An Altered Physiological State of *Pseudomonas aeruginosa* in the Biofilm Environment: Effect on the *algD* Promoter and a New Attachment-Inducible Regulatory Element

Christopher James Cooper

For Courtney: On the 23rd of April 1999 the birth of a baby girl, whom we aptly named Courtney, had a mind numbing effect on my life and my outlook thereon. The inspiration provided by this life-altering experience is reflected in this work. May you one day achieve what your heart desires.

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An Altered Physiological State of *Pseudomonas aeruginosa* in the Biofilm Environment: Effect on the *algD* Promoter and a New Attachment-Inducible Regulatory Element

by

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the requirements for the degree

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in

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in it's entirety or in part been submitted at any university for a degree.

Signature:

Date:

An Altered Physiological State of *Pseudomonas aeruginosa* in the Biofilm Environment: Effect on the *algD* Promoter and a New Attachment-Inducible Regulatory Element

by

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Degree: MSc (Microbiology)

SUMMARY

Biofilm-associated bacterial cells are known to display a unique phenotype distinct from that of free-living or planktonic cells. Suspended cells of *P. aeruginosa* PAO (DSM 1707) growing in the presence of a biofilm (surface-influenced planktonic or SIP cells) were compared to planktonic cells. The biofilm and SIP phenotypes were different to each other, and both differed from the planktonic population. The SIP population was not a mixture of planktonic and detached biofilm cells but rather a distinct physiological state. Furthermore, indirect evidence is presented for the presence of diffusible signals produced by the biofilm that give rise to the SIP phenotype. The physiological effects of a *lacZ*-based reporter vector pALacZsd on the planktonic, SIP and biofilm populations of *P. aeruginosa* were 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 be lower in total attached cultures when compared to planktonic cultures.

The attachment of *P. aeruginosa* to a surface with the subsequent formation of a biofilm as well as environmental stimuli causes 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 data presented indicate that at least five separate factors, *i.e.* osmolarity, water availability, glucose, growth as a biofilm and growth in the presence of a biofilm, influence the regulation of *algD*, either individually or in combination. In a previous study, putative attachment-inducible regulatory elements of *Pseudomonas aeruginosa* PAO were identified. One of these regulatory elements was further characterised in this study. The effect of the different physicochemical conditions found to up-regulate *algD* promoter activity were also investigated for this regulatory element. The data presented indicate that the regulatory element may contain a promoter sequence, or part thereof, that is influenced by detachment of *P. aeruginosa* from a surface.

PREFACE

The following aspects of this work have been submitted for publication:

1. Submitted for publication in *FEMS* Microbiology Letters:
   Pseudomonas aeruginosa displays two biofilm-related phenotypes distinct from the planktonic state.

The following aspects of this work have been presented as talks or posters, at international and national conferences:

Conference:	Biofilms 2000, Big Sky, Montana, USA, July 2000
Posters:	1. Characterisation of a novel biofilm regulatory element of *Pseudomonas aeruginosa* (PAO1) 2. Expression of the *Pseudomonas aeruginosa* (PAO1) *algD* promoter is affected when grown as a biofilm
Presenter:	C. J. Cooper
Conference:	101st General Meeting of the ASM, 2001, Orlando, Florida, USA, May 2001
Poster:	Comparison of a novel putative promoter (clone 65) to *algD*, a biofilm attachment-inducible promoter of *Pseudomonas aeruginosa* PAO1
Presenter:	V. S. Brözel
Conference:	Eleventh Biennial Conference of the SASM, Grahamstown, South Africa, January 2000
Presentation:	Characterisation of the *Pseudomonas aeruginosa* (PAO1) *algD* promoter
Presenter:	C. J. Cooper
Conference:	The Seventeenth Congress of the South African Genetics Society (SAGS), Pretoria, South Africa, June 2000
Presentation:	Characterisation of the *algD* promoter in *Pseudomonas aeruginosa* (PAO1) growing as a biofilm
Poster:	Comparison of a novel putative promoter to *algD*, a biofilm attachment-inducible promoter of *Pseudomonas aeruginosa* PAO1
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LIST OF ABBREVIATIONS

A	absorbance
AHL	acylated homoserine lactone
AMP	adenosine mono-phosphate
ATP	adenosine tri-phosphate
α	alpha
ca.	approximately
bp	base pair
β	beta
BSA	bovine serum albumin
cm	centimetre
μm^3	cellular volume in cubic micrometers
cfu	colony forming units
CSLM	confocal scanning laser microscopy
cAMP	cyclic-AMP
CF	cystic fibrosis
Da	Dalton
d	day
°C	degrees Celsius
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleic-5'-triphosphate
DLVO	Derjaguin, Landau, Verwey, and Overbeek
DSM	Deutsche Sammlung von Mikroorganismen
DMSO	dimethyl sulphoxide
DOT	dissolved oxygen tension
ddH_2O	double distilled water
etc.	etcetera
EtOH	ethanol
EPS	extracellular polymeric substance
Fig.	figure
FISH	fluorescent *in situ* hybridisation
g	gram
GFP	green fluorescent protein
V_0	cellular volume at zero growth rate
G	guanine
h	hour
kDa	kiloDalton
LPS	lipopolysaccharide
l	litre

LB	Luria Bertani
LB-S	Luria Bertani broth without NaCl
LB + EtOH	LB-S containing 2.5 or 2.9% (v/v) ethanol
LB + NaCl	LB-S containing 0.7 M added NaCl
mRNA	messenger RNA
µl	microlitre
µm	micrometre
$mg.l^{-1}$	milligrams per litre
ml	millilitre
mm	millimetre
MMG	minimal M63 salts containing glucose
min	minute
M	molar
MWCO	molecular weight cut off
ONPG	2-nitrophenyl-β-D-galactopyranoside
OD	optical density
%	percent
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
P	probability
RT-PCR	reverse transcription followed by polymerase chain reaction
rpm	revolutions per minute
RNA	ribonucleic acid
s	second
σ	sigma
SDS	sodium dodecyl sulphate
±	plus and minus one standard deviation from the mean
cm^2	surface area in square centimetres
SIP	surface-influenced planktonic
X	times
× g	times gravity
TCA	tri-carboxylic acid
UV	ultra violet
U	unit
v/v	volume per volume
w/v	weight per volume

CHAPTER 1

INTRODUCTION

It is widely believed that the majority of bacteria persist attached to surfaces within structured biofilm ecosystems and not as free-floating organisms (Costerton *et al.*, 1987). Moreover it is becoming clear that these natural assemblages of bacteria within the biofilm matrix function as a cooperative consortium, in a relatively complex and coordinated manner (Dalton & March, 1998).

Remarkable discoveries have been made in biofilm research in the past decade. The application of new microscopic and molecular techniques to biofilm investigations has opened our eyes to this under-appreciated area of microbial ecology. Using these technologies, researchers have shown that biofilms are not simply organism-containing slime layers on surfaces; instead, biofilms represent biological systems with a high level of organisation where bacteria form structured, coordinated, functional communities (Caldwell *et al.*, 1993). Since a surface-attached lifestyle is ubiquitous, it is likely that this type of sessile community-based existence is a critical characteristic for persistence of the bacteria. Organisms can exist in an environment independently, but in many cases they seem to proliferate more effectively by interacting and forming communities (Davey & O'Toole, 2000).

A fundamental concept that requires refining in the context of biofilm studies is the suspended state. Planktonic cells have been defined as cells that have never existed in the attached mode before (Rice *et al.*, 2000). In contrast, suspended cultures that have been in contact with a surface during their recent history, or grown in the proximity of a surface have been termed surface-influenced planktonic (SIP) cells. These biofilm-associated bacterial cells or SIP cells have recently been shown to display a unique phenotype distinct from that of both free-living (planktonic) cells and biofilm cells (Steyn *et al.*, 2001).

The attachment of *Pseudomonas aeruginosa* to a surface and subsequent biofilm formation has been well documented due to the importance of this microorganism in

the medical field and is currently one of four "model" biofilm microorganisms, the others being *Escherichia coli*, *Vibrio cholera* and *Pseudomonas fluorescens*. The up-regulation of genes involved in the production of the exopolysaccharide alginate has been shown to be of fundamental importance in both initial attachment of *P. aeruginosa* to a solid surface as well as production of the biofilm matrix during biofilm development (Davies & Geesey, 1995). The alginate biosynthetic genes are regulated in a complex manner determined by, amongst others, environmental signals. Due to the large amount of data available in this field of research, *P. aeruginosa* is an ideal microorganism for the characterisation of the newly recognised SIP mode of bacterial growth.

The objectives of this study were:

1. To study the physiological parameters of biofilm and surrounding suspended (SIP) cells grown in batch culture in the presence of glass wool, and to compare these to planktonic cells under conditions of low and high salt concentrations as well as in the presence of a dehydrating agent;
2. To investigate the phenotypic effect that an extrachromosomal plasmid-based reporter construct has on *P. aeruginosa* planktonic, SIP and biofilm populations;
3. To investigate how transcription of the *algD* promoter of *P. aeruginosa* PAO (DSM1707) is influenced when cells are grown as a biofilm as well as in various chemical environments; and
4. To more closely examine a suspected biofilm regulatory element, namely pALacZsd65.

CHAPTER 2

LITERATURE REVIEW

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

2.1 THE BIOFILM MODE OF GROWTH

Zobell (1943) was one of the first to suggest, "Bacteria are not free-floating organisms but are attracted to surfaces". Zobell described the irreversible attachment of microorganisms to a surface and described the need for microorganisms to produce "extracellular adhesive materials". It was not, however, until 1971 that Marshal *et al.* (1971) published a model mechanism for the formation of bacterial biofilms. Since then, a wide array of knowledge has been accumulated dealing with the formation and control of bacterial biofilms. In the last 20 years, advances in molecular and microscopic techniques have made in-depth investigations in the field of biofilm physiology more attainable. It has become increasingly clear that the natural assemblage of bacteria within the biofilm matrix functions as a cooperative consortium in a relatively complex and co-ordinated manner (Davey & O'Toole, 2000).

2.1.1 Biofilm development

Biofilm development has been shown to depend on collective bacterial behaviour (Davey & O'Toole, 2000) in order to develop from a single, surface-adherent cell into structured consortia embedded in a gelatinous polymer matrix (Davey & O'Toole, 2000). The view of bacteria as interactive organisms capable of significant collective activity as a general bacterial trait was conceived over a decade ago. Since then complex differentiation and collective behaviour have been demonstrated for a number of different organisms under a variety of different situations (Shapiro & Dworkin, 1997). Whether single or multispecies, the development of biofilms requires multicellular behaviour. Biofilm formation may also require coordination with, interactions of, and communication between multiple bacterial species. Biofilms in general may include such phenomena as microorganisms that form natural assemblages at air-liquid interfaces and in suspensions in which they preferentially aggregate to form flocs or granules (McLeod *et al.*, 1995).

2.1.1.1 The planktonic cell

Planktonic cells have been defined as cells that have never existed in the attached mode before (Rice *et al.*, 2000). Our perception of bacteria as unicellular life forms is deeply rooted in the pure culture paradigm. Since bacteria can, in a strict sense, be diluted to a single cell and studied in liquid culture, this mode of operation has been exploited and used to study many bacterial activities. Although this traditional way of culturing bacteria in liquid medium has been instrumental in the study of microbial pathogenesis and enlightening as to microbial physiology, pure culture planktonic growth is rarely how bacteria exist in nature (Davey & O'Toole, 2000).

2.1.1.2 The attachment of microorganisms to a surface

The majorities of bacteria in natural environments are not found in the suspended state, but are widely believed to be either attached to surfaces or sediment particles (Fletcher & Loeb, 1979). Some bacteria have special attachment structures, e.g. pilli but many lack such structures (Fletcher & Loeb, 1979). Exopolysaccharide production, however, is not involved in the initial attachment process but is involved in the development of surface films (Allison & Sutherland, 1987). In bacterial strains capable of producing polysaccharide the cells that attach, develop into microcolonies (Allison & Sutherland, 1987).

The first stage in the development of a biofilm is the irreversible attachment of a bacterium to a surface. The nature of the attachment process is believed to be dependent, amongst other factors, on the type of organism, type of surface (biotic or abiotic) and surface properties. Not all bacteria coming into contact with a solid surface, however, form biofilms. The vast majority of cells in fact are believed to leave the surface again after a short period of contact. To distinguish between reversible and irreversible attachment of microorganisms to surfaces, the following definitions have been proposed (Marshal *et al.*, 1971). Namely:

- Reversible sorption is defined as an instantaneous attraction of a bacterium to a surface. The bacterium is weakly held near the surface while still exhibiting such phenomena as Brownian motion.
- Irreversible sorption is defined as the firm adhesion of the bacterium to the surface, which no longer exhibits Brownian motion.

More recently initial attachment to the surface has been studied in greater detail. Numerous

studies have reported on the effects of nutrient accumulation at the surface and the activity of attached versus free-living bacteria. Earlier work in this area is summarised by Marshall *et al.* (1971) and more recently by Fletcher (1987) and Van Loosdrecht *et al.* (1990). Results reported by various authors ranges from stimulation or inhibition to no effect of surface attachment on the activity of microorganisms, depending on the properties of the organism, the substrates and the substratum surfaces involved. The processes involved in biofilm formation include substratum-conditioning, transport of cells to the surface, adsorption of the cells to the surface, switching of these cells from reversible to irreversible adsorption, detachment of some of the irreversibly adsorbed cells, and erosion / detachment of cells resulting from growth.

In general, the initial step of biofilm formation is the adhesion of the microorganisms to surfaces by non-specific physicochemical interactions. These interactions are governed by the particular charges and hydrophobicities of both bacteria and the materials involved (Marshall, 1986; Fletcher & Loeb, 1979). Bacteria and most natural and artificial surfaces investigated thus far are negatively charged (Hancock, 1991; Neu & Marshall, 1991; Norde & Lyklema, 1989; Van Loosdrecht *et al.*, 1987). Therefore, adhesion will take place only when attractive forces, such as Van der Waal's forces or hydrophobic interactions, between bacterial surface polymers and the solid surface overcome the electrostatic repulsion (Marshall, 1986; Rijnaarts *et al.*, 1995). The deposition of colloidal particles on solid surfaces can be calculated by applying the Derjaguin, Landau, Verwey, and Overbeek (DLVO) theory of colloidal stability (James, 1991; Rutter & Vincent, 1980). This calculation accounts for two forces, the sum of the Van der Waal's attraction and electrostatic interaction. This colloid-model calculation has already been applied successfully to the deposition of bacterial cells on solid surfaces (Rijnaarts *et al.*, 1995).

The charge on the cell surface of a bacterium is usually inferred from its electrokinetic (zeta) potential (ζ) (Van Loosdrecht *et al.*, 1987), which, in turn, can be calculated from the mobility (u) of the bacterium in an electric field. The charge results from anionic and cationic (acidic and basic) groups on the cell surface (Jucker *et al.*, 1996). Consequently, the effective charge is influenced by the specific adsorption of ions (Jucker *et al.*, 1996) and strongly depends on the pH and the ionic strength (I) of the medium. The dependence of u on I is due to the diffuse layer of counter ions formed around charged particles. The higher I, the more ions are available to shield and neutralise the charge of the particle. The number of charged acidic or

basic groups present in bacterial surface polymers depends on the pH of the medium. At pH values below the whole cell bioelectric point (iep), the net charge of the bacterium is positive. The iep of most bacteria reported so far is in the range of pH 1.5 to 4.5 (Jucker *et al.*, 1996). In most models the spatial and temporal variability of the cell envelope is not considered, even though different cell surface structures that contact the surface give rise to different adhesion mechanisms (Otto *et al.*, 2001). Attachment of *E. coli* to abiotic surfaces leads to alteration in the composition of outer membrane proteins, which suggests that physical interactions with the surface may lead to an alteration of the surface characteristics of the cell envelope and may, therefore, influence adhesion (Otto *et al.*, 2001).

Sauer *et al.* (2002) and Rice *et al.* (2000) have demonstrated that during initial attachment of *P. aeruginosa*, some planktonic bacteria contacted the substratum, via the cell pole, and became transiently fixed. The initial attachment was reversible as some cells were observed to detach or attach only briefly. Initial surface attachment for many bacteria requires flagella (O'Toole & Kolter, 1998) or surface adhesins and depends on nutritional signals from the environment. In *P. aeruginosa*, the transcription of *algC*, a key gene involved in the biosynthesis of alginate, is induced soon after bacteria attach to the surface (Davies & Geesey, 1995). The involvement of flagella in the initial attachment process (under conditions of continuous flow) was supported by visual observations made Sauer *et al.* (2002), as some cells rotated about the polar axis while fixed to a single spot on the surface, resulting in a longitudinal irreversible attachment. It has previously been demonstrated that adhesion to a surface initiates the phenotypic expression of a range of genes not used by planktonic organisms (Costerton *et al.*, 1995). Evidence of this type of behaviour was first presented by Dagostino *et al.* (1991). Using a *lacZ* reporter system, two marine bacteria were shown to express certain genes upon attachment to surfaces.

These primary (reversibly attached) biofilm cells have been shown to experience a lag in their growth rate when compared to both biofilm and planktonic cell populations, which may be attributed to adapting to a sessile environment or undergoing a phenotypic change. The expression of the alginate biosynthetic operon may result in the observed lag in growth, since cells would be directing metabolic energy into EPS, rather than into replication (Rice *et al.*, 2000). Irreversible attachment is postulated to occur once cells commence with their development. This phenomenon can be visualised as multiple cells in contact with each other and the surface (Sauer *et al.*, 2002). Motility has been observed to cease in irreversibly

attached cells, and the Las quorum sensing system (*lasB* gene) was activated (Sauer *et al.*, 2002). The cell clusters formed by irreversibly attached cells remained attached to the substratum through to the last stage of biofilm development. Cells of *P. aeruginosa* that were in the planktonic and early attachment stage did not display substantially different physiologies (Sauer *et al.*, 2002).

Among the many factors believed to be involved in the initial attachment of microorganisms to surfaces are surface hydrophobicities. Cell morphology and biofilm structure of the Gram-negative marine isolate SW5 are markedly influenced by the surface hydrophobicity of the substratum (Dalton *et al.*, 1994). SW5 at hydrophobic glass surfaces was characterised by the formation of tightly packed biofilms consisting of single and paired cells. In contrast SW5 at hydrophilic glass surfaces was characterised by the sparse colonization and formation of chains of cells anchored at the surface by the colonizing cell (Dalton *et al.*, 1994). The adhesion rate of the negatively charged bacterium *Enterococcus faecalis* has been demonstrated to be higher on a hydrophilic glass surface than on a hydrophobic silicone rubber surface (Gallardo-Moreno *et al.*, 2001). In another study by Jucker *et al.* (1996) the adhesion of the positively charged bacterium *Stenotrophomonas* (*Xanthomonas*) *maltophilia* was shown to favour adhesion to negatively charged surfaces such as Teflon and glass. While adhesion of negatively charged bacteria is impeded in media of low ionic strength, because of a thick negatively charged diffuse layer, adhesion of *S. maltophilia* was particularly favoured in dilute medium. The adhesion efficiencies of *S. maltophilia* at various ionic strengths could be explained in terms of long-range interaction energies between *S. maltophilia* and glass or Teflon.

As is the case with other developmental systems, it is essential that a microorganism senses and responds to environmental signals during both initial attachment and biofilm formation. Each step leading to the formation of a mature biofilm is a potential control point in the developmental process and represents an opportunity for a bacterium to alter the regulation of its gene expression. For example, it is clear that the nutritional status of the environment has an impact on the decision of bacteria to initiate the formation of a biofilm; however, nutrient status may also play a role in determining the type of attachment process genetically as is demonstrated in *V. cholera* (Yildiz & Schoolnik, 1999; Watnick *et al.*, 1999). Although many researchers have proposed models for the initial attachment of microorganisms to surfaces the question "How does the microorganism sense, respond to, or attach to the surface?" has by no

means been fully answered.

2.1.1.3 Growth of the biofilm

Since microbial biofilms, consisting of either single or multiple species, are structurally organised and dependent on several factors such as nutrient supply, flow rate, pH and temperature, it is important to understand the mechanisms of biofilm formation, growth and maintenance. In such dynamic systems, the individual cells experience conditions determined by the outer environment, already existing structures, and the local microbial activities. Sub-populations, which are completely different from the majority of the community, may form locally (Sternberg *et al.*, 1999). This was substantiated by Sauer *et al.* (2002) who observed that bacteria having physiologies from more than one stage of biofilm growth were present simultaneously within a single biofilm. Furthermore, Whiteley *et al.* (2001) demonstrated that *P. aeruginosa* displays multiple phenotypes with distinct physiological characteristics (structural and metabolic) that can be correlated with the various stages of biofilm development.

Biofilm experiments are performed in many different ways, and no standardised set-up for experiments exists. Many different physical factors are known to influence biofilm formation, and it is therefore difficult to present general models of biofilm development based on reports from different laboratories. Furthermore, the development of biofilm structures is, to a certain extent, a stochastic process and independent rounds of biofilm experimentation never result in exact structural copies, even if the experimental conditions are kept constant (Heydorn *et al.*, 2002). The different descriptions of *P. aeruginosa* biofilms, to a large extent, reflect the different experimental set-ups used by different laboratories, but they also illustrate the capacity of *P. aeruginosa* to adapt to a wide variety of different conditions by changing the structure of its biofilms.

Many species have shown distinct developmental steps in biofilm formation, which include (1.) initial attachment to a surface (as discussed above), (2.) the formation of microcolonies (which by many is believed to form part of the irreversible attachment process), and (3.) maturation of microcolonies into an EPS-encased mature biofilm. Many authors, however, fail to highlight that (4.) once the attached population has grown to a multicellular conglomerate; a system of biofilm maintenance is essential for the survival of the mature biofilm.

Once initial attachment to the surface has taken place it seems that cells become irreversibly attached and biofilm formation proceeds. The idea that *P. aeruginosa* displays multiple physiologies during development as a biofilm was proposed when it was noted that this organism progresses through multiple developmental stages, each having a unique phenotype. The formation of biofilms can be considered as a developmental cycle (Fig. 2.1). It begins when free-swimming (planktonic) bacteria recognise a surface and firmly (irreversibly) attach as described above. Subsequently the attached cells grow and divide and at the same time may recruit additional planktonic cells that attach to the existing biofilm. During early phases, single cells and small microcolonies are seen. Virtually all of the bacteria in these phases have relatively high growth rates (Sternberg *et al.*, 1999). Sauer *et al.* (2002) made the observation that as the biofilm became progressively more layered, it was accompanied by the activation of the Rhl quorum-sensing system.

Left unchecked, simple growth of the bacteria on the surface would eventually lead to extreme crowding, possibly starving many cells that might not be able to obtain nutrients. At the same time, toxic metabolic wastes could accumulate among the densely packed cells. The solution to these problems is to create space between loosely packed clusters of cells (Kolter & Losick, 1998). Attached bacteria migrate slightly from the surface as they excrete extracellular polysaccharides that serve as the matrix for the biofilm. As the biofilm architecture develops, cells cluster in pillar- and mushroom-like structures, with water channels between them through which nutrients can flow in and waste products out, function very much like a primitive circulatory system (Costerton *et al.*, 1995). Type IV pilli have been shown to be required for maturation of *P. aeruginosa* biofilm under quiescent conditions (O'Toole & Kolter, 1998); however, they are not necessary for development under conditions of flow (De Kievit *et al.*, 2001). The mechanical basis for this flagellum-independent type of surface motility is believed to be extension and retraction of the type IV pilli, which propel the cells along the surface (Palmer, 1999). Twitching motility has been suggested to be necessary for microcolony formation and thus for normal biofilm development (O'Toole & Kolter, 1998). After attachment, cells are able to move along the surface by using twitching motility instead of flagellum-mediated swimming. The cells multiply and form a continuous layer covering the substrate, and later, groups of bacteria begin to form microcolonies. With time, microcolonies develop into a mature biofilm that is often associated with the production of EPS. RpoS is also believed to be involved in the control of biofilm growth in *P. aeruginosa*

(Heydorn *et al.*, 2002).

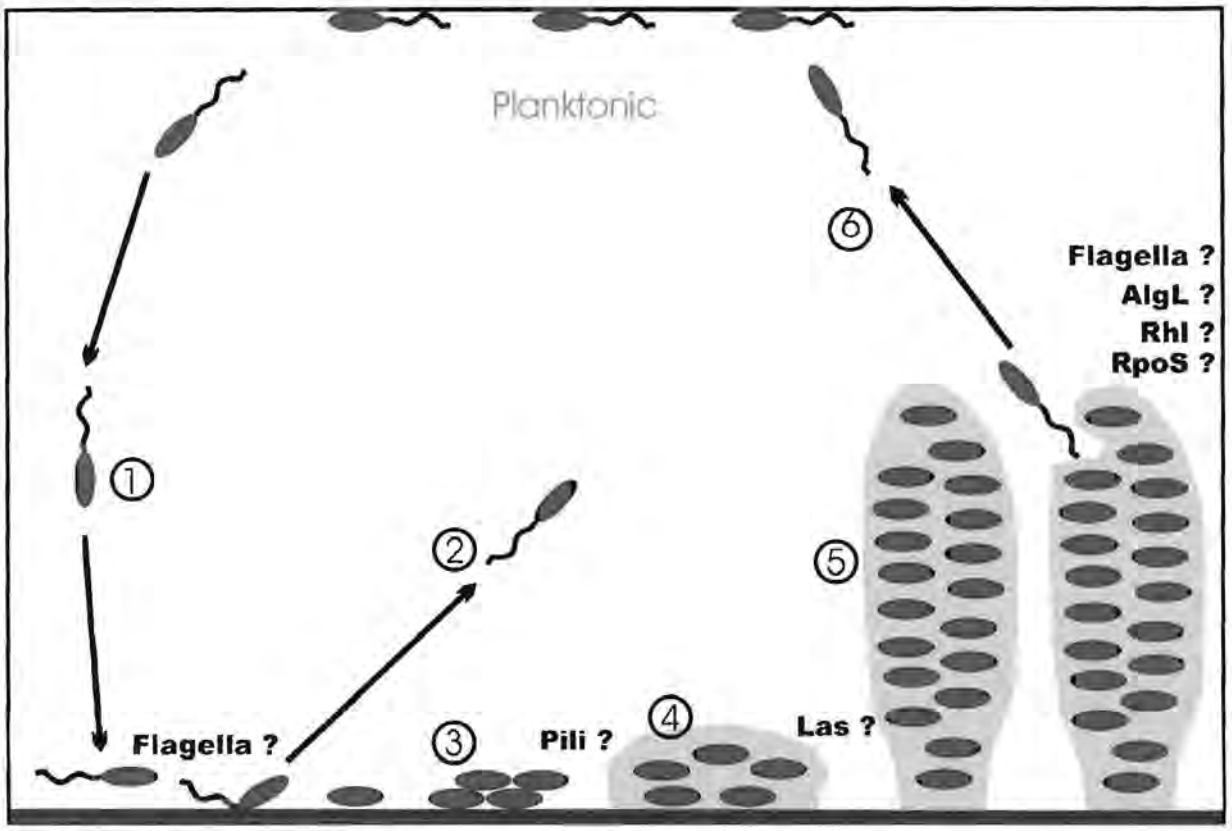

Fig. 2.1: Outline of the current model of biofilm development in *P. aeruginosa* showing the involvement of some of the known processes and genes. (1.) Sensing of the surfaced by the planktonic cell, (2.) reversible attachment, (3.) irreversible attachment, (4.) microcolony development (development of the polymer matrix), (5.) development of classic biofilm architecture and maintenance of the mature biofilm, and (6.) detachment.

Numerous conditions, such as surface and interface properties, nutrient availability, the composition of the microbial community, and hydrodynamics, can affect biofilm structure (Stoodley *et al.*, 1998). Biofilms have been examined under various hydrodynamic conditions such as laminar and turbulent flows, and it was shown that biofilm structures are altered in response to flow conditions (Stoodley *et al.*, 1998). Biofilms grown under laminar flow conditions were found to be patchy and consisted of rough round cell aggregates separated by interstitial voids. Biofilms grown in turbulent flow cells were also patchy, but elongated "streamers" that oscillated in the bulk fluid were observed. Furthermore, biofilm development under continuous flow was polymorphic and structurally adapted to changes in nutrient availability.

The interstitial voids or channels are an integral part of biofilm structure. Using particle-tracking techniques, researchers have been able to demonstrate water flow through these channels (Stoodley *et al.*, 1994). The channels are, in essence, the lifelines of the system, since they provide a means of circulating nutrients as well as exchanging metabolic products with the bulk fluid layer (Costerton, 1995). Oxygen is available in the biofilm as far down as the substratum, indicating that the channels facilitate the transport of oxygenated bulk fluid throughout the biofilm to the surface (DeBeer *et al.*, 1994). This phenomenon was further demonstrated in a toluene-degrading multi-species biofilm. In this set of experimental data toluene was available to cells deep within the biofilm, indicating transport through channels (Møller *et al.*, 1996). There are likely to be mechanisms for the formation as well as the maintenance of these structures. The elaborate architecture provides the opportunity for metabolic cooperation, and niches are formed within these spatially well-organised systems. Consequently the bacteria are exposed to an array of distinct environmental signals within a biofilm. For example, cells situated near the centre of a microcolony are more likely to experience low oxygen tensions.

Detachment of individual cells (or clumps of cells) from the biofilm completes the developmental cycle. Generally bacterial cells are thought to leave the biofilm after division of cells in the outer layers of the biofilm matrix. This phenomenon is not thought to occur in large numbers. Recent evidence presented by Sauer *et al.* (2002), however, suggests that the detachment process may be more complex than originally thought. Cell clusters / aggregates within the biofilm were observed to undergo alterations in their structure due to the dispersion of bacteria from their interior portions. They hypothesised that this phenomenon may be due to reduced growth activity caused by decreased penetration of nutrients through the biomass, as the biofilm gets older and thicker (Sternberg *et al.*, 1999). These detaching bacteria were motile and were observed to swim away from the inner portions of the cell clusters through openings in the cluster and enter the bulk liquid (Sauer *et al.*, 2002). Bacteria remaining in the void were motile. The ability of bacteria to swim freely within the void spaces indicated the absence of dense polymers or other gel-like material in the void space and two-dimensional protein profiles indicated that portions of the biofilm bacteria were reverting back to the planktonic phenotype.

The detachment phenomenon described above may give some indication as to how bacterial biofilms maintain differentiated structure. Frequently, various authors have reported a lack of

growth in the central zones of the biofilm. This lack of growth may be similar to the lag observed by Rice *et al.* (2000) for bacteria initially attaching to the surface where they hypothesised that this lag was due to an alteration of the phenotype of the attaching bacterium. Similarly a lack of nutrients and oxygen availability in the central biofilm regions may cause a phenotypic differentiation into a detachment phenotype where cells are released from the biofilm with the subsequent formation of a new water (reticulation) channel. This would allow for further development of the two microcolonies left behind at the surface. The hypothesis presented would, however, require substantiation. Apoptosis of cells inside the biofilm is also thought to be in some way involved in biofilm maintenance (S. Kjelleberg, personal communication). This phenomenon may provide the means of recycling biofilm components (nutrients).

Mature biofilms have a specialised architecture that is believed to ensure the well being of the individual cells that comprise it. In a recent review, Davey & O'Toole (2000) further supported the idea that biofilm formation is a process of microbial development and is not unlike that observed in cell-cycle controlled swarmer-to-stalk cell transition in *Caulobacter crescentus*, sporulation in *Bacillus subtilis*, and fruiting body formation in *Myxococcus xanthus*. This view is gaining increasing acceptance as studies on initial events in biofilm development reveal alterations in bacterial cell physiology that hint that changes may occur throughout the developmental cycle (Sauer *et al.*, 2002). Despite the enormous amount of research done on this developmental system, relatively little is know about biofilm development and maintenance.

2.1.2 Physiology of bacteria in biofilms

Cells in the bacterial biofilm are thought to be more resistant to ultra-violet light (Degiorgi *et al.*, 1996), heat (Deog-Hwan & Marshall, 1995), and a host of other environmental factors. 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, these *P. aeruginosa* isolates have been biofilm organisms as is generally observed in *P. aeruginosa* isolates of a medical origin. It is not, however, for this reason that biofilms show an increased resistance to antimicrobial agents. When cells exist in a biofilm they can become 10-1000 times more resistant to the effects of antimicrobial agents (Nickel *et al.*, 1985; Gristina *et al.*, 1987). The development of biocide resistance is not understood, but

recent studies have used a variety of model systems to determine how and why biofilms are so resistant to antimicrobial agents. These mechanisms include slow growth (Kinniment & Wimpenny, 1992) and / or inductions of an RpoS-mediated stress response, the physical and / or chemical structure of exopolysaccharides or other aspects of biofilm architecture (de Beer *et al.*, 1994; Hoyle *et al.*, 1993) and the development of biofilm-specific biocide-resistant phenotypes (Mah & O'Toole, 2001). In *P. aeruginosa*, it has been suggested that *algU* acts in concert with *rpoS* to control the stress response. Due to the heterogeneous nature of the biofilm, it is likely that there are multiple resistance mechanisms at work within a single community (Mah & O'Toole, 2001).

Reports by Kolter & Losick (1998) and Watnick & Kolter (2000) emphasize the diversity, movement and dynamic nature of the pure culture biofilm and pose the question "Is there such a thing as the biofilm phenotype?" In the past, the biofilm was assumed to be comprised of cells with similar phenotypic characteristics, whereas it is becoming more evident (at least in pure culture biofilms) that in fact a number of phenotypically different cells seem to work together in order to build and maintain the biofilm matrix. This should not be so surprising, as at any one time free-living cells may become a part of the biofilm, biofilm cells are detaching, biofilm cells are dividing and some biofilm cells are dying. These phenomena together with known differences in ages of the biofilm cells within the biofilm, oxygen availability, nutrient status and other differences in environmental conditions throughout the biofilm, the parameters are set to ensure a variety of different "biofilm phenotypes".

It should, therefore, not be unexpected that in screening for biofilm regulatory elements many authors have found a variety of different genes that are in some way involved in the regulation of the biofilm phenotype. It is widely accepted that cells growing in a biofilm exhibit marked physiological differences from their planktonic counterparts. Nevertheless, the regulatory mechanism underlying the switch to the biofilm phenotype and the actual cellular metamorphoses that occur remain unknown.

2.1.2.1 Gradients

The structural heterogeneity of bacterial biofilms, as observed by CSLM, has serious implications for mass transfer within biofilms (Costerton *et al.*, 1995). The use of microsensors to measure the concentration of substrates directly in various regions of the biofilm has demonstrated that dissolved oxygen concentrations decrease in microcolonies as

the centre of the microcolony is approached, reaching almost anoxic conditions in the centre of the microcolony (Costerton *et al.*, 1995; Xu *et al.*, 1998). Diffusion, therefore, seems to limit the dissolved oxygen concentration within microcolonies; however, the outer layers of the biofilm do not experience decreases in dissolved oxygen concentration due to the biofilm channels (Costerton *et al.*, 1995). It has been shown by CSLM that convective flow occurs in the water channels within living biofilms (Stoodley *et al.*, 1994).

Evidence obtained from the examination of living biofilms has shown that cells may produce surface roughness that increases turbulence and mass transport at the colonised surface (Costerton *et al.*, 1995). Mass transport within biofilms is further enhanced by convective flow of the bulk fluid through the biofilm water channels. Similarly, differences in pH have been observed within dense cell aggregates (Caldwell *et al.*, 1992). Lawrence *et al.* (1994) demonstrated that diffusion in the biofilm was hindered relative to diffusion in the bulk solution. The observation made by many authors that cells in the centre of microcolonies have a slower growth rate (are metabolically less active) than those in close proximity to the bulk solution indicates that diffusion of nutrients into microcolonies may play a significant role in biofilm formation. This phenomenon has, however, never been directly substantiated.

2.1.2.2 Gene regulation and metabolism of biofilm-associated bacteria

The metabolism of a bacterium isolated from a surface is often studied in liquid culture, although this condition represents only one of a range of real physical conditions occurring in the original environment. To show that there are significant differences between free-living and attached cells, some authors have compared bacterial growth (Irriberri *et al.*, 1990) as well as assimilation and degradation activity in the presence or absence of a surface in the reaction system (Manz *et al.*, 1998; Møller *et al.*, 1997; Møller *et al.*, 1996). Unfortunately, the results obtained do not always give rise to the same conclusions. This may be due to the complexity of factors influencing the bacterial attachment process (i.e. the nature and concentration of the growth medium, the structure and composition of the surface, the physicochemical parameters of the medium and the physiology of the bacterial cells).

Where surface-induced stimulation of bacterial activity has been observed, a number of mechanisms have been proposed that invoke a direct effect of the surface on the bacterium (Marshall *et al.*, 1971). Van Loosdrecht *et al.* (1990) have stated, however, that evidence for surfaces directly influencing bacterial metabolism is inconclusive, because all such

observations can be attributed to indirect effects of surfaces on the surroundings of the bacterial cells. Dagostino *et al.* (1991), using transposon mutagenesis, however, showed the up-regulation of genes at a solid surface where no up-regulation occurred either in liquid or agar media. The up-regulation of these genes could not be attributed to nutrient accumulation at the surface (Van Loosdrecht *et al.*, 1990) as the experiments were carried out in a nutrient-rich milieu. Furthermore, Dagostino *et al.* (1991), using a *lacZ* reporter system, were able to demonstrate that two marine bacteria expressed certain genes only upon attachment to a surface.

Gene fusion experiments have clearly established that expression of the *P. aeruginosa* alginate biosynthetic genes *algC* (Davies *et al.*, 1993 & Davies & Geesey, 1995) and *algD* (Hoyle *et al.*, 1993 & Rice *et al.*, 1995) are upregulated following attachment to a surface, with a concomitant increase in alginate production. EPS biosynthesis is not, however, the only metabolic change that occurs during the transition from the planktonic to the attached state, but is just one of a number of co-ordinately regulated events leading to the formation of a biofilm. There are significant changes in the profile of outer-membrane proteins and in the expression of virulence factors such as elastase (*lasB*). Recent studies have shown the up-regulation of a number of genetic elements that may be necessary for *P. aeruginosa* biofilm formation and maintenance including *crc* (O'Toole *et al.*, 2000), *lasI* and *rhlI* (Davies *et al.*, 1998; De Kievit *et al.*, 2001), *flgK*, *lasI* and *rhlA* (Sauer *et al.*, 2002).

Using a *lacZ*-based reporter library, Weyers (1999) showed more than a three-fold transcriptional upregulation for a number of biofilm regulatory elements when comparing planktonic populations to total attached populations. Whiteley *et al.* (2001) were able to demonstrate the activation or inactivation of 73 genes when comparing biofilm and planktonic cell populations. In all, 34 genes were activated and 39 were repressed in the biofilm population. Approximately 34% of the 73 genes were found to code for proteins of unknown function. A gene coding for a probable fimbrial protein was found to be downregulated between 7- and 16-fold, a gene coding for a flagellar hook protein was downregulated 2- to 2.5-fold, *tolA* was induced 3- to 4-fold and *rpoS* was downregulated 2- to 2.3-fold. The most highly activated biofilm genes were found to be those of a temperate bacteriophage Pf1. The genes for the synthesis of pilli and flagella were repressed in biofilms, indicating that these appendages may not be required for maintenance of a mature biofilm, but that they are involved in early steps in biofilm development. More importantly, this work points out that

phage induction may be important for gene transfer within the biofilm

Using a combination of CSLM and deletion mutants, Heydorn *et al.* (2002) demonstrated that an *rpoS* mutant of *P. aeruginosa* formed densely packed biofilms that were significantly thicker than those produced by the wild-type strain. This has been confirmed by Whiteley *et al.* (2001). A null mutation in the *rpoS* gene of *P. aeruginosa* (FRD1) has been shown to decrease alginate production by approximately 70% and a null mutation in the *rpoS* gene of *P. aeruginosa* (PAO1) has been shown to display an altered twitching motility phenotype (Suh *et al.*, 1999). O'Toole & Kolter (1998) have shown that flagellar and twitching motility are necessary for *P. aeruginosa* biofilm formation, indirectly implying that *rpoS* is necessary for efficient expression of some biofilm regulatory mechanisms and biofilm formation. This data has, however, been contradicted by Heydorn *et al.* (2002) where twitching motility was not required for microcolony formation in *P. aeruginosa* biofilms and microcolonies were not formed through twitching motility-mediated cell aggregation. Heydorn *et al.* (2002) were further able to demonstrate that biofilm formation in a *lasI* deletion mutant was indistinguishable from the wild-type biofilm indicating that a functional *lasI* gene was not necessary for biofilm formation.

Due to differential gene expression in the biofilm environment, one would expect to find substantial differences in the proteome of biofilm and planktonic cells. Sauer *et al.* (2002), using two-dimensional gel electrophoresis, claimed an average of 35% difference in the protein profiles of the various stages of biofilm development as well as a change in expression levels of more than 50% of the proteome when comparing mature biofilms to planktonic cells. Furthermore, it has been recently demonstrated that surface-influenced planktonic or SIP cells of *P. aeruginosa* grown in batch culture in the presence of glass wool as substratum display a proteome distinct to both the planktonic and biofilm proteomes (Steyn *et al.*, 2001). The data presented by Steyn *et al.* (2001) found four expression patterns for planktonic, SIP or biofilm populations using two dimensional gel electrophoresis namely: (1) expression only occurs in a particular population; (2) genes that are expressed in planktonic cells, are under-expressed in SIP cells, but over-expressed in biofilm cells; (3) genes that are expressed in planktonic cells, that are not expressed in SIP cells, but over-expressed in biofilm cells and; (4) genes that are expressed in planktonic cells but are unchanged in either SIP or biofilm populations. They reported that genes expressed in planktonic cells, while being under-expressed in SIP cells, but over-expressed in biofilm populations appear to be the most common.

The transition to surface-attached growth requires a complex developmental pathway involving a temporal series of events that are regulated in response to environmental and bacterial-derived signals. It is not surprising, therefore, that diverse changes in gene expression are necessary to develop the complex architecture and unique physiological properties of a mature biofilm (Møller *et al.*, 1996). *V. cholera* has been demonstrated to utilise different pathways for initial attachment to a surface depending on the nature of the surface itself. *In vivo*, the Tcp pilus is required for colonisation of the intestine; however, Tcp appears to play no role in attachment to abiotic surfaces (Davey & O'Toole, 2000).

2.1.2.3 Cell - cell interactions in biofilms

Although most laboratory biofilm studies involve a single species, it is important to note that biofilms formed in nature often include multiple bacterial species. Using microscopic and molecular labelling techniques, direct monitoring of gene expression in *Pseudomonas putida* as influenced by *Acinetobacter* has been accomplished, demonstrating a metabolic cooperation between the two bacterial species when degrading benzyl alcohol (Kuchma & O'Toole, 2000). The same authors demonstrated an altered biofilm structure due to the advantages of the metabolic cooperation between the two bacterial species.

A hallmark of a mature *P. aeruginosa* biofilm is its complex architecture characterised by large EPS-enclosed colonies separated by fluid-filled channels. The ordered structure of the mature biofilm indicates that cells must aggregate to become organized. Such organization suggests that cell-to-cell communication is required during biofilm maturation. A well-studied form of intercellular communication involves the phenomenon known as quorum sensing and response. In this signalling system, cells monitor levels of autoinducer signal molecules, typically N-acylated homoserine lactones (AHLs), which act as signals that mediate population-density-dependent gene expression (Kuchma & O'Toole, 2000). Davies *et al.* (1998), using a *lasI* mutant, demonstrated the formation of an abnormal biofilm in the absence of the corresponding AHL. The cells failed to maintain the spaces between each other, and failed to form the elaborate architecture of mature biofilms. Wild-type biofilm formation was restored upon addition of the AHL synthesized by *lasI*. Furthermore, studies have shown that naturally occurring biofilms formed on submerged stones and on in-dwelling urethral catheters produce AHLs, suggesting that this may be a general phenomenon of biofilm development in AHL-producing organisms. Both active efflux and diffusion have been shown

to be involved in the transport of *P. aeruginosa* cell-to-cell signals.

There is, however, much confusion over the involvement of *lasI* in biofilm formation as a recently published article by Heydorn *et al.* (2002) has convincingly demonstrated that *lasI* is in fact not required for adequate biofilm formation in *P. aeruginosa*. Collectively, these results may suggest that cell-to-cell communication may not be necessary for *P. aeruginosa* biofilm formation and maintenance under all conditions and, furthermore, that other "signalling systems" or mechanisms of cellular communication may exist.

2.1.2.4 **Pseudomonas aeruginosa** *as a biofilm organism*

Published reports 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 formation of biofilms by single species is a well-regulated developmental process that results in a complex population of cell types. Although much species-specific behaviour exists that reflects the unique requirement of each microorganism, some general concepts hold true in the formation of most bacterial biofilms. *P. aeruginosa* will form biofilms under most conditions that allow growth and therefore makes it an ideal candidate for the study of biofilm formation. Despite the great interest in alginate and its role in *P. aeruginosa* biofilms, there is little direct evidence that lack of alginate production leads to alterations in the structure of the biofilm.

2.2 METHODOLOGIES FOR THE STUDY OF BACTERIAL BIOFILMS

The formation of a mature biofilm appears to require a regulatory cascade that controls the temporal and spatial expression of biofilm-specific genes. Genetic and molecular techniques used in combination with advanced microscopic techniques are necessary to unravel these regulatory cascades. This marked heterogeneity of gene expression in biofilms presents new challenges in this area of study and will require future methodological innovation (Kuchma & O'Toole, 2000).

2.2.1 Culturing systems

One of the key advances in the study of microbial communities has been the development of various tools for cultivating communities, such as chemostats, continuous flow slide cultures, microstats, and colonisation tracks (Davey & O'Toole, 2000). Biofilms of *P. aeruginosa* have been studied under conditions of flow, primarily by using various flow cells (Zinn *et al.*, 1999), or in stagnant batch culture using microtitre plates (O'Toole *et al.*, 1999). A fundamental concept that requires refining in the context of biofilm studies is the suspended state. Planktonic cells have been defined as cells that have never existed in the attached mode before (Rice *et al.*, 2000). In contrast, suspended cultures that have been in contact with a surface during their recent history, or grown in the proximity of a surface have been termed surface-influenced planktonic (SIP) cells (Steyn *et al.*, 2001). Each biofilm-culturing system facilitates the study of specific parameters while excluding others, thus necessitating an understanding of the ramifications of each specific culturing platform.

2.2.1.1 Microtitre plates

Microtitre plates have been used primarily for purposes of screening of genetic mutants (O'Toole & Kolter, 1998; O'Toole *et al.*, 1999). In microtitre plate-grown cultures, the suspended cells are exposed to the surrounding biofilm for the duration of the experiment, therefore being SIP and not planktonic cultures. The resulting biofilm therefore develops under quiescent and batch conditions.

2.2.1.2 Agar embedded populations

Agar-embedded bacterial colonies as well as colonies growing on the agar have previously been referred to as "biofilm" (Jouenne *et al.*, 1994; Tresse *et al.*, 1997). This approach implies that a semi-solid surface such as agar is able to induce the biofilm phenotype. Various researchers have had some success in identifying biofilm-specific proteins in this manner

(Jouenne *et al.*, 1994); however, it is doubtful whether this mode of "biofilm" growth is capable of inducing the full spectrum of genes involved in biofilm development and maintenance. Cells cultured in this manner also develop under batch and quiescent conditions.

2.2.1.3 Chemostats

Chemostats can be a valuable tool in the culturing of biofilm populations; however, it is essential that biofilm and planktonic populations be grown in separate chemostats due to the effect exerted on the planktonic population by biofilm bacteria. Biofilm bacteria may cause interference in various assays due to detachment into the planktonic phase of growth. An example of the effective use of chemostat-grown biofilm cultures is the study of Sauer *et al.* (2002). In this study, planktonic populations were grown in a separate chemostat where the influence of surface area or the biofilm on the planktonic population was negligible. In other instances (continuous flow re-circulating systems) bias is introduced into the experimental procedure, as the cells used as the planktonic population are in fact SIP populations (Whiteley *et al.*, 2001).

2.2.1.4 Flow cells

Flow cells support biofilm growth under continuous conditions while enabling both a range of flow rates and real–time microscopic investigation (Wolfaardt *et al.*, 1994). Suspended cells leaving flow cells have all been shed from the biofilm, as the cells are fed with sterile broth following an initial inoculation and a short quiescent period to allow for attachment. Cells leaving flow cells are therefore detached cells and not planktonic in the strict sense.

2.2.1.5 Glass wool

Glass wool has been successfully used as a surface for attachment (Steyn *et al.*, 2001). The high surface-to-volume ratio of glass wool yields sufficient biomass for a range of quantitative biochemical analyses. Attached biomass can easily be separated from the surrounding SIP cells, and biofilm cells could then be removed from the glass wool and dispersed by vortexing. Cells cultured in this manner similarly develop under batch conditions; however, flow can also be introduced by varying the size of the culture vessel.

2.2.2 Methodologies for the characterisation and identification of biofilm genes

2.2.2.1 Quantification of biofilm bacteria and biofilm-components

Numerous methods have been applied to the quantification of biofilm bacteria and biofilm

components (Fernandes *et al.*, 2002; Wyffels *et al.*, 2001), each suffering from drawbacks due to the nature of biofilms. Most of the quantification methodologies thus far have been developed with planktonic bacteria in mind. Biofilm bacteria have a tendency, due to the large amounts of EPS produced, to form clumps when removed from surfaces, which have proven difficult to disperse (personal observation). Due to this phenomenon, methodologies such as plate count techniques, which rely on the acquisition of single cells, suffer severe drawbacks. Methodologies such as biomass quantification have proven more useful and are used more frequently; however, one should keep in mind that most of these biochemical techniques were also developed based on the pure culture planktonic principles initially adopted by microbiologists.

2.2.2.2 Gene regulation

Since the metabolic activity of biofilm cells and planktonic cells has been proposed to differ (Costerton *et al.*, 1994), it has been shown that there is a large difference in gene expression in the two environments. Gene regulation is altered in biofilms on a transcriptional (Whiteley *et al.*, 2001; Weyers, 1999) as well as a translational level (Sauer *et al.*, 2002) showing major differences in the phenotypes of planktonic and biofilm populations. Very little is known about the extent and function of the many genes that may be involved in the formation and regulation of *P. aeruginosa* biofilms, let alone those involved in the detachment process.

2.2.2.2.1 Reporter gene-based methods

A plethora of different cloning vectors is now available both commercially and in the public domain, many of which are based on plasmids and bacteriophage genomes. 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 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 are thought to represent no problem (Andersen *et al.*, 1998), unlike the analysis of heterogeneous and complex biofilm populations.

Downstream fusion of the reporter gene *lacZ* coding for β-galactosidase to the promoter region of a structural gene is a frequently used technique to study the expression of the gene. The rate of transcription, inferred from the β-galactosidase activity of the cells, is equated to

"intrinsic promoter strength" but, more importantly, also measures the action of *cis*- and *trans*-acting elements involved in induction and / or repression of the gene. Considering that these promoter fusions are evaluated in growing cells, usually in the exponential phase in batch culture, the rate of transcription is not always the same as the level of expression. Furthermore, it should be remembered that it takes some time for the level of expression to reach a steady-state value (Warner & Lolkema, 2002).

Two important points that must be considered when interpreting data obtained from plasmid reporter constructs are: (1) the copy number of the plasmid in planktonic and biofilm cells should remain constant and (2) when comparing β-galactosidase activities, the growth rates in the two situations must be the same. To date most efforts to provide a suitable reporter system for use in biofilm, or for that matter even planktonic studies, have paid very little attention to the effect that the vector construct, or integrated reporter system, has on the physiology of the organism of interest. An important point to remember when interpreting data from plasmid-based reporter systems is the effect of plasmid maintenance and antibiotic selection pressure on the host cell.

2.2.2.2.2 Transcriptomic approaches

The favoured approach to global profiling of the transcriptome is the use of DNA microarrays (Marshall & Hodgson, 1998). The only report of microarray-based analysis of biofilms was by Whiteley *et al.* (2001) on *P. aeruginosa*. They were, however, only able to detect a mere 1% of genes that showed differential expression in the biofilm and planktonic modes of growth. This data, considering the vast array of genes that are currently known to be expressed differentially in the two environments, points out the disadvantages of highly technical procedures in which a number of small margins of error may lead to significant distortions.

2.2.2.2.3 Use of mutants

Many bacterial mutants are now being screened and analysed to determine the effect of the specific genes on biofilm development (Davies *et al.*, 1998; O'Toole & Kolter, 1998). A good example of the productive results of this approach is the work of Davies *et al.* (1998), who used specific mutants of *P. aeruginosa* to demonstrate that a signal molecule provides a form of cell-to-cell communication that is an essential component for the normal development of biofilms. The use of mutants to characterise biofilm development should, however, be

approached with caution as it is often difficult to predict the effect that a mutation may have on other physiological processes within the bacterial cell. The absence of many gene products can be compensated for by secondary systems of suppressor mutations, therefore, the use of mutants only provides a means for the study of genes that have "essential" roles in biofilm development.

2.2.2.2.4 Proteomics

The field of proteomics has changed the understanding of molecular biology and supplements sequence data with protein information about where, in which ratio and under what conditions proteins are expressed (Kellner, 2000). Sauer *et al.* (2002) were able to detect outer membrane proteins by two-dimensional PAGE; however, they were unable to detect proteins from the inner membrane. They hypothesised that this was probably due to the higher hydrophobicity of the inner membrane proteins resulting in poor solubilisation during sample preparation and isoelectric focusing. In addition, the analysis of membrane proteins from total cell extracts by two-dimensional gel electrophoresis may have been hampered by other components such as lipids, which may interfere with solubilisation in the rehydration buffer (Sauer *et al.*, 2002).

2.2.3 Microscopy

Microscopy is the only technique whereby single cells can be studied within biofilms *in situ*. The understanding of biofilm structure, physiology and control hinges on the application of confocal scanning laser microscopy (CSLM) and other related microscopic techniques. A great obstacle to the study of biofilms, i.e. difficulty in obtaining clear photomicrographs of the densely packed microorganisms under hydrated conditions, was overcome by this major advance in microscope instrumentation (Palmer & Sternberg, 1999). Bacterial biofilm studies began with the study of living biofilms as they grew on the surfaces of glass-walled flowcells (Lawrence & Neu, 1999; Palmer, 1999) permitting semi-continuous non-destructive documentation of temporal changes in biofilm structure and physiology.

The application of CSLM to biofilm research radically altered the perception of biofilm structure and function (Lawrence *et al.*, 1991). Before the use of CSLM, electron microscopy was the method of choice to examine microbial biofilms under high resolution. Unfortunately, sample preparation for electron microscopy results in dehydrated samples. Consequently, this approach provided a deceivingly simplistic view of biofilms, since the biofilm collapsed when water was removed. On the other hand, CSLM, which allows the visualisation of fully

hydrated samples, has revealed the elaborate three-dimensional structure of biofilms (Lawrence *et al.*, 1991). Biofilms formed from single species *in vitro* and those produced in nature by mixed species consortia exhibit similar overall structural features.

The early application of CSLM to biofilm research yielded descriptive reports. For the first time, the arrangement of single cells and of microcolonies within living hydrated biofilms was described (Lawrence *et al.*, 1991). Key studies in which nutrient composition was demonstrated to affect biofilm architecture were those of Wolfaardt *et al.* (1994) and Møller *et al.* (1997). A multidisciplinary approach taken by Singleton *et al.* (1997) contributed to models of flow within biofilms and signalling molecules have been shown to play an important role in the architecture of *P. aeruginosa* biofilms using CSLM (Davies *et al.*, 1998). The development of species-specific fluorescently-labelled oligonucleotide probes has allowed the distinction between morphologically similar cells within a biofilm (Manz *et al.*, 1998), albeit requiring prior fixation and therefore not facilitating real-time studies.

Application of confocal microscopy to three-dimensional localisation of non-enzyme reporter systems, notably GFP, has brought significant advances to the understanding of gene activity in biofilms. The physiological state of cells in biofilms can be assessed using specially designed promoter-reporter systems, which fluoresce only in actively dividing cells. It is important to recognise that confocal microscopy cannot generally be used to visualise unstained materials, such as the exopolymer matrix, in which biofilm cells are embedded. Furthermore, the experiments required to perform a statistical analysis must be based on high-resolution images, and such images are obtained only from fluorescently-labelled specimens (Heydorn *et al.*, 2002). The preferred method of tagging has been to construct chromosomal insertions to ensure a stable gene dosage of the tag sequence, and mini-Tn5 has so far been the carrier of choice.

2.3 ALGINATE SYNTHESIS IN *P. aeruginosa*

Bacteria experience a certain degree of shelter and homeostasis when residing within a biofilm, and one of the key components of this micro-niche is the surrounding exopolymeric substance matrix. This matrix is composed of a mixture of components such as EPS, protein, nucleic acids, and other substances. The best studied of these components is EPS. Most bacteria are able to produce polysaccharides, either as wall polysaccharides (capsules) or as extracellular excretions into the surrounding environment (EPS). The formation of *P.*

aeruginosa biofilms is accompanied by the formation of a mucoid exopolysaccharide (Davies *et al.*, 1993) 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, amongst others, *P. aeruginosa*. With prolonged infection, *P. aeruginosa* shifts to a mucoid form 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). The production of alginate and the genetic mechanism thereof have therefore been extensively studied. A number of genes and gene products have been identified in the alginate biosynthetic pathway, the two studied in terms of biofilm formation being the *algC* (Davies *et al.*, 1993; Davies & Geesey, 1995) and *algD* genes (Hoyle *et al.*, 1993; Rice *et al.*, 1995). The initial attachment of *P. aeruginosa* to a surface causes up-regulation of the *algC* gene, while *algD* is up-regulated shortly thereafter.

2.3.1 Biosynthesis of exopolysaccharides

Bacterial surface polysaccharides come in two general forms, those bound to the cell surface by attachment to lipid A lipopolysaccharide (LPS), and those associated with the cell surface as a capsule, exopolysaccharide (EPS). EPS are very hydrated polymers with 99% of their wet weight comprised of water (Weiner *et al.*, 1995). They have considerable heterogeneity, from the simple α 1-4 linked, unbranched glucose polymers called dextrans, to the highly complex, branched and substituted heteropolysaccharides made up of oligosaccharide repeating subunits such as xanthan gum and colanic acid (Weiner *et al.*, 1995).

Alginate is a copolymer of β-D-mannuronic acid and α-L-guluronic acid (GulA), linked together by 1-4 linkages (Rehm & Valla, 1997). The monomers are distributed in blocks of continuous mannuronate residues (M-blocks), guluronate residues (G-blocks), or alternating residues (MG-blocks). Alginates isolated from different natural sources vary in length and distribution of the different block types. The polymer is a well-established industrial product obtained commercially by harvesting brown seaweeds. Some bacteria, mostly derived from

the genus *Pseudomonas* and belonging to the RNA superfamily I, are also capable of producing copious amounts of this polymer as an exopolysaccharide. The intermediate precursor of polymerisation is GDP-mannuronic acid. The sugar residues in this compound are polymerised into mannuronan, which is further modified by acetylation at positions O-2 and / or O-3 and by epimerisation of some of the residues, leading to a variable content of acetyl groups and GulA residues. The GulA residue content and distribution strongly affect the physicochemical properties of alginate. All *Pseudomonas* alginates, however, lack G-blocks (Rehm & Valla, 1997). The high molecular mass of bacterial alginate and the negative charge ensure that the polysaccharide is highly hydrated and viscous (Gacesa, 1998).

The biosynthesis of *P. aeruginosa* alginate has been reviewed by Gacesa (1998). The genes for alginate biosynthesis are all located on the bacterial chromosome. At least 24 genes have been directly implicated in alginate biosynthesis in *P. aeruginosa* and there is good evidence that others may be involved (Gacesa, 1998). Some of the control genes act globally and encode proteins such as alternative sigma factors. Other "*alg*" genes such as *algC* are also involved in LPS biosynthesis (Gacesa, 1998). The majority of the genes involved in alginate biosynthesis are organised into three major clusters located at approximately 9, 34, and 68 min on the *P. aeruginosa* chromosome. Most of the biosynthetic genes, with the exception of *algC*, which is transcribed independently, are directly under control of the *algD* promoter in the 34 min region (Gacesa, 1998). The role of the *algL* gene, encoding an alginate lyase in the 34 min region, is thought to be that of cellular detachment (Boyd & Chakrabarty, 1994). It is, however, not clear why a degradative enzyme should be expressed concurrently with the biosynthetic enzymes.

A convincing pathway for alginate biosynthesis in *P. aeruginosa* has proved difficult to ascertain, but has been elucidated using a combination of complementation analyses and gene cloning / over-expression studies. Although the initial steps in the pathway are indisputable (Fig. 2.2), there is still considerable debate about the final stages of biosynthesis and export of alginate.

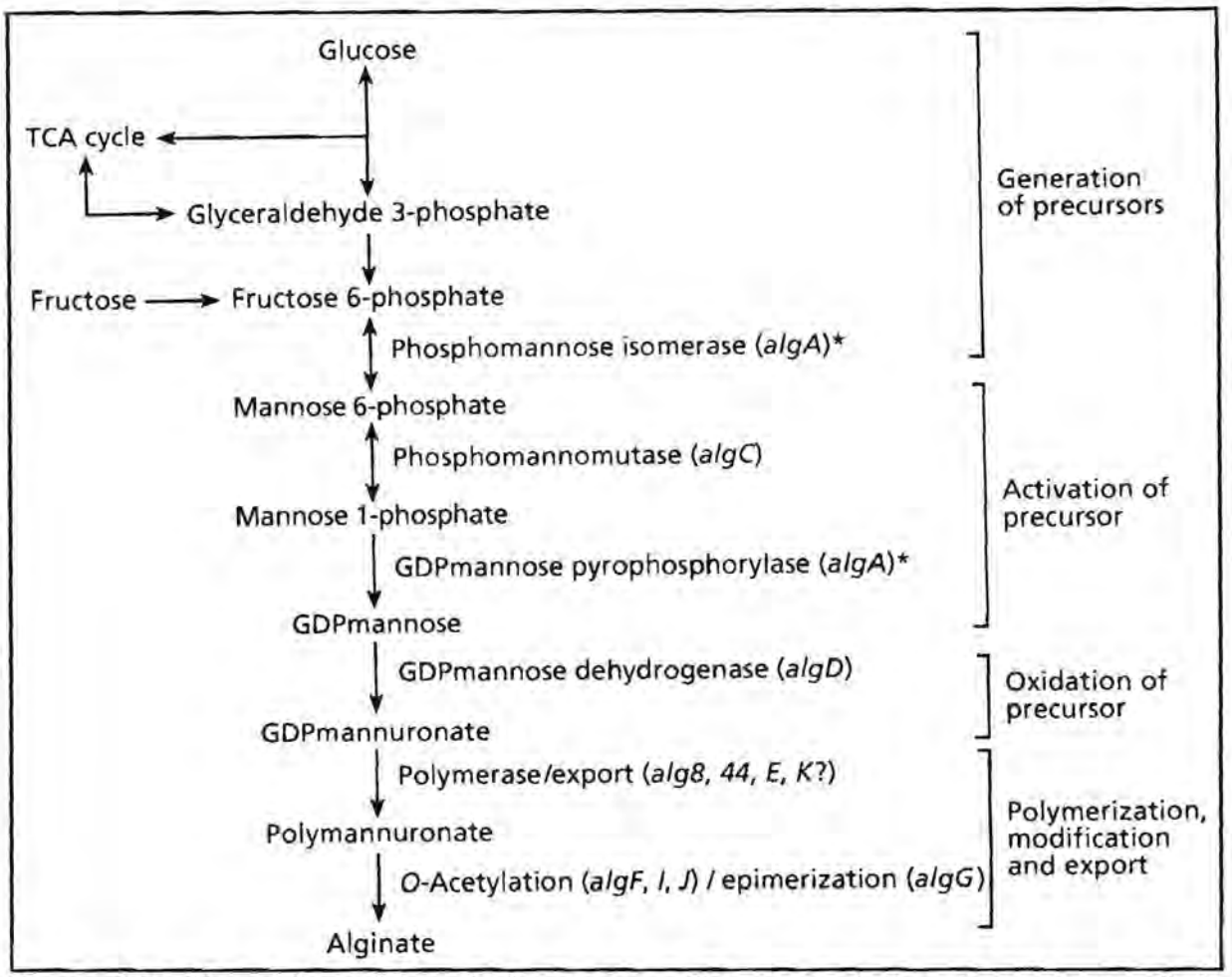

Figure 2.2: The alginate biosynthetic pathway. The genes indicated are those of *P. aeruginosa*. **algA* encodes the bifunctional enzyme phosphomannose isomerase / GDP-mannose pyrophosphorylase. Taken from Gacesa (1998).

The initial steps in the alginate biosynthesis pathway are essentially those of general carbohydrate metabolism and the intermediates are widely utilized. In particular, the steps up to and including GDP-mannose are common to both alginate and LPS biosynthesis. This and other evidence clearly indicates a crucial role for GDP-mannose dehydrogenase (*algD*) in the biosynthesis of alginate. The *algD* gene is proximal to the promoter on the alginate operon and expression is tightly controlled. GDP-mannose dehydrogenase is thought to be the kinetic control point in the alginate pathway.

2.3.2 Regulation of *P. aeruginosa* alginate biosynthetic genes

The synthesis and export of alginate 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). The committal step remains the conversion of GDP-mannose to GDP-

mannuronate by the *algD* gene product (Gacesa, 1998). The chemical environment has been found to have a considerable affect on transcription from *algD*. The regulation of alginate biosynthesis is extremely complex and involves specific gene products and those that act more globally. The five genes *algU* (*algT*), *mucA*, *mucB* (*algN*), *mucC* (*algM*) and *mucD* (*algY*) located at 68 min on the chromosome comprise the main switch controlling the conversion between non-mucoid and mucoid forms of *P. aeruginosa*. Genes located at the region spanning 9-13 min modulate the production of alginate and have been described as auxiliary regulators of mucoidy (Gacesa, 1998). The best characterised of these regulators is *algR*, which is transcribed in response to the protein AlgU. AlgR binds to three sites upstream of the *algD* promoter and, in conjunction with AlgU, up-regulates transcription of *algD* and the downstream genes. AlgR also promotes expression of *algC*. AlgB also modulates *algD* expression and based on sequence analysis is a member of the NtrC subclass of two-component regulators, but the *algB* and *algR* regulatory systems appear to operate independently of each other (Gacesa, 1998).

Two-component signal transduction pathways comprising the putative sensor proteins AlgQ (kinase) and AlgZ, interacting with regulatory proteins like AlgR and AlgB have 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). A number of additional genes, including *rpoS* (Suh *et al.*, 1999), are also involved in alginate regulation.

2.3.2.1 The algC gene

The *algC* promoter, similar to the *algD* promoter, is regulated by the response regulator protein AlgR (Davies *et al.*, 1993). Davies *et al.* (1993) showed *algC* to be upregulated in biofilm cells compared to their planktonic counterparts and the degree of up-regulation was found to correlate with alginate biosynthesis. The *algC* promoter was shown to have maximum activity in planktonic cultures during the late lag and early log phases of cell growth. In biofilm cells, *algC* activity was found to exceed planktonic activity except during the period immediately following inoculation into fresh medium (Davies *et al.*, 1993). Similarly, single cells have been shown to up-regulate *algC* in the initial stages of biofilm development, however, initial (reversible) cell attachment to a substratum (Teflon® and glass) appeared to be independent of *algC* promoter activity (Davies & Geesey, 1995).

2.3.2.2 The algR and algB genes

The *algD* promoter is believed to be controlled by the *algR* (an environmentally responsive regulatory gene) and *algU* (*algT*) gene products. The *algR* gene is a transcriptional regulator of *algD*. DNA sequence analysis of *algR* revealed homology of its gene product with a class of environmentally responsive bacterial regulatory genes, including *ompR*, *phoB*, *sfrA*, *ntrC*, *spoOA*, *dctD*, and *virG*: these transcriptional activators control cellular reactions to osmotic pressure, phosphate limitations, or specific chemical compounds present in the medium or released from wounded host tissue (Deretic *et al.*, 1989). *algR* has been found to map in a chromosomal region that is capable of inducing mucoidy when chromosomally amplified (Gacesa, 1998).

The transcriptional activation of *algD* and a high level production of alginate in *P. aeruginosa* also requires the response regulator AlgB (Selvaraj *et al.*, 1998). AlgB is analogous to proteins from the NtrC (NRII) subfamily of signal transduction systems that are known to activate transcription specifically in concert with the σ^{54}-RNA polymerase holoenzyme (Nifa *et al.*, 1995; Stock *et al.*, 1995). The NtrC-type response regulators are required for the ATP-dependent isomerization of the closed promoter complexes formed by the σ^{54}-holoenzyme into open transcriptional complexes (Lee *et al.*, 1993; Wang *et al.*, 1995; Perez-Martin & de Lorenzo, 1996; Wang & Gralla, 1996; Syed & Gralla, 1998). Woolwine & Wozniak (1999) demonstrated that loss of a gene of unknown function, *phpA*, in an *algB* genetic background had a positive effect on *algD* transcription.

2.3.2.3 The algU *gene and* muc *loci*

algU, which encodes the alternative sigma factor σ^{22}, undergoes a strong transcriptional activation in mucoid *P. aeruginosa* cells (Deretic *et al.*, 1987a; Deretic *et al.*, 1987b). The most recently 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 *algU* gene is auto-regulated and has two negative regulators encoded by the *mucA* and *mucB* (anti-σ factor) genes, which repress the activity of the AlgU protein. Edwards and Saunders (2001) were able to demonstrate a small difference in *algU* expression when comparing non-mucoid and mucoid strains of *P. aeruginosa* using real-time PCR.

Mucoid CF isolates frequently have a defective *mucA* allele and inactivation of *mucA* or *mucB* results in the mucoid phenotype due to interruption of inactivation of AlgU and enhanced

expression of *algU* (Martin *et al.*, 1993b). Spontaneous reversion to the non-mucoid phenotype is often due to a suppressor mutation in *algU* (DeVries & Ohman, 1994). In another set of strains (muc23) conversion to mucoidy occurs in the absence of *mucA* alterations. The gene(s) that correspond to the *muc23* mutation and their nature are not known at present (Boucher *et al.*, 2000). The periplasmic protein MucB (AlgN) was proposed to act 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. Edwards and Saunders (2001) have further suggested that the *mucB* gene may play a role in the recognition of stress conditions, and that having a disrupted *mucA* gene does not always result in a mucoid phenotype. Furthermore, they were able to demonstrate that levels of *mucB* mRNA did not vary consistently between the mucoid and non-mucoid phenotypes, while mRNA copy number was consistently higher in mucoid *P. aeruginosa* strains grown on LB agar. From this data they concluded that the mucoid phenotype on LB agar was not mediated via significant differences in the transcription of *mucA* and *mucB*.

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. *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, 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. AlgU has also been shown to be involved in the negative control of flagellum synthesis. In the absence of *muc* mutations, activation of the *algD* promoter most likely represents a major decision in the life cycle of *P. aeruginosa* possibly linked to the establishment of biofilms (Davies *et al.*, 1998). Boucher *et al.* (2000) have demonstrated that transcription of *algD* under nitrogen-poor conditions is dependent on AlgU while under nitrogen-rich conditions RpoN or RpoN-dependent processes repress AlgU-dependent transcription of *algD*.

2.3.2.4 The algD gene

algD expression depends on regulators from the superfamily of bacterial two-component signal transduction systems (Kato & Chakrabarty, 1991; Yu *et al.*, 1997) and a specialised alternative sigma factor, σ^E (also known as AlgU) (Martin *et al.*, 1993a; DeVries & Ohman, 1994), functionally equivalent to the extreme heat shock sigma factor σ^E (RpoE) in *E. coli* (Yu *et al.*, 1995). a*lgD* is responsible for the production of GDP-mannose dehydrogenase, which oxidises GDP-mannose to GDP-mannuronate (Gacesa, 1998). Expression from the *algD* promoter has been found to be strongly upregulated on attachment to a surface and during the initial stages of biofilm formation (Hoyle *et al.*, 1993). Edwards & Saunders (2001) were able to demonstrate a 100-fold difference in the copy number of the *algD* mRNA when comparing mucoid and non-mucoid *P. aeruginosa* strains.

2.3.2.4 RpoN (σ^{54})

Conversion of *P. aeruginosa* to mucoidy can occur via a second, *algU*-independent pathway, in which alginate production and transcription of the critical *algD* promoter depend on another alternative σ factor, RpoN (σ^{54}). The *algD* promoters dependent on σ^{54} and σ^E show a complete overlap, resulting in identical mRNA 5' ends (Boucher *et al.*, 2000). The two pathways are, however, not independent as σ^{54} also represses σ^E-dependent transcription of *algD*. The negative regulatory effect of σ^{54} on σ^E-dependent *algD* expression is based on σ^{54} binding to the *algD* promoter and its interference with σ^E-dependent transcription. σ^{54} lacks an intrinsic ability to form open transcription initiation complexes and the repression of *algD* by σ^{54} also depends on environmental conditions. It therefore appears that σ factor antagonism plays a physiological role in controlling alginate production in *P. aeruginosa* during adaptation to different ecological states (e.g. biofilm development, stress and other growth conditions). There is a strong possibility that *algD* transcription is directed by σ^E (AlgU) in *mucA* mutant mucoid strains and by σ^{54} (RpoN) in muc23 mutant mucoid *P. aeruginosa* from promoters with overlapping recognition sequences (Boucher *et al.*, 2000).

2.3.3 Physicochemical factors regulating *algD* expression

The regulation of alginate synthesis has been found to be influenced by a number of environmental factors including the presence of ethanol in the growth medium (DeVault *et al.*, 1990), the presence of glucose in the growth medium (Ma *et al.*, 1997; DeVault *et al*, 1991), growth medium osmolarity (Berry *et al.*, 1989), the presence of a surface for

attachment (Dalton *et al.*, 1994; Davies *et al.*, 1993), oxygen tension (Leitão & Sá-Correia, 1997), growth temperature (Leitão *et al.*, 1992; Leitão & Sá-Correia, 1993), heat shock (Schurr *et al.*, 1995), growth phase (Leitão & Sá-Correia, 1995), pH and nitrogen or phosphate limitation (Berry *et al.*, 1989). These environmental factors have been found to regulate alginate biosynthesis either by acting directly or indirectly on transcription from the *algD* promoter.

2.3.3.1 Ethanol

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). Prolonged exposure to ethanol was shown to be capable of inducing the conversion to mucoidy of the *P. aeruginosa* PAO1 or 8821 strains. The mechanism by which ethanol exerts its effect on the cells is believed to be dependent on DNA gyrase (DeVault *et al.*, 1990) causing localised supercoiling of the *algD* promoter region. The mechanism by which ethanol exerts this effect is, however, believed to differ from that observed for osmotically-shocked cells as ethanol-induced activation of the *algD* promoter is not observed in *E. coli*, presumably because of the lack of a signal-transducing protein in *E. coli* (DeVault *et al.*, 1990). Edwards & Saunders (2001) were able to demonstrate that there was no significant change in the expression levels of either *algU* or *mucA* when *P. aeruginosa* PAO1 was grown in 3% ethanol. However, an increase in the level of *algD* and decrease in the level of *mucB* expression was observed. *P. aeruginosa* showed a log difference in *algD* mRNA levels with the copy number increasing from 1.7×10^5 copies to 6.5×10^6 copies. The NCTC *alg-* strain used in the same study showed a two log increase with *algD* mRNA increasing from 2.2×10^5 copies to 2.7×10^7 relative copies. A decrease of half a log in *mucB* expression was observed in both type strains. This suggests that *mucB* may not have a regulatory effect on *algU* transcription. Exposure to 1% ethanol had no effect on transcription of any of the alginate genes.

2.3.3.2 Oxygen tension

Alginate production and an increase in *algD* transcription have been demonstrated for *P. aeruginosa* FRD1 under strict anaerobic conditions (Hasset, 1996). Alginate production and *algD* transcription levels were found to increase when nitrate was used as the terminal electron acceptor and the addition of purified alginate was found to cause a decrease in

growth, suggesting that alginate further contributes to oxygen limitation for mucoid *P. aeruginosa* strains. Conversely Leitão & Sá-Correia (1997) have demonstrated up-regulation of transcription of the alginate genes *algA*, *algC* and *algD* with increasing oxygen tension. The mRNA levels of *algA*, *algC* and *algD* genes were found in this study to increase, co-ordinately, in cells of the highly mucoid *P. aeruginosa* 8821M strain grown under increasing dissolved oxygen tensions (DOT) of up to 70% of air saturation. Despite the up-regulation of alginate gene transcription by DOT above 10% of air saturation, the activities of the encoded enzymes were either maintained or decreased at high oxygen tensions, leading to a slight decrease in alginate synthesis. This may be due to the oxidative inactivation of alginate enzymes.

2.3.3.3 Growth phase

Alginate synthesis by a highly mucoid *P. aeruginosa* strain has been found to be growth phase dependent (Leitão & Sá-Correia, 1995). Alginate production per unit of biomass was found by the aforementioned authors to reach maximum values in the deceleration phase of growth; however, the degree of polymerisation was found to increase as batch growth proceeded, reaching maximum values at the stationary phase of growth. The activity of the four enzymes leading to GDP-mannuronic acid formation, phosphomannose isomerase, phosphomannomutase, GDP-mannose pyrophosphorylase and GDP-mannose dehydrogenase peaked earlier in the late exponential phase of growth. Growth phase dependent activity of alginate biosynthetic enzymes correlated with the level of transcription of the encoding alginate genes *algA*, *algC*, and *algD* during growth. The pattern of coordinate transcriptional growth phase regulation of these alginate structural genes concurs with the growth dependent transcription of the regulatory gene *algR*.

2.3.3.4 Growth temperature

Leitão & Sá-Correia (1993) were able to demonstrate a change in both the yield and the properties of alginate synthesised by *P. aeruginosa* by both genetic and growth temperature manipulations. An increase in growth temperature was found to significantly affect alginate synthesis in a mucoid *P. aeruginosa* variant (8821M) while alginate synthesis in a nonmucoid isolate (8821) was found to be temperature independent (Leitão *et al.*, 1992). Furthermore, the nonmucoid isolate presented the highest specific growth rates over the range of temperatures used indicating the possibility of competition between growth and alginate synthesis for precursors and energy. The specific growth rates of cells expressing *algA* or *algD* genes from

recombinant plasmids were also found to be lower than those obtained from control cells harbouring only the cloning vector (Leitão & Sá-Correia, 1993). This is comprehensible as the result of the metabolic burden associated with the deviation of cell resources (precursors, energy and enzymes) for plasmid replication and transcription of the genes present. This burden was found to vary with environmental conditions. Under conditions of high osmolarity, growth at elevated temperature induces alginate synthesis in wild-type nonmucoid *P. aeruginosa* PAO1 (Schurr *et al.*, 1995).

2.3.3.5 Heat shock

One of the principle regulatory elements governing the conversion to mucoidy of *P. aeruginosa* is AlgU, an alternative sigma factor that is 66% identical to and functionally interchangeable with σ^E from *E. coli* and *Salmonella typhimurium* (Martin *et al.*, 1994). σ^E has been implicated in the expression of systems enhancing bacterial resistance to environmental stress. Two of the five *P. aeruginosa algU* promoters have been found to be inducible by heat shock in wild-type nonmucoid *P. aeruginosa* PAO1 (Schurr *et al.*, 1995). Furthermore, another AlgU-dependent promoter, the proximal promoter of *algR*, was also found to be induced by heat shock (Schurr *et al.*, 1995). Edwards & Saunders (2001), using RT-PCR, demonstrated that there was no response of *algD* or *algU* expression after heat-shocking conditions were applied, and no conversion to mucoidy was observed.

2.3.3.6 High osmolarity

Transcriptional activation of *algD* increases due to increases in osmolarity [with the exception of cells 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). Sequence homology between the *P. aeruginosa algR* gene and the *E. coli ompR* gene, which regulates the cellular response to changes in osmolarity of the growth medium, together with the abnormally high levels of Na^+ and Cl^- in respiratory tract fluid in CF patients suggested that high osmolarity in the lung of the CF patient might be a signal contributing to the induction of alginate synthesis (mucoidy) in infecting *P. aeruginosa*. This prompted Berry *et al.* (1989) to conduct a range of experiments in which they found that transcriptional activity of *algD* increased as the osmolarity of the culture medium increased. The increased activation of *algD* at high osmolarity was not, however, sufficient to induce alginate synthesis in nonmucoid strains suggesting that other environmental factors are involved in full activation of the alginate genes. Maximum transcription of *algD* was obtained at a concentration of 0.35 M NaCl or

KCl in YTG medium (5 g of yeast extract, 10 g of tryptone, 2 g glucose per litre). Velasco *et al.* (1995) have shown that *P. aeruginosa* PAO1 resists NaCl concentrations as high as 0.7 M, with K^+ accumulation and glutamate synthesis being the first mechanisms involved in adaptation to osmotic stress. Furthermore, the same authors demonstrated that betaine modulates intracellular glutamate levels in osmotically stressed *P. aeruginosa* PAO1.

2.3.3.7 Glucose

DeVault *et al.* (1991) suggested that growth of nonmucoid *P. aeruginosa* PAO1 in glucose-containing medium represses *algD* gene transcription. From the results of this experiment, it was postulated that the environmentally responsive *algD* promoter was interacting with a CRP (cyclic AMP receptor)-like analogue, similar to that in *Xanthomonas campestris*, causing a bending of the DNA that could influence *algD* transcriptional activity. Ma *et al.* (1997) found that growth of a mucoid *P. aeruginosa* isolate (FRD1) in rich medium containing glucose (1 to 5%) or gluconate increased alginate production and *algD* transcription. Conversely, *P. aeruginosa* PAO1 was found to exhibit an increase in *algD* transcription at concentrations of 0.5 and 1 %, while growth in media containing 2 % and to a greater extent, 5 % glucose, caused a decrease in *algD* transcription.

P. aeruginosa does not metabolise glucose via the EMP pathway due to lack of 6-phosphofructokinase (Leslie & Phibbs, 1984). Glucose is converted by one of two routes; either by oxidation to gluconate and 2-ketogluconate in the periplasm by membrane-bound glucose and gluconate dehydrogenase, or by uptake by an inducible transport system and phosphorylation by glucokinase to glucose 6-phosphate and then to 6-phosphogluconate by glucose-6-phosphate dehydrogenase – the *zwf* gene product (Hunt & Phibbs, 1983). *P. aeruginosa* therefore uses a cyclic version of the Entner-Doudoroff pathway to metabolise carbohydrates (Hager *et al.*, 2000). The enzymes are clustered in three operons around 39 min on the chromosome. They all appear to be under the control of the *hexR* repressor, and are therefore termed the *hex* regulon (Hager *et al.*, 2000; Ma *et al.*, 1998). HexR is a repressor inactivated by carbohydrates such as glycerol, fructose, mannitol, glucose and gluconate. Those members of the pathway responsible for the metabolism of glucose to glyceraldehyde-3-phosphate and pyruvate are co-ordinately regulated by these sugars. Where succinate is the sole energy source, the *hex* regulon is repressed (Hager *et al.*, 2000). TCA cycle intermediates (organic acids such as succinate) are used preferentially to sugars, but the regulation is in a cAMP-independent manner. Adenylate cyclase activity does not fluctuate with the carbon

source, and the only phosphotransferase system known is for fructose (Hester *et al.*, 2000). A repressor, Crc, was found to repress carbohydrate metabolism in the presence of TCA intermediates, although its mechanism is unknown to date (MacGregor *et al.*, 1996; Wolff *et al.*, 1991).

It is clear from the findings above that alternate pathways for catabolism of glucose to the central metabolite 6-phosphogluconate exist in *P. aeruginosa*. Ma *et al.* (1997) clearly showed that growth on glucose or gluconate, leading to the production of 6-phosphogluconate, increases alginate biosynthesis and *algD* transcriptional activity. The conflicting results of these two authors may be the result of differences between laboratory strains that have been passaged multiple times or alternatively due to catabolite repression control.

CHAPTER 3

Pseudomonas aeruginosa DISPLAYS TWO BIOFILM-RELATED PHENOTYPES DISTINCT FROM THE PLANKTONIC STATE

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

3.1 SUMMARY

Biofilm-associated bacterial cells are known to display a unique phenotype distinct from that of free-living or planktonic cells. Suspended cells of *P. aeruginosa* PAO (DSM 1707) growing in the presence of a biofilm (surface-influenced planktonic or SIP cells) were compared to planktonic cells. The yield (culturable count, optical density and total protein), cell volume and alginate levels of biofilm, SIP and planktonic cells grown in batch culture for 16 h in LB broth without NaCl (LB-S), with 0.7 M NaCl (LB + NaCl) and with 2.5% (v/v) ethanol (LB + EtOH) in the presence of glass wool were determined. The biofilm and SIP phenotypes were different to each other, and both differed from the planktonic population. The SIP population was not a mixture of planktonic and detached biofilm cells but rather a distinct physiological state. Furthermore, evidence is presented for the presence of diffusible signals produced by the biofilm that give rise to the "detachment" (SIP) phenotype.

3.2 INTRODUCTION

The biofilm mode of bacterial growth has gained increased attention, and has become a core paradigm in the study of bacterial behaviour (Watnick & Kolter, 2000). Biofilms are believed to develop from single, surface-adherent cells to form structured consortia embedded in a gelatinous polymer matrix (O'Toole *et al.*, 2000). Bacteria growing, as biofilms are known to display a unique phenotype distinct from that of free-living or planktonic cells. They are generally more resistant to antimicrobial agents (Stewart, 1996), express a range of genes differentially to their planktonic counterparts (O'Toole *et al.*, 2000), and consequently have a unique proteome (Steyn *et al.*, 2001). Recent evidence indicates that bacteria have a range of environment-specific approaches to develop biofilms. For example, cell morphology and biofilm structure of the Gram-negative marine isolate SW5 are markedly influenced by the surface hydrophobicity of the substratum (Dalton *et al.*, 1994). Whereas type IV pilli is required for maturation of *P. aeruginosa* biofilm under quiescent conditions (O'Toole *et al.*, 1998), they are not necessary for development under conditions of flow (De Kievit *et al.*, 2001).

A fundamental concept that requires refining in the context of biofilm studies is the suspended state. Planktonic cells have been defined as cells that have never existed in the attached state before (Rice *et al.*, 2000). By contrast, suspended cultures that have been in contact with a surface during their recent history, or have grown in the proximity of a surface have been termed surface-influenced planktonic (SIP) cells (Steyn *et al.*, 2001). Biofilms of *P. aeruginosa* have been studied under conditions of flow, primarily by using various flow cells (Zinn *et al.*, 1999), or in stagnant batch culture using microtitre plates (O'Toole *et al.*, 1999). The latter approach has been used primarily for purposes of screening of genetic mutants. In microtitre plate-grown cultures, the suspended cells are exposed to the surrounding biofilm for the duration of the experiment, therefore being SIP and not planktonic cultures. By contrast, suspended cells leaving flow cells have all been shed from the biofilm, as the cells are fed with sterile broth following an initial inoculation and a short quiescent period to allow for attachment. Cells leaving flow cells are therefore detached cells and not planktonic in the strict sense.

It has been recently demonstrated that SIP cells of *P. aeruginosa* grown in batch culture in the presence of glass wool as substratum display a proteome distinct to both the planktonic and biofilm proteomes (Steyn *et al.*, 2001). The data presented by Steyn *et al.* (2001) indicated

four expression patterns for planktonic, SIP or biofilm populations using two-dimensional gel electrophoresis. These consisted of: (1) genes that are expressed only in a particular population; (2) genes that are expressed in planktonic cells, but are unchanged in either SIP or biofilm populations; (3) genes that are expressed in planktonic cells, that are not expressed in SIP cells, but over-expressed in biofilm cells; and (4) genes that are expressed in planktonic cells, are under-expressed in SIP cells, but over-expressed in biofilm cells. They reported that the latter appears to be the most common expression pattern.

The objectives of this work were therefore to study the physiological parameters of biofilm and surrounding suspended (SIP) cells grown in batch culture in the presence of glass wool, and to compare these to planktonic cells under conditions of low and high salt concentrations, as well as in the presence of a dehydrating agent. In the data presented, the SIP population is demonstrated to have unique physiological characteristics that are in part controlled by the biofilm population. This study lays the groundwork necessary for the future study of this unique mode of growth.

3.3 MATERIALS AND METHODS

3.3.1 Bacterial strains, media and growth conditions

P. aeruginosa PAO (DSM 1707) (Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany) was grown in Luria-Bertani (LB) broth (0.5% [w/v] yeast extract; 1% [w/v] tryptone) in the absence of NaCl (LB-S), unless specifically indicated. When required, NaCl was added to a final concentration of 0.7 M (LB + NaCl) and ethanol to a final concentration of 2.5% (v/v) (LB + EtOH). The NaCl and EtOH concentrations used were based on initial experimentation using planktonic cultures (Fig 5.3 & 5.4). Cultures were incubated at 37°C with agitation at 200 rpm until mid-exponential phase (OD_{540} = *ca.* 0.5) was reached and then diluted to OD_{540} = 0.1 in pre-warmed (37°C) LB-S. Aliquots (4 ml) were transferred to 10 ml glass test tubes with or without 0.2 g of glass wool (Merck, Darmstadt, Germany; mean standard diameter = 10 μm). All cultures were incubated at 37°C with agitation for 16 h.

Cultures grown in the presence of glass wool (total attached biomass) were separated into SIP and biofilm biomass by gently removing the liquid phase from the test tubes with a 1 ml micropipette. Fresh sterile broth (4 ml) was added to the glass wool-attached biomass matrix and then vortexed vigorously 3 times for 30 s each to remove the biofilm biomass from the glass wool substrate. Removal of the biofilm from the glass wool and separation of the

biofilm matrix into single cells was confirmed by bright-field microscopy using a Zeiss Axioskop microscope and Zeiss Achrostigma 40X or 100X objective (Zeiss, Oberkochen, Germany). Images were captured using a COHU monochrome CCD camera (RS-170, Cohn Inc., San Diego, California), and processed using Psion Image and CorelDRAW 7™ software.

3.3.2 Analytical Methods

3.3.2.1 Spectrophotometric determination of biomass yield

All analytical determinations were performed on three separate samples and on three separate occasions so that all values given represent the average of nine separate samples. The OD_{540} of 1 ml of culture was determined in a 1 ml cuvette with a path length of 1 cm using a Spectronic 20 Genesis™ spectrophotometer. The respective sterile culture media were used as blanks. Aliquots of the 1 ml of culture used here were subsequently used in assays to determine total culturable counts, total protein concentration and cellular volume.

3.3.2.2 The culturable count

Serial dilutions were performed immediately in ddH_2O and plated in triplicate on LB-agar plates. The colonies were counted after incubation of the plates at 37°C for 18 h.

3.3.2.3 Determination of total protein concentrations

Samples of the cultures (500 µl) were collected by centrifugation at 16 000 × g for 5 min, and the supernatant removed. The bacterial cell pellets were resuspended in 500 µl of ddH_2O, sonicated 3 times for 20 s each using a Cole Palmer Ultrasonic Homogeniser 1700 Series probe and boiled at 95°C for 10 min. Protein concentrations were determined by the Bradford assay (Bradford, 1976) using the Coomassie® Plus Protein Assay Reagent (Pierce, Rockford, IL, USA). Briefly, 50 µl of sample was mixed with 1.5 ml of the Coomassie® Plus Protein Assay Reagent and the A_{595} measured. Protein concentrations were determined from a standard curve prepared for bovine serum albumin (BSA) ranging from 0 to 1 500 $\mu g.ml^{-1}$.

3.3.2.4 Determination of cellular volumes

Cell suspensions (10 µl) were pipetted on to glass slides and covered with a cover slip. Three different fields for each of three replicate cultures were viewed using 1000 X magnification and images were captured as described above. For calibration purposes, a micrometer was

photographed under the same magnification. Three cells in each image were randomly chosen and their length and width (excluding extracellular polymeric substances) measured. The cellular volume was then calculated using the following formula:

$$4/3\ \pi r^3 + (l\text{-}2r)\ \pi r^2$$

The dimensions of cells from a planktonic culture grown to mid-exponential phase (A_{540} = *ca.* 0.5) were determined in an identical manner. Cell volumes following the various growth conditions were compared to each other by multifactor ANOVA (Statgraphics 7.0) at the 95 % confidence level.

3.3.3 Growth rate

An increase in cellular volume of *Escherichia coli* has been reported to be accompanied by an increase in the growth rate of the cell (Hussain *et al.*, 1987), and can be calculated using the following equation:

$$V = V_o e^{(\mu \ln 2)}$$

where μ is the specific growth rate, e is a constant and V_o corresponds to a hypothetical cell volume at $\mu = 0\ h^{-1}$, and can be determined experimentally. Under controlled conditions, cellular volume (V) is directly proportional to the growth rate of the culture. For *E. coli*, V_o is 0.4 μm^3 (Hussain *et al.*, 1987), for *Pseudomonas putida* V_o is 0.9 μm^3 (Møller *et al.*, 1995), but V_o for *P. aeruginosa* is not known.

3.3.4 Alginate assay

3.3.4.1 Sample preparation

Alginate assays were carried out as reported by May & Chakrabarty (1994), with the following minor modifications. Samples (500 µl) were centrifuged at 16 000 × g and the supernatant containing the alginate recovered. The cell pellet was washed 3 times in 0.5 ml of saline solution (0.9%), the supernatants pooled, and added to the culture supernatant. The cell pellet was used for determination of total protein concentrations. The supernatant obtained from each culture was divided into 500 µl aliquots, supplemented with 3 volumes of ice-cold 95% ethanol and incubated overnight at -70°C. The samples were then centrifuged for 15 min at 13 700 × g and the supernatant discarded. The pellet was washed twice with 95% ethanol and once with absolute ethanol before being vacuum dried. Each pellet was resuspended in 250 µl of ddH_2O and the replicate samples combined. Samples were dialysed (Pierce, Snakeskin pleated dialysis tubing, MWCO 10 000 Da) for 24 h against 3 changes of ddH_2O, lyophilised and resuspended in 70 µl of ddH_2O.

3.3.4.2 Determination of uronic acid concentrations

Alginate concentrations were determined using a modified carbazole assay (Knutson & Jeanes, 1968). A borate stock solution was prepared by dissolving 12.37 g of H_3BO_3 in 45 ml of 4 M KOH and diluting to 100 ml with ddH_2O. A borate working solution was prepared daily by diluting the above stock solution 1:20 with concentrated H_2SO_4. A 0.1% (w/v) carbazole (Sigma, Atlasville, South Africa) solution was prepared in absolute ethanol. The borate working solution was equilibrated in an ice-water bath and the sample, prepared as described above, was then carefully layered on top of 600 µl of ice-cold borate working solution. The sample mixture was cooled, vortexed for 4 s, and immediately returned to the ice-water bath. Following the addition of 20 µl of the carbazole solution on top of the reaction mixture, it was again cooled, vortexed for 4 s, and returned to an ice-water bath until all samples were processed. The reaction mixture was finally incubated at 55°C for 30 min and the uronic acid concentration was determined from a standard curve obtained for seaweed alginate (BDH Chemicals, Poole, England) ranging from 10 to 1 000 $\mu g.ml^{-1}$. Uronic acid content was expressed per femtogram cell protein.

3.3.5 Investigation of the presence of a diffusible signal molecule

P. aeruginosa PAO (DSM 1707) was grown in LB-S to mid-exponential phase and then diluted as described above under growth conditions. Aliquots (2 ml) were transferred to 20-ml McCartney bottles with or without 0.1 g of glass wool. For cultures to be grown in the presence of glass wool, a dialysis chamber (Pierce, Snakeskin pleated dialysis tubing, MWCO 10 000D) containing 2 ml of the starter culture in LB-S was added. As a negative control, an identical dialysis chamber was also added to cultures grown in the absence of glass wool. All cultures were incubated for 16 h with shaking at 37°C. The cultures were analysed by bright-field microscopy, and cellular volumes of cells inside the dialysis chamber were determined as described above. Cultures from dialysis chambers exposed to glass wool biofilms were termed artificial SIP populations. The experimental setup for these investigations is indicated in Fig. 3.1.

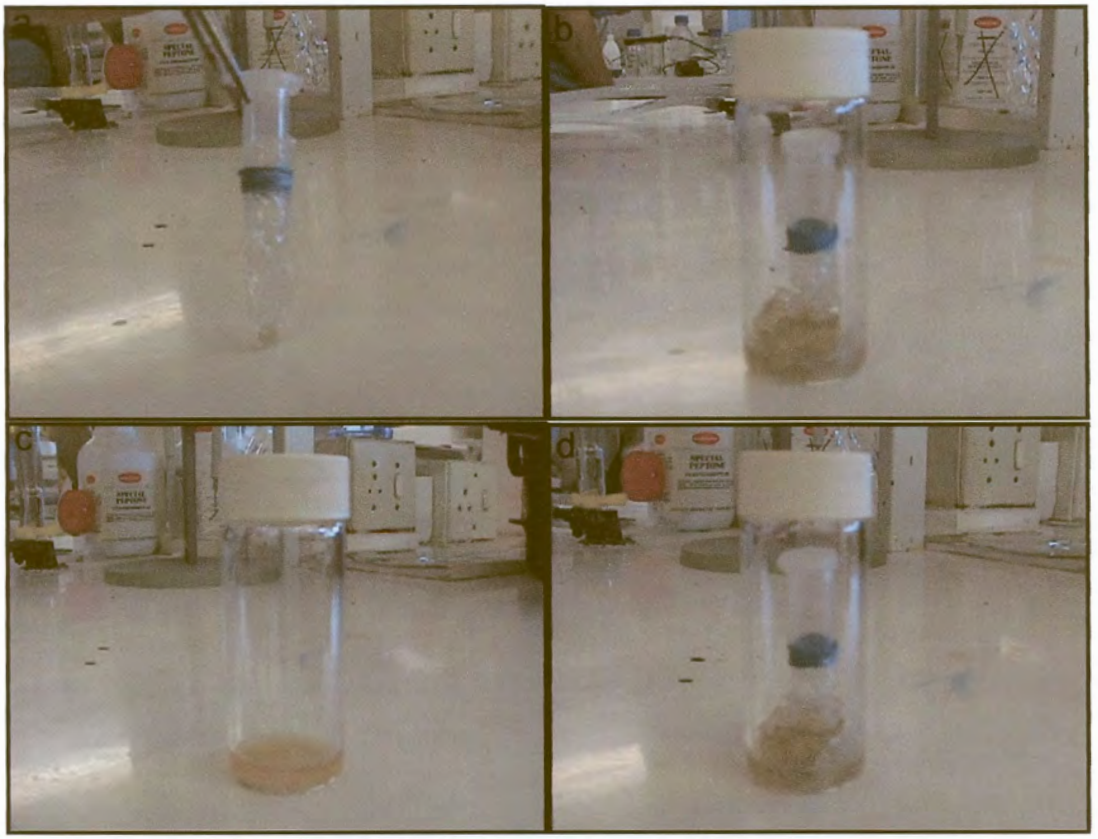

Fig. 3.1: Photographs of (a) the dialysis chamber, (b) negative control without glass wool, (c) planktonic cells, and (d) artificial SIP cells used to determine the possible involvement of a diffusible signal molecule in the physiological differentiation of SIP populations in LB-S.

3.4 RESULTS

3.4.1 Biofilm formation by *P. aeruginosa* after culturing in LB-S, LB + NaCl and LB + EtOH

P. aeruginosa formed biofilm within 16 h following inoculation into LB-broth with and without NaCl or ethanol (Figs. 3.2, 3.3 and 3.4), showing that glass wool is a suitable surface for studying biofilms of this organism. The high surface-to-volume ratio of glass wool (70 cm^2. ml^{-1} broth) yielded sufficient biomass for a range of quantitative biochemical analyses. Attached biomass could easily be separated from the surrounding SIP cells, and biofilm cells could then be removed from the glass wool and efficiently dispersed by vortexing, with no cells remaining on the glass wool (Fig. 3.2c and d).

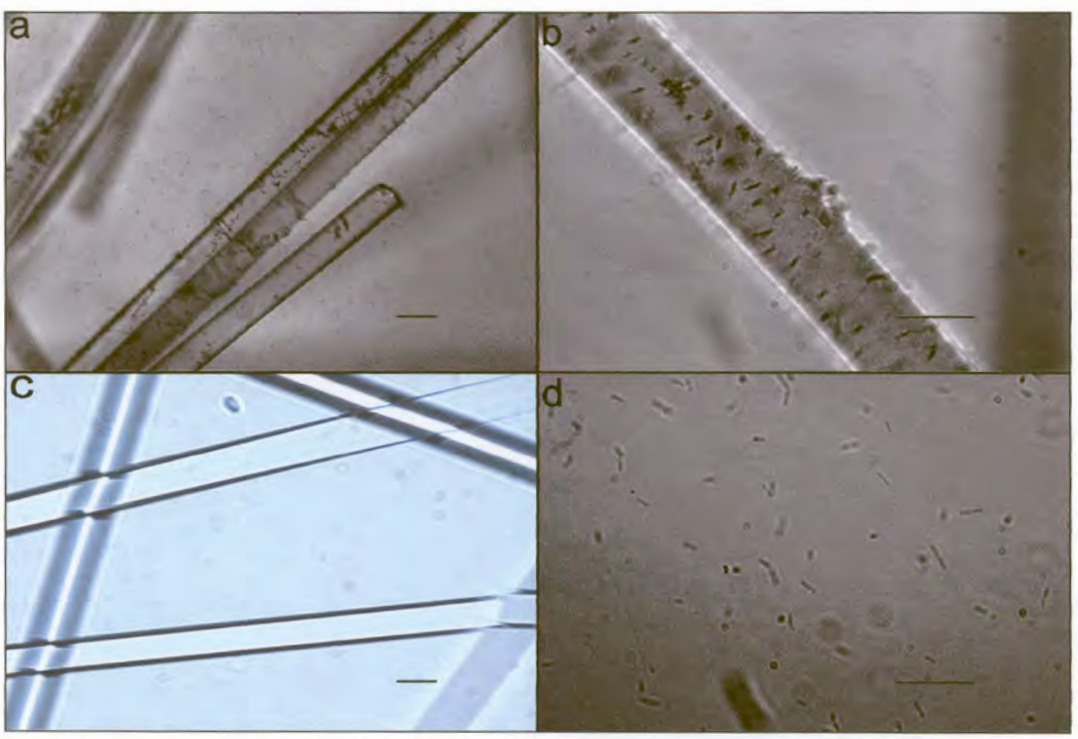

Fig. 3.2: Photomicrographs showing *P. aeruginosa* PAO (DSM 1707) biofilm on glass wool after 16 h of incubation in LB-S. Fig. 3.2a shows biofilm and SIP cells. Fig. 3.2b shows a *P. aeruginosa* biofilm after separation of the attached biomass from SIP cells. The efficacy of removal and dispersal of biofilm from glass wool by vortexing is demonstrated in Figs. 3.2c and 3.2d. Bars = 10 µm.

Compared to the biofilm cells of *P. aeruginosa* cultures, the biofilm cells, of cultures grown in LB-broth containing 0.7 M NaCl (Fig. 3.3) appeared to be smaller and shorter.

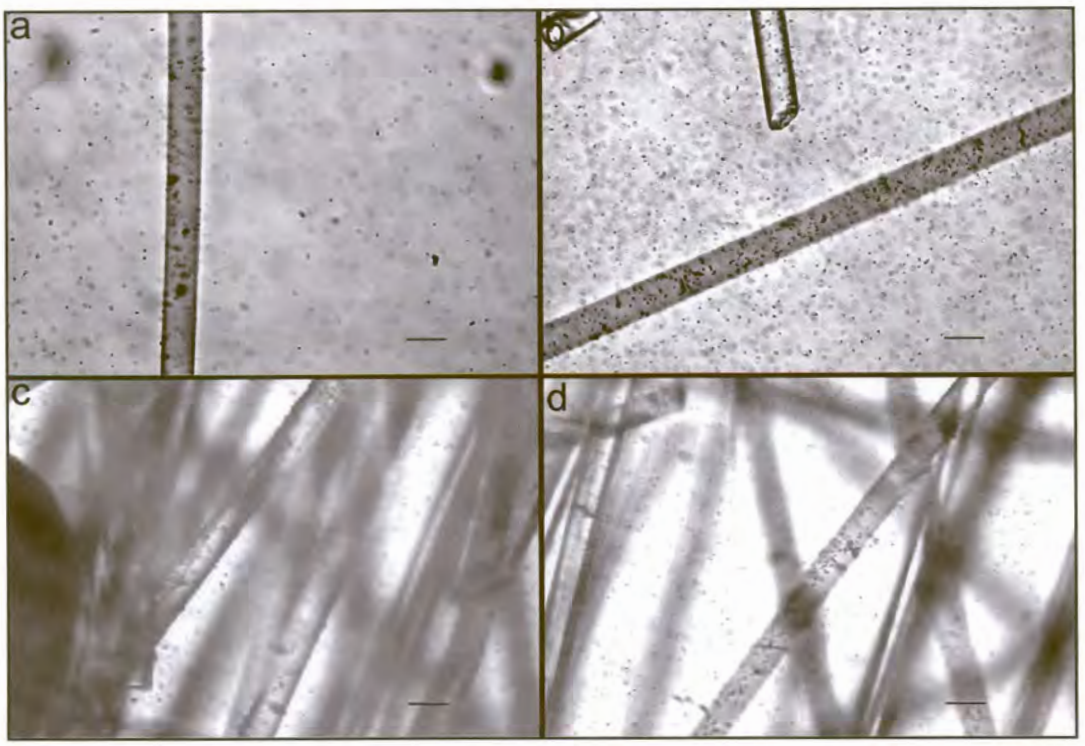

Fig. 3.3 (a - d): Photomicrographs showing *P. aeruginosa* biofilm on glass wool after 16 h of incubation in LB + NaCl. Bar = 10 µm.

Planktonic and biofilm cells grown in LB + EtOH appeared at first to be larger than cells cultured in either LB-S or LB + NaCl. This was, however, misleading as the cells occurred in chains (Fig. 3.4). Septa in these chains of cells were clearly visible using fluorescence microscopy after staining of cells with either a Gamma Proteobacterial- or *Pseudomonas* Group 1-specific 23S rRNA probe (Amman *et al.*, 1995; Amman *et al.*, 1996; Appendix 2). Biofilm formation was drastically affected by the production of copious amounts of EPS (Fig. 3.4g). The cells were found to form large mats within the EPS matrix, covering parts of the surface and also extending into the liquid phase (Fig. 3.4c and h). These mats apparently originated from one microcolony that grew in size and sent fronds out into the medium (Fig. 3.4d, e and f).

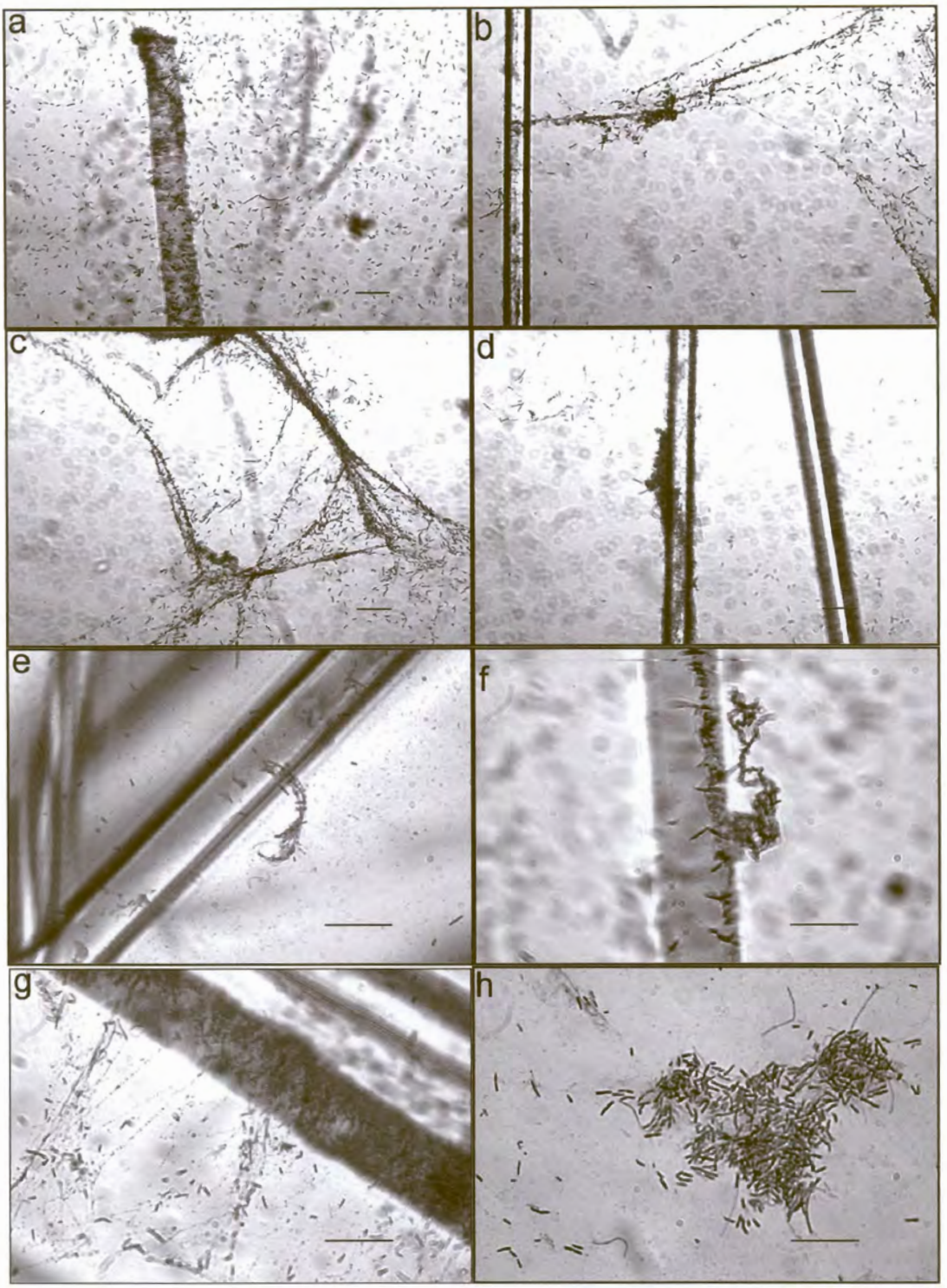

Fig. 3.4 (a-h): Photomicrographs showing *P. aeruginosa* biofilm on glass wool after 16 h of incubation in LB + EtOH. Bar = 10 µm.

3.4.2 Variation in biomass

As seen by the standard deviation in the culturable count (Fig. 3.5), optical density (Fig. 3.6), total cellular protein concentration (Fig. 3.7) and uronic acid concentration (Table 3.1), 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 trends seen in single samples were the same as those seen in the triplicate samples (data not shown). 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.

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

P. aeruginosa grown in LB-S and in the presence of glass wool (total attached biomass) yielded double the culturable count compared to planktonic cultures (9.22E+09 compared to 4.71E+09). There were 1.8-fold more cells in the SIP environment compared to the planktonic environment. The biofilm population corresponded to 16% of the planktonic population (Fig. 3.5), and represents 8.5% of the total cell population grown in the presence of glass wool (Fig. 3.6). Although the culturable counts correlated well with the obtained optical density values (Fig. 3.6), the observed differences may have been due to differences in cell volume under the different growth conditions (Fig. 3.8). The larger the difference in cellular volume between growth phases, the larger the difference in the correlation was found to be.

Similar to the results obtained for *P. aeruginosa* cultures grown in LB-S, the SIP population grown in LB + NaCl also displayed a higher culturable count when compared to the planktonic population grown in LB + NaCl. This correlated with the increase in optical density (Fig. 3.6). However, high culture broth osmolarity appeared to have affected either the culturability of both planktonic and biofilm cells or the $cfu.ml^{-1}$ due to the formation of chains of cells (Appendix 2). Culturable counts therefore did not correlate with the optical density of these cultures (Fig. 3.6). The planktonic population had an inferred approximate 30% decrease and the biofilm population an approximate 65% decrease in culturability compared to the SIP population (calculations are explained below under cellular volume as a function of total cellular protein).

Approximately 50% of the cells grown in the presence of a glass wool surface and 2.5% (v/v) ethanol (LB + EtOH) were attached to the surface, a significantly higher proportion than

when grown in either LB-S or LB + NaCl (Fig. 3.6). The SIP population displayed a lower culturable count than the planktonic population (1.6-fold), unlike those grown in LB-S and LB + NaCl (Fig. 3.5). These differences correlated well with the differences observed in the optical density of the cultures (Fig. 3.6). The biofilm population had 1.3-fold less cells than the planktonic population. Ethanol did not seem to have any effect on the culturability of the cells, however, overall cfu. ml^{-1} of planktonic and SIP cultures was substantially less than for cultures grown in either LB-S or LB + NaCl (Fig. 3.5).

3.4.4 Total cellular protein

The total protein concentration of cultures grown in LB-S in the presence of glass wool (total attached biomass) was twice that of planktonic cultures, but the biofilm population had a biomass equivalent to *ca.* 16% of the total attached biomass (Fig. 3.7). This is not an unusual phenomenon in LB-S as biomass accumulation at the surface reaches a threshold at *ca.* 15% of the total biomass, or 8-9% of the total cell population. This is evidenced both in minimal medium (Steyn *et al.*, 2001) and LB (R. MacDonald, pers. communication) at any time point between 2 and 26 h of growth. Furthermore, Rice *et al.* (2000) demonstrated that a significant proportion of attached cells detach following the first and second cell division events. This would, however, not be expected to correlate efficiently with the increase in the culturable counts of these samples due to the differences in cellular volume. Compared to the planktonic population, the biofilm population exhibited a 3.5-fold lower total protein concentration, but a 6-fold lower culturable count. The SIP population exhibited a 1.7-fold higher total protein concentration and culturable count when compared to planktonic cultures.

For cultures grown in LB + NaCl, there was 1.3-fold more protein biomass in the SIP population, and 3.6-fold less protein biomass in the biofilm population than in the planktonic population (Fig. 3.7). Cultures grown in the presence of glass wool (total attached biomass) showed a 1.6-fold increase in protein biomass when compared to the planktonic culture. The biofilm population had a biomass *ca.* equivalent to 17.5% of the total biomass of the population grown in the presence of glass wool. The picture presented is very similar to that for cultures grown in LB-S, with the exception of SIP cells. The SIP population did not show as much of an increase in protein biomass compared to the planktonic population, but they also exhibited less of an increase in cell volume.

For cultures grown in LB + EtOH, there was a 1.6-fold decrease in total cellular protein concentration from the planktonic to SIP populations, while the biofilm population showed a

1.7-fold decrease in protein biomass compared to the planktonic population (Fig. 3.7). The biofilm population had a biomass equivalent to *ca.* 48% of the total biomass of the population of cells grown in the presence of a surface. Biomass accumulation at the surface was expected to be only slightly higher than under normal circumstances due to a slight up-regulation of alginate, and possibly other attachment-related genetic elements.

3.4.5 Cellular volume as a function of total cellular protein

Planktonic cultures grown in LB-S to an OD_{540} of 0.45 had a cell volume of 3.254 ± 1.345 μm^3. After 16 h of growth, SIP and biofilm cells showed an increase in cellular volume compared to planktonic populations (Fig. 3.8). The 3.5-fold lower protein concentration of biofilm population (Fig. 3.7) and 6-fold lower yield in culturable counts (Fig. 3.6) were thus compensated for by a 1.8-fold increase in cellular volume compared to planktonic cells. SIP cells, however, had 35% less protein per μm^3 (Table 3.1). This has implications for total biomass measurements, implying that protein concentrations in SIP populations, according to the volume of the cell, should have been closer to 744 $\mu g.ml^{-1}$ (calculated as: [SIP protein concentration ÷ 65 × 100]), if the observed 35% decrease in total protein concentrations per μm^3 is accounted for.

Planktonic cultures grown in LB + NaCl had a slightly larger cell volume (1.08-fold) than those grown in LB-S. Biofilm cells had 1.2-fold less volume than planktonic cells and 1.6-fold less volume than SIP cells (Fig. 3.8). This may have been as a result of the higher osmotic potential at the surface due to accumulation of NaCl at the liquid-surface interface (Marshall, 1992).

In LB + EtOH, as in the case of LB + NaCl, planktonic cells were found to be larger than those grown in LB-S and had a cell volume closer to that of SIP cells cultured in LB-S. There was no significant difference seen in the cell volume between the SIP and planktonic populations (Fig. 3.8). SIP cells did, however, increase in width (Appendix 1). Biofilm populations showed a substantial difference in cell volume, being less than both planktonic and SIP populations. They had 1.5-fold less volume than biofilm cells grown in LB-S. SIP cells were again different from those grown in LB-S and LB + NaCl. The difference in fg protein per μm^3, as seen for LB + NaCl, between SIP and biofilm or planktonic cells was lost

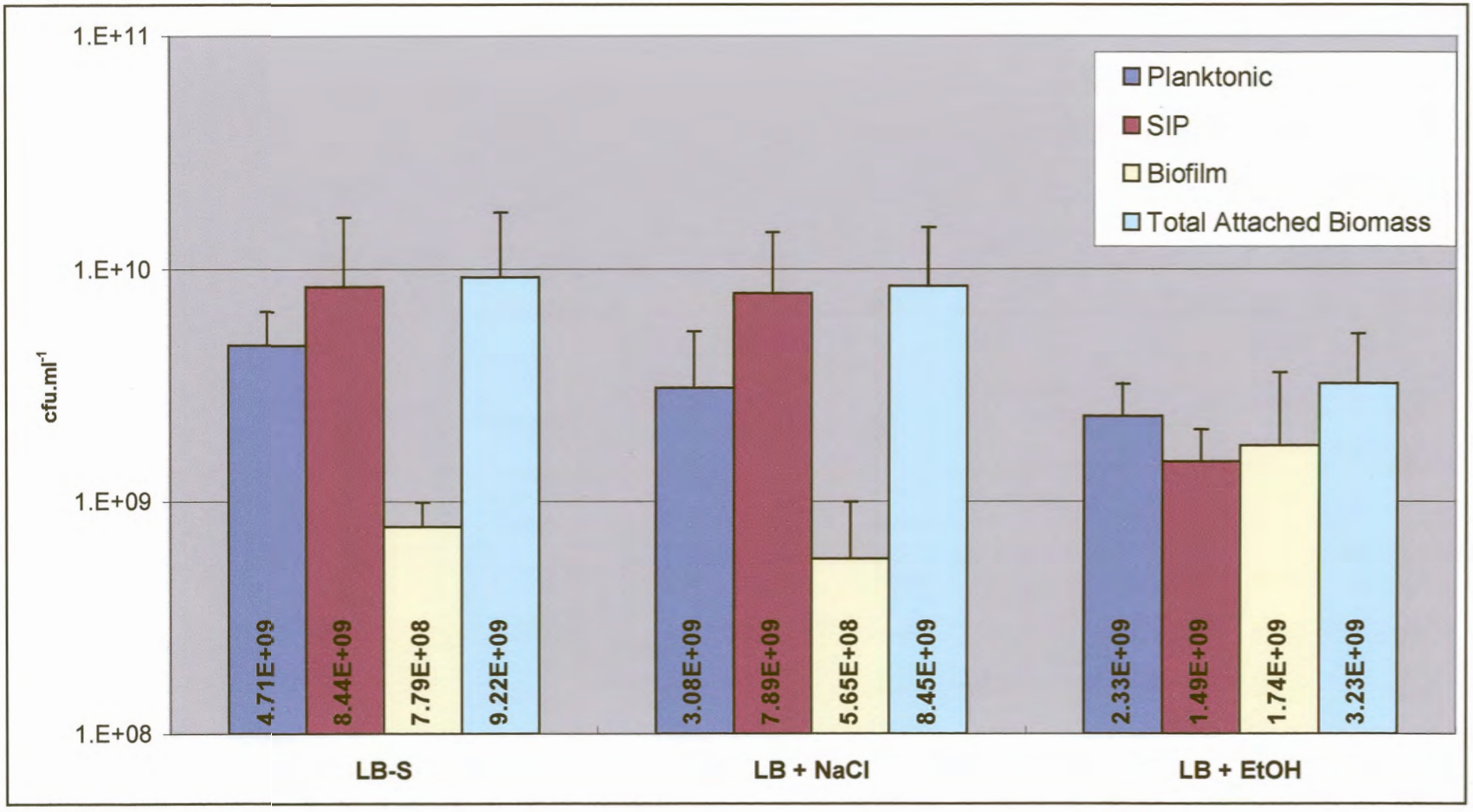

Fig. 3.5: Culturable count of *P. aeruginosa* cultured for 16 h in LB broth without salt (LB-S), with 0.7 M NaCl (LB + NaCl) and 2 % (v/v) ethanol (LB + EtOH). Error bars denote one standard deviation of the mean.

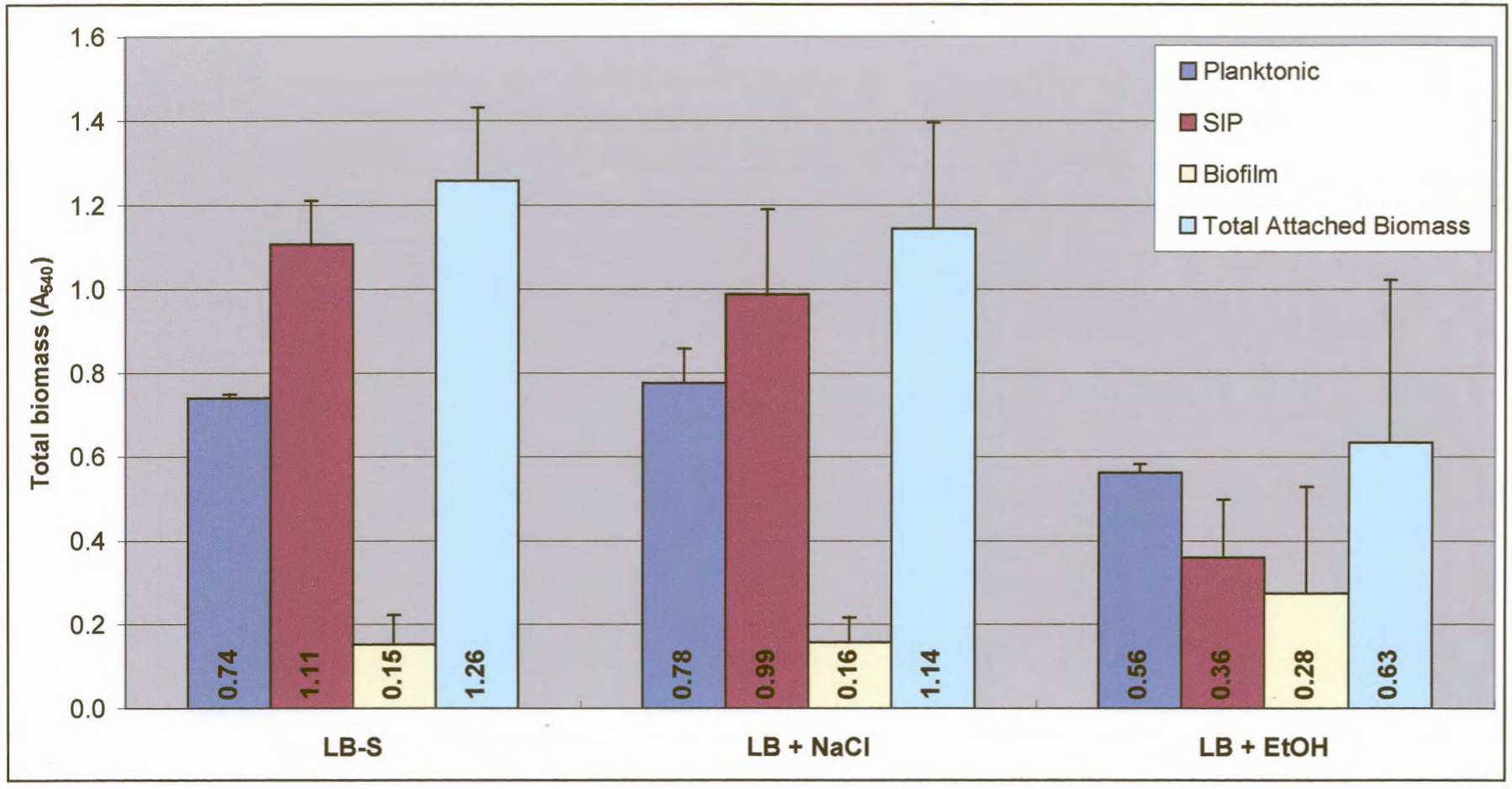

Fig. 3.6: Optical density (A_{540}) of *P. aeruginosa* cultured for 16 h in LB broth without salt (LB-S), with 0.7 M NaCl (LB + NaCl) and 2.5% (v/v) ethanol (LB + EtOH). Error bars denote one standard deviation of the mean.

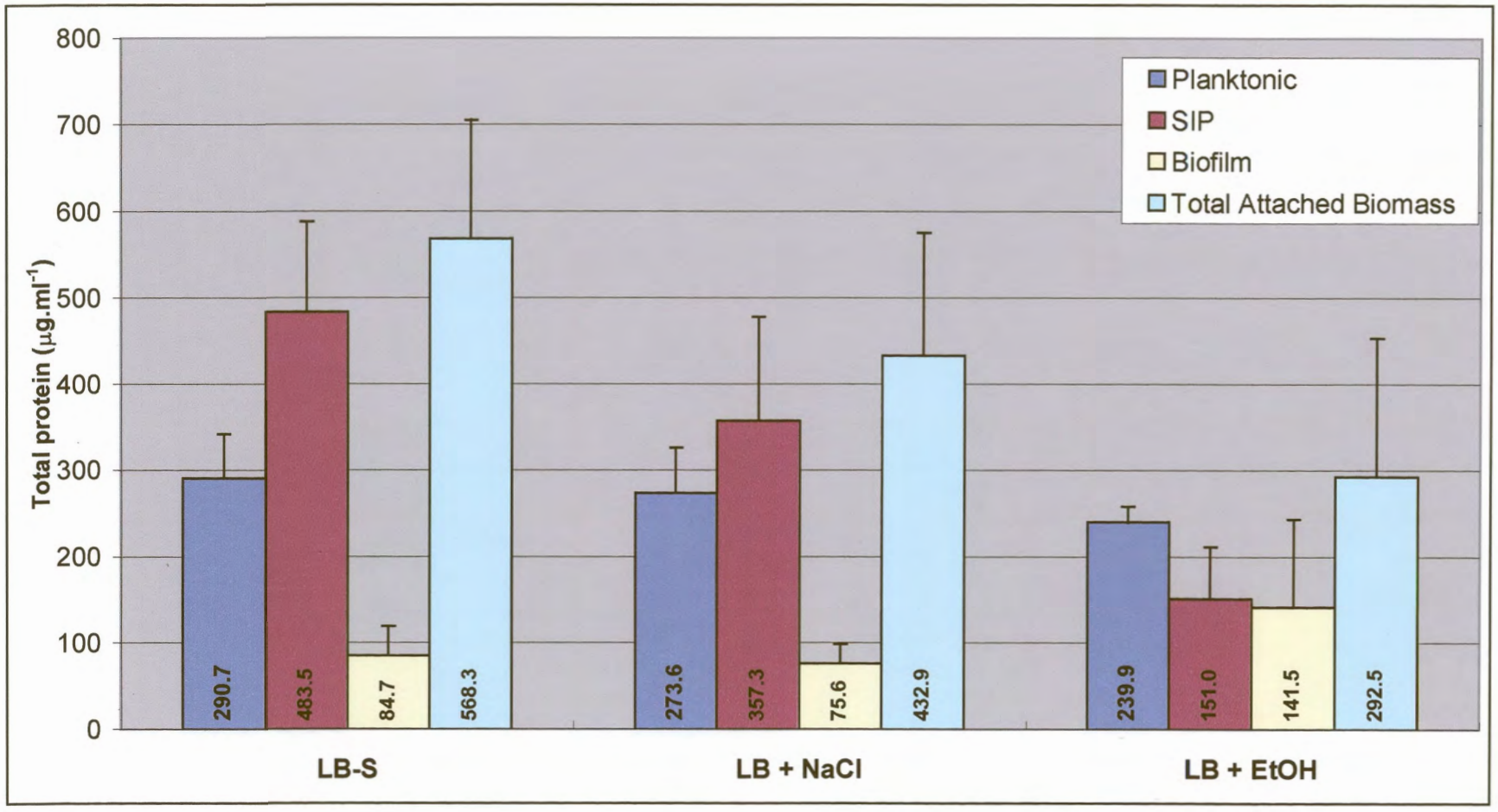

Fig. 3.7: Total cellular protein of *P. aeruginosa* cultured for 16 h in LB broth without salt (LB-S), with 0.7 M NaCl (LB + NaCl) and 2.5% (v/v) ethanol (LB + EtOH). Error bars denote one standard deviation of the mean.

Table 3.1: Protein concentrations per cell and per cell volume, and alginate per unit of cell protein and cell volume for *P. aeruginosa* grown for 16 h in the presence of glass wool in LB broth without salt (LB-S), with 0.7 M NaCl (LB + NaCl) and 2.5% (v/v) ethanol (LB + EtOH)

Culture Medium	**Growth Phase**	**fg protein. cfu^{-1}**	**fg protein. μm^{-3}**	**fg uronic acid. fg protein^{-1}**	**fg uronic acid. μm^{-3}**	**Hypothetical growth rate**
LB-S	Planktonic	61.70 +/- 10.83	33.24 +/- 5.83	0.38 +/- 0.07	12.53	1.00†
	SIP*	57.26 +/- 12.38	21.61 +/- 4.67	0.18 +/- 0.11	3.87	1.43
	Biofilm	108.71 +/- 44.12	33.86 +/- 13.74	0.62 +/- 0.05	21.01	1.73
LB + NaCl	Planktonic	88.90 +/- 16.91	43.32 +/- 8.24	0.44 +/- 0.03	19.11	
	SIP	45.30 +/- 15.22	18.92 +/- 6.36	0.28 +/- 0.08	5.27	
	Biofilm	133.8 +/- 40.03	86.21 +/- 25.8	0.84 +/- 0.49	72.38	
LB + EtOH	Planktonic	103.00 +/- 7.74	34.75 +/- 2.61	1.44 +/- 0.36	50.05	
	SIP	101.64 +/- 40.30	32.54 +/- 12.90	0.93 +/- 0.11	30.09	
	Biofilm	81.20 +/- 58.42	33.44 +/- 24.06	0.87 +/- 0.25	28.95	

* Surface-influenced planktonic culture.

† The smallest volume in each culture medium is assumed to have a growth rate of 1.00.

and there was no overall decrease or increase observed compared to cultures grown in LB-S (Table 3.1).

3.4.6 Growth Rate

Hypothetical growth rates based on cellular volume were used as an indication of the differences in growth rates between growth conditions. This holds true only if V_o remains constant in planktonic, SIP and biofilm samples. There was a 1.7-fold increase in the deduced growth rate of the biofilm population compared to that of planktonic populations in LB-S. SIP cells had an intermediate growth rate, 1.4-fold higher than planktonic populations, or 1.3-fold slower than the biofilm population (Table 3.1). Growth rates could not be calculated or compared for either the LB + NaCl or LB + EtOH environments due to the osmotic effect of NaCl (Zielinski *et al.*, 1992) and the dehydrating effect of ethanol (DeVault *et al.*, 1990).

3.4.7 Uronic acid concentrations

Uronic acid concentrations are more correctly expressed per μm^3 cell volume than per fg protein (a measure of biomass) due to the 35% decrease in protein concentration per μm^3 seen in SIP cells when cultured in LB-S (Table 3.1). The biofilm population showed an approximate 40% increase in alginate compared to the planktonic population, and an 80% increase compared to the SIP population. When taken individually, the SIP population had 70% less alginate than the planktonic population (calculated as: 100% - [fg uronic acid μm^{-3} in the population of interest / fg uronic acid μm^{-3} in the population being compared × 100]).

Uronic acid concentrations of cultures grown in LB + NaCl (Table 3.1) were higher than those grown in LB-S with the planktonic population showing a 17% increase in alginate. The SIP population showed the greatest increase in alginate (56%) and the biofilm population showed a 35% increase over the corresponding populations grown in LB-S. These results are in contrast to those presented by Berry *et al.* (1989) who were unable to induce alginate synthesis, even though *algD* was upregulated in non-mucoid planktonic cells with concentrations of up to 0.5 M NaCl in YTG medium. When compared to other populations grown in LB + NaCl, SIP cells had 3.6-fold less alginate than planktonic populations, while the biofilm population showed a 3.8-fold increase compared to the planktonic population, and a 13.7-fold increase compared to the SIP population.

Uronic acid concentrations of cultures grown in LB + EtOH (Table 3.1) were higher in

planktonic, biofilm and SIP populations compared to those of cultures grown in both LB-S and LB + NaCl. The highest up-regulation, compared to cultures grown in LB-S, was observed in the planktonic and SIP populations. The biofilm population grown in LB + EtOH had 40% less alginate than the planktonic population and 6% less alginate than the SIP population. These lower alginate concentrations in biofilm and SIP populations may be due to ethanol accumulation at the surface. Whereas planktonic cells are grown in the optimal ethanol concentration for expression of *algD* (Chapter 5), SIP cells experience a lower concentration and biofilm populations experience a higher concentration of ethanol. The higher localised ethanol concentration in the biofilm environment is seen to be more deleterious to alginate production than the lower concentration in the SIP environment.

3.4.8 Investigation of the presence of a diffusible signal molecule

Using the experimental setup as described under Materials and Methods (Section 3.3.5) and indicated in Fig. 3.1, the planktonic cells had a calculated cellular volume of 0.803 ± 0.251 μm^3 after 16 h of growth. By contrast, artificial SIP cells, which were able to receive putative diffusible proteins (signal molecules) produced by the biofilm, had a volume of 1.297 ± 0.555 μm^3. The cell volumes observed were smaller than those obtained previously (Fig. 3.8) due to a higher growth rate in McCartney bottles than in test tubes. Nevertheless, the data represented a 1.6-fold difference in cellular volume when comparing planktonic populations to artificial SIP populations after 16 h of growth. In test tubes, SIP cells were found to be 1.4-fold bigger than planktonic cells (Fig. 3.8), which was comparable to the increase observed in McCartney bottles. The increase in cellular volume was found to be statistically significant at a 95% confidence interval using ANOVA. The difference in cellular volume observed in control samples that had planktonic cells only on the outside of the dialysis chamber was found not to be statistically significant (data not shown).

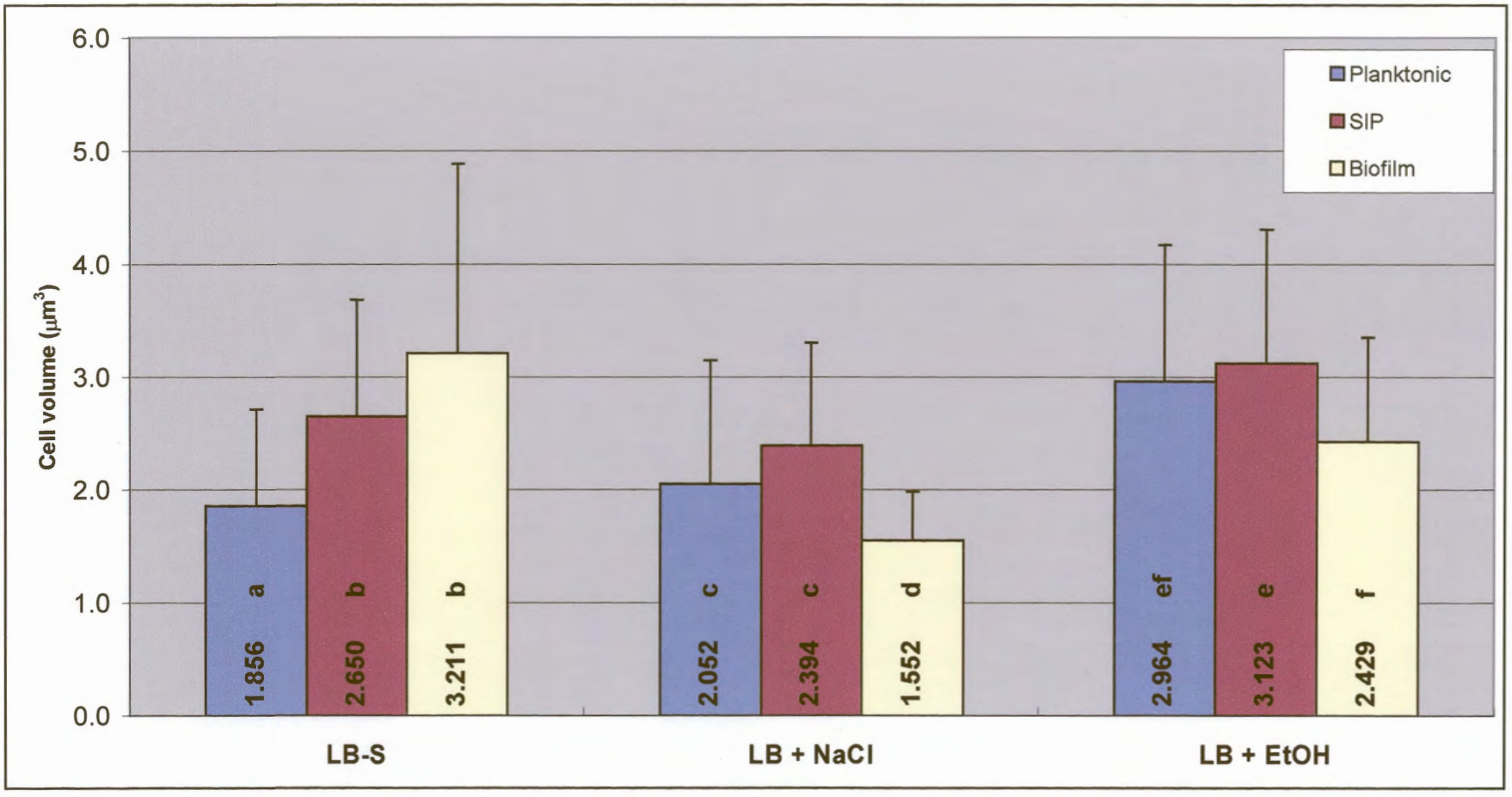

Fig. 3.8: Cell volumes of planktonic, surface-influenced planktonic (SIP) and biofilm cells of *P. aeruginosa* grown for 16 h in LB broth without salt (LB-S), with 0.7 M NaCl (LB + NaCl) and 2.5% (v/v) ethanol (LB + EtOH). Means with different superscripts indicate statistically significant differences ($P<0.05$). Error bars denote one standard deviation of the mean.

3.5 DISCUSSION

The opportunistic pathogen *P. aeruginosa* has become the primary Gram-negative model organism for the study of bacterial biofilms (O'Toole *et al.*, 2000). *P. aeruginosa* biofilms have been cultured under a variety of conditions and on a number of substrates (Fletcher & Loeb, 1979; Black *et al.*, 1988; Jucker *et al.*, 1996; Wainwright *et al.*, 1999). However, the efficient separation and subsequent quantitative analysis of biofilm and surrounding suspended (SIP) populations as well as separation of the biofilm material from these substrates has proven to be extremely difficult. In the experimental protocols followed in this study, glass wool was used as a substratum for the attachment and subsequent biofilm formation of *P. aeruginosa*. Glass wool was found to provide an ideal surface for attachment as it yielded sufficient biomass for biochemical (quantitative) analyses (Fig. 3.2a, 3.2b and Steyn *et al.*, 2001) and allowed the separation of attached biomass from SIP cells with relative ease and accuracy.

The biofilm-grown cells were phenotypically different to planktonic cells grown in the same medium, supporting the well-documented difference between these two modes of growth (O'Toole *et al.*, 2000; Costerton *et al.*, 1995). When cells of *P. aeruginosa* attach to a glass surface they undergo an extensive period of adaptation before they begin to divide (Rice *et al.*, 2000). This process is reminiscent of that of nutrient up- and downshift, whereby bacteria adapt to a different growth rate as dictated by the available nutrient source. Biofilm cells of *P. putida* in a chemostat environment grow at a fixed rate, independent of the growth rate dictated by the dilution rate, indicating that a constant physiological state is established in biofilm cells (Møller *et al.*, 1995). The differences in cellular volume reported here may therefore indicate a higher growth rate of biofilm than planktonic cells. Previous work with both marine and freshwater bacterial consortia has shown that the attached cells have larger biomass per cell ratio than free-living cells, as well as a higher metabolic activity per cell (Iriberri *et al.*, 1990), indicating that this is a phenomenon not only restricted to *P. aeruginosa*.

The yield of SIP cells was clearly greater than the yield of planktonic cells in broth with or without NaCl. The growth rate of cells growing in the liquid phase should be similar to that of planktonic cells growing in the same broth, indicating that the

increased yield in SIP cells was due to cells detaching from the biofilm into the liquid phase. This implied that the SIP population would constitute a mixture of cells growing in the planktonic phase and those detached from the biofilm at some stage, therefore a mixture of two physiological states. However, the analyses of the SIP population did not reflect properties intermediate between those of planktonic and biofilm cells, as would be expected upon analysis of a mixture of the two populations. Proteomic analysis of SIP biomass revealed that 10 proteins present in both planktonic and biofilm biomass were absent in the SIP cells, indicating a unique physiological state (Steyn *et al.*, 2001). The apparently homogenous phenotype of cells originating from two separate environments indicated an underlying regulatory mechanism controlling both. This could be by way of a diffusible signal molecule produced by biofilm cells and released into the liquid phase, signalling the suspended cells to alter their mode of growth.

Two of the early steps in alginate biosynthesis are encoded by *algC* and *algD*, and both these genes are upregulated in *P. aeruginosa* growing at a surface, concomitant with higher alginate concentration per cell (Hoyle *et al.*, 1993; Davies & Geesey, 1995). The data presented here confirms that biofilm cells have more alginate per unit biomass than do planktonic cells when grown in the presence or absence of NaCl. However, corresponding SIP cells have less alginate than the planktonic cells, indicating a near shutdown of alginate biosynthesis in this population. This may indicate that SIP cells have switched to a non-attachment mode as opposed to planktonic cells, which are intermediate between the SIP and biofilm state.

Cultures grown in LB + EtOH were seen to be physiologically different to those grown in broth with or without NaCl. Ethanol changed the dynamics of the system, not purely by imposing stress on the cells, but also by causing a shift in alginate biosynthesis and cell differentiation. Alginate biosynthesis was strongly upregulated in all populations; however, biofilm cells seemed to adhere more strongly to the surface by the large amounts of alginate produced, and by aggregation in chains. Thus, very few cells may leave the biofilm to form a part of the SIP population due to chain formation (Fig. 3.4b), hence the observed increase in biofilm biomass and decrease in SIP biomass compared to those cultures grown in LB-S and LB + NaCl.

The expression of specific genes in bacteria is often studied using promoter-reporter gene fusions such as the β-galactosidase gene, and dividing total expression by the amount of biomass as determined by the optical density (Miller, 1992). This approach assumes that the various clones or mutants have the same overall protein expression per cell, so that alterations in β-galactosidase per OD indicate alterations in expression of the specific gene in question. The data presented here shows that *P. aeruginosa* populations growing in the three phases and in various media have differing ratios of protein per cell and protein per OD unit. This implies that expression studies of biofilm-related genes should rather employ total protein than cell number or optical density to determine their regulation against a background of altered general cell physiology.

In conclusion, the data presented shows that the SIP population is physiologically different to both biofilm and planktonic populations growing under otherwise identical physicochemical conditions. It is therefore not merely a combination of planktonic and biofilm cells. The SIP population constitutes a rather homogenous physiological state, implying concerted regulation by a common signal such as a diffusible signal molecule secreted by the biofilm.

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.

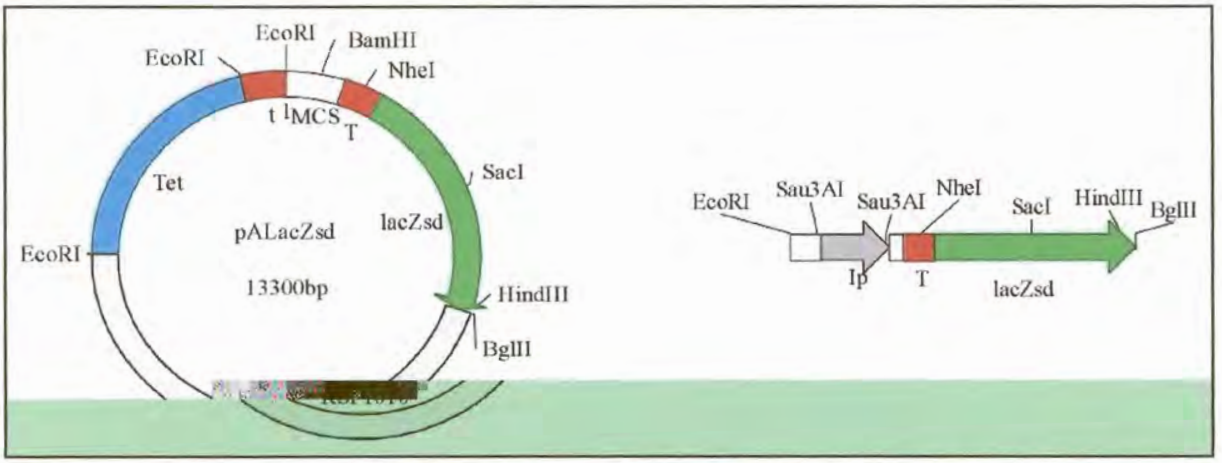

Figure 4.1: Schematic representation of the pALacZsd vector (left) and the insert used to create the pALacZsdIP vector (right). The pALacZsd vector contains a multiple cloning site (MCS), a *lacZ* gene (lacZsd), part of the RSF1010 replicon (RSF1010), a *tetA* tetracycline resistance gene (Tet), a transcriptional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

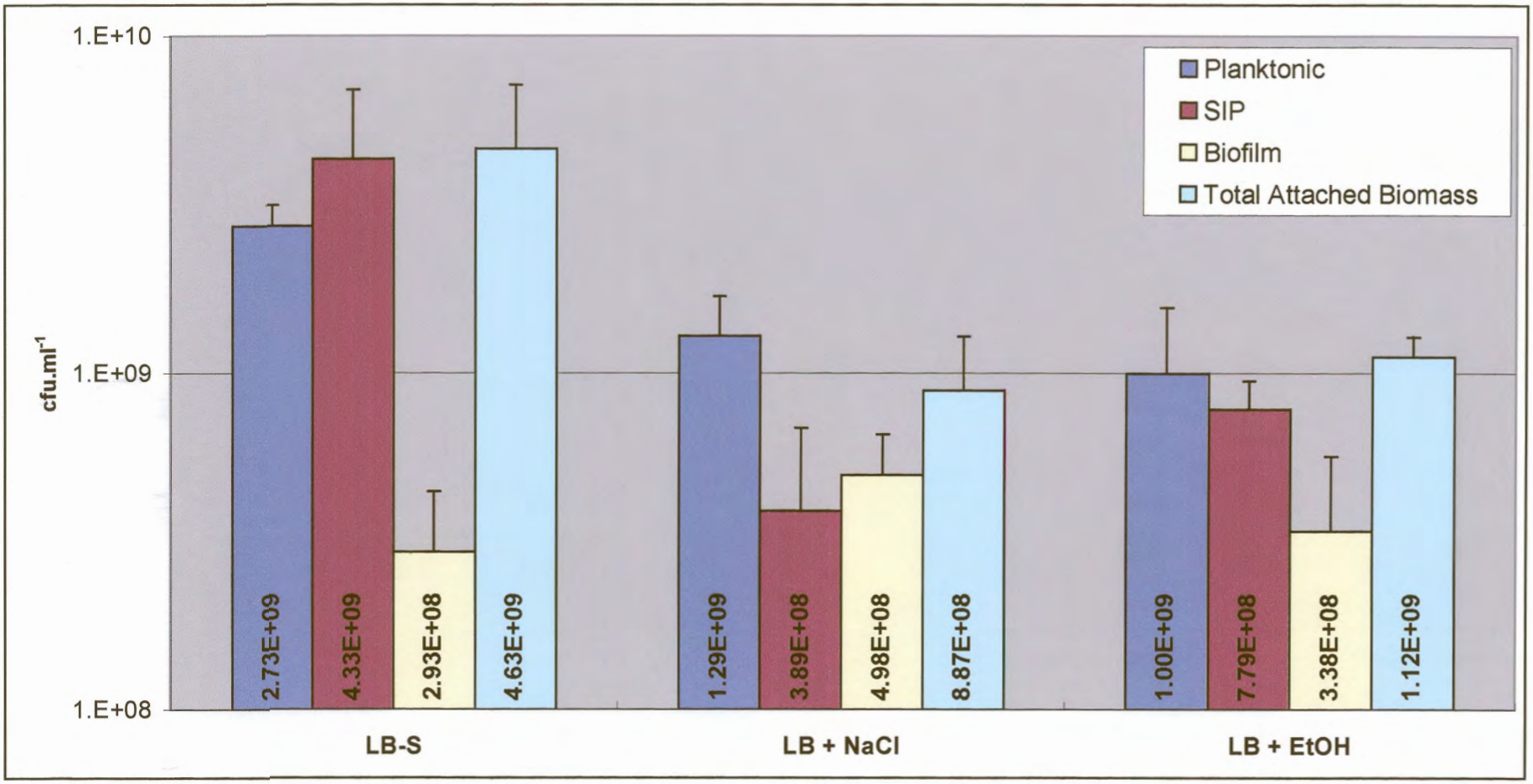

Fig. 4.2: Culturable count of *P. aeruginosa* transformed with pALacZsd cultured for 16 h in LB broth without salt (LB-S), with 0.7 M NaCl (LB + NaCl) and 2.9% (v/v) ethanol (LB + EtOH). Error bars denote one standard deviation of the mean.

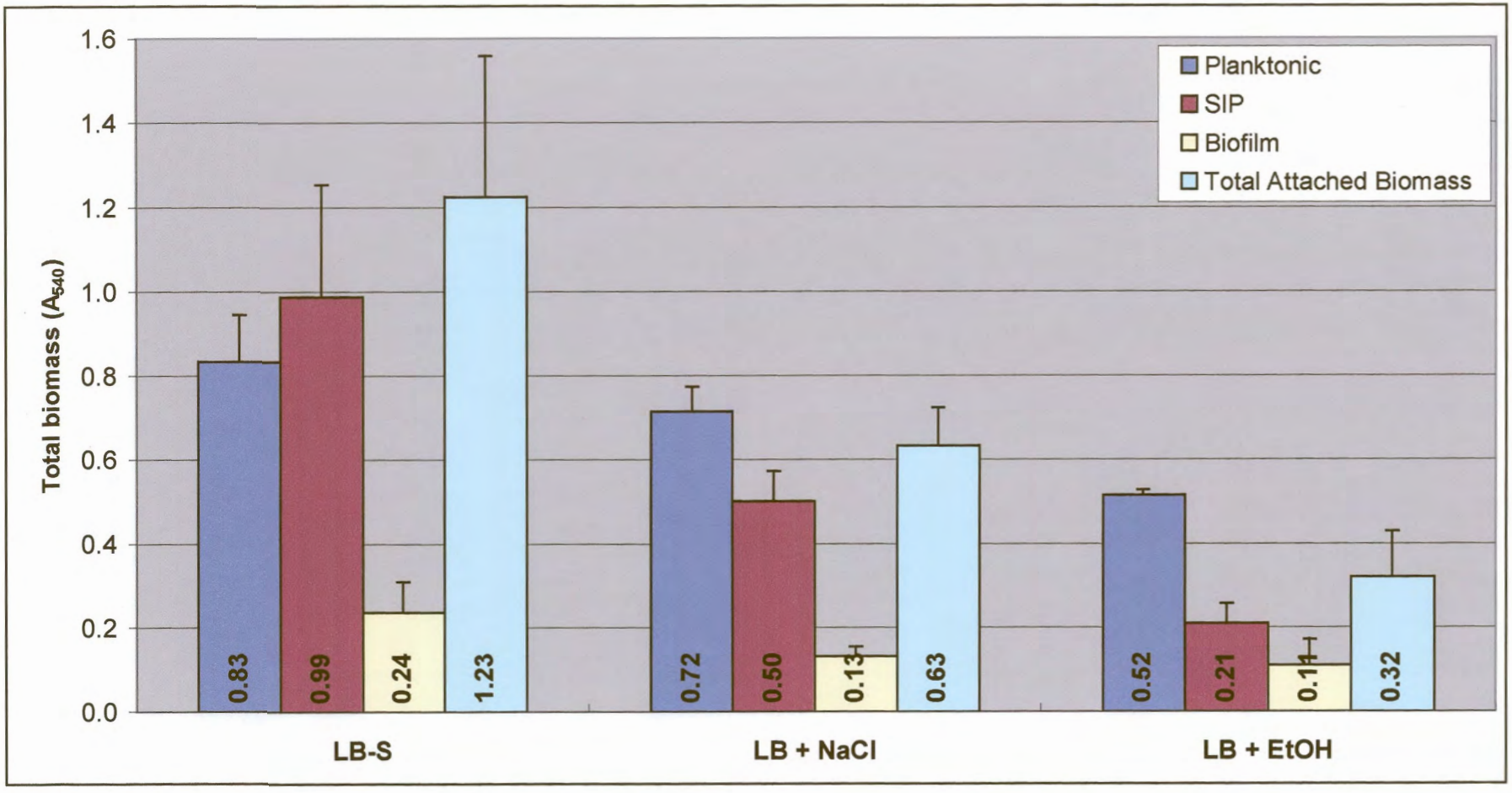

Fig. 4.3: Optical density (A_{540}) of *P. aeruginosa* transformed with pALacZsd cultured for 16 h in LB broth without salt (LB-S), with 0.7 M NaCl (LB + NaCl) and 2.9% (v/v) ethanol (LB + EtOH). Error bars denote one standard deviation of the mean.

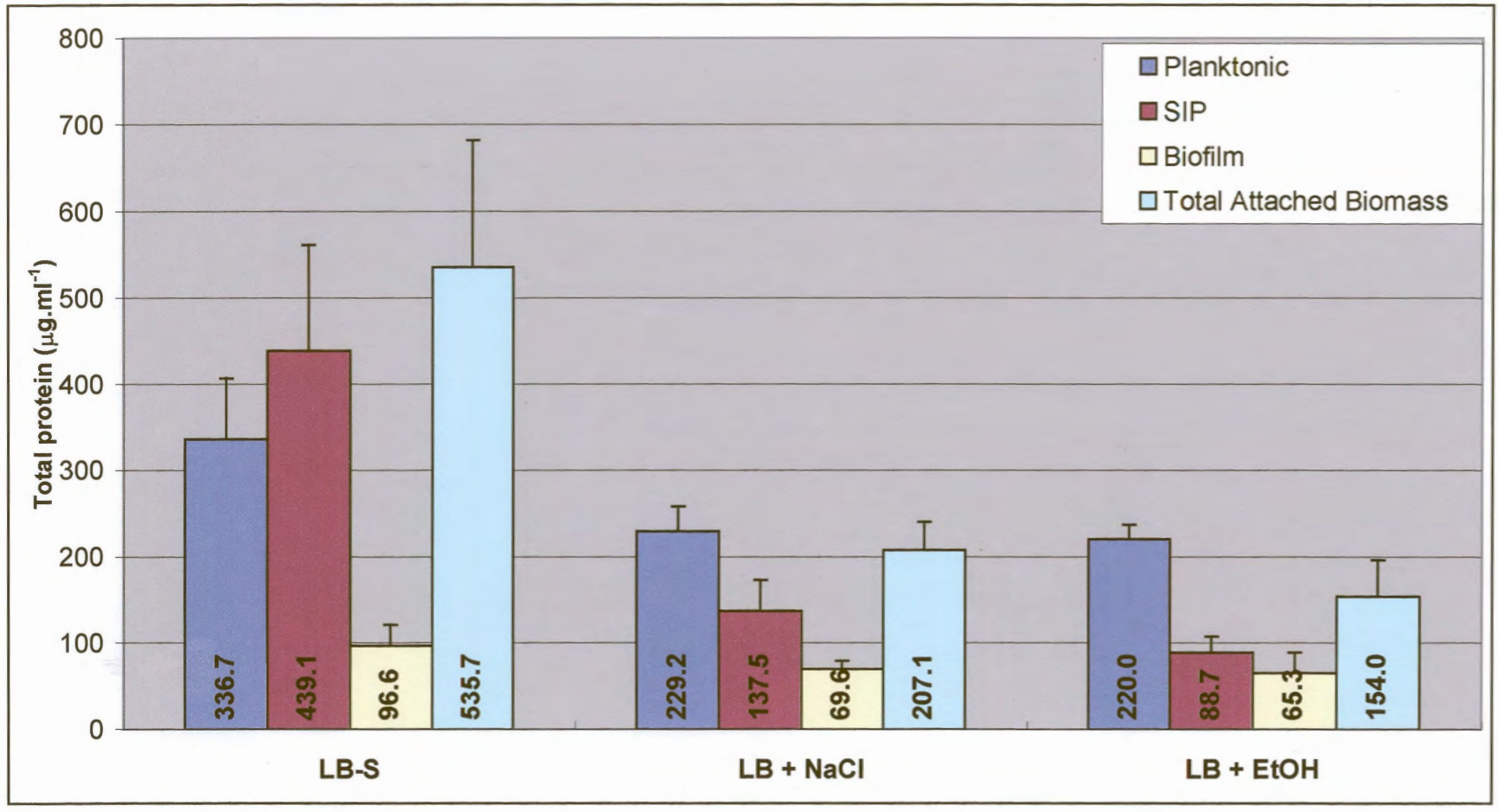

Fig. 4.4: Total cellular protein of *P. aeruginosa* transformed with pALacZsd cultured for 16 h in LB broth without salt (LB-S), with 0.7 M NaCl (LB + NaCl) and 2.9% (v/v) ethanol (LB + EtOH). Error bars denote one standard deviation of the mean.

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.

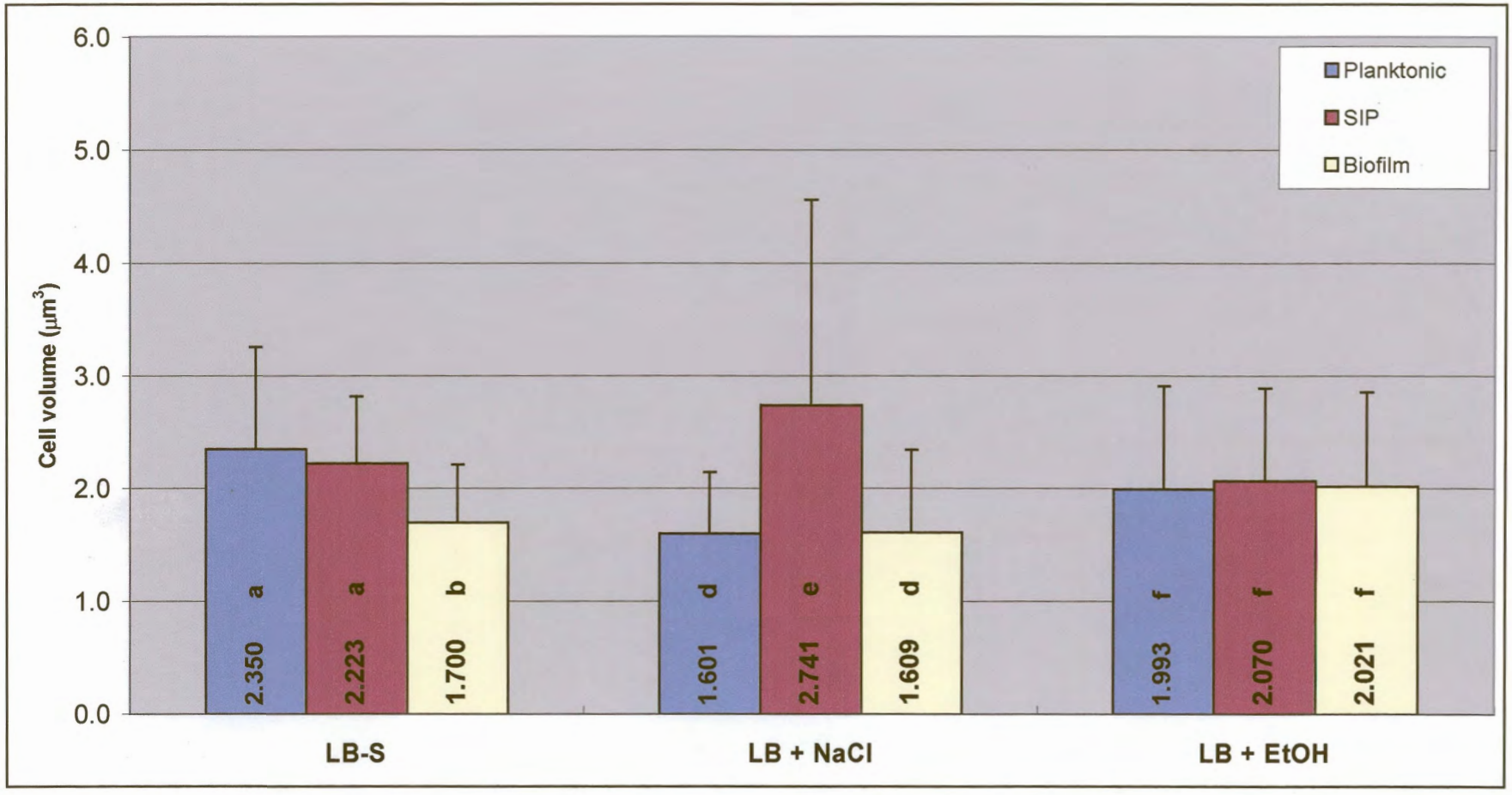

Fig. 4.5: Cell volumes of planktonic, surface influenced planktonic (SIP) and biofilm cells of *P. aeruginosa* transformed with pALacZsd grown for 16 h in LB broth without salt (LB-S), with 0.7 M NaCl (LB + NaCl) and 2.9% (v/v) ethanol (LB + EtOH). Means with different superscripts indicate statistically 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).

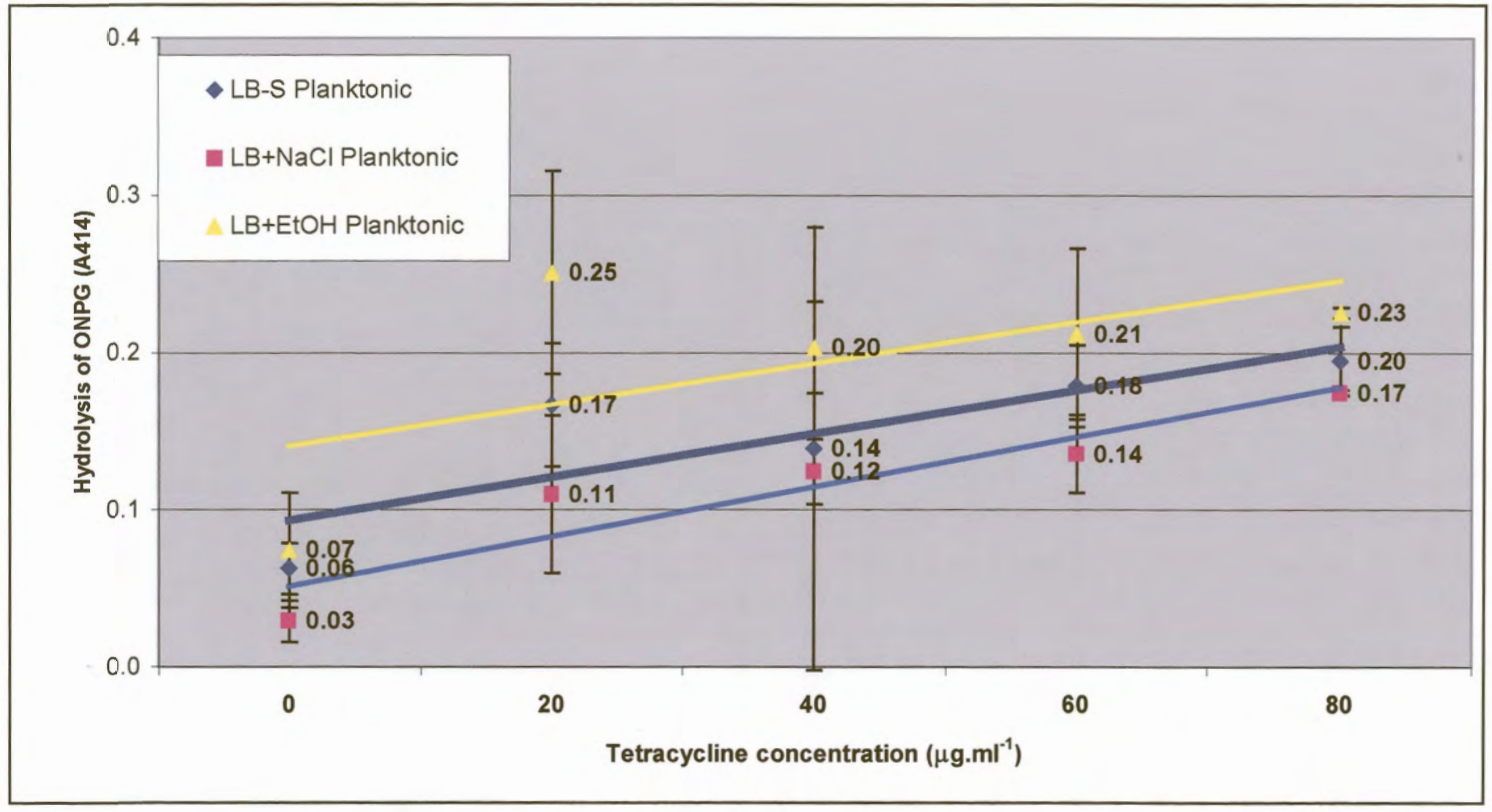

Fig. 4.6: The effect of tetracycline concentration (in $\mu g.ml^{-1}$) and growth conditions on basal β-galactosidase levels in planktonic cells. Error bars denote one standard deviation of the mean.

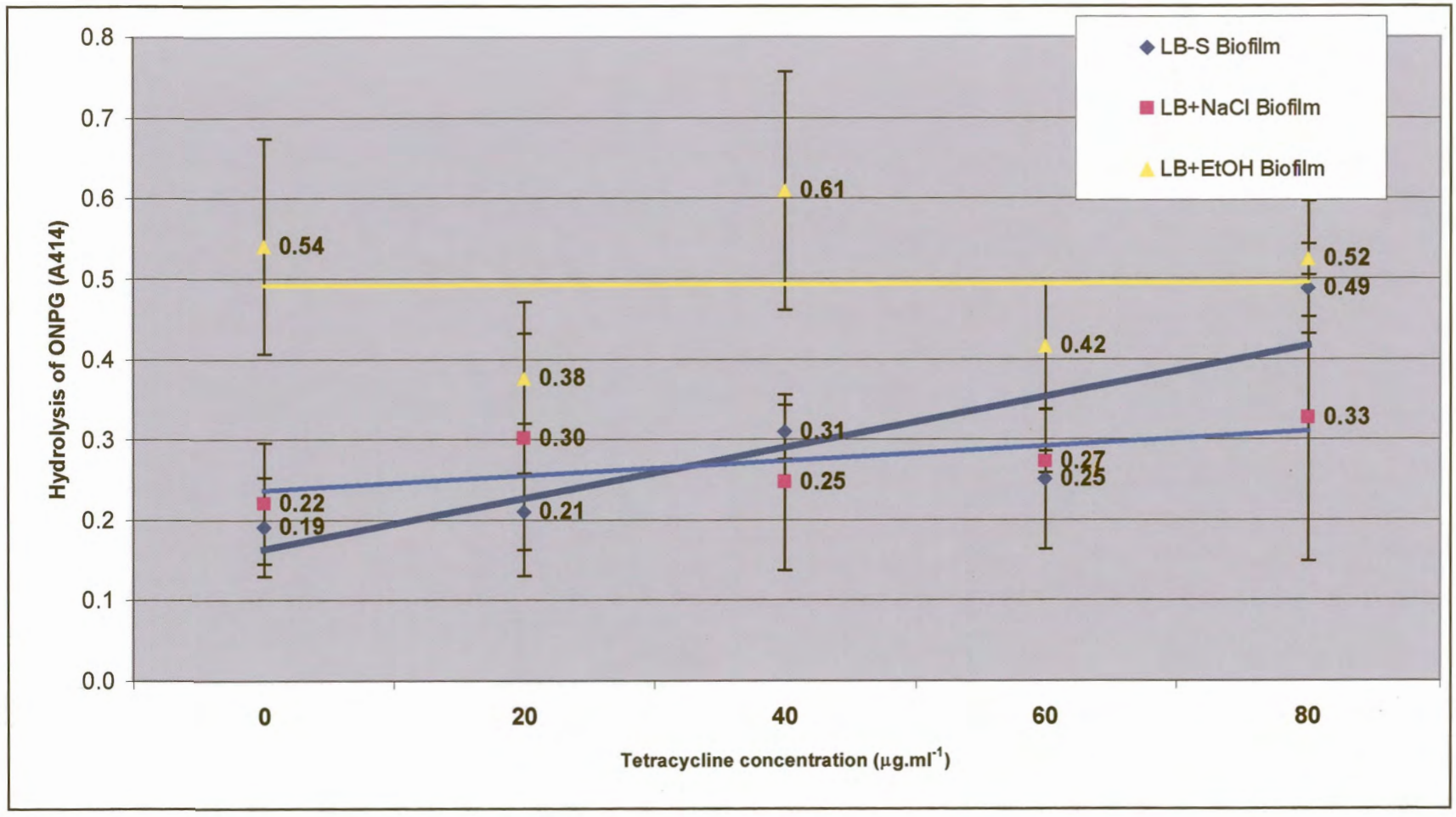

Fig. 4.7: The effect of tetracycline concentration (in $\mu g.ml^{-1}$) and growth conditions on basal β-galactosidase levels in total attached (attached + SIP) cultures. Error bars denote one standard deviation of the mean.

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.

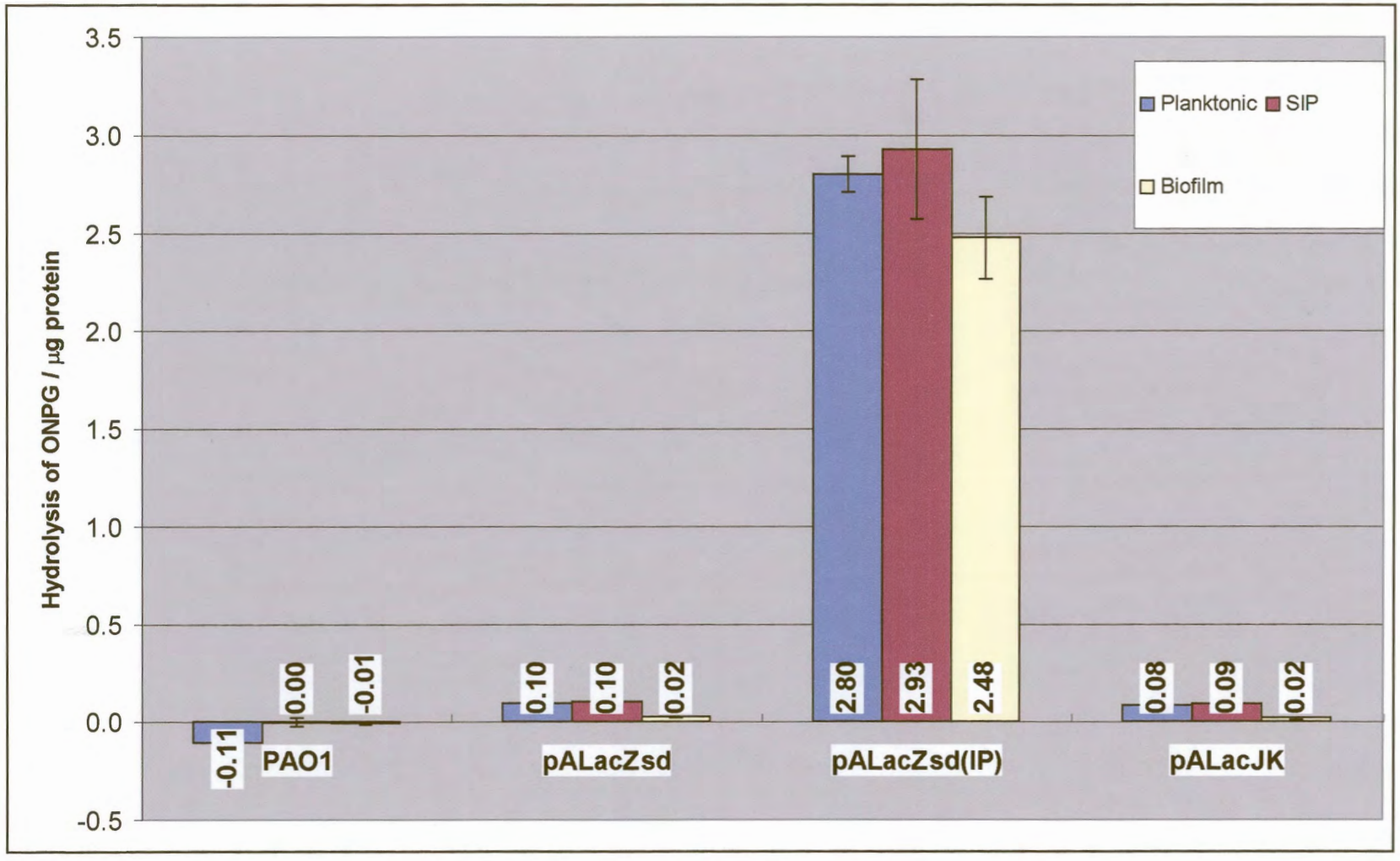

Fig. 4.8: ONPG hydrolysis per µg protein for *P. aeruginosa* pALacJK cultured for 16 h in LB broth without salt (LB-S). Error bars denote one standard deviation of the mean.

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.

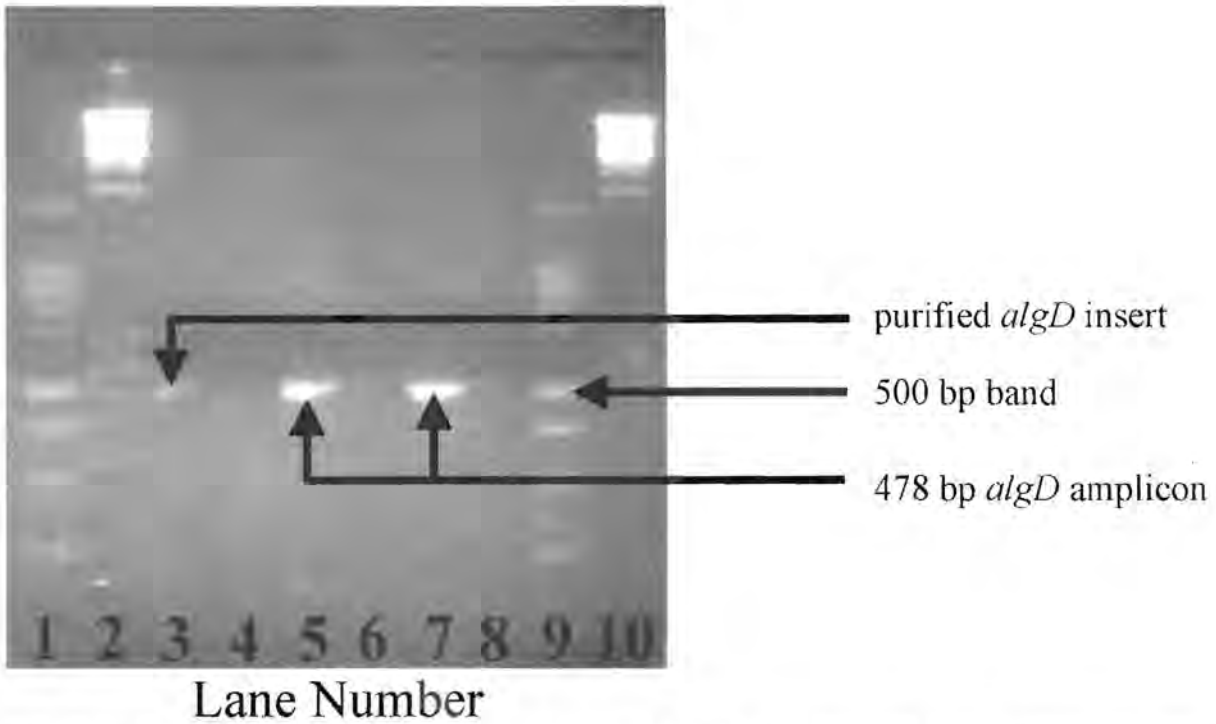

Fig. 5.1: A purified *algD* promoter (lane 3) was ligated into the pALacZsd reporter vector and recombinant clones were identified following PCR amplification of a 478-bp *algD* amplicon (lanes 5 and 7). Lanes 1 and 9, 100 bp DNA Ladder (Promega, Madison, USA) and lanes 2 and 10, phage λ DNA digested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

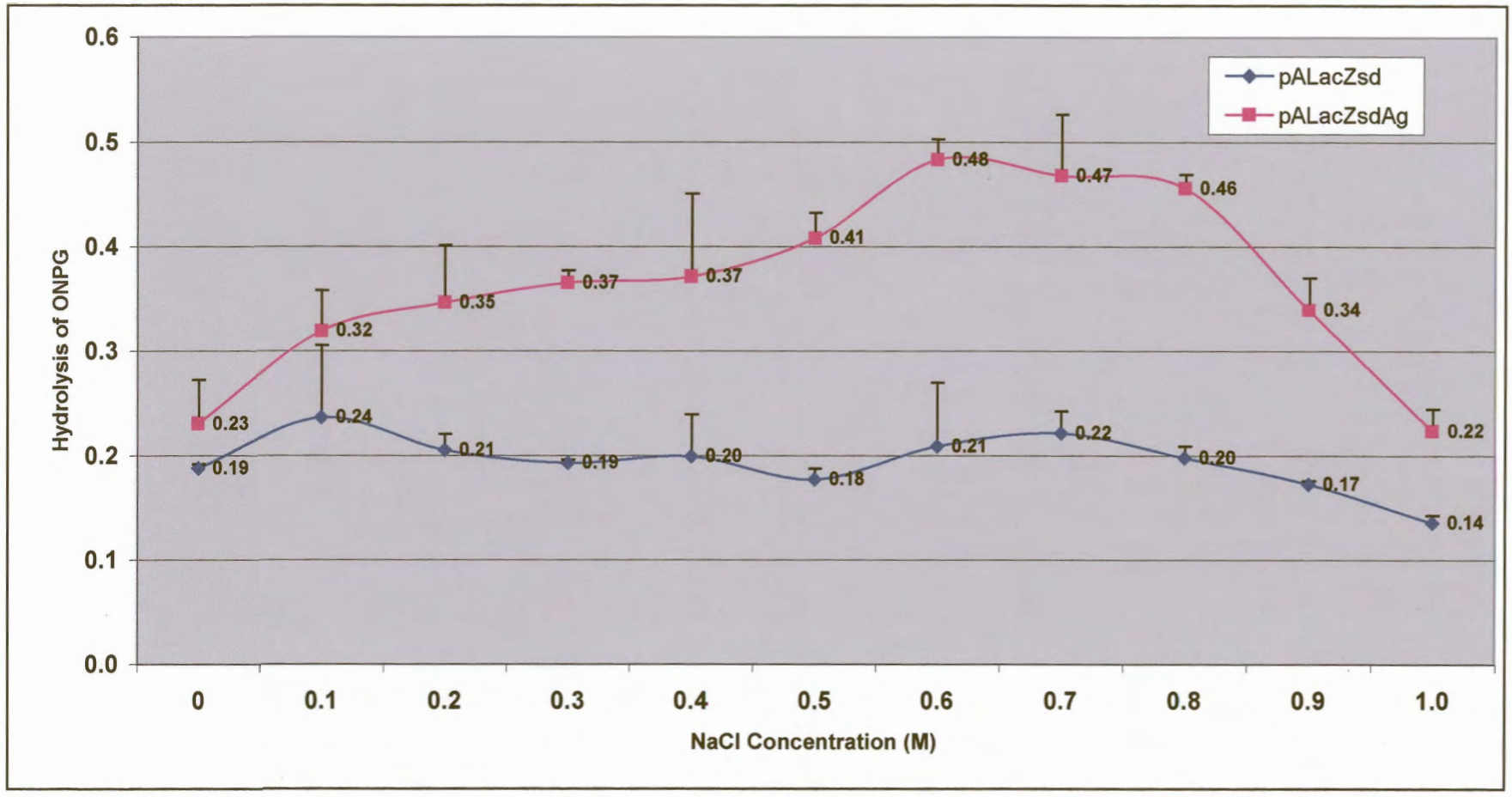

Fig. 5.2: *algD* promoter activity as measured by β-galactosidase activity assays on 16 h planktonic cultures of *P. aeruginosa* (pALacZsdAg) grown at various NaCl concentrations in LB, using ONPG as substrate. Error bars denote one standard deviation of the mean.

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.

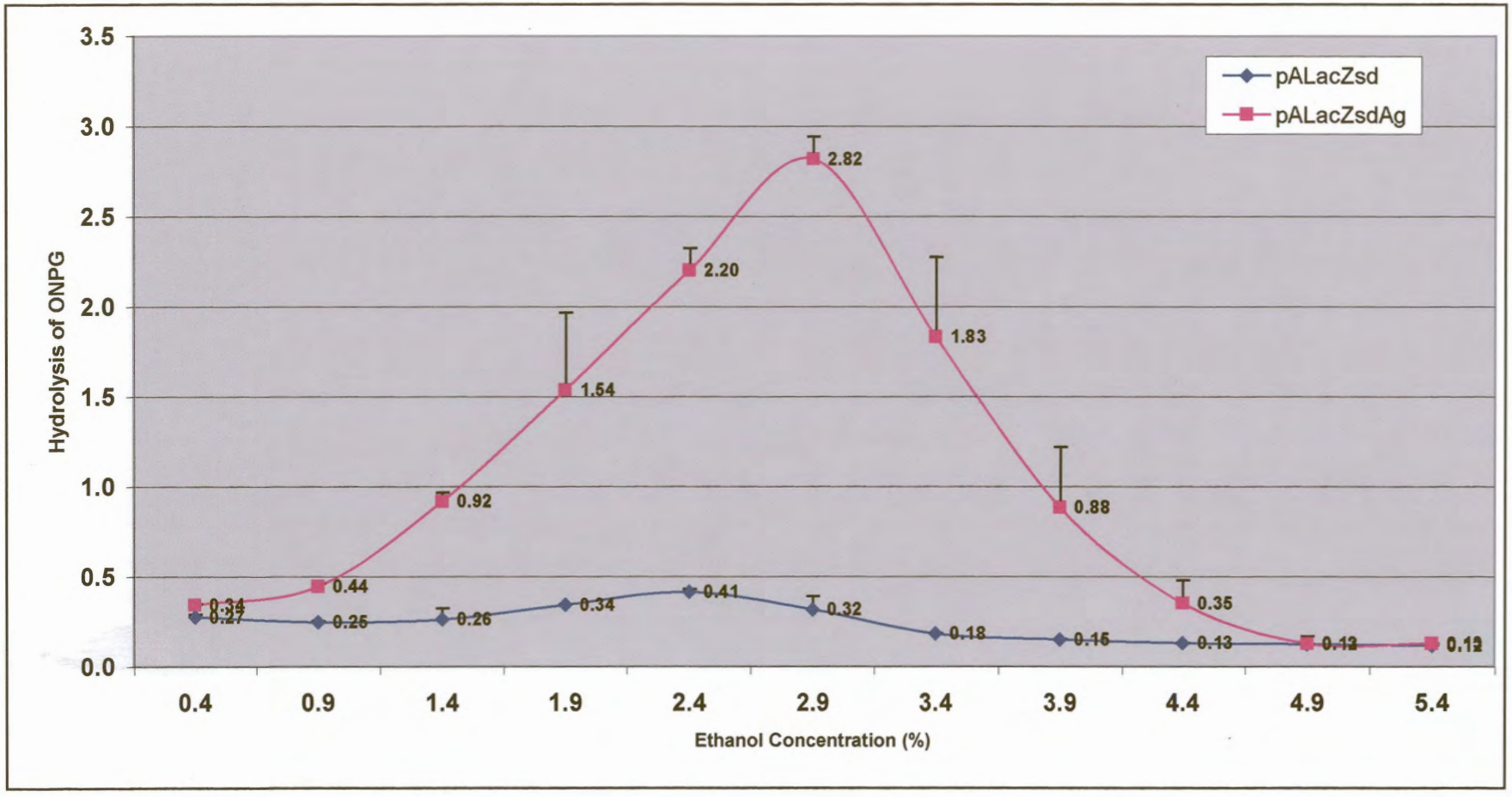

Fig. 5.3: *algD* promoter activity as measured by β-galactosidase activity assays on 16 h planktonic cultures of *P. aeruginosa* (pALacZsdAg) grown at various ethanol concentrations in LB, using ONPG as substrate. Error bars denote one standard deviation of the mean.

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.

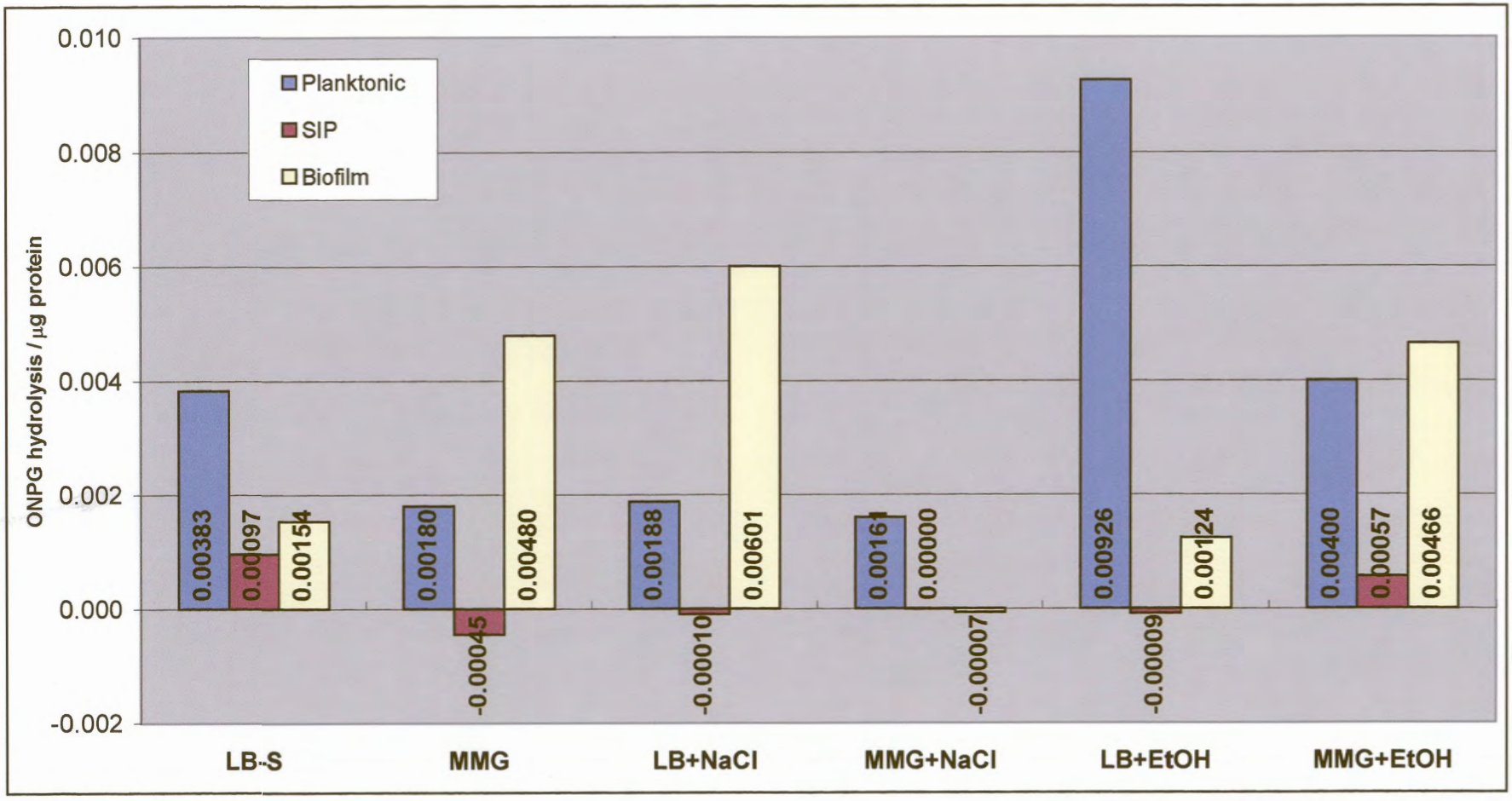

Fig. 5.4: *algD* promoter activity after 16 h of growth as influenced by LB-S and MMG media with and without 0.7 M NaCl or 2.9% (v/v) ethanol.

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).

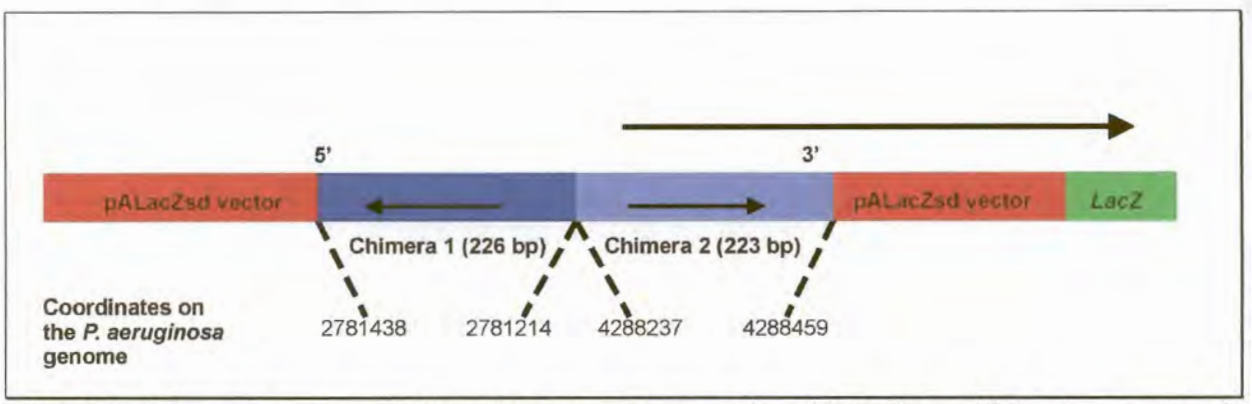

Fig. 6.1: Schematic representation of the two chimeras cloned into the multiple cloning site of the pALacZsd vector. Arrows indicate the putative transcriptional direction in which the insert DNA was cloned into the vector.

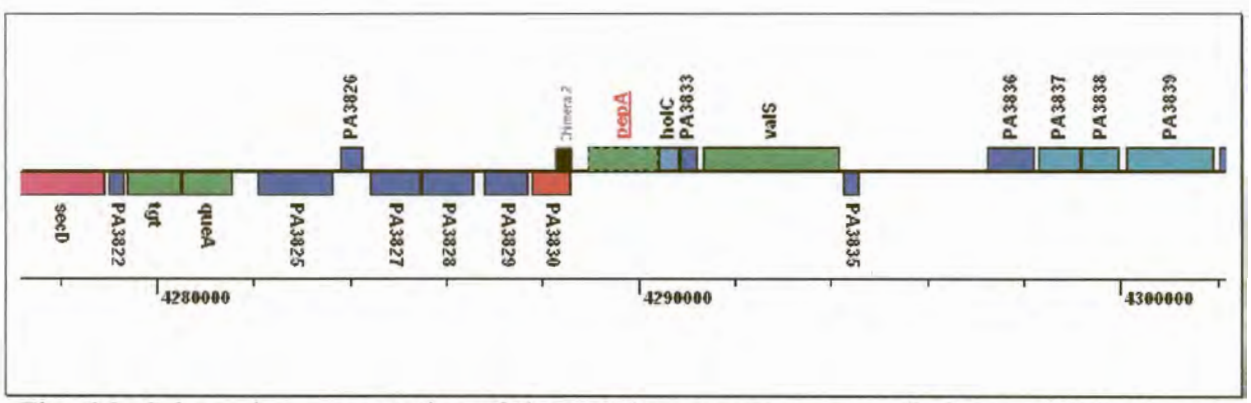

Fig. 6.2: Schematic representation of the putative operon structure in the *pepA (phpA)* gene region as well as the location, on the *P. aeruginosa* genome, of the second chimera present in the pALacZsd65 vector (adapted from http://www.pseudomonas.com/AnnotationByPAU.asp?PA=PA3831).

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.

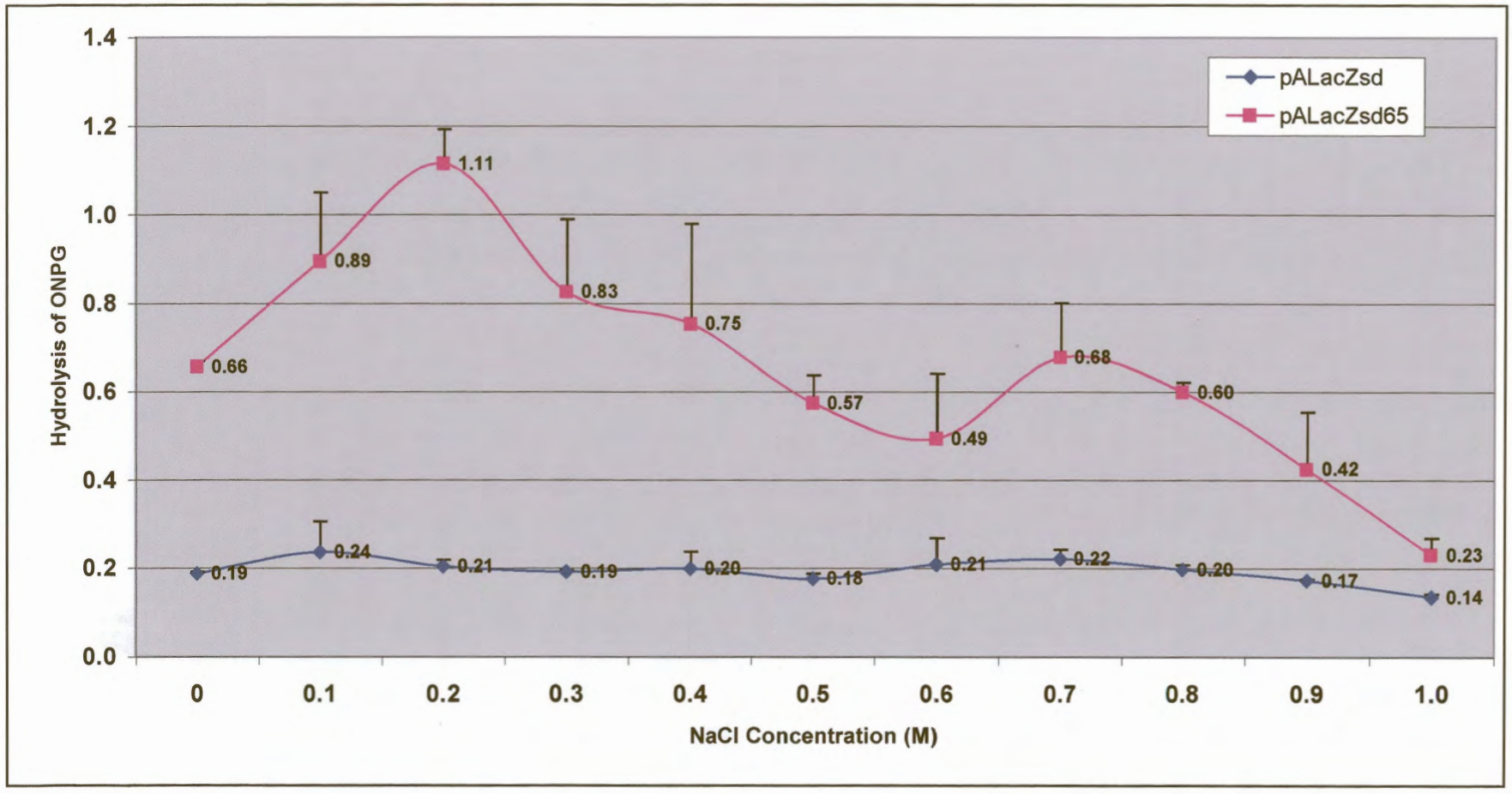

Fig. 6.3: pALacZsd65 promoter activity as measured by β-galactosidase activity assays on 16 h planktonic cultures of *P. aeruginosa* (pALacZsd65) grown at various NaCl concentrations in LB, using ONPG as substrate. Error bars denote one standard deviation of the mean.

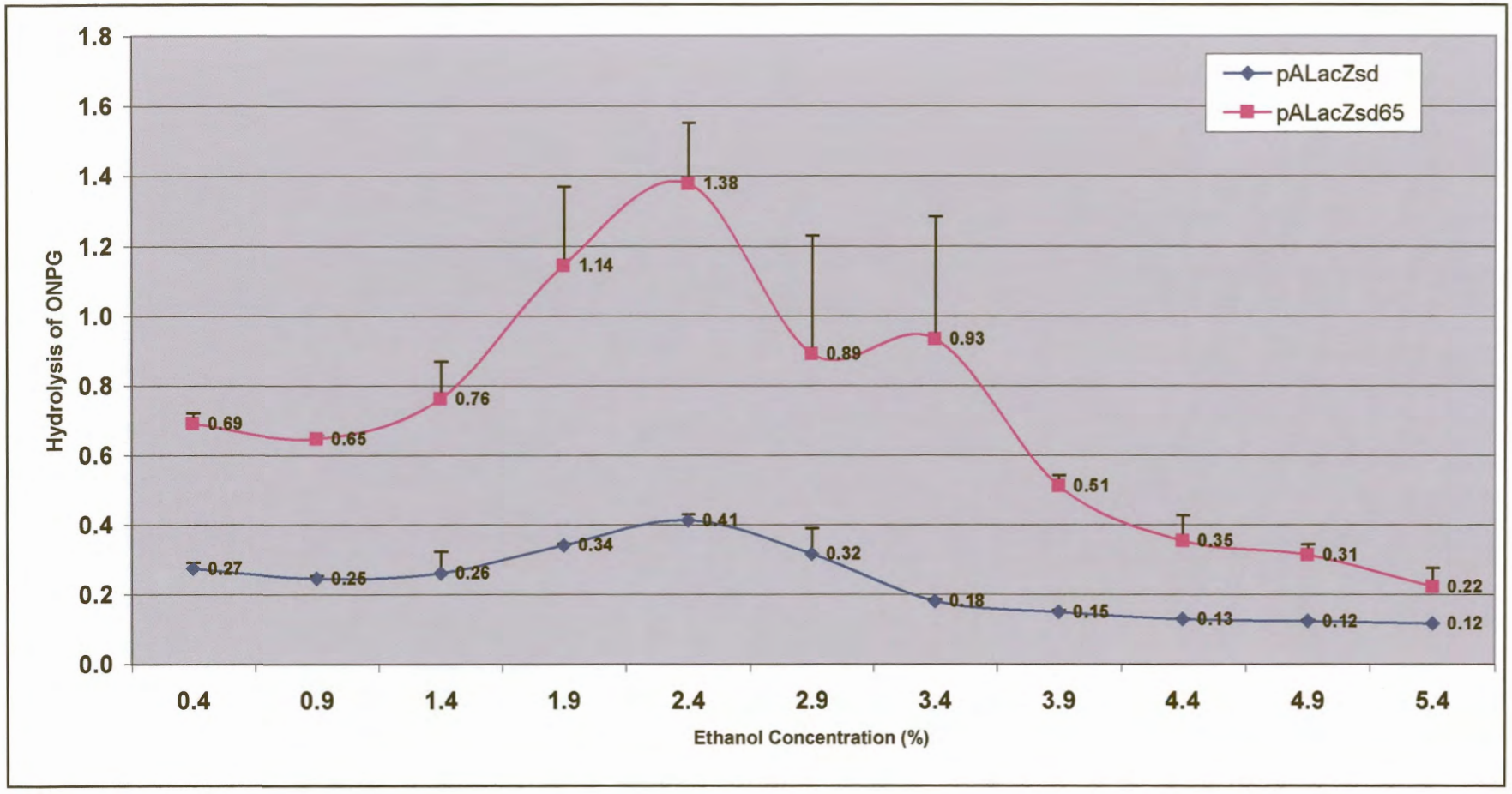

Fig. 6.4: pALacZsd65 promoter activity as measured by β-galactosidase activity assays on 16 h planktonic cultures of *P. aeruginosa* (pALacZsd65) grown at various ethanol concentrations in LB, using ONPG as substrate. Error bars denote one standard deviation of the mean. Activity could not be determined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

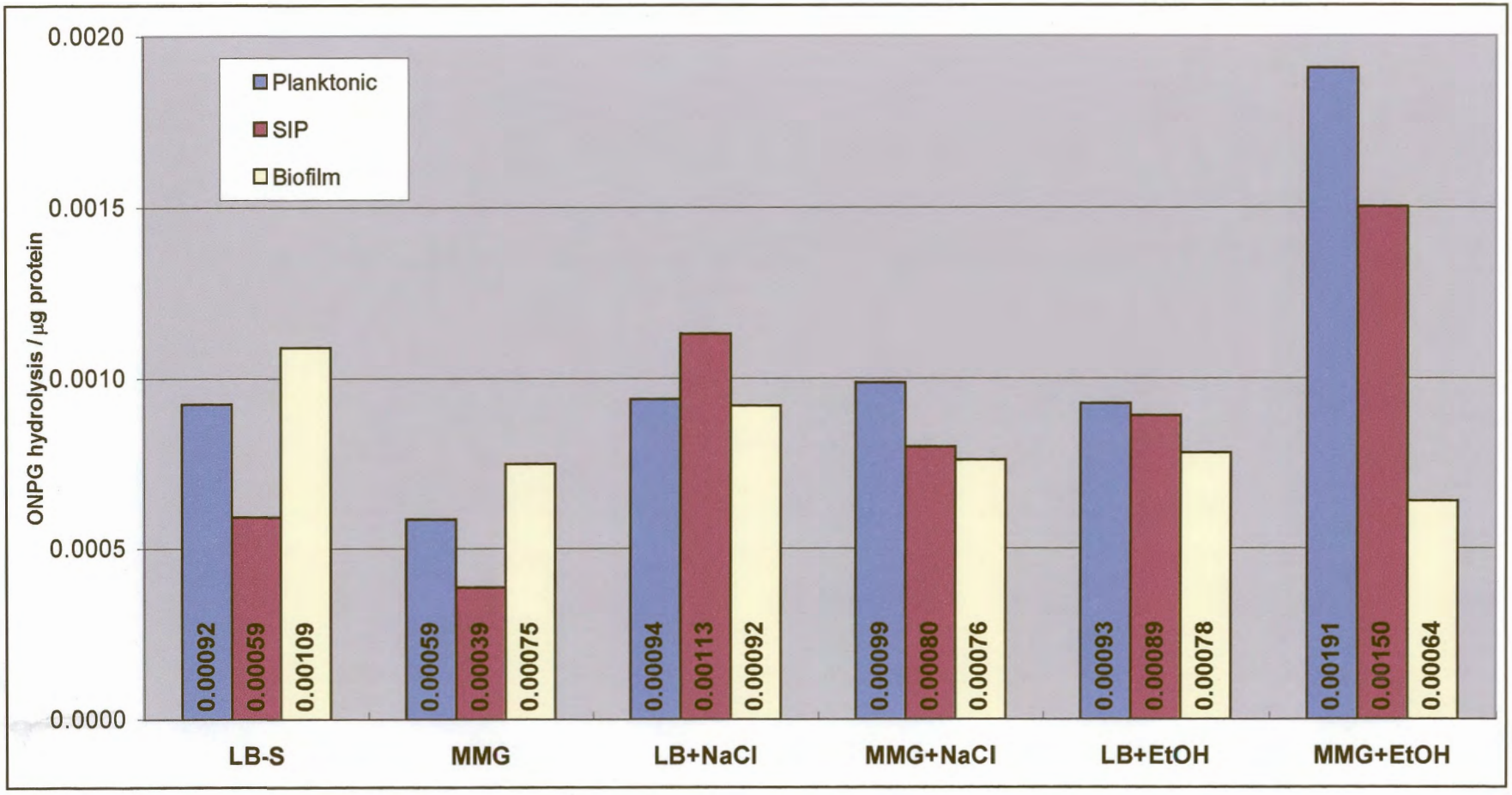

Fig. 6.5: pALacZsd65 promoter activity after 16 h of growth as influenced by LB-S and MMG media with and without 0.2 M NaCl or 2.4% (v/v) ethanol.

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.

APPENDIX 1

CELLULAR WIDTH, LENGTH AND VOLUME MEASUREMENTS FOR *Pseudomonas aeruginosa* PAO (DSM 1707) AND *Pseudomonas aeruginosa* TRANSFORMED WITH THE pALacZsd REPORTER VECTOR

Table 7.1: Cell length, cell width and cell volume for *P. aeruginosa* planktonic, SIP and biofilm cells grown for 16 h in the presence of glass wool in LB broth

	Planktonic Culture			SIP Culture			Biofilm Culture		
P. aeruginosa PAO (DSM 1707)	Length	Width	Volume	Length	Width	Volume	Length	Width	Volume
	3.423	0.952	2.383	3.624	0.949	2.494	4.103	1.028	3.385
	3.526	0.795	1.595	3.923	1.044	3.101	3.693	0.997	2.639
	3.457	0.789	1.591	3.703	0.929	2.354	4.291	1.063	3.609
Average	3.468	0.846	1.856	3.750	0.974	2.650	4.029	1.029	3.211
SD	0.712	0.145	0.858	0.619	0.152	1.033	0.612	0.235	1.674
P. aeruginosa pALacZsd	Length	Width	Volume	Length	Width	Volume	Length	Width	Volume
	4.138	1.024	3.152	3.257	0.962	2.162	3.224	0.850	2.259
	3.902	0.849	2.045	3.810	0.890	2.209	3.247	0.791	1.765
	4.041	0.785	1.853	3.919	0.896	2.299	3.476	0.883	1.954
Average	4.027	0.886	2.350	3.662	0.916	2.223	3.316	0.841	1.993
SD	0.714	0.148	0.903	0.635	0.105	0.592	0.588	0.123	0.917

Table 7.2: Cell length, cell width and cell volume for *P. aeruginosa* planktonic, SIP and biofilm cells grown for 16 h in the presence of glass wool in LB broth with 0.7 M NaCl (LB + NaCl)

	Planktonic Culture			SIP Culture			Biofilm Culture		
P. aeruginosa PAO (DSM 1707)	Length	Width	Volume	Length	Width	Volume	Length	Width	Volume
	3.234	0.836	1.596	3.070	1.067	2.456	3.032	0.926	1.813
	3.484	0.885	1.992	3.390	0.974	2,377	2.694	0.822	1.288
	4.325	0.864	2.568	3.552	0.954	2.348	3.078	0.844	1.554
Average	3.681	0.861	2.052	3.337	0.998	2.394	2.935	0.864	1.552
SD	1.137	0.108	1.095	0.768	0.154	0.909	0.537	0.110	0.432
P. aeruginosa pALacZsd	Length	Width	Volume	Length	Width	Volume	Length	Width	Volume
	3.418	0.761	1.404	4.985	0.823	2.573	3.547	0.754	1.540
	3.705	0.798	1.746	5.417	0.873	3.356	3.657	0.747	1.592
	3.071	0.865	1.653	4.256	0.857	2.294	3.550	0.811	1.695
Average	3.398	0.808	1.601	4.904	0.851	2.741	3.585	0.771	1.609
SD	0.923	0.101	0.544	2.181	0.159	1.818	1.101	0.112	0.738

Table 7.3: Cell length, cell width and cell volume for *P. aeruginosa* planktonic, SIP and biofilm cells grown for 16 h in the presence of glass wool in LB broth with 2.5% (v/v) ethanol (LB + EtOH)

	Planktonic Culture			SIP Culture			Biofilm Culture		
P. aeruginosa PAO (DSM 1707)	Length	Width	Volume	Length	Width	Volume	Length	Width	Volume
	4.534	0.950	3.050	4.556	0.966	3.146	4.157	0.933	2.700
	5.804	0.881	3.346	4.937	0.948	3.263	3.818	0.889	2.214
	4.177	0.867	2.497	4.692	0.928	2.960	4.005	0.881	2.372
Average	4.838	0.899	2.964	4.728	0.947	3.123	3.993	0.901	2.429
SD	1.457	0.142	1.205	1.354	0.130	1.185	0.844	0.124	0.924
P. aeruginosa pALacZsd	Length	Width	Volume	Length	Width	Volume	Length	Width	Volume
	5.422	0.739	2.259	4.078	0.700	1.503	4.053	0.997	2.868
	4.183	0.746	1.765	5.092	0.802	2.426	3.377	0.814	1.608
	3.747	0.826	1.594	4.371	0.830	2.280	3.544	0.785	1.587
Average	4.451	0.771	1.993	4.514	0.777	2.070	3.658	0.865	2.021
SD	1.512	0.124	0.917	1.097	0.113	0.822	0.699	0.106	0.836

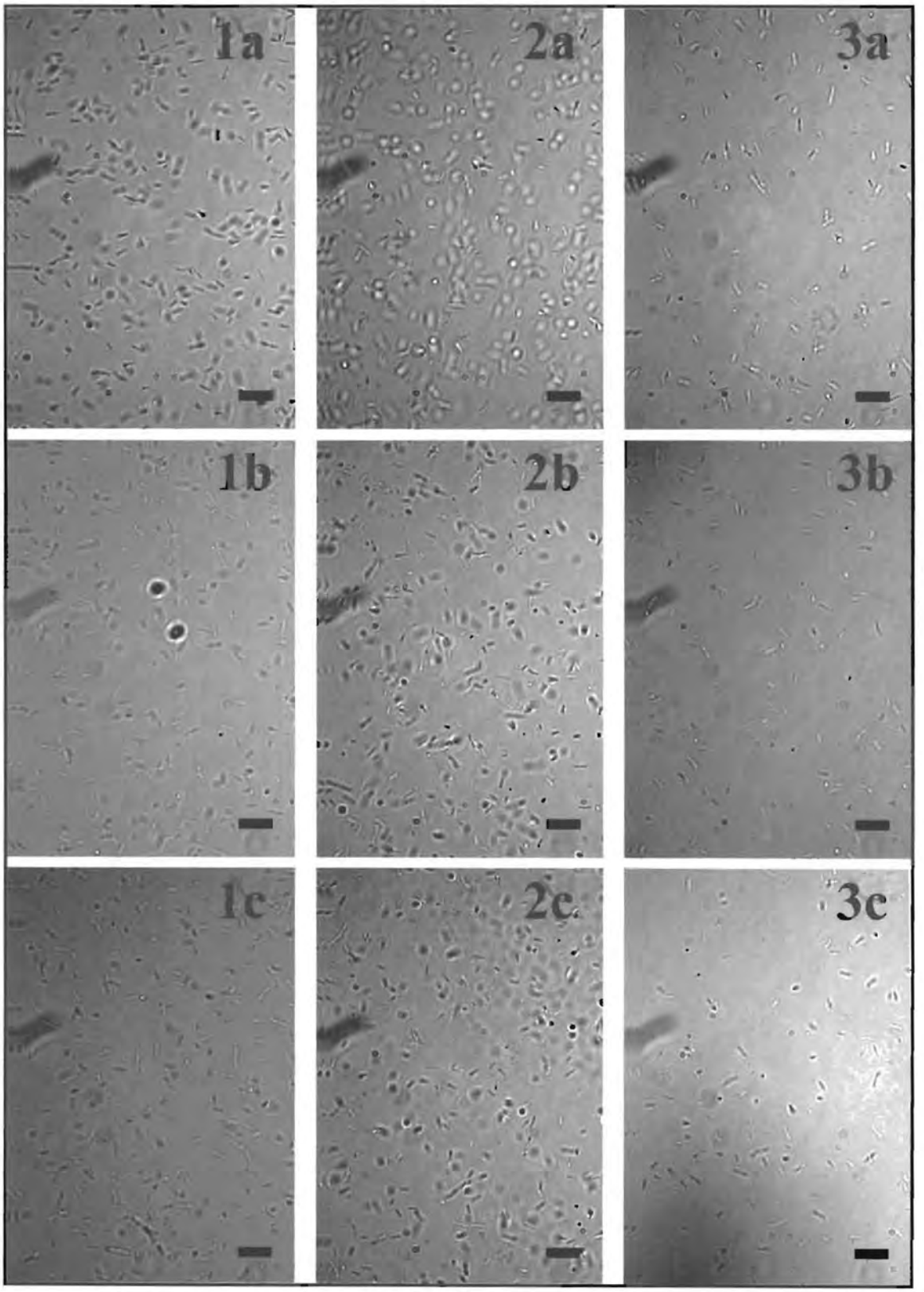

Fig. 7.1: Triplicate photomicrographs (a – c) showing *P. aeruginosa* PAO (DSM 1707) planktonic cells after 16 h of incubation in LB-S. All photomicrographs were taken from triplicate cultures (1 – 3). Bars = 10 µm.

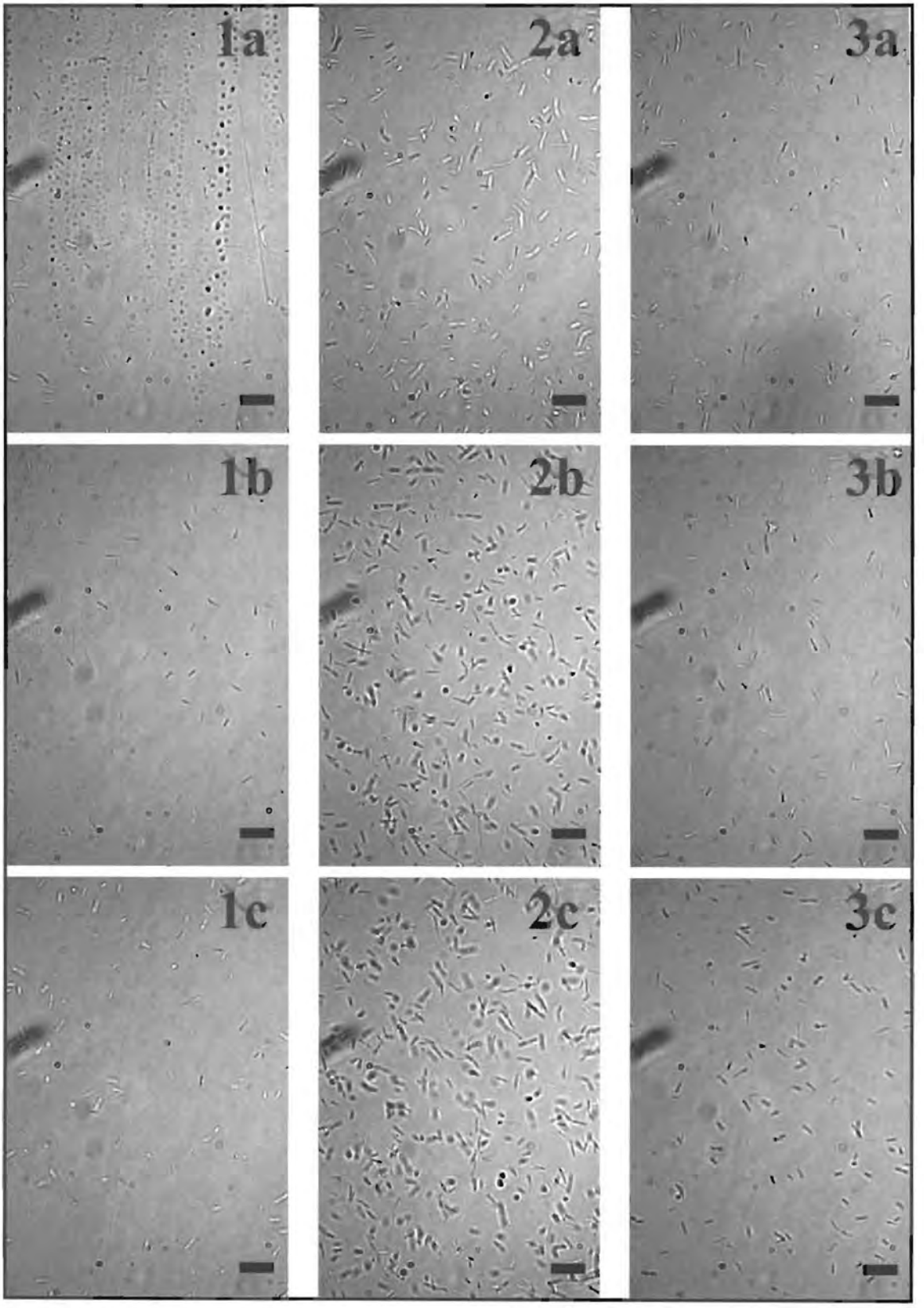

Fig. 7.2: Triplicate photomicrographs (a – c) showing *P. aeruginosa* PAO (DSM 1707) SIP cells after 16 h of incubation in LB-S. All photomicrographs were taken from triplicate cultures (1 – 3). Bars = 10 μm.

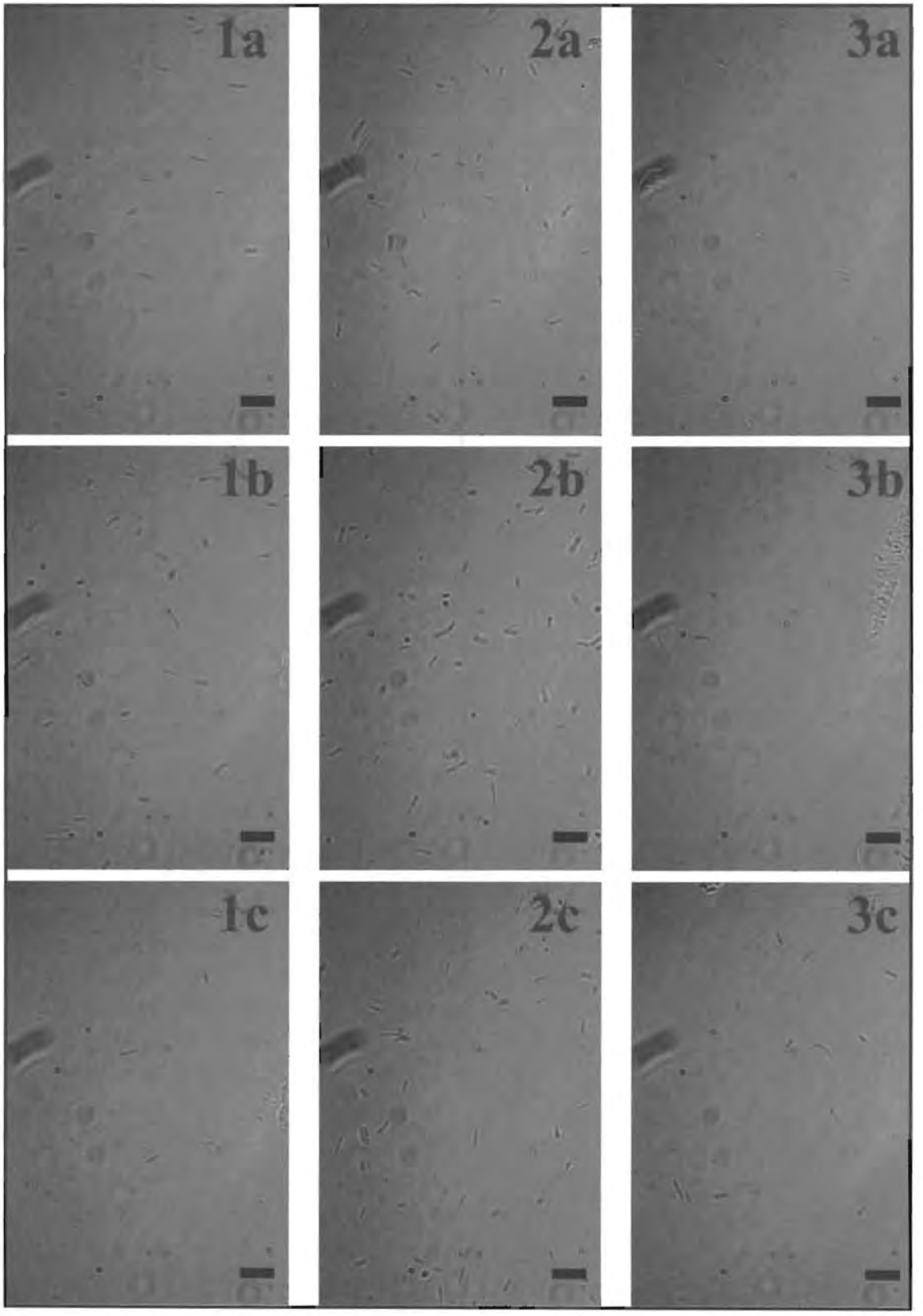

Fig. 7.3: Triplicate photomicrographs (a – c) showing *P. aeruginosa* PAO (DSM 1707) biofilm cells after 16 h of incubation in LB-S. All photomicrographs were taken from triplicate cultures (1 – 3). Bars = 10 µm.

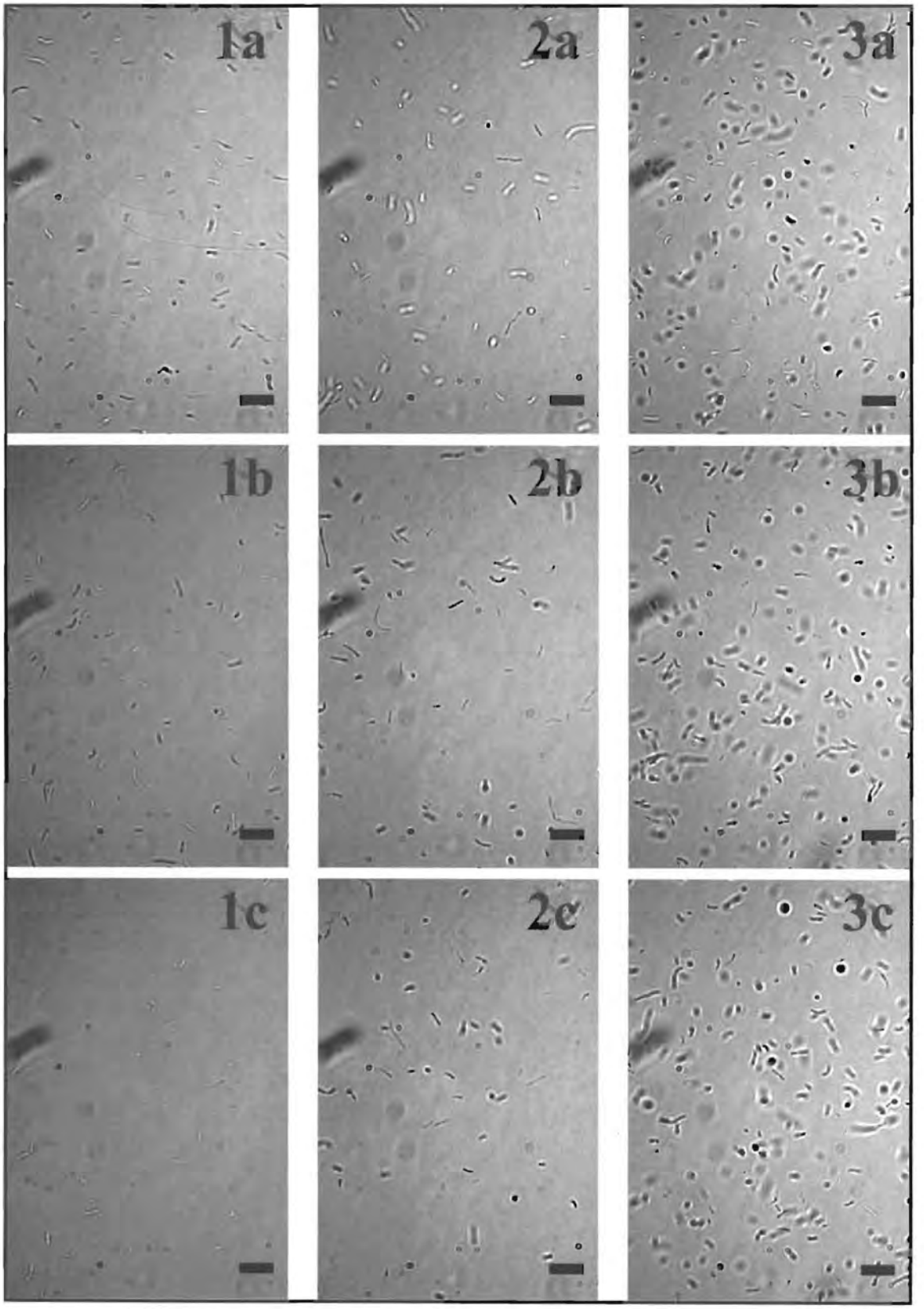

Fig. 7.4: Triplicate photomicrographs (a – c) showing *P. aeruginosa* PAO (DSM 1707) planktonic cells after 16 h of incubation in LB + NaCl. All photomicrographs were taken from triplicate cultures (1 – 3). Bars = 10 µm.

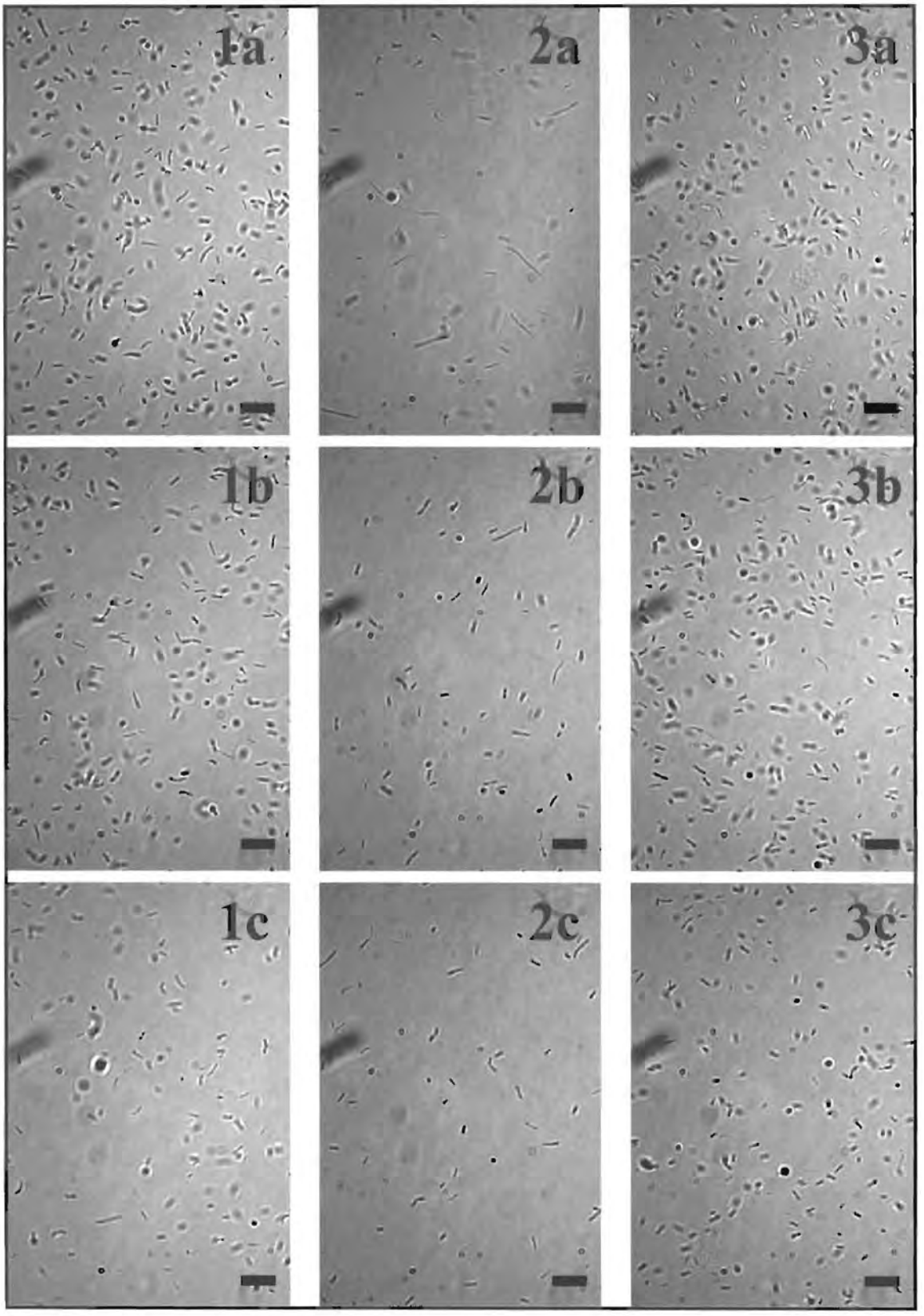

Fig. 7.5: Triplicate photomicrographs (a – c) showing *P. aeruginosa* PAO (DSM 1707) SIP cells after 16 h of incubation in LB + NaCl. All photomicrographs were taken from triplicate cultures (1 – 3). Bars = 10 μm.

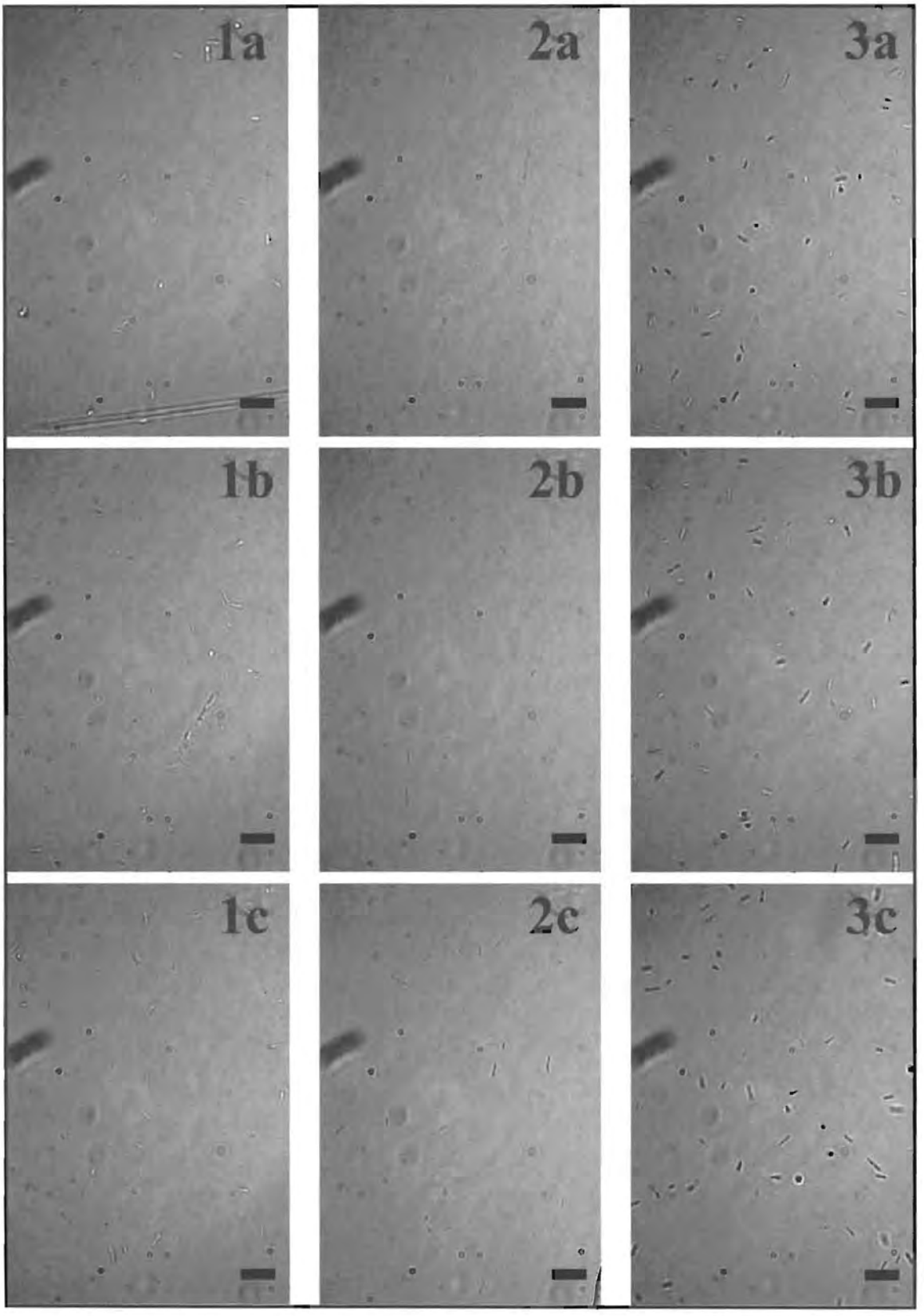

Fig. 7.6: Triplicate photomicrographs (a – c) showing *P. aeruginosa* PAO (DSM 1707) biofilm cells after 16 h of incubation in LB + NaCl. All photomicrographs were taken from triplicate cultures (1 – 3). Bars = 10 µm.

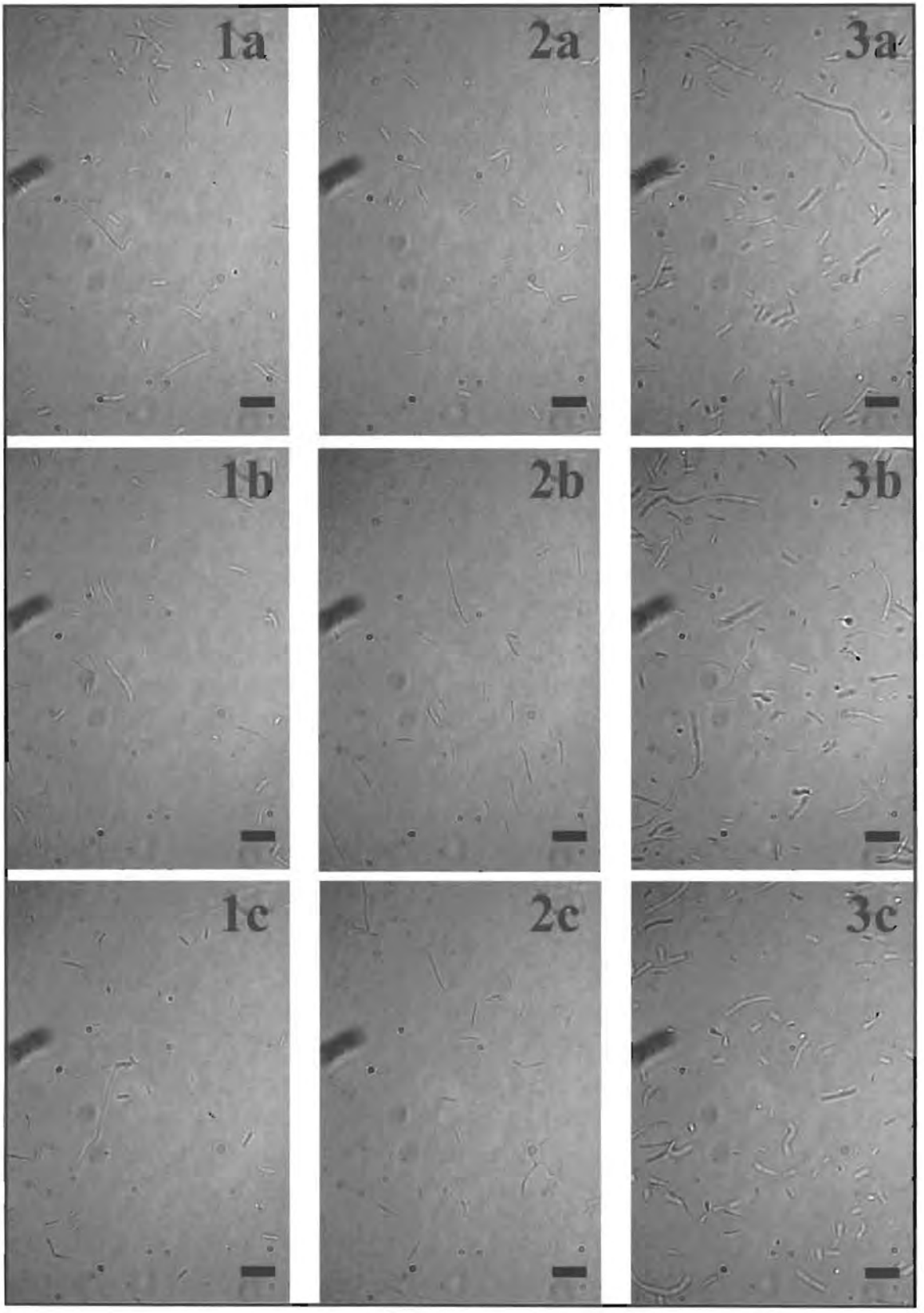

Fig. 7.7: Triplicate photomicrographs (a – c) showing *P. aeruginosa* PAO (DSM 1707) planktonic cells after 16 h of incubation in LB + EtOH. All photomicrographs were taken from triplicate cultures (1 – 3). Bars = 10 µm.

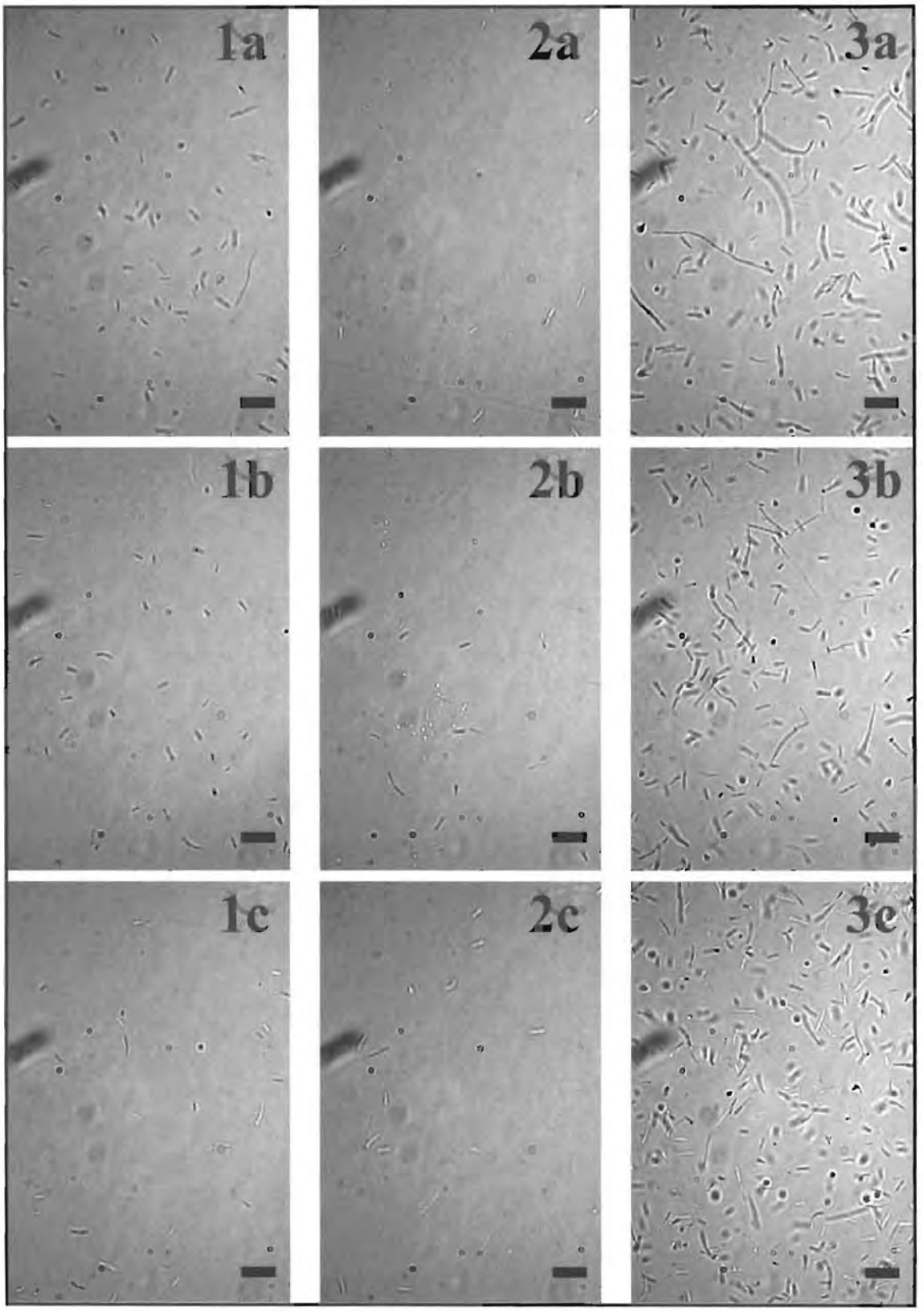

Fig. 7.8: Triplicate photomicrographs (a – c) showing *P. aeruginosa* PAO (DSM 1707) SIP cells after 16 h of incubation in LB + EtOH. All photomicrographs were taken from triplicate cultures (1 – 3). Bars = 10 µm.

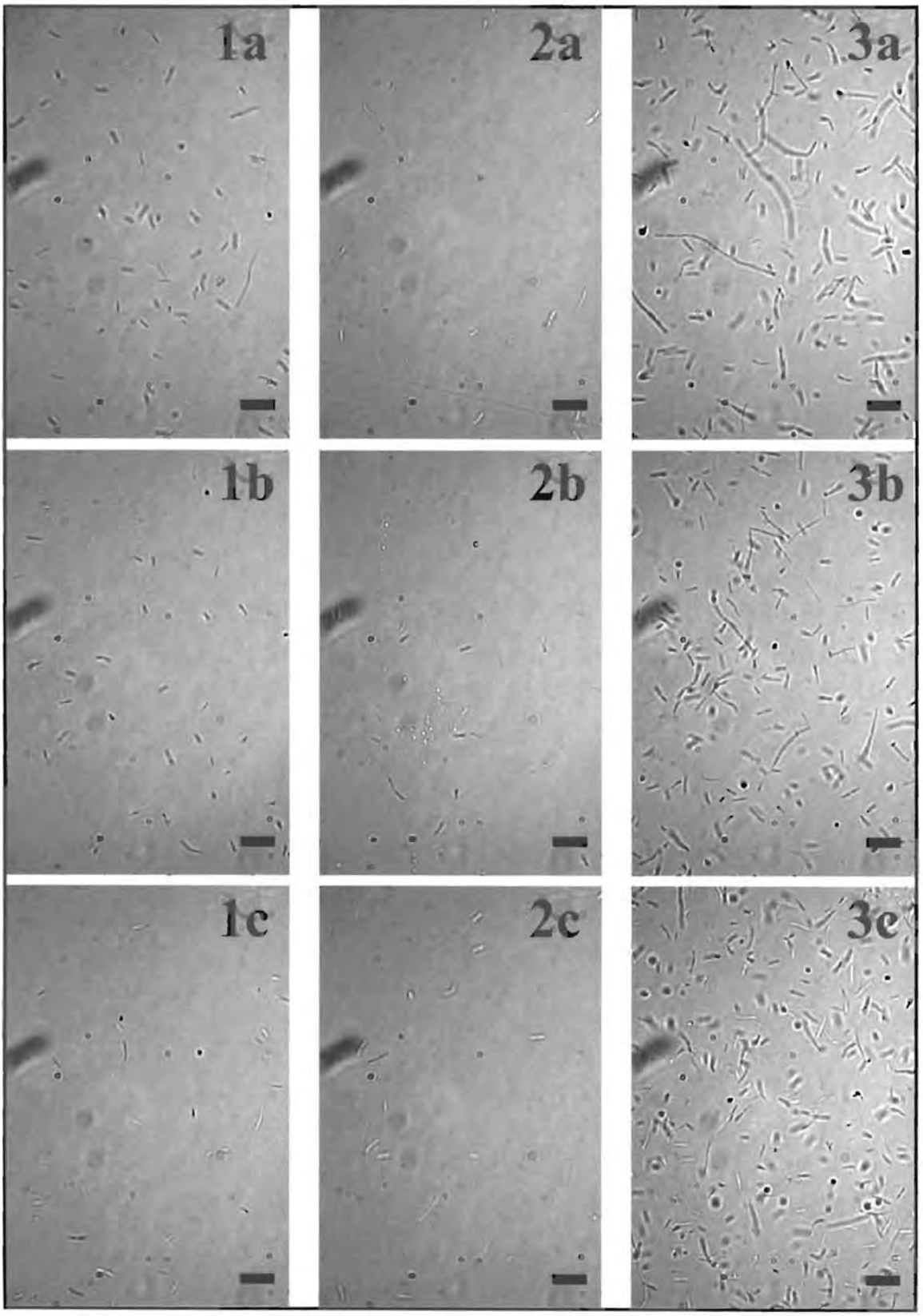

Fig. 7.9: Triplicate photomicrographs (a – c) showing *P. aeruginosa* PAO (DSM 1707) biofilm cells after 16 h of incubation in LB + EtOH. All photomicrographs were taken from triplicate cultures (1 – 3). Bars = 10 µm.

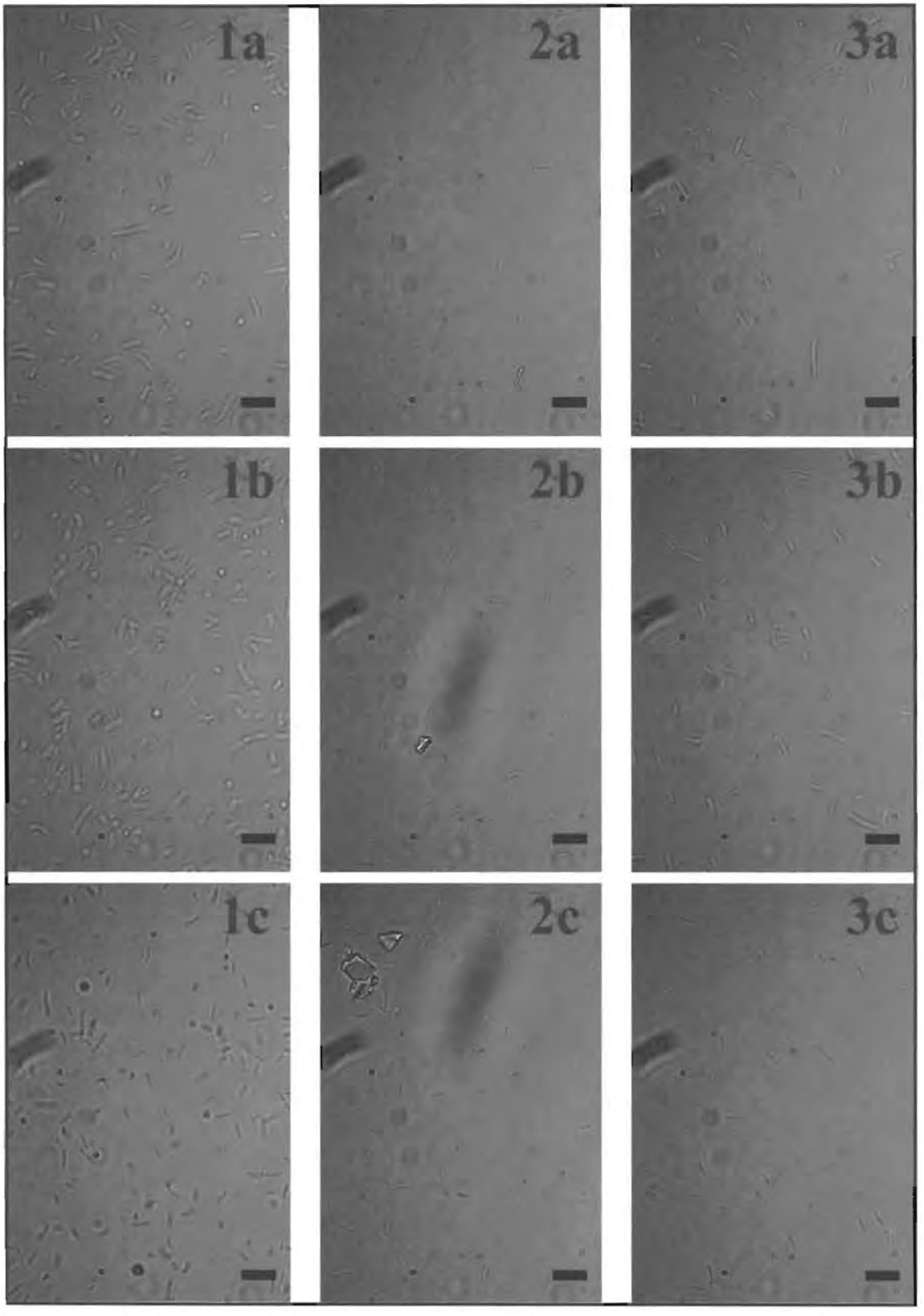

Fig. 7.10: Triplicate photomicrographs (a – c) showing *P. aeruginosa* pALacZsd planktonic cells after 16 h of incubation in LB-S. All photomicrographs were taken from triplicate cultures (1 – 3). Bars = 10 μm.

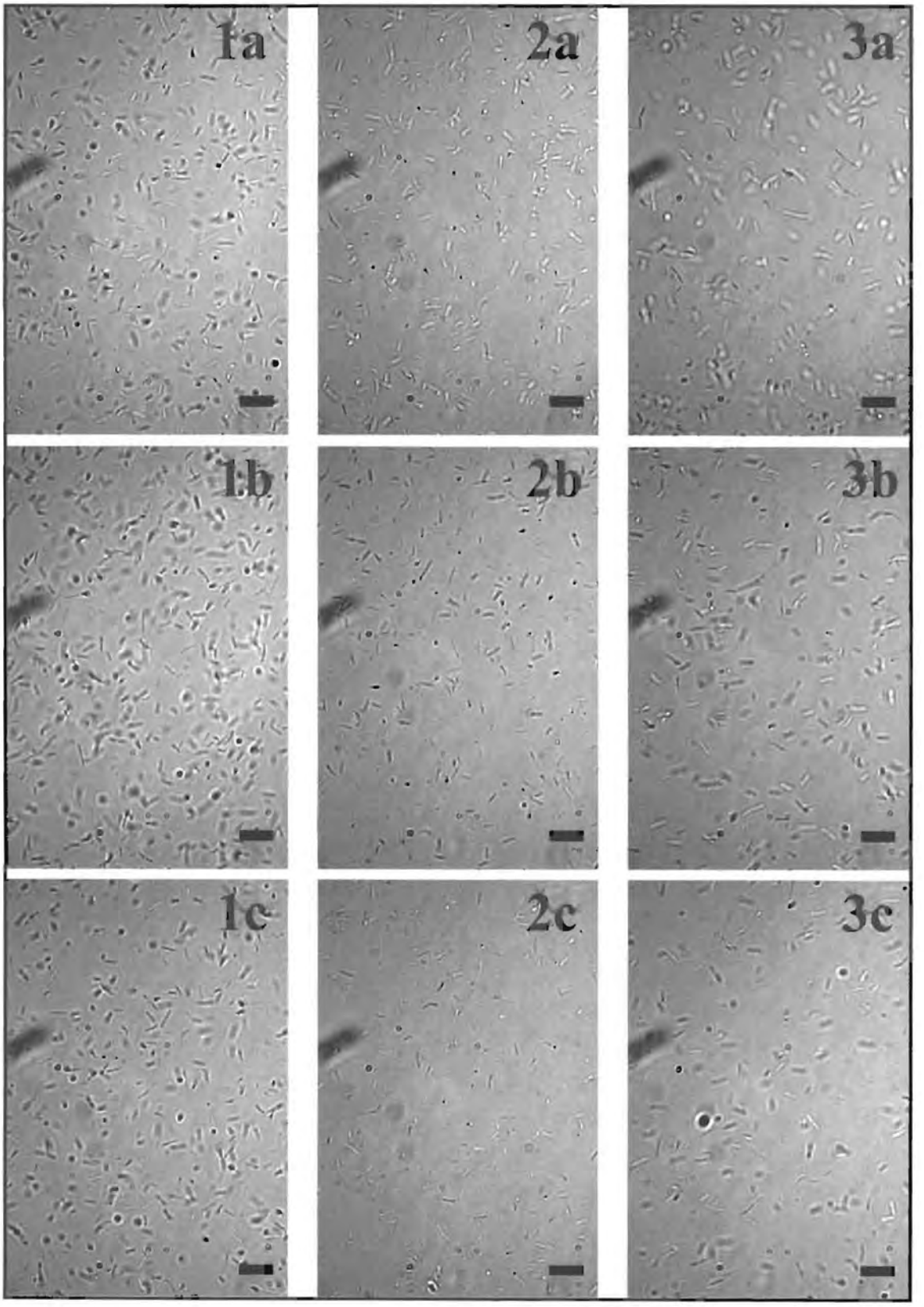

Fig. 7.11: Triplicate photomicrographs (a – c) showing *P. aeruginosa* pALacZsd SIP cells after 16 h of incubation in LB-S. All photomicrographs were taken from triplicate cultures (1 – 3). Bars = 10 μm.

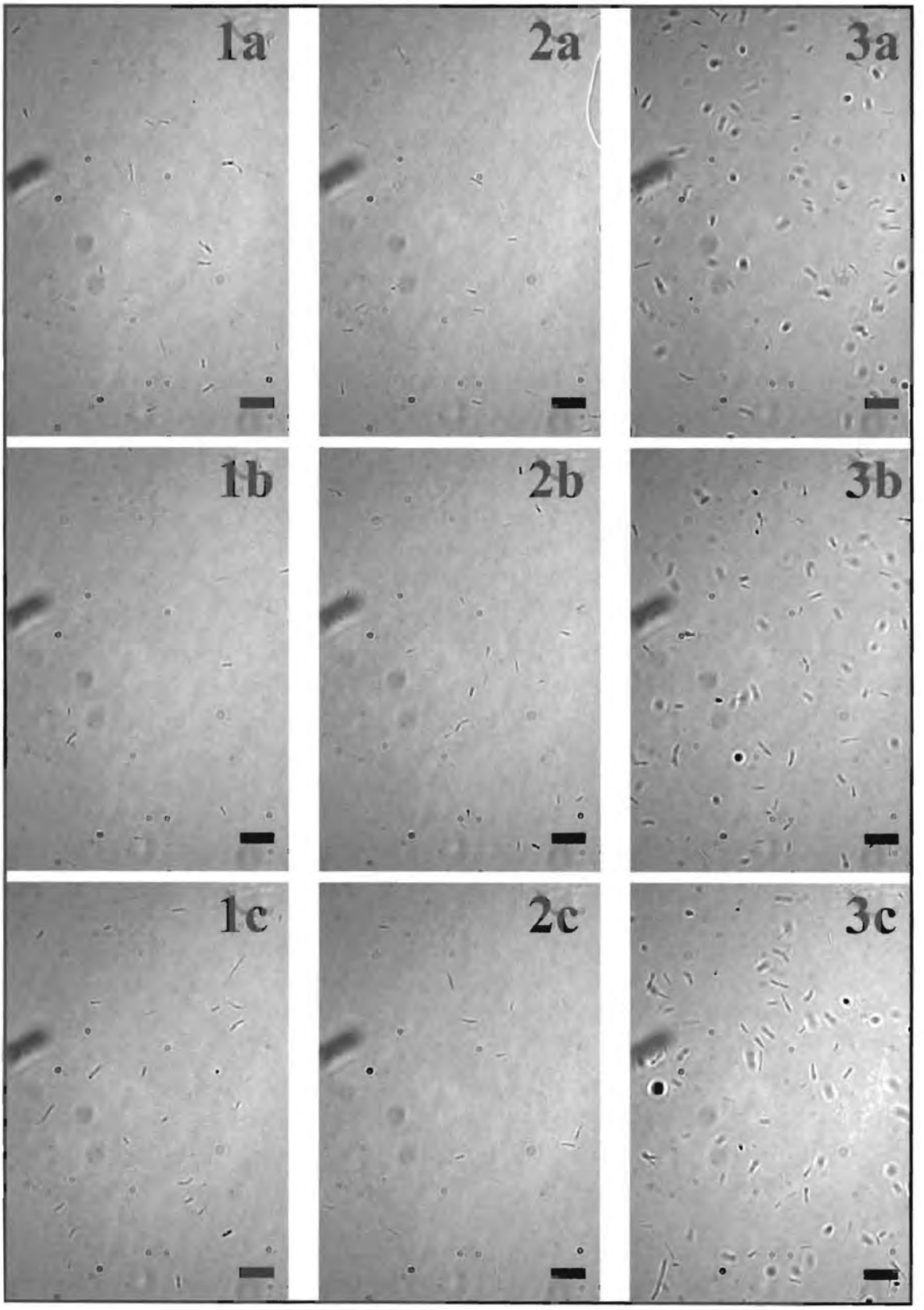

Fig. 7.12: Triplicate photomicrographs (a – c) showing *P. aeruginosa* pALacZsd biofilm cells after 16 h of incubation in LB-S. All photomicrographs were taken from triplicate cultures (1 – 3). Bars = 10 µm.

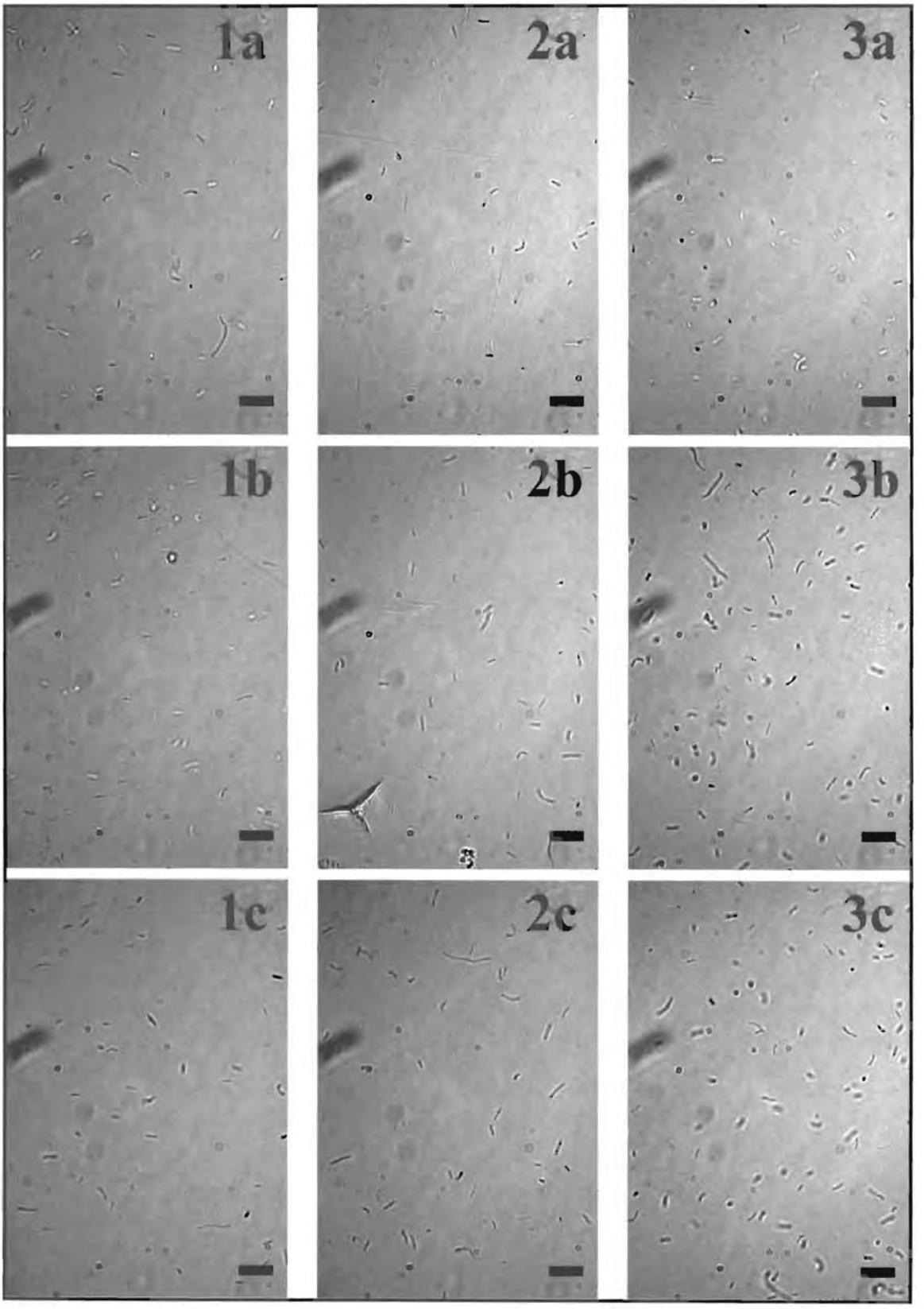

Fig. 7.13: Triplicate photomicrographs (a – c) showing *P. aeruginosa* pALacZsd planktonic cells after 16 h of incubation in LB + NaCl. All photomicrographs were taken from triplicate cultures (1 – 3). Bars = 10 μm.

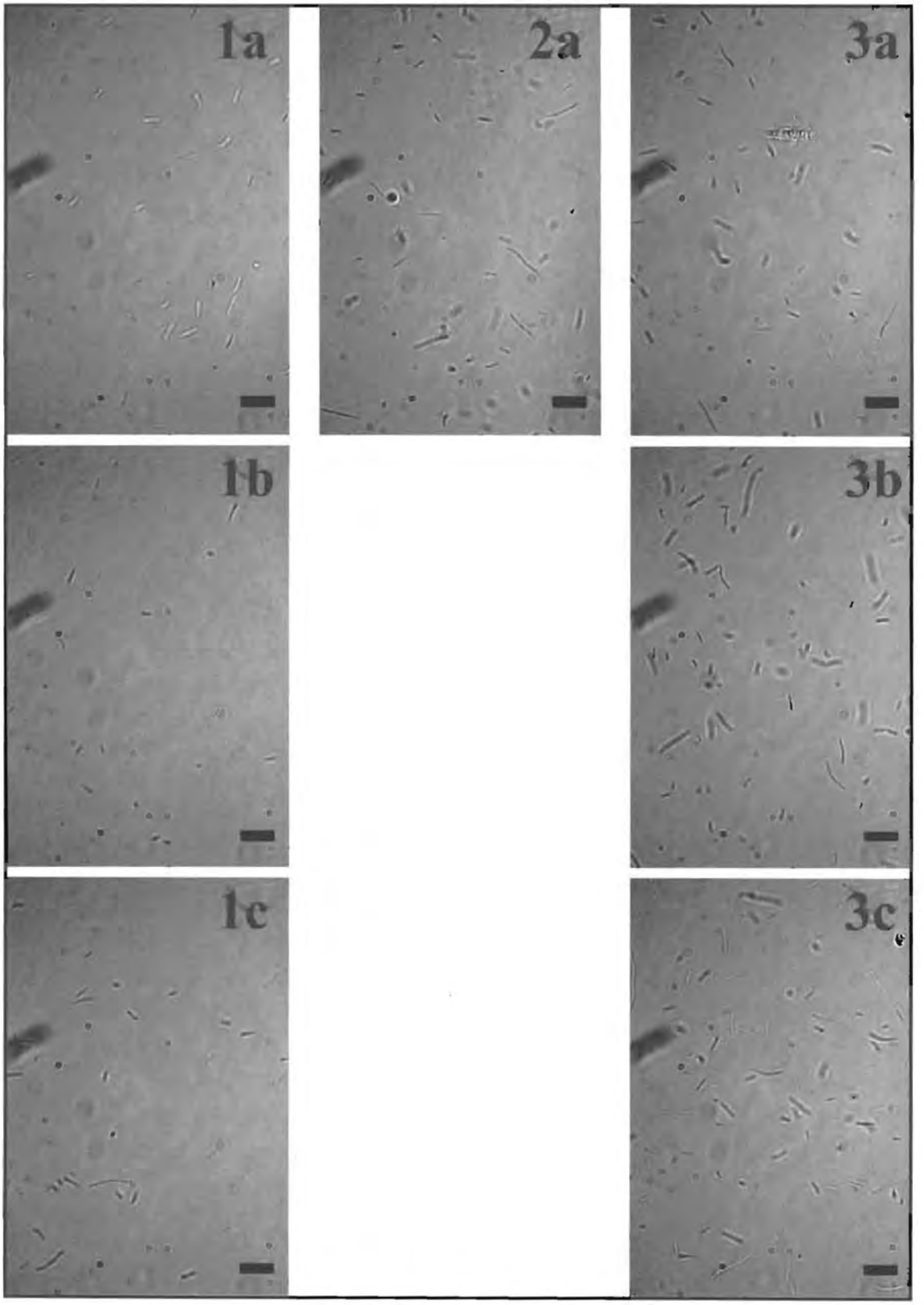

Fig. 7.14: Triplicate photomicrographs (a – c) showing *P. aeruginosa* pALacZsd SIP cells after 16 h of incubation in LB + NaCl. All photomicrographs were taken from triplicate cultures (1 – 3). Bars = 10 μm.

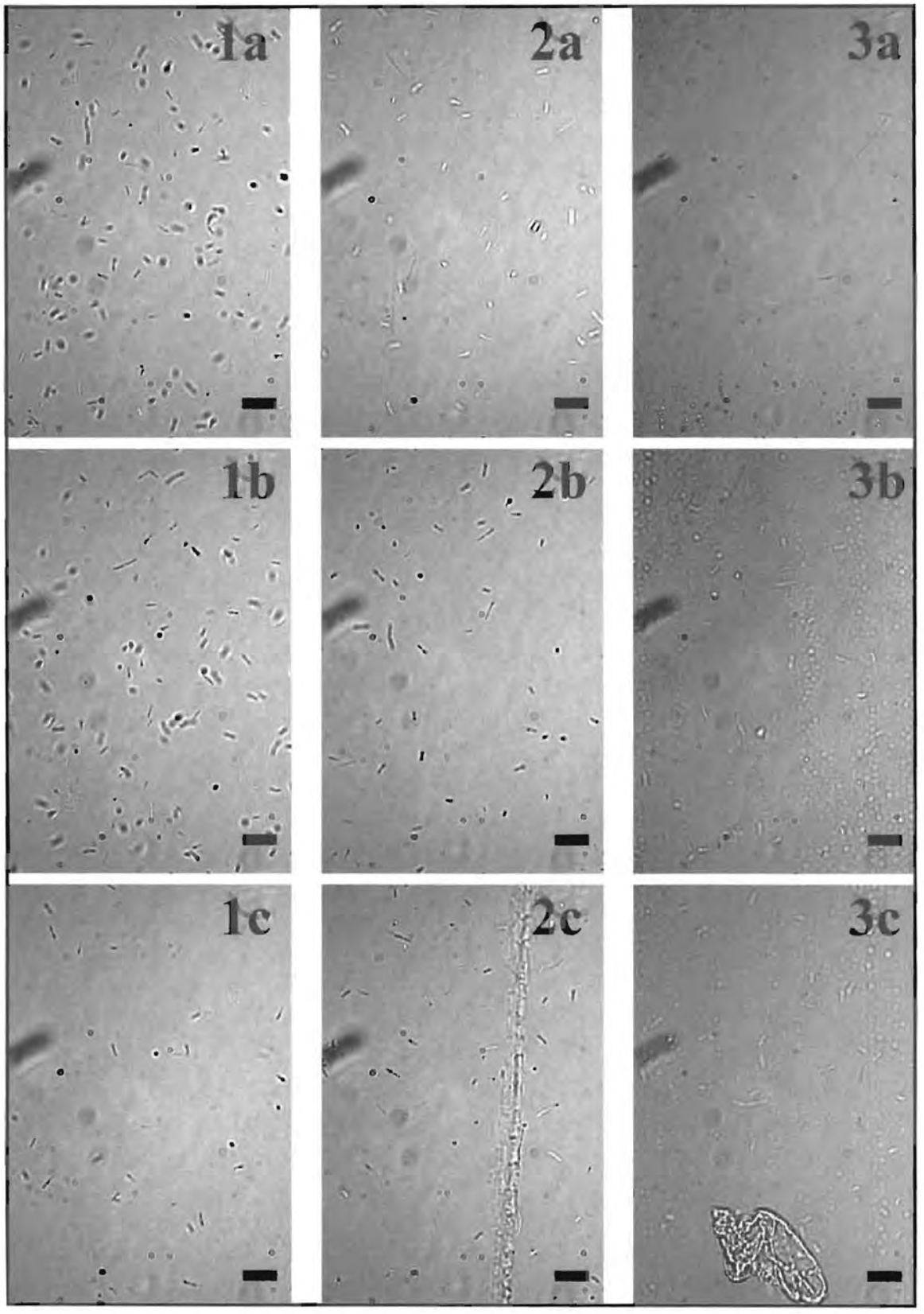

Fig. 7.15: Triplicate photomicrographs (a – c) showing *P. aeruginosa* pALacZsd biofilm cells after 16 h of incubation in LB + NaCl. All photomicrographs were taken from triplicate cultures (1 – 3). Bars = 10 μm.

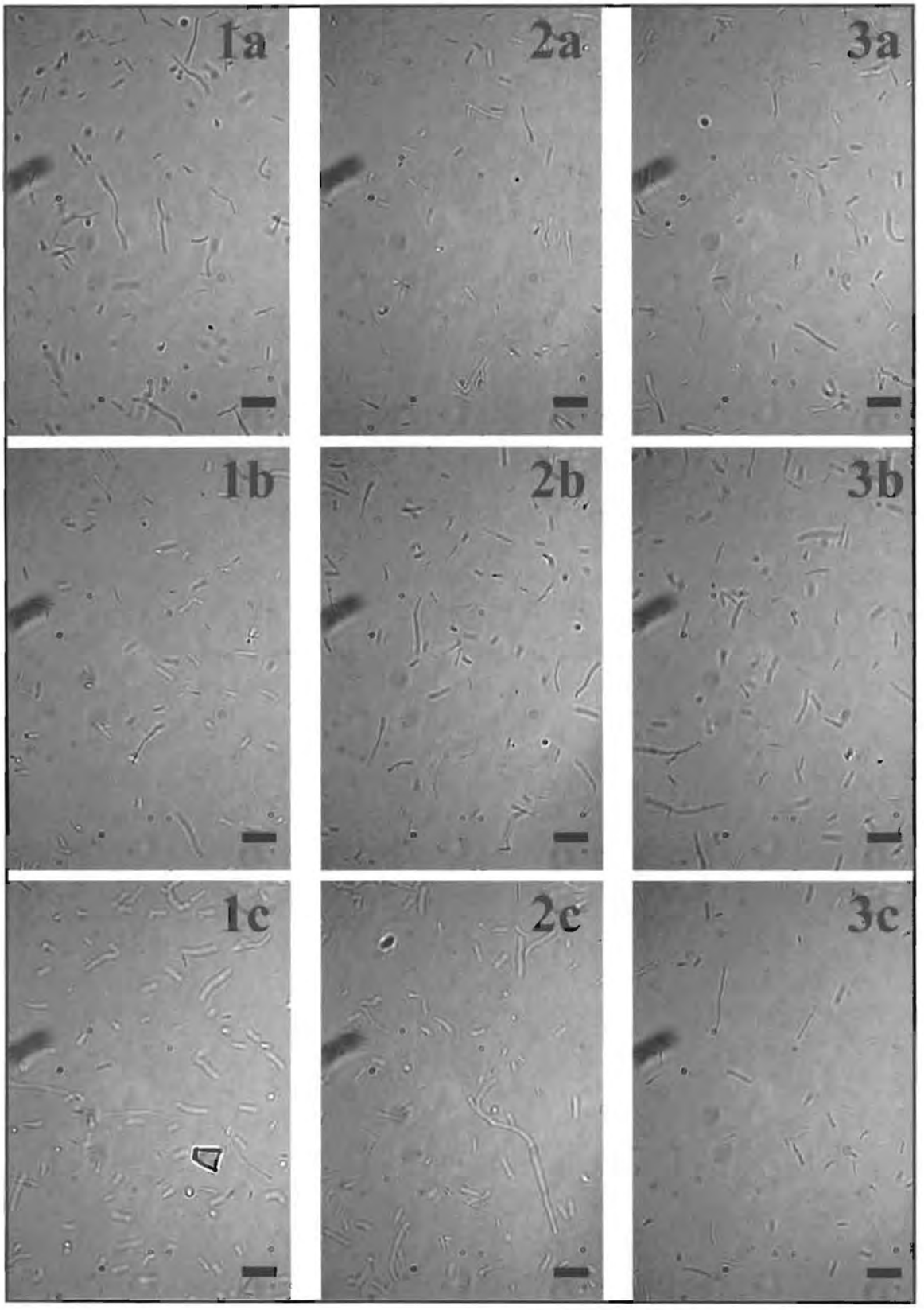

Fig. 7.16: Triplicate photomicrographs (a – c) showing *P. aeruginosa* pALacZsd planktonic cells after 16 h of incubation in LB + EtOH. All photomicrographs were taken from triplicate cultures (1 – 3). Bars = 10 μm.

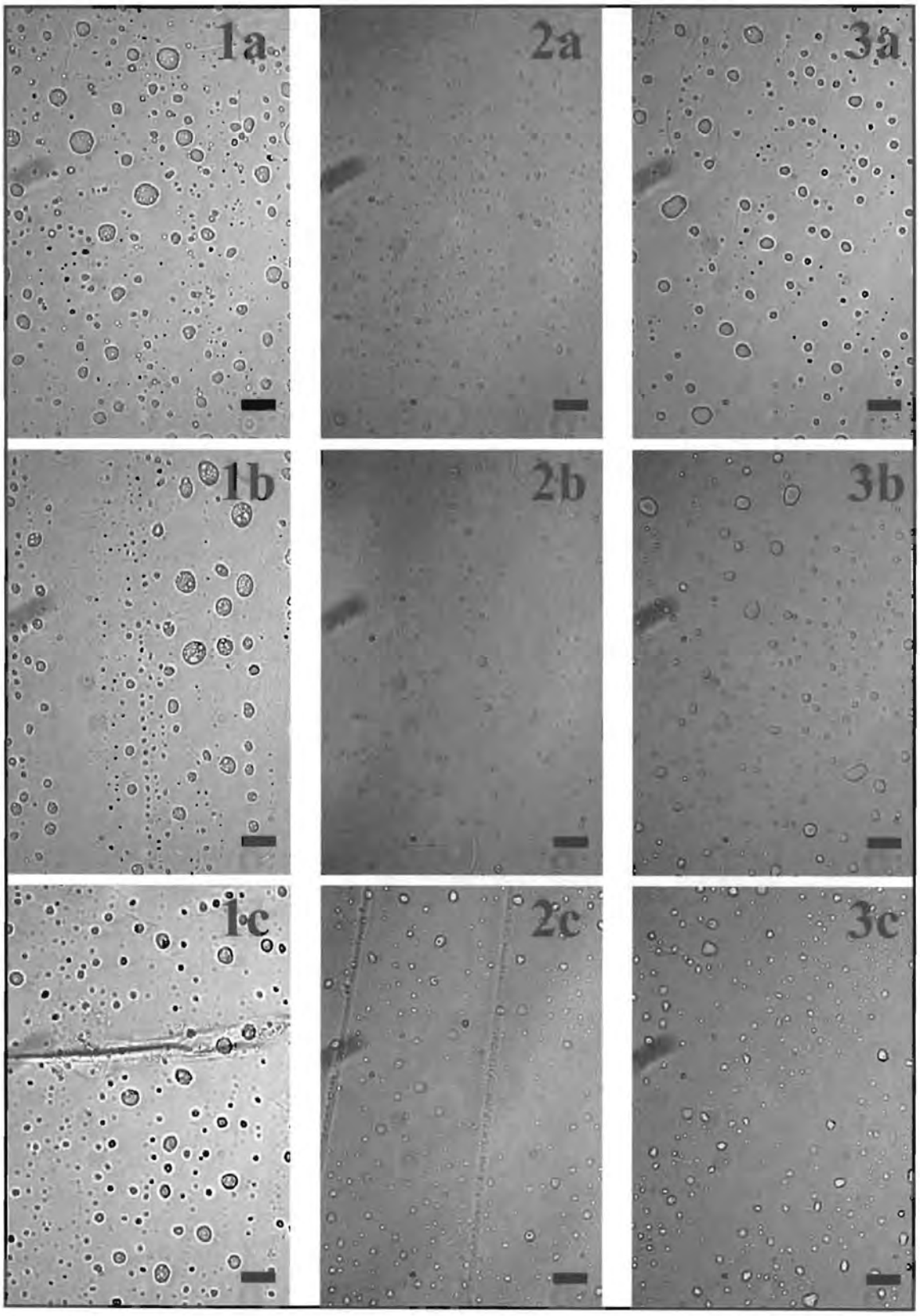

Fig. 7.17: Triplicate photomicrographs (a – c) showing *P. aeruginosa* pALacZsd SIP cells after 16 h of incubation in LB + EtOH. All photomicrographs were taken from triplicate cultures (1 – 3). Bars = 10 μm.

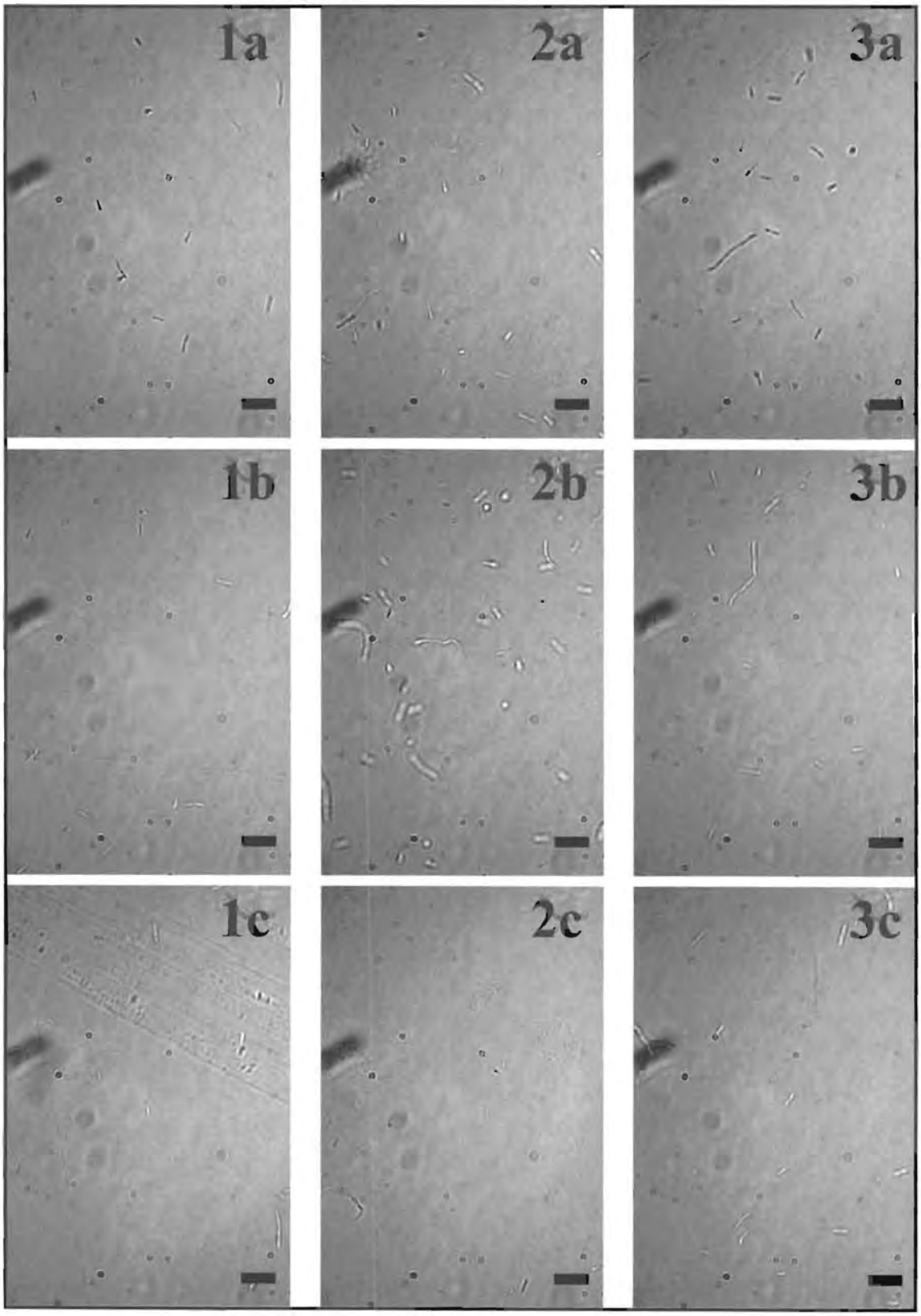

Fig. 7.18: Triplicate photomicrographs (a – c) showing *P. aeruginosa* pALacZsd biofilm cells after 16 h of incubation in LB + EtOH. All photomicrographs were taken from triplicate cultures (1 – 3). Bars = 10 μm.

APPENDIX 2

FLUORESCENCE MICROSCOPY OF *Pseudomonas aeruginosa* PAO (DSM 1707) AFTER STAINING OF CELLS WITH 23S rRNA GAMMA PROTEOBACTERIAL OR *Pseudomonas* GROUP 1 SPECIFIC PROBES

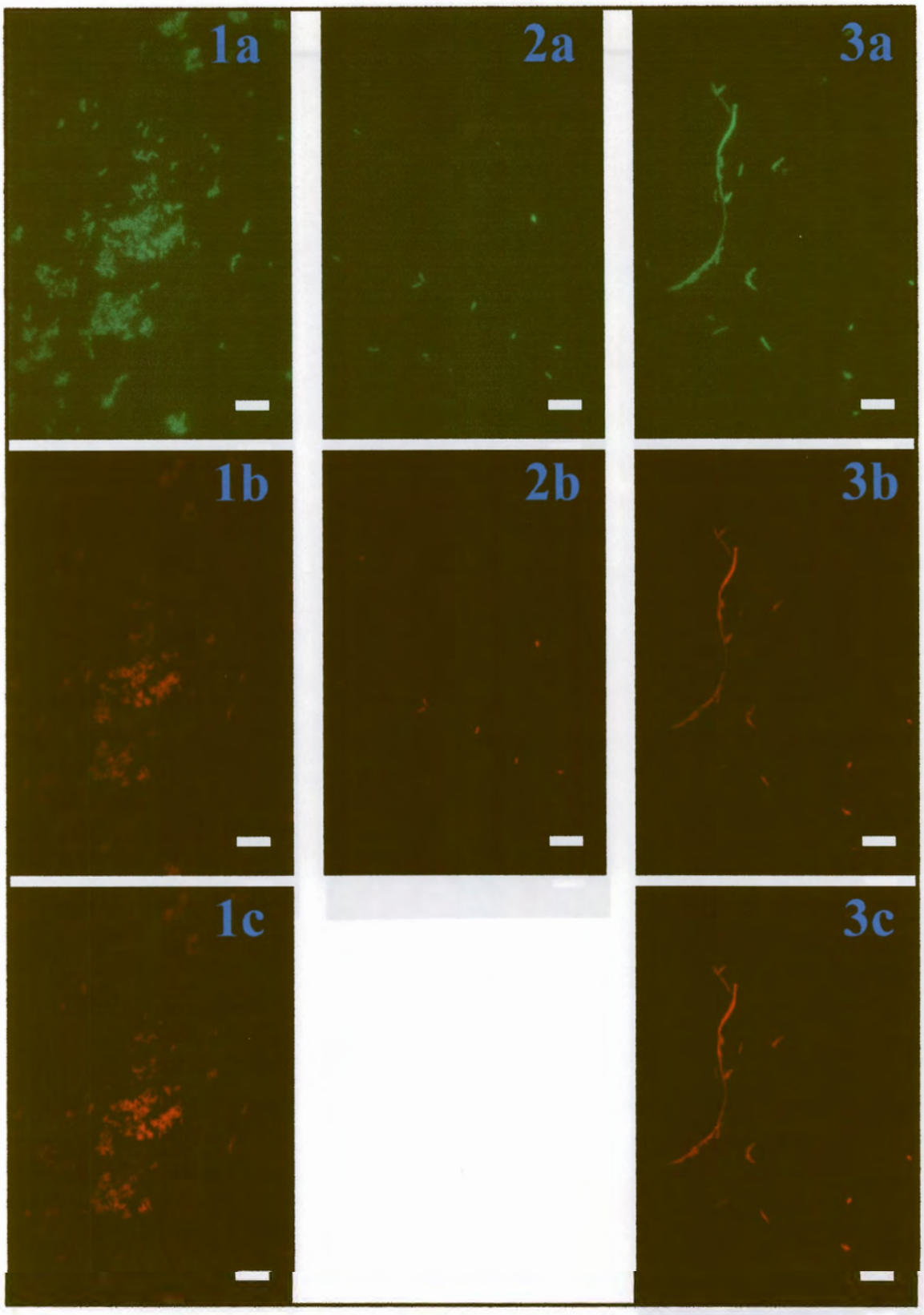

Fig. 8.1: Photomicrographs showing *P. aeruginosa* (1) planktonic cells grown in LB-S, (2) total attached cells grown in LB + NaCl and (3) total attached cells grown in LB + EtOH after 16 h incubation. Cells have been probed with a Gamma proteobacterial (green) or a *Pseudomonas* group 1 specific (red) probe. Bars = 10 µm.

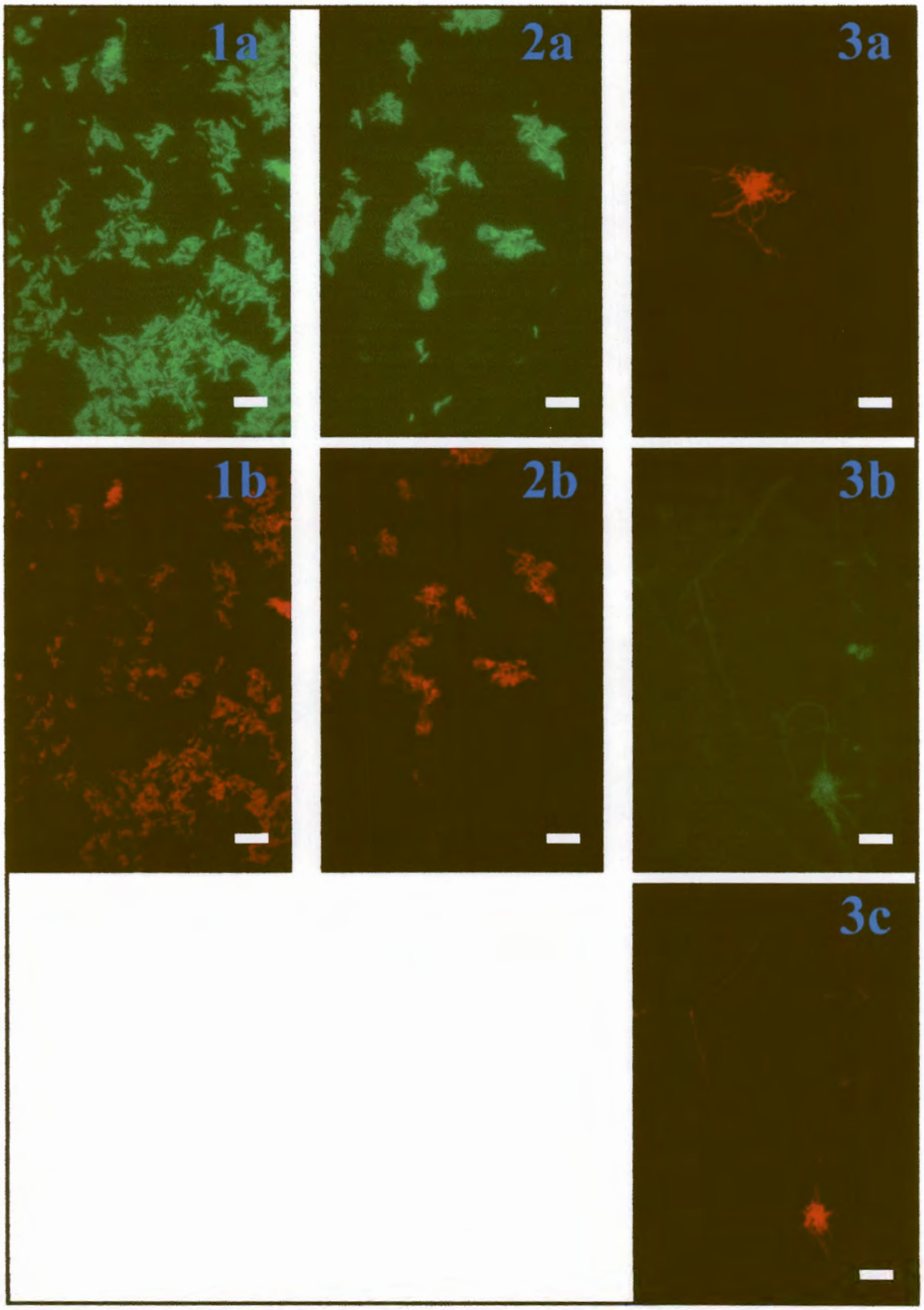

Fig. 8.2: Photomicrographs showing *P. aeruginosa* pALacZsd (1) planktonic cells grown in LB-S, (2) total attached cells grown in LB + NaCl and (3) total attached cells grown in LB + EtOH after 16 h incubation. Cells have been probed with a Gamma proteobacterial (green) or a *Pseudomonas* group 1 specific (red) probe. Bars = 10 µm.

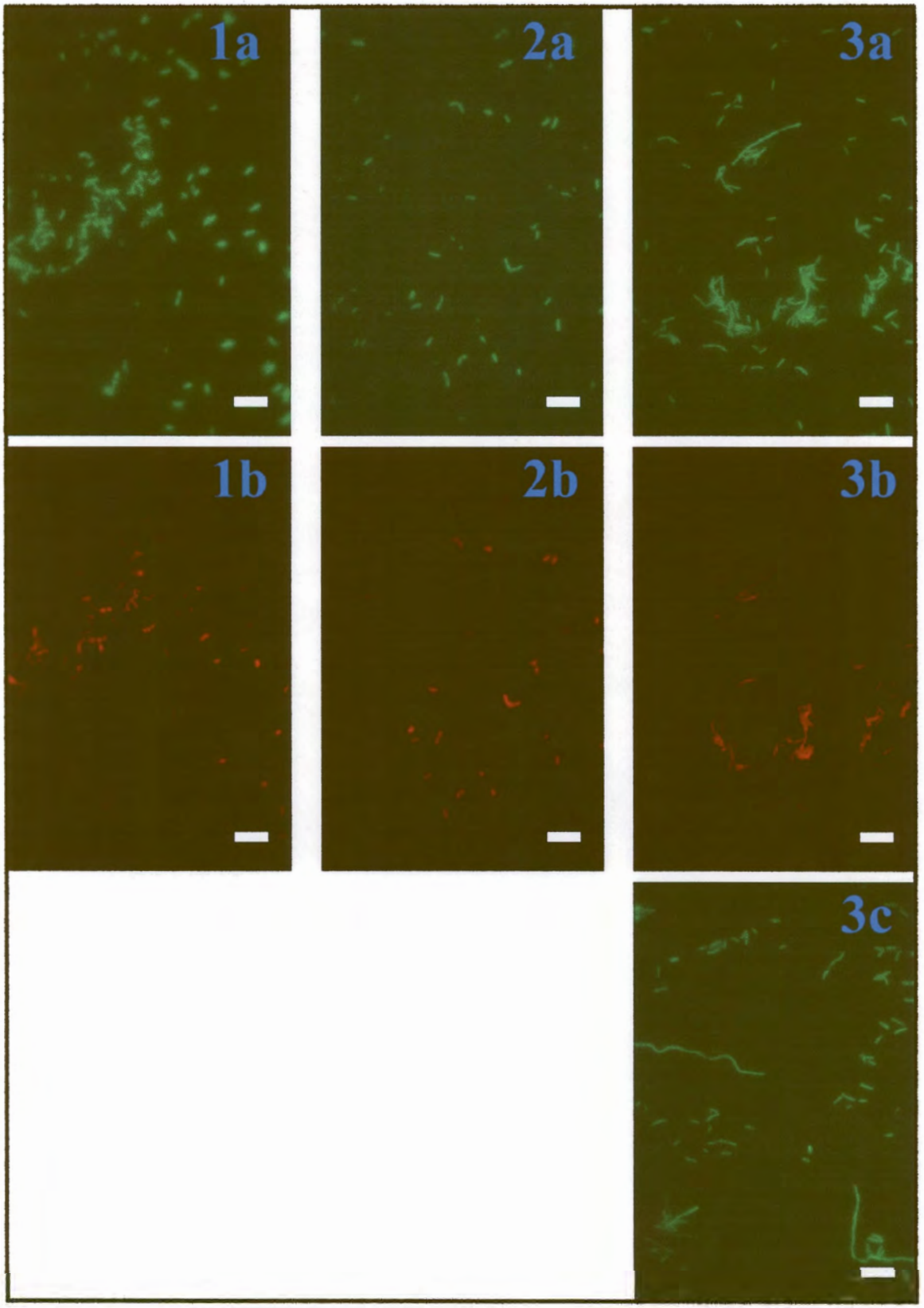

Fig. 8.3: Photomicrographs showing *P. aeruginosa* pALacZsdAg (1) planktonic cells grown in LB-S, (2) total attached cells grown in LB + NaCl and (3) total attached cells grown in LB + EtOH after 16 h incubation. Cells have been probed with a Gamma proteobacterial (green) or a *Pseudomonas* group 1 specific (red) probe. Bars = 10 μm.

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