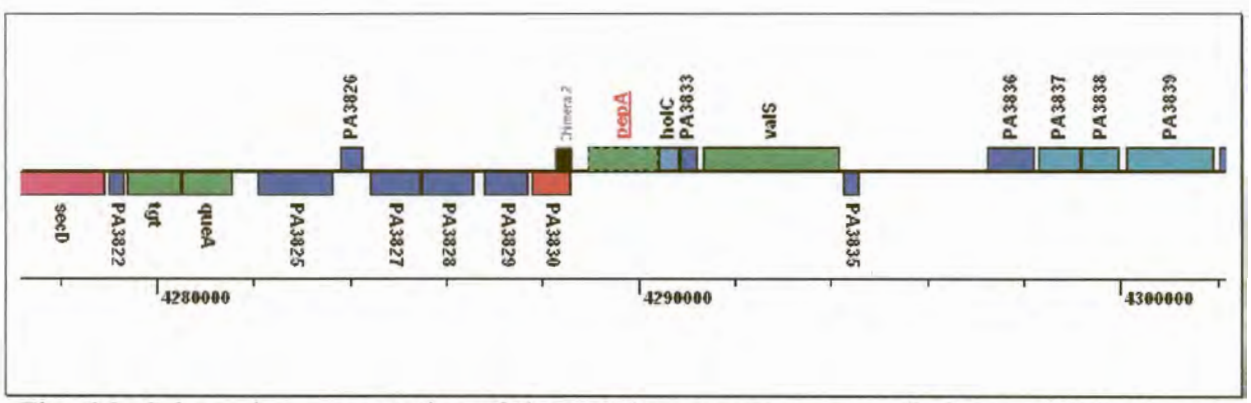

Fig. 6.2: Schematic representation of the putative operon structure in the *pepA (phpA)* gene region as well as the location, on the *P. aeruginosa* genome, of the second chimera present in the pALacZsd65 vector (adapted from http://www.pseudomonas.com/AnnotationByPAU.asp?PA=PA3831).

6.4.2 Determination of the optimal NaCl and EtOH concentrations for maximal pALacZsd65 promoter activity in planktonic populations

β-galactosidase expression from pALacZsd65 increased as the osmolarity of the growth medium increased and then subsequently decreased, but the relationship between NaCl concentration and promoter activity was not as defined as that previously seen for the *algD* promoter (Fig. 5.2). The NaCl concentration for maximal pALacZsd65 promoter activity was found to be 0.2 M, with a second lower peak of promoter activity at 0.7 M NaCl (Fig. 6.3). The optimal NaCl concentration was found to up-regulate promoter activity *ca.* 2-fold.

The EtOH concentration for maximal pAlacZsd65 promoter activity was found to be 2.4% (Fig. 6.3), which is very similar to the optimum ethanol concentration previously observed or maximal *algD* promoter activity (Fig. 5.3). However, the effect was rather mild in comparison to the effect of ethanol observed on the *algD* promoter. Whereas the *algD* promoter was up-regulated 8-fold at the optimal ethanol concentration, the pALacZsd65 promoter activity was up-regulated 2-fold.

6.4.3 pALacZsd65 promoter activity

6.4.3.1 Promoter activity of pALacZsd65 in low salt broth

The pALacZsd65 promoter displayed weak activity in the SIP population of cultures grown in LB-S (Fig. 6.5). The promoter activity in the biofilm population was 1.2-fold higher than that in the planktonic population, and 1.85-fold higher than the activity observed in the SIP population. These results indicate that direct association of the cells with the surface leads to up-regulation of the promoter in pALacZsd65, while growth in close proximity to a surface (SIP cells) leads to down-regulation of the pALacZsd65 promoter. The presence of 0.2% (w/v) glucose in combination with minimal M63 salts (MMG) was found to down-regulate pALacZsd65 promoter activity in planktonic, SIP and biofilm populations when compared to the levels obtained in cultures grown in LB-S (Fig. 6.5). Comparative levels of pALacZsd65 promoter activity were maintained in planktonic and biofilm populations of cultures grown in MMG, but the promoter activity was slightly downregulated in the SIP population. By contrast, results obtained for the *algD* promoter showed promoter activity was abolished in the SIP population only (Fig. 5.4).

6.4.3.2 Promoter activity of pALacZsd65 in high salt broth

The addition of 0.2 M NaCl enhanced the level of pALacZsd65 promoter activity in the SIP population of cultures grown in LB + NaCl, while promoter activity in planktonic cultures was unaffected when compared to the corresponding populations grown in LB-S (Fig. 6.5). However, the pALacZsd65 promoter activity was not upregulated in the presence of a glass wool surface and 0.2 M NaCl. Transcription from the pALacZsd65 promoter in cultures grown in MMG with and without NaCl was similar for biofilm populations, while both planktonic and SIP populations were upregulated in cultures grown in MMG + NaCl. As seen for the *algD* promoter (Fig. 5.4), the metabolic flux dictated by the different nutrient make-up of MMG medium influenced regulation of the pALacZsd65 promoter in biofilm-cultured cells differently than in either planktonic or SIP cells.

6.4.3.3 Promoter activity of pALacZsd65 in broth containing 2.4% (v/v) ethanol

The pALacZsd65 promoter activity in the planktonic population grown in LB + EtOH was not upregulated when compared to planktonic cultures grown in LB-S (Fig 6.5). However, promoter activity in the SIP population was upregulated 1.5-fold, while the promoter activity was downregulated 1.4-fold in the biofilm population. By contrast, pALacZsd65 promoter activity in the planktonic and SIP populations of cultures grown in MMG + EtOH was seen to be upregulated by as much as 2- and 1.7-fold, respectively, when compared to the corresponding populations grown in LB + EtOH. The respective biofilm populations remained unaffected when compared to the biofilm population of cultures grown in MMG.

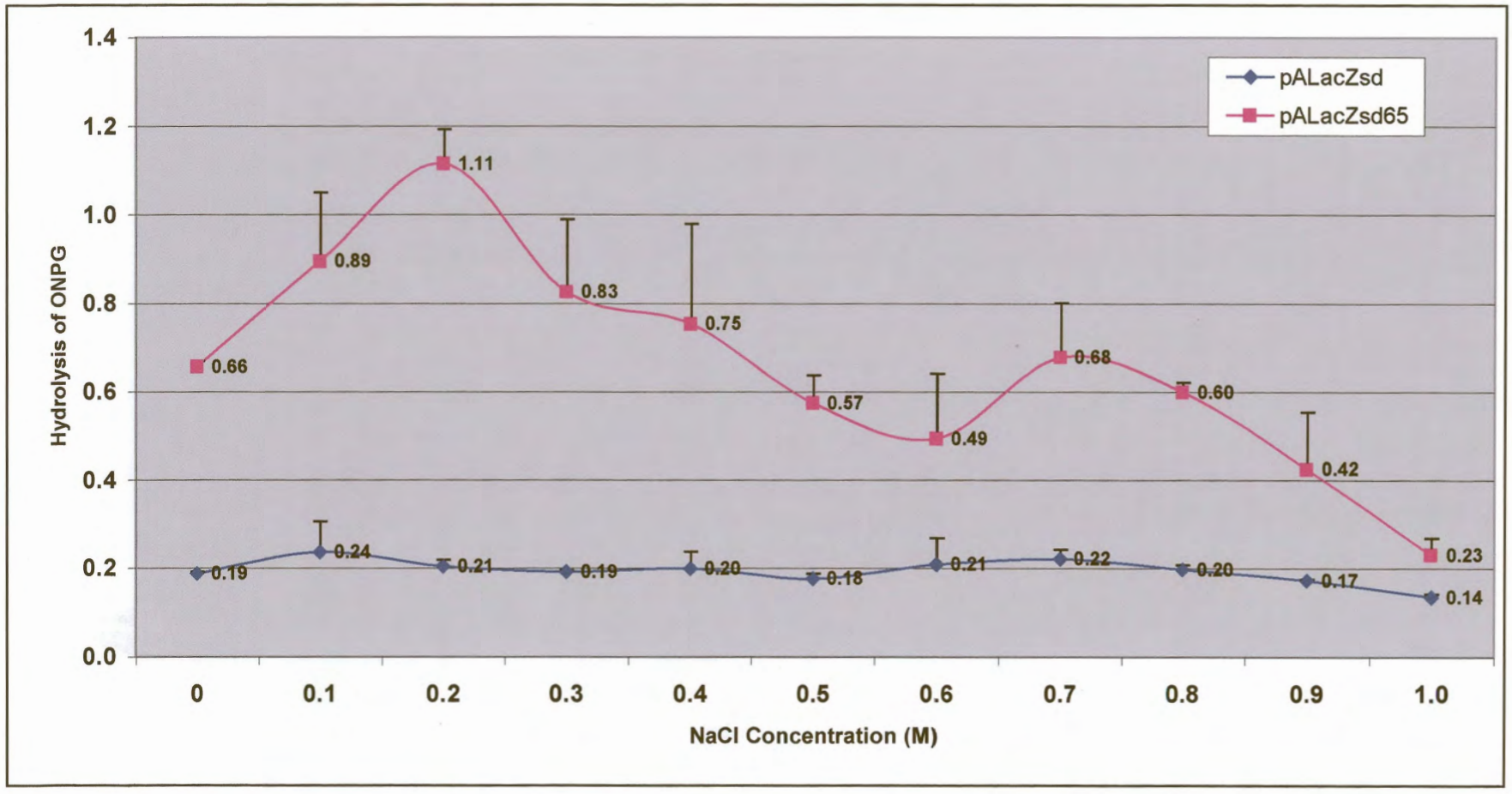

Fig. 6.3: pALacZsd65 promoter activity as measured by β-galactosidase activity assays on 16 h planktonic cultures of *P. aeruginosa* (pALacZsd65) grown at various NaCl concentrations in LB, using ONPG as substrate. Error bars denote one standard deviation of the mean.

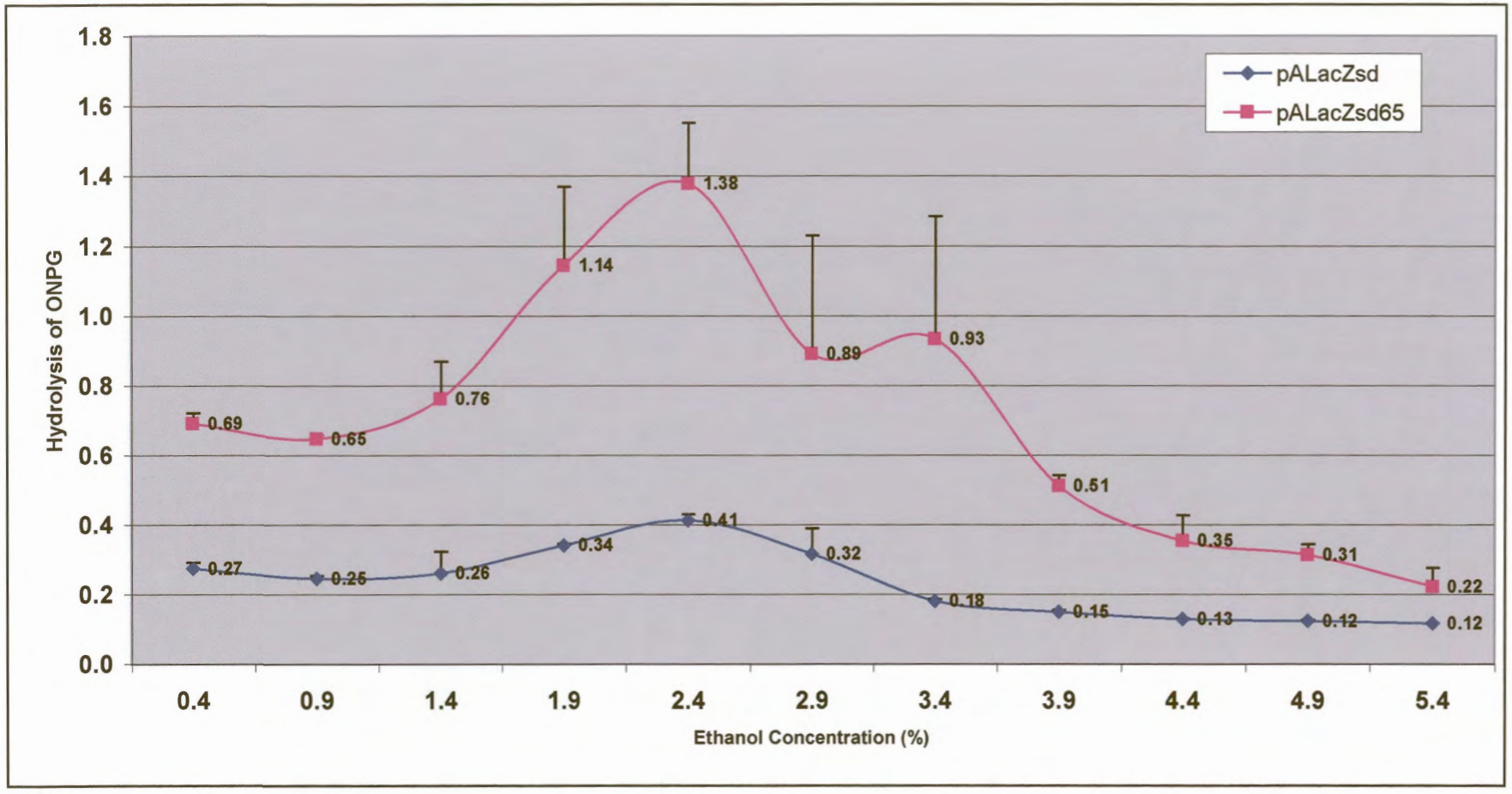

Fig. 6.4: pALacZsd65 promoter activity as measured by β-galactosidase activity assays on 16 h planktonic cultures of *P. aeruginosa* (pALacZsd65) grown at various ethanol concentrations in LB, using ONPG as substrate. Error bars denote one standard deviation of the mean. Activity could not be determined in the absence of ethanol due to the requirement for tetracycline in the culture broth.

6.5 DISCUSSION

Transcription from pALacZsd65 in planktonic cultures was shown to be influenced by both salinity (Fig. 6.3) and ethanol (Fig. 6.4) in a concentration-dependant manner. However, growth in the vicinity of a biofilm (SIP) and nutrient availability (Fig. 6.5) also influenced pALacZsd65 transcriptional activity. The 1.3-fold up-regulation of pALacZsd65 promoter activity in the biofilm population of cultures grown in MMG was similar to the 1.2-fold up-regulation of the promoter in the biofilm population cultured in LB-S (Fig. 6.5). Growth in MMG was found to repress expression of the reporter gene in planktonic, biofilm and SIP populations when compared to the corresponding populations grown in LB-S. The reporter gene expression profile of pALacZsd65 in the respective SIP populations is clearly different to that obtained for the *algD* promoter (Fig. 5.4). The pALacZsd65 promoter activity was found to be the lowest in the SIP population of cultures grown in both MMG and LB-S (Fig. 6.5); however, at no point was expression abolished.

Compared to cultures grown in MMG and LB-S broth, the pALacZsd65 promoter activity in SIP populations was found to be enhanced by NaCl and the β-galactosidase expression levels were higher than the levels obtained for the biofilm populations. Similarly, ethanol was also able to increase pALacZsd65 transcription levels in planktonic and SIP populations. Interestingly, NaCl and ethanol decreased pALacZsd65 transcription in the biofilm populations grown in LB + NaCl and LB + EtOH, respectively, while the promoter activity in the biofilm populations of cultures grown in MMG media remained very similar. These experimental findings may be explained as follows. The pALacZsd65 promoter activity in the biofilm population of cultures grown in LB-S was approximately 18% higher than in the planktonic population, while a substantial amount of the biofilm biomass has been shown to leave the surface at each cell division (Section 3.5). This seems to indicate a correlation between transcription levels in the biofilm environment and the amount of biomass detaching from the surface. Assuming that pALacZsd65 promoter activity is upregulated prior to or during detachment from the surface, and not attachment, one would indeed expect to see a substantial increase in pALacZsd65 promoter activity in the SIP populations of cultures grown in LB + NaCl and LB + EtOH. Thus, the pALacZsd65 gene product may be involved in the detachment of cells from the surface as well as in the regulation of the "detachment phenotype" seen in SIP populations once biomass accumulation at the surface has reached "quota".

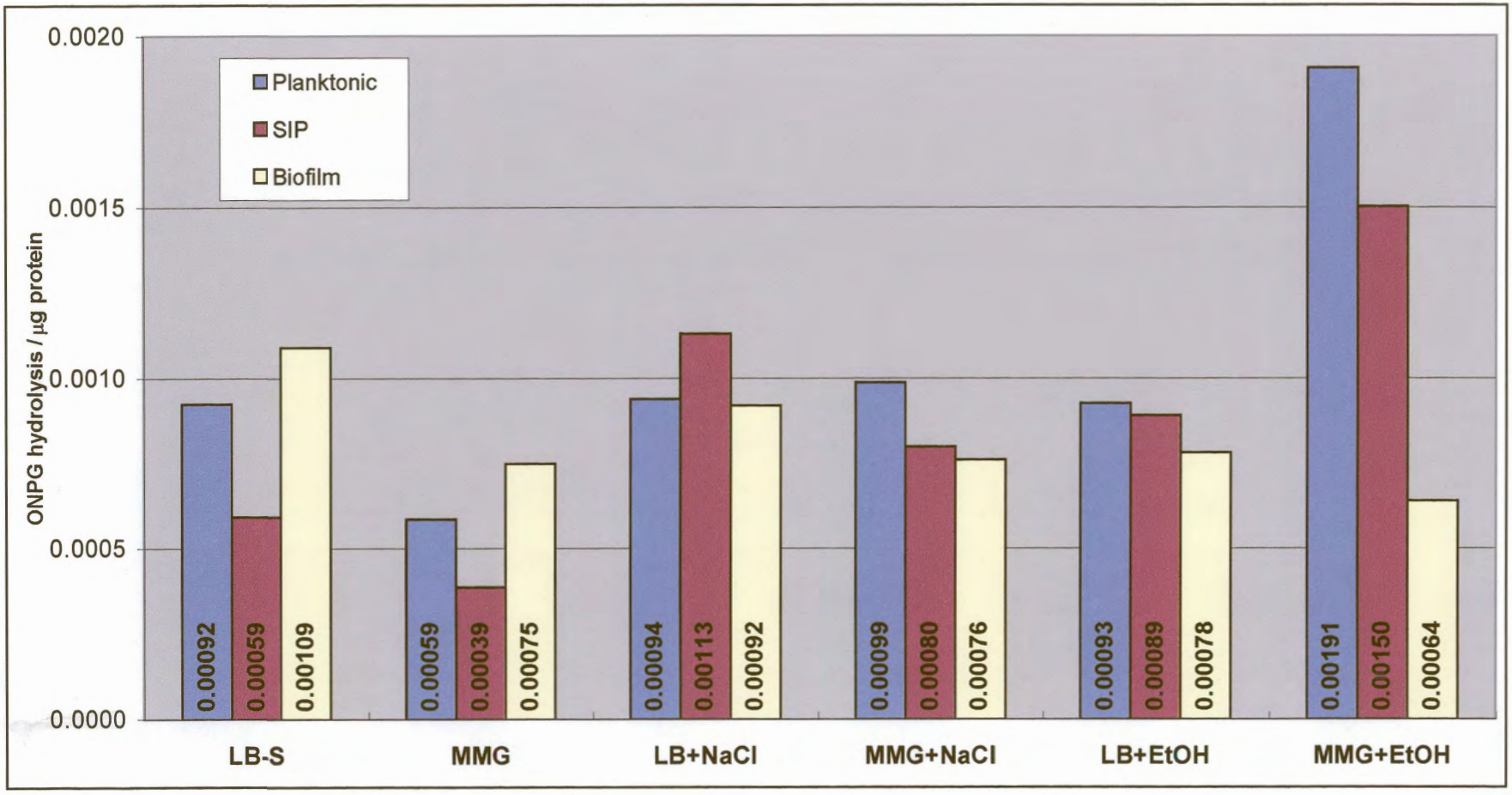

Fig. 6.5: pALacZsd65 promoter activity after 16 h of growth as influenced by LB-S and MMG media with and without 0.2 M NaCl or 2.4% (v/v) ethanol.

Taking into consideration that chimera 2 contained in pALacZsd65 maps upstream of the *pepA (phpA)* gene, and may contain the promoter sequence (or part thereof), and that insertional inactivation of the *P. aeruginosa* (FRD1) *pepA (phpA)* gene correlated with an increase in transcription of *algD* and the mucoid phenotype (Woolwine & Wozniak, 1999; Woolwine *et al.*, 2001), the explanation that detachment is regulating pALacZsd65 is plausible. O'Toole *et al.* (2000) have shown the global carbon metabolism regulator (*crc*) to be essential for biofilm formation in *P. aeruginosa* PA14. Biofilm cultures grown in MMG that are not supplemented with TCA cycle intermediates would therefore be expected to show less up-regulation of promoters involved in the attachment process. Transcription patterns of pALacZsd65 in cultures grown in MMG were shown to be the same as those for cultures grown in LB-S with the exception of transcription levels being approximately one third lower in all populations (Fig. 6.5). These results provide further indirect evidence that pALacZsd65 may contain a detachment-inducible promoter, as the promoter activity in biofilm populations remained the same regardless of whether TCA cycle intermediates were present in the media or not.

In conclusion, the data presented outlines the existence of more than one biofilm regulatory mechanism. The regulatory mechanisms used for biofilm formation and maintenance as well as detachment appears to be determined by environmental signals. NaCl, ethanol, medium constituents and growth as a member of the SIP population influence transcription from the pALacZsd65 promoter separately. The increases in pALacZsd65 promoter activity in SIP populations may be due to the "detachment phenotype" in these environments caused by accumulation of cells at the surface, which is thought to be a direct result of up-regulation of alginate production.

CHAPTER 7

CONCLUDING REMARKS: PLASTICITY OF THE *Pseudomonas aeruginosa* PAO (DSM1707) BIOFILM

The data presented in the preceding chapters of this thesis bring to light a number of questions, which warrant further discussion. The implications of the data presented as a whole provide further insight into the flexibility of *P. aeruginosa* biofilm formation and maintenance.

Biomass accumulation at the glass wool surface for both untransformed and transformed *P. aeruginosa* cultures, grown in LB-S medium, were shown to reach a "threshold" value of *ca.*18% of the total protein biomass, or 8-9% of the total culturable count (Sections 3.4.3, 3.4.4, 4.4.2 & 4.4.3). This phenomenon may be explained by the hypothetical growth rates (cellular volumes) of planktonic, SIP and biofilm populations calculated for untransformed cultures (Section 3.4.6), as well as a propensity for attached cells to detach and / or suspended cells not to attach. Hypothetical growth rates were calculated from 16 h old cultures, in the late log phase of growth (data not shown). Protein biomass measurements for untransformed planktonic, SIP and biofilm cultures indicated that the SIP population should have a higher growth rate than that obtained. This may have been due to the continual shedding of biofilm cells (i.e. a smaller average volume was obtained due to the smaller size of shed biofilm cells). In essence, the SIP population should consist of a mixture of (1) planktonic cells that have not yet been in contact with the surface, (2) planktonic cells that have attached reversibly to the surface and left again, and (3) biofilm cells that have detached from the surface. Assuming the surface has reached "threshold" at 9% of the total culturable count of the culture and that the relationship between μ and V (Section 3.3.3) is the same for planktonic and biofilm populations, the implications are that at each doubling of the biofilm population, 10% of the total culturable population or 19% of the total protein biomass detaches forming part of the SIP population (calculated as total protein biomass of the biofilm and SIP populations according to their respective growth rates at the next division of the SIP population less 19% of the calculated protein biomass measurement which remains at the surface). The calculation does not take into account that a small fraction of the SIP population would be expected to attach to the surface; however, after 16 h of growth a large percentage

of the SIP population should theoretically consist of cells that have at some stage been in contact with or formed a part of the biofilm population.

The hypothesis presented above is evidenced statistically by analysis of cellular volumes using ANOVA. At a 90% confidence interval SIP cells were found to be different from biofilm and planktonic cells, however, biofilm and SIP cells were not found to be significantly different at a confidence interval of 95%. This is hypothesized to be due to the continual shedding of 10% of the biofilm population that are becoming part of the SIP population and the higher "hypothetical growth rate". This indicates that the SIP population is in fact physiologically different and that at a confidence interval of 95% shed biofilm cells mask the physiological difference between the biofilm and SIP populations.

The increase in attached biomass of untransformed *P. aeruginosa* biofilm populations grown in LB + NaCl medium is thought to be a consequence of up-regulation of alginate biosynthesis (52% up-regulation in planktonic cells, 36% up-regulation in SIP cells and 244% up-regulation in biofilm cells [Table 3.1]). This makes detachment from the biofilm more time-consuming in relation to the achievable growth rate of the population. Cultures grown in LB + NaCl medium, however, appear to maintain more control over alginate biosynthesis than those grown in LB + EtOH medium. NaCl, unlike EtOH, is seen to only have a substantial effect on alginate biosynthesis when coupled with the glass wool surface, whereas EtOH causes large amounts of alginate to be synthesized by *P. aeruginosa* in planktonic, but not attached populations.

In untransformed *P. aeruginosa* populations grown in LB + NaCl medium large differences in SIP protein concentrations per unit cellular volume were observed (Table 3.1) when compared to the corresponding population grown in LB-S. This difference in protein concentration may be due to the 35% decrease in protein per volume [as evidenced in LB-S grown cells (Table 3.1)] together with an additional 30% decrease in the culturability of planktonic cells due to the presence of NaCl. SIP cells may not be as severely affected by NaCl concentrations due to accumulation of NaCl at the glass wool surface. Similarly, there may be a 65% decrease in the culturability of biofilm cells due to the higher NaCl concentration at the surface. By making use of 1D-PAGE, the decrease in protein concentrations in SIP cells was found to be due to a lowering of global protein concentrations and not of specific or selected proteins (data not shown).

Presumably, the formation of chains of cells by untransformed *P. aeruginosa* grown in LB + EtOH may be due to DNA damage caused by DNA supercoiling in the *algD* promoter region but also elsewhere on the genome. The presence of ethanol in the growth medium causes localized supercoiling at the *algD* promoter region (DeVault *et al.*, 1990), which is presumed to be responsible for up-regulation of the *algD* promoter. The data presented indicates that this supercoiling may cause DNA damage which in turn causes production of the FtsZ protein (Margolin, 1999). The FtsZ protein acts at an early stage of cell division, and is a target of the cell division inhibitor SulA (SfiA) that is in turn produced in response to DNA damage (the SOS system) (Margolin, 1999). Build-up of critical FtsZ protein concentrations, needed for cell division to complete may be retarded as evidenced by chain formation in cultures grown in LB + EtOH medium. Overproduction of alginate may then hold cells together in the end-to-end manner seen (Fig. 3.4f). For this reason a large amount of biomass was seen to accumulate at the glass wool surface (Fig. 3.4h). Ethanol may change the dynamics of the system, not purely by imposing stress on the cells, but also by causing a shift in expression patterns and cell differentiation into SIP cells as evidenced by cellular volume measurements of untransformed *P. aeruginosa* cultures (Fig 3.8).

The physiological differentiation of the SIP population may be coupled to differential regulation of alginate lyase (Monday & Schiller, 1996) (along with other detachment regulated genetic elements) in response to the putative signal produced by the biofilm population (Section 3.4.8). These genetic elements would be responsible, for among other things, aiding cell detachment from the biofilm in a manner that keeps the biofilm population at 15% of the total attached protein biomass as evidenced in Chapter 3, and a "genetic shift" of the SIP population to the detachment genotype as evidenced by alginate concentrations (Table 3.1). Transcription of many other biofilm regulatory elements should be abolished or down-regulated in the SIP population. This was recently demonstrated by comparing the 2-dimensional protein profiles of *P. aeruginosa* planktonic, SIP and biofilms populations (Steyn *et al.*, 2001).

It may be possible that planktonic populations for the first few hours of growth have an attachment phenotype until "threshold" is reached (which differs according to environmental constraints) after which a diffusible signal molecule is produced by the biofilm that changes the physiology of the planktonic population to that of SIP. In the case demonstrated for untransformed *P. aeruginosa* grown in LB-S medium (which is more likely to represent a

natural environment than either LB + NaCl or LB + EtOH), SIP populations are thought to represent a "detachment phenotype". The presence of a surface initially causes planktonic cells to colonize the surface. This is evidenced indirectly by work done in type 1-fimbriated *E. coli*, which shows an altered composition of outer membrane proteins in response to attachment (Otto *et al.*, 2001). Once "threshold" (equivalent to 18% of the protein biomass or 9% of the total culturable count) levels have been reached, the planktonic population is by way of a biofilm signal molecule phenotypically changed to SIP.

Data obtained for the relationship between cellular volume and total cellular protein or culturable count for plasmid-bearing *P. aeruginosa* cultures was found to be significantly different to data obtained for untransformed *P. aeruginosa* cultures (Table 7.1). Similar to the biofilm population in untransformed *P. aeruginosa* LB + NaCl medium (Fig 3.5) the culturability of plasmid bearing *P. aeruginosa* planktonic, SIP and biofilm populations in the presence of 0.7 M NaCl and 2.9% ethanol may be adversely affected (Fig 4.2). This is evidenced both by protein per volume and protein per total culturable count (Table 7.1). SIP populations grown in LB + EtOH medium were, however, not affected. The observed increase in the protein per volume and protein per total culturable count due to the decreased cellular volume, decreased culturable count and maintenance of total protein concentrations is very high when compared to untransformed *P. aeruginosa*. The most likely explanation for the higher values obtained may be a decrease in the culturability of the biofilm population, which in turn may be due to the biofilm population being more susceptible to tetracycline or being more readily damaged when removed from the glass wool by vortexing. SIP cells maintain a lower protein per volume ratio than planktonic and biofilm cells, however, apparent compactness of the planktonic, SIP and biofilm populations was found to have increased compared to cultures grown in LB-S. The difference in compactness in the SIP population compared to planktonic and biofilm populations has decreased to *ca.* 15% (Table 7.1) compared to untransformed *P. aeruginosa's* 30%. (Table 7.1)

Table 7.1: Protein concentrations per cell and per cell volume for *P. aeruginosa* (untransformed) and *P. aeruginosa* containing the pALacZsd vector construct (plasmid-bearing) grown for 16 h in the presence of glass wool in LB broth without salt (LB-S) and with 0.7 M NaCl (LB + NaCl) and 2.9% ethanol (LB + EtOH).

Culture Medium	**Growth Phase**	**fg protein. cfu^{-1} (untransformed)**	**fg protein. cfu^{-1} (plasmid-bearing)**	**fg protein. μm^{-3} (untransformed)**	**fg protein. μm^{-3} (plasmid-bearing)**
LB-S	Planktonic	61.70 +/- 10.83	123.19 +/- 25.73	33.24 +/- 5.83	56.91 +/- 24.33
	SIP*	57.26 +/- 12.38	101.33 +/- 28.19	21.61 +/- 4.67	48.59 +/- 11.16
	Biofilm	108.71 +/- 44.12	329.41 +/- 81.30	33.86 +/- 13.74	132.97 +/- 13.88
LB + NaCl	Planktonic	88.90 +/- 16.91	177.80 +/- 22.42	43.32 +/- 8.24	114.82 +/- 35.89
	SIP	45.30 +/- 15.22	353.57 +/- 91.43	18.92 +/- 6.36	151.58 +/- 45.12
	Biofilm	133.8 +/- 40.03	139.91 +/- 18.68	86.21 +/- 25.8	92.61 +/- 28.68
LB + EtOH	Planktonic	103.00 +/- 7.74	220.04 +/- 17.27	34.75 +/- 2.61	120.36 +/- 35.18
	SIP	101.64 +/- 40.30	113.87 +/- 23.87	32.54 +/- 12.90	59.13 +/- 21.38
	Biofilm	81.20 +/- 58.42	192.94 +/- 69.87	33.44 +/- 24.06	86.88 +/- 7.67

*: Surface influenced planktonic culture.

The drastic increase in protein biomass per total culturable count and protein biomass per volume in the plasmid-bearing *P. aeruginosa* LB-S biofilm environment is thought to be an overestimation, which may be due to one of two reasons. Firstly, the biofilm population may naturally contain a large number of dead cells or the cells may have a tendency to clump together. Microscopic analysis of BacLight-stained biofilm populations of *P. aeruginosa* transformed with pALacZsd did not, however, reveal a large number of dead cells either in the biofilm itself or once the biofilm had been removed from the glass wool surface (data not shown). In my opinion, the data suggests a decrease in the culturability of the biofilm population, which may result from clumping of these cells together after removal from the glass wool matrix or alternatively due to the biofilm population being more susceptible to the tetracycline present in the culture media. This hypothesis is not in agreement with classical biofilm models, as biofilm-associated cells are thought to be more resistant to antibiotics. However, a recently published article by Drenkard & Ausubel (2002) has reported that biofilms are not more resistant to certain antibiotics than planktonic populations. In LB-S grown cultures tetracycline, therefore, in combination with transformation with the pALacZsd vector system and 0.4% additional ethanol causes cells to be more compact by about 20 ng protein. μm^{-3}, the remainder of the difference may be due to clumping of the biofilm cells.

A decrease in the biofilm biomass yield is seen in plasmid-bearing *P. aeruginosa* grown in LB + NaCl (Fig. 4.4) when compared to untransformed *P. aeruginosa* grown in LB + NaCl (Fig 3.7a). This may be a consequence of accumulation of tetracycline and ethanol at the surface in combination with the higher NaCl concentration at the glass wool surface. Similar to the biofilm population of plasmid-bearing *P. aeruginosa* cultures grown in LB-S, the culturability of the planktonic and biofilm populations in the presence of 0.7 M NaCl or 2.5% additional ethanol are adversely affected. The culturability of the various SIP populations remains similar to untransformed *P. aeruginosa*. This may be as a result of lower NaCl or ethanol concentrations in this environment due accumulation of NaCl and ethanol at the surface.

The data presented in this thesis allows for a hypothesis on the mechanism of the regulation of alginate synthesis in non-mucoid *P. aeruginosa*. The conversion from non-mucoid to mucoid and reversion back to non-mucoid phenotypes of *P. aeruginosa* appears to be involved in the differentiation of biofilm, SIP and planktonic phenotypes. Inactivation of the *mucA* locus has previously been shown to induce the mucoid phenotype, while suppressor mutations in *algU*

have been shown to restore the non-mucoid phenotype (DeVries and Ohman, 1994). These conversion mechanisms are, however, not thought to play a role in the phenomena observed here. In the data presented (Chapter 5), it seems more likely that planktonic cells regulate alginate biosynthesis in the traditional *algU*-regulated manner, while biofilm cells up-regulate alginate biosynthesis in an RpoN-dependent manner. This would explain the additive effect seen due to environmental conditions in combination with attachment to a surface. The mechanism of conversion to the SIP (detachment) phenotype may be due to sigma factor antagonism between AlgU and RpoN leading to a decreased recognition of the *algD* promoter binding sites. The result would be lower alginate levels in SIP populations than in planktonic populations. This hypothesis would result in three phenotypes identical to those observed for planktonic, biofilm and SIP populations.

The data obtained for the regulation of the *algD* promoter correlates with the observed increases and decreases in alginate concentrations per μm^3 (Table 3.1), with the exception of the SIP population grown in LB-S, which has less alginate per μm^3 than planktonic populations. This is thought to be due to a relationship between cell size (Fig. 3.8) and the amount of alginate per volume of cell. Cell size in the SIP population grown in LB-S is intermediate to that of the biofilm and planktonic populations, with the planktonic cells being the smallest and the biofilm cells being the largest. Populations grown in LB + NaCl or LB + EtOH, however, are seen to exhibit the largest cell size in the SIP population with the planktonic population being intermediate in size and the biofilm cells being smallest. The lower alginate concentrations for SIP cells grown in LB-S can therefore simply be due to a larger external surface area needing to be covered due to the larger volume of the cells. Single cell microscopic analysis of an integrated *algD-LacZ* fusion should be carried out using a fluorescent β-galactosidase substrate to confirm the regulation patterns observed, and use of a short half life GFP system could provide the timing behind expression of the *algD* promoter in biofilm, SIP, and planktonic populations.

Future experimentation with the pALacZsd65 clone should include sub-cloning and re-assaying of the two chimeras independently. Single cell microscopic analysis of each chimera should be carried out using a short half life GFP system to confirm the regulation patterns observed, as well as to determine the location of the cells in the biofilm that are thought to be detaching. Levels of mRNA should be studied by Northern blot analysis to validate up-regulation of the *pepA (phpA)* gene. This data will make the argument presented in Chapter 6

(where chimera 2 is hypothesized to be involved in the cellular detachment process) more concrete, confirming that the promoter element behaves as predicted by the pALacZsd65 clone and that chimera 2 is in fact the promoter of, or contains a regulatory region of, the *pepA (phpA)* gene.