



Regulation of alginate biosynthesis in *Pseudomonas aeruginosa* biofilms
by David Gwilym Davies

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology

Montana State University

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Abstract:

Recombinant gene technology was used to evaluate the regulation of promoters for the alginate biosynthesis genes *algC* and *algD* and the degradative enzyme alginate lyase in a mucoid strain of *Pseudomonas aeruginosa*. These investigations were performed on suspended cell cultures and on developing and mature biofilms in continuous culture on Teflon and glass substrata. Activity of the *algC* promoter was shown to be up-regulated in biofilm cells compared with planktonic cells in liquid medium. Promoter up-expression correlated with alginate biosynthesis as measured by infrared spectroscopy, uronic acid accumulation and alginate-specific antibodies. The *algC* promoter was shown to have maximum activity in planktonic cultures during the late lag and early log phases of the cell growth cycle. During a time course experiment, biofilm *algC* activity exceeded planktonic activity except during the period immediately following inoculation into fresh medium.

Detection of *in vivo* expression of *algC* and *algD* promoter activity was performed using a fluorogenic substrate for a plasmid-borne *lacZ* gene product, evaluated by microscopy coupled with image analysis. Using this technique, cells were tracked over time and analyzed for *algC* or *algD* activity. During the initial stages of biofilm development, cells already attached to a glass surface exhibited up-expression of both *algC* and *algD*. However, initial cell attachment to the substratum appeared to be independent of either *algC* or *algD* promoter activity. Furthermore, cells not exhibiting either *algC* or *algD* up-expression were shown to be less capable of remaining at a glass surface under flowing conditions than were cells in which up-expression was detected.

Alginate lyase activity was studied in biofilm cultures of *P. aeruginosa* 8830 containing a *tac/algL* transcriptional fusion plasmid. Using IPTG these bacteria could be induced to produce elevated levels of alginate lyase. Results demonstrated that alginate lyase alone was not sufficient to influence sloughing or detachment of biofilm material. When either NaCl or SDS was used to release overproduced lyase from the bacteria, sloughing was observed. The addition of purified lyase to the menstruum flowing past developed cell clusters resulted in no sloughing or detachment.

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Doctor of Philosophy

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Microbiology

MONTANA STATE UNIVERSITY-BOZEMAN
Bozeman, Montana

January 1996

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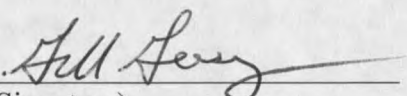
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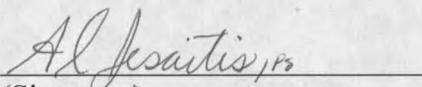
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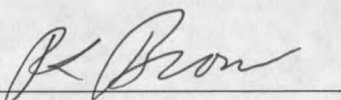
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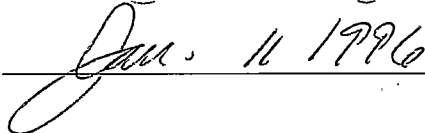
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ACKNOWLEDGMENTS

I would like to express my appreciation to my academic and research advisor, Dr. Gill Geesey for his support, invaluable guidance and encouragement during my studies as a graduate student under his supervision. I will miss working under him and will never forget the opportunities that he has provided me. I would also like to thank Dr. A. M. Chakrabarty for his contributions to research design, his helpful criticisms and his generosity in providing the genetic constructs without which this thesis research would have been impossible. Dr. Aoife Boyd (former fellow graduate student) was a wonderful working partner who helped enrich my graduate experience as well as my thesis. My committee deserves thanks for their contributions of advice, direction, and for not being too hard on me during my "comps". Thank you Gordon, Barry, Sam and Joan.

Working at the Center for Biofilm Engineering has been one of the more enriching experiences in my life. The people here have been like family and no simple acknowledgment can do justice to the influence all of the people at "The Center" have had on me. Three friends, comrades, teachers, supporters and major influences in my life must be thanked by name; these are Anne Camper, Warren Jones and Paul Stoodley. Without these three, I hesitate to guess where my life would have led.

I would finally like to thank the people who, above all others, have supported and encouraged me in my educational endeavors. These are my family, Mom, Dad, Joshua and Diana. Thank you. You were with me every step of the way.

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ABSTRACT

Recombinant gene technology was used to evaluate the regulation of promoters for the alginate biosynthesis genes *algC* and *algD* and the degradative enzyme alginate lyase in a mucoid strain of *Pseudomonas aeruginosa*. These investigations were performed on suspended cell cultures and on developing and mature biofilms in continuous culture on Teflon and glass substrata. Activity of the *algC* promoter was shown to be up-regulated in biofilm cells compared with planktonic cells in liquid medium. Promoter up-expression correlated with alginate biosynthesis as measured by infrared spectroscopy, uronic acid accumulation and alginate-specific antibodies. The *algC* promoter was shown to have maximum activity in planktonic cultures during the late lag and early log phases of the cell growth cycle. During a time course experiment, biofilm *algC* activity exceeded planktonic activity except during the period immediately following inoculation into fresh medium.

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CHAPTER 1

INTRODUCTION

Biofilms are biological films that develop and persist at interfaces in aqueous environments in natural and artificial aquatic systems. These biological films are composed of microorganisms embedded in an organic gelatinous structure composed of one or more matrix polymers which are secreted by the resident microorganisms.

Biofilm bacteria have been shown to predominate in numbers and in metabolic activity in natural (Geesey *et al.*, 1977; Costerton *et al.*, 1994), industrial (Boivin *et al.*, 1991), and medical (Khoury *et al.*, 1992) ecosystems. The involvement of extracellular polymers in bacterial biofilms has been documented for both aquatic (Jones *et al.*, 1969; Sutherland, 1980) and marine bacteria (Floodgate, 1972), and the association of exopolysaccharides with attached bacteria has been demonstrated using electron microscopy (Geesey *et al.*, 1977; Dempsey, 1981) and light microscopy (Zobell, 1943; Allison and Sutherland, 1984). The presence of such exopolysaccharides is believed to be involved in the development of the microbial biofilm (Wardell *et al.*, 1983; Allison and Sutherland 1987). Analysis of biofilm bacteria isolated from freshwater and marine environments has shown that the polymers they produce are composed largely of acidic polysaccharides (Fletcher, 1980; Sutherland, 1980; Christensen and Charaklis, 1990).

The acidic polysaccharide alginate has been shown to be involved in biofilm formation by *Pseudomonas aeruginosa* in pulmonary infections (Dogget, 1977). This

extracellular substance is composed of β -1,4-linked D-mannuronic acid and its C-5 epimer, L-guluronic acid (Figure, 1.1).

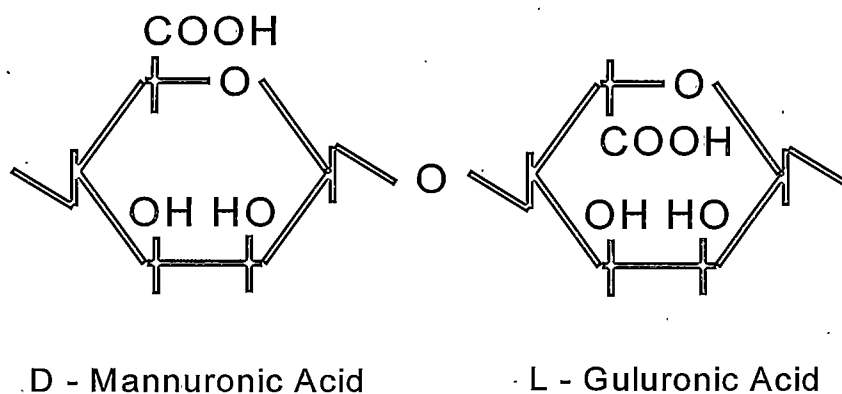


Figure 1.1. Structure of alginate.

One organism commonly isolated from biofilms in the environment, industrial plumbing systems and from infections, is the bacterium *Pseudomonas aeruginosa*. In 1966, Linker and Jones reported that this organism has the ability to produce alginate. Alginate overproducing strains of *P. aeruginosa* generate copious quantities of the acidic polysaccharide and as a result have a distinctive mucoid colony morphology. The term mucoid when applied to *P. aeruginosa* is restricted to those strains producing large slimy colonies during 24 hour incubation on common agar-based media and whose appearance results from the overproduction of alginate as a major component of the bacterial glycocalyx (Govan, 1990). Mucoid variants of *P. aeruginosa* are most commonly

isolated from the respiratory tract infections that accompany the genetic disease, cystic fibrosis (Dogget, 1977).

Cystic Fibrosis (CF), is the most prevalent lethal genetic disease among people of European descent. It is inherited as an autosomal recessive trait at a rate of 1 in 2,000 live births among Caucasians (May *et al.*, 1991). Clinical manifestations of the disease include chronic pulmonary disease with a persistent cough, elevated sweat electrolytes and pancreatic abnormalities that result in problems with nutrient absorption and recurrent diarrhea (Chartrand and Marks, 1983). CF is caused by a genetic defect resulting in an abnormal electrolyte transport and mucous secretion from exocrine glands and secretory epithelia (McPherson and Dormer, 1987).

During initial phases of CF lung infections, *P. aeruginosa* isolates display a nonmucoid colony morphology which is not associated with alginate production. However, over time and with the initiation of antibiotic therapy, the nonmucoid form begins to over-produce alginate (Doggett, *et al.*, 1966). This mucoid form predominates in the cystic fibrosis-damaged lung and is the major pathogen isolated from sputum cultures of patients in advanced stages of the disease. The mucoid form has also been identified in approximately 2% of non-CF *P. aeruginosa* infections (Doggett, 1969). Such mucoid isolates have not been recovered from the environment.

An interesting aspect of the mucoid phenotype is that it is unstable when cells are grown in laboratory culture; such bacteria have an unusually high reversion rate to the nonmucoid phenotype (Govan, 1975; Govan *et al.*, 1979). It is because of the high

frequency of spontaneous mutations to nonmucoidy that it has been difficult to characterize and genetically map structural gene mutations in the alginate pathway.

Linker and Jones in 1964, were the first to characterize the alginate produced by *P. aeruginosa*. This alginate was later found to be similar to the commercially useful polymer obtained from marine algae (Linker and Jones, 1966; Lin and Hassid, 1966a) and to the polysaccharide later identified with the capsular material of *Azotobacter vinelandii* (Gorin and Spencer, 1966).

Preliminary studies on the alginate biosynthesis pathway were performed by Lin and Hassid (1966a, 1966b), following investigations performed on the exopolysaccharide produced by the alga *Fucus gardneri* and by Pindar and Bucke (1975), in studies on the slime layer polysaccharide of *A. vinelandii*. This seminal work demonstrated that the first alginate precursor was fructose 6-phosphate which was recruited from the carbohydrate pool of the Entner-Doudoroff pathway and fructose 1,6-bisphosphate aldolase. Similar recruitment of this precursor has since been shown to take place in *P. aeruginosa* (May *et al.*, 1991).

The first alginate biosynthesis enzymes were reported by Piggott *et al.* (1981). They were able to demonstrate the presence of very low levels of phosphomannose isomerase (PMI), GDP-mannose pyrophosphorylase (GMP), and GDP-mannose dehydrogenase (GMD). Padgett and Phibbs (1986), detected another alginate biosynthesis enzyme, phosphomannomutase (PMM).

In continuing studies into the regulation of alginate biosynthesis, the research group headed by Dr. A. M. Chakrabarty, at the University of Illinois at Chicago, was able

to isolate a number of stable alginate mutants derived from the lung of a CF patient (Figure 1.2).

The original isolate was mucoid on solid, rich, nutrient medium, with a high background reversion rate. One of the revertant mutants was found to be stably nonmucoid and was designated strain 8822. This strain was mutagenized with ethyl methanesulfonate (EMS) to produce the stable mucoid derivative, strain 8830. Again, following EMS mutagenesis, a fourth strain was produced, 8852 which is nonmucoid but can be complemented back to mucoidy by the addition of AlgR1, a DNA binding protein. To determine that the mutational changes resulting from these procedures were not the

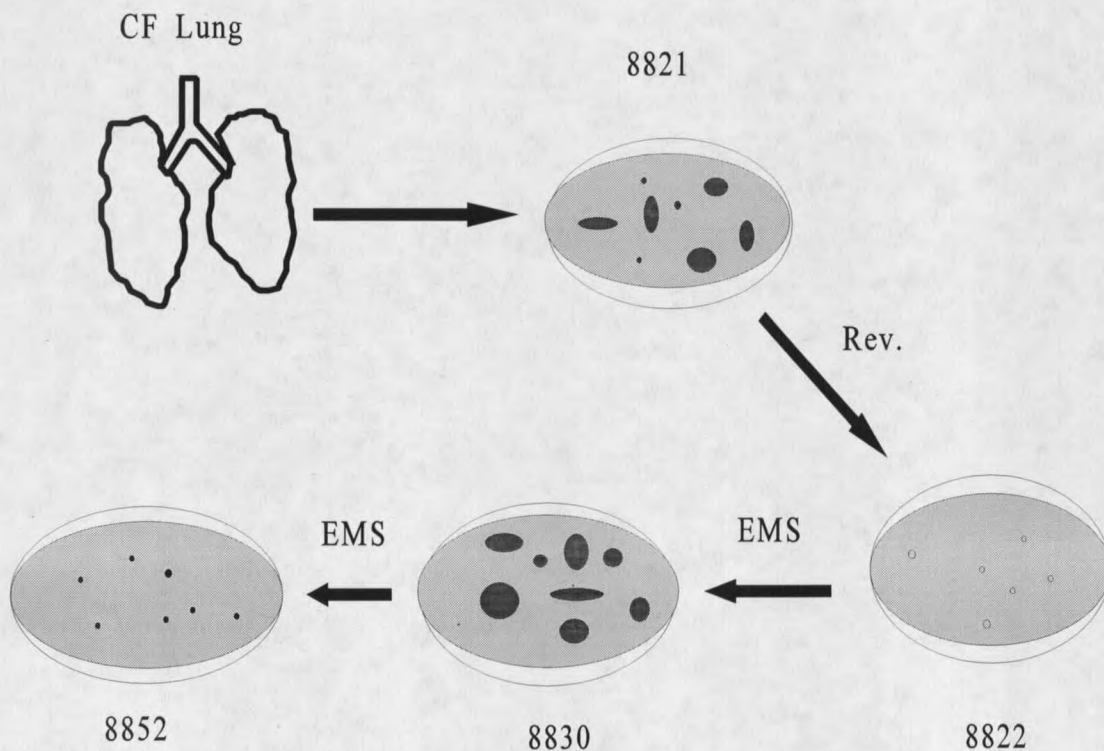


Figure 1.2. Production of stable *Pseudomonas aeruginosa* mutants of a strain isolated from the lung of a cystic fibrosis patient.

result of lesions in the carbohydrate metabolism genes, each mutant was tested for its ability to grow on minimal medium supplemented with different carbon sources. No growth aberrations were noted in the presence of glucose, gluconate, glycerol, mannitol, fructose, glutamate, or succinate (Darzins and Chakrabarty, 1984).

Other useful mutants have since been made from this stock of four strains. Using these strains and their derivatives, it has been possible to isolate individual structural gene mutants defective in alginate synthesis. This work (particularly in the case of strain 8830) has been instrumental in developing our current understanding of the genes involved in alginate biosynthesis in *Pseudomonas aeruginosa* (Banerjee *et al.*, 1985; Darzins *et al.*, 1985; Roychoudhury *et al.*, 1989; Sá Correia *et al.*, 1987). This has been due to the increased activity of the alginate genes that occurs only in mucoid strains.

A schematic representation of the alginate biosynthesis pathway is diagramed in Figure 1.3. The first enzyme to be isolated and identified from *Pseudomonas aeruginosa* was the gene product for *algA* (Darzins *et al.*, 1985). This is the first enzyme required for alginate synthesis and has been found to be bifunctional, having phosphomannose isomerase (PMI) activity, as well as GDP-mannose pyrophosphorylase (GMP) activity (Sà-Correia *et al.*, 1987). PMI is responsible for interconversion of fructose 6-P and mannose 6-P. Mannose 6-P is converted to mannose 1-P by phosphomannomutase (PMM). GDP-mannose pyrophosphorylase converts mannose 1-P to GDP-mannose, and GDP-mannose dehydrogenase converts GDP-mannose to GDP-mannuronic acid (GMD). The remaining steps of the *P. aeruginosa* alginate pathway have not been fully elucidated.

Following the activity of GMD, acetylation, polymerization, epimerization and export are known to occur.

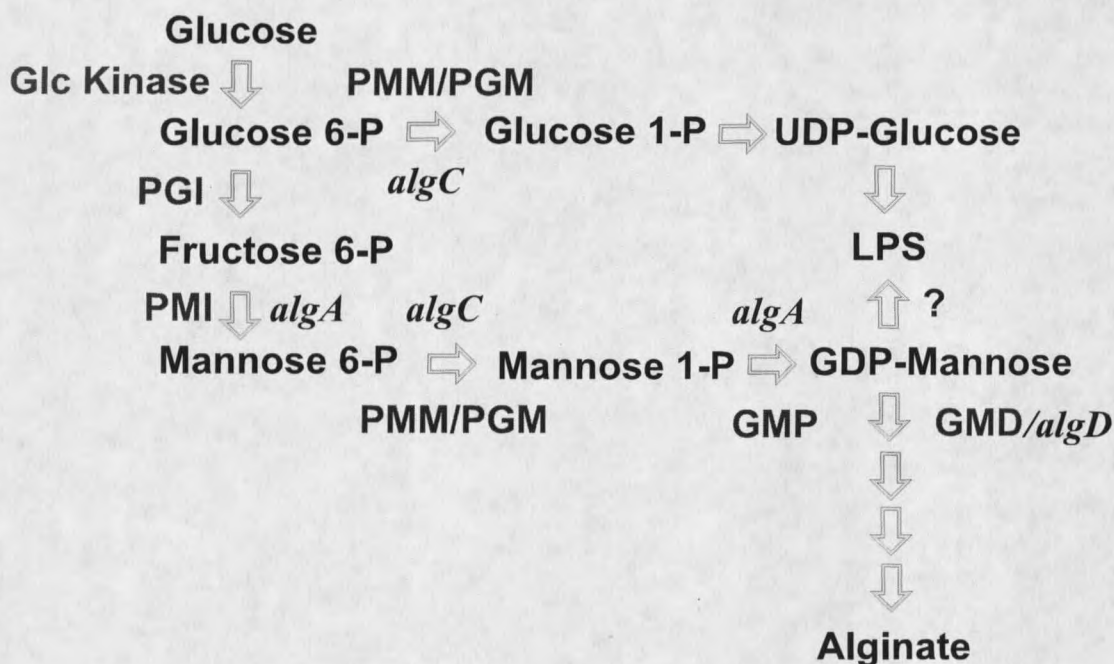


Figure 1.3. Alginate biosynthesis pathway. The gene encoding each enzyme is given in italics. Abbreviations for enzymes: PGI, phosphoglucose isomerase; PMI, phosphomannose isomerase; PMM, phosphomannomutase; PGM, phosphoglucosyltransferase; GMP, GDP-mannose pyrophosphorylase; GMD, GDP-mannose dehydrogenase. The remaining steps in the production of alginate, include polymerization, epimerization, acetylation and export. A number of remaining alginate genes are of unknown function.

Acetylation is carried out by *algF* (Shinabarger *et al.*, 1993). This enzyme is believed to be active only on mannuronic acid, and *O*-acetyl modification is proposed to regulate the degree of epimerization by shielding mannuronic acid groups from the epimerase enzyme. Acetylation of mannuronate residues has also been shown to be protective against enzymatic cleavage by alginate lyases (Gacesa, 1992). Epimerization interconverts D-mannuronic acid and L-guluronic acid. The incorporation of L-guluronic

acid into alginate may result in changes in the rheological properties of the finished polymer, as well as provide a protective role against cleavage by alginate lyase (Boyd and Chakrabarty, 1995). Alginate produced by *P. aeruginosa* 8830 has been shown to be composed of 95% mannuronic acid and 5% guluronic acid (Abrahamson *et al.*, 1995). None of the guluronate residues have been shown to be arranged into poly-guluronate blocks. *O*-Acetylation has been found on approximately 69% of the mannurate residues; guluronate residues are not acetylated.

The production of alginate by *P. aeruginosa* is known to be regulated by transcriptional activators as well as by a σ^{54} recognition sequence. A schematic representation of the known alginate genes is shown in Figure 1.4. The DNA binding protein AlgR1 has been shown to be a positive transcriptional activator which interacts with the *algD* promoter region at Three separate regions upstream from the mRNA start site (Mohr *et al.*, 1992). Similar binding sites have been postulated for the *algC* promoter region (May *et al.*, 1991).

The *algD* promoter regulates transcription of *algD*, *alg8*, *alg44*, *algE*, *algG*, *alg60*, *algL*, *algF* and *algA*. The *aglC* promoter is known to regulate only the *algC* gene (Boyd *et al.*, 1993; Chitnis and Ohman, 1990; May *et al.*, 1991). Other regulatory proteins have been found. AlgR2 has been shown to increase *algD* promoter activity when in the presence of AlgR1 (Kato *et al.*, 1989). AlgR3, a highly basic regulatory protein has not been assigned a known function, however, it has been shown to have significant sequence homology (44% identity) with a sea urchin histone protein believed to regulate DNA supercoiling in response to changes in osmolarity (Kato *et al.*, 1990; Hulton *et al.*, 1990).

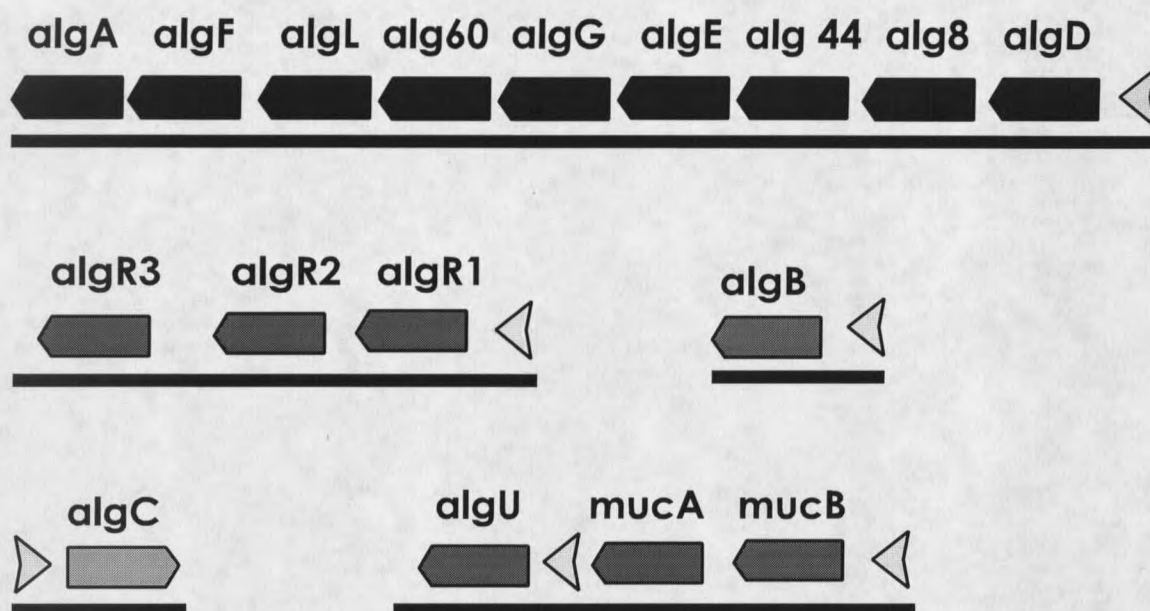


Figure 1.4. Genes involved in alginate biosynthesis and regulation. The function of the gene products are as follows: AlgD, GDP-mannose dehydrogenase; Alg8 and Alg44, probable membrane proteins involved in alginate polymerization; AlgE, probable outer membrane porin; AlgG, epimerase; Alg60, required gene product of unknown function; AlgL, alginate lyase; AlgF, alginate acetylase; AlgA, phosphomannose isomerase/GDP-mannose pyrophosphorylase; AlgC, phosphomannomutase/phosphoglucomutase. AlgR1, AlgR2 and AlgR3 are positive response regulators for the alginate biosynthesis genes. MucA, MucB and AlgU, regulate the switch to mucoidy. AlgB has been shown to enhance alginate production. Triangle indicates promoter region and direction of transcription. For a complete discussion of the alginate genes, refer to the text.

AlgR1, AlgR2 and AlgR3, appear to regulate the level of transcription of the alginate biosynthesis genes. In addition to these response regulators, recent studies concerning the molecular mechanisms for conversion to mucoidy have defined a cluster of genes identified as *algU*, *mucA* and *mucB*. The mucoid condition of *P. aeruginosa* has been shown to be dependent upon the presence of AlgU (also known as AlgT), a σ^{54} -like protein which interacts with RNA polymerase core to activate the promoters of the

alginate biosynthesis genes (Martin *et al.*, 1993a; DeVries and Ohman, 1994). The activity of *algU* is controlled by two downstream accessory genes, *mucA* and *mucB* (also described as *algN*) (Martin *et al.*, 1993b; Martin *et al.*, 1993c). MucA and MucB act to suppress AlgU activity, and mutations in these genes can cause conversion to mucoidy by relieving AlgU from negative regulation (Martin *et al.*, 1994).

In laboratory studies, it has been shown that the mucoid strain *P. aeruginosa* 8830 is capable of regulation of alginate production, even though there appeared to be constitutive expression of the alginate operons. When strain 8830 was grown in L-broth medium in the laboratory of Dr. A. M. Chakrabarty, it failed to produce detectable levels of alginate, despite the addition of a wide variety of supplemental carbon sources. However, when utilizing the same substrates on solid medium, the organism was able to produce large amounts of alginate (Darzins and Chakrabarty, 1984). Strain 8830 has also been shown to be responsive to medium osmolarity, demonstrating maximum up-regulation when grown on 0.3 M - 0.4 M NaCl (A. M. Chakrabarty, personal communication). We have been able to reproduce these observations in our laboratory.

Extensive genetic and biochemical studies have been performed on the family of bacteria derived from the CF isolate, *P. aeruginosa* 8821, and observations have demonstrated that strain 8830, while capable of producing large amounts of alginate, still has the ability to regulate alginate production in a way that is consistent with wild-type mucoid and nonmucoid isolates (Berry *et al.*, 1989).

Alginate biosynthesis in strains of *P. aeruginosa* isolated from environments other than the CF lung has not been well characterized. Transcription of their alginate

biosynthesis genes is usually not detectable (May *et al.*, 1991). It is for these reasons, and because of the unusual stability of its mucoid phenotype (Darzins and Chakrabarty, 1984), that *P. aeruginosa* 8830 has been used in the present work to evaluate alginate biosynthesis in a biofilm mode of growth.

While *P. aeruginosa* 8830 is derived from a medical isolate, the alginate genes that it carries are presumed to be under similar control as those that affect alginate biosynthesis in environmental strains of *P. aeruginosa* and perhaps in other bacteria. Past investigations have suggested that the production of alginate by *P. aeruginosa* was unique to the environment of the cystic fibrosis lung (Fialho *et al.*, 1990). Yet, it is unlikely that these bacteria, which are found predominantly in the soil and water, developed the ability to produce alginate in order to inhabit the lungs of humans having the genetic disorder leading to cystic fibrosis. It is also unlikely that infection of the CF lung by *P. aeruginosa* results from contact with other patients having CF. Deretic *et al.* (1993), has suggested that the mucoid phenotype of *P. aeruginosa* is probably present in the environments other than in chronically infected patients. Recent investigations have shown that alginate genes are widespread within the group *Pseudomonas*, even though they are not normally expressed (Wallace *et al.*, 1994). Examples of 23 *Pseudomonas* species were probed for the presence of *Pseudomonas aeruginosa* alginate genes. Of these, all group I pseudomonads except *P. stutzeri* were shown to contain homologous sequences to the gene probes: *algA*, *algD* and *algR* (Fialho *et al.*, 1990). The presence of these principal alginate genes indicated that these organisms were capable of producing

alginate, and that the pathway for alginate synthesis by these organisms incorporated similar elements to those found in *P. aeruginosa* 8830.

Additional studies by Grobe *et al.*, (1995) demonstrated the presence of mucoid *Pseudomonas* species isolated from the surfaces of various technical water systems. In their study, ten out of 81 *Pseudomonas* isolates demonstrated a mucoid phenotype when grown on *Pseudomonas* Isolation Agar. This work further indicated that attached *Pseudomonas* species in the environment possess the biosynthetic capability to produce alginate.

The above information suggests that the study of alginate gene regulation using the mutant strain 8830 will provide insight into the behavior of other non-mutant *P. aeruginosa* strains which are found in natural environments.

The aim of the research described in this thesis has been to characterize the behavior of alginate gene regulation during biofilm development by *P. aeruginosa* strain 8830. This research was performed using reporter gene technology to follow the behavior of specific alginate promoters. It was hoped that by examining critical alginate genes during the development of a biofilm, insight might be gained into the manner in which matrix polymer production is managed by the bacterium. Two regulatory and one structural gene were studied during the course of this thesis research. These genetic loci were chosen because they are believed to play central roles in the development of biofilms due to their importance in regulation of alginate biosynthesis and processing.

All of the alginate biosynthesis genes are clustered in two separate operons; the *algC* operon and the *algD* operon. The promoters for both were shown to contain a σ^{54}

recognition sequence for RNA polymerase binding. This indicated that both promoters were environmentally responsive genetic elements (DeVault *et al.*, 1989; Zielinski *et al.*, 1992). However, the promoters for each of the two operons differ significantly and are not considered to respond to identical regulation. For these reasons, and because the *algC* promoter is believed to be involved both in alginate biosynthesis and in LPS biosynthesis (Zielinski *et al.*, 1991; Ye *et al.*, 1994), it was considered necessary to study promoter activity for both the *algC* and the *algD* operons.

The third genetic locus to be studied was *algL*, a gene which encodes the degradative enzyme alginate lyase. This gene was chosen because the regulation and activity of its gene product, AlgL indicated that it may play an important role in mediating detachment or sloughing in *P. aeruginosa* biofilms (Boyd *et al.*, 1994).

Taken as a group, these Three genetic loci were predicted to be critical components governing the initial events of biofilm development (*algC*), the formation of matrix polymer required for biofilm maturation (*algD*), and the ultimate breakdown of a biofilm (*algL*).

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CHAPTER 2

EXOPOLYSACCHARIDE PRODUCTION IN BIOFILMS: SUBSTRATUM

ACTIVATION OF ALGINATE GENE EXPRESSION

BY *Pseudomonas aeruginosa*Introduction

In 1966, Linker and Jones first reported the production of alginate in *Pseudomonas aeruginosa* (Linker and Jones, 1966). This organism has been shown to preferentially assume an attached state, embedding itself in exopolymer composed of alginate (Costerton *et al.*, 1987). Alginate is a linear copolymer of β -1-4-linked D-mannuronic acid and its C-5 epimer L-guluronic acid. Alginate as a biofilm matrix polymer plays an important role in diseases such as cystic fibrosis (CF) (Chartrand and Marks, 1983; Govan and Harris, 1986) and urinary tract infections (Nickel *et al.*, 1985), in the fouling of man-made materials (Christensen and Characklis, 1990), and at surfaces in the natural environment (Geesey *et al.*, 1978).

The pathway of alginate biosynthesis by *P. aeruginosa* is summarized in Fig 1.3. The alginate genes *algA* (encoding the enzyme phosphomannose isomerase-guanosine diphosphomannose pyrophosphorylase), *algC* (encoding the enzyme phosphomannomutase), and *algD* (encoding the enzyme GDP-mannose dehydrogenase) are responsible for conversion of fructose 6-phosphate to GDP-mannuronic acid. These

genes have been completely sequenced and the locations of the promoters have been determined (May *et al.*, 1991). Little is known about the subsequent conversion of GDP-mannuronic acid to alginate, however, the process is known to require polymerization and epimerization of mannuronic acid residues as well as acetylation and export of the polymer.

The activation of a critical alginate promoter, *algD*, by *P. aeruginosa* has been shown to take place during nitrogen limitation, membrane perturbation induced by ethanol and when cells were exposed to media of high osmolarity (DeVault *et al.* 1989; DeVault *et al.*, 1990). Similar to the *algD* promoter, the *algC* promoter has been shown to be activated by environmental signals such as high osmolarity and this activation is dependent on the presence of the response regulator protein AlgR1, which has been shown to bind a consensus AlgR1-binding site (CCGTTCGTCN5) centered at -87 of the *algC* promoter (Zielinski *et al.*, 1992).

The activities of the alginate biosynthetic enzymes are extremely low, even in mucoid strains of *P. aeruginosa* and are either greatly reduced or absent in nonmucoid strains (Padgett and Phibbs, 1986; Piggot *et al.*, 1981; Sá-Correia *et al.*, 1987). Due to the low activity of these enzymes, reporter genes have been used to detect the activity of the alginate promoters, rather than measure the enzymes directly. The gene *AlgC*, encoding phosphomannomutase was examined in the present study because it is a key regulation point in the alginate biosynthetic pathway. The promoter sequence for *algC* along with 1.0 kb of upstream DNA was fused to a promoterless *lacZ* (β -galactosidase)

reporter gene to form the plasmid designated pNZ63. The plasmid was introduced into the stable mucoid *P. aeruginosa* strain 8830 (Zielinski *et al.*, 1991).

The strain 8830 was selected for the present study due to the large amount of alginate it produces and its very low rate of reversion to the nonmucoid form (Darzins and Chakrabarty, 1984). Strain 8830 was isolated as a stable mucoid variant following ethyl methanesulfonate mutagenesis of strain 8822, a spontaneous nonmucoid revertant of a CF isolate strain 8821 (Darzins and Chakrabarty, 1984). The exopolysaccharide produced on solid media by strain 8830 has been shown by Darzins, *et al.* (1984) to contain alginate as determined by the method of Knutson and Jeanes, (1968).

In view of the preference of *P. aeruginosa* to form biofilms composed of cells and associated exopolymer, it was of interest to determine the effect that attachment to surfaces had on alginate production. Teflon was chosen as the substratum for this study due to its low reactivity and the fact that it does not interact with metal ions. In the current effort, substratum activation of the *algC* promoter is demonstrated in *P. aeruginosa* strain 8830 and related to alginate synthesis.

Materials and Methods

Strains and Media

The bacterium used in this study was *Pseudomonas aeruginosa* strain 8830 containing the *algC-lacZ* transcriptional fusion plasmid pNZ63. This reporter plasmid as well as its characteristics have previously been described (DeVault *et al.*, 1989; DeVault *et al.*, 1990; Zielinski *et al.*, 1991).

All experiments were conducted using a defined culture medium (modified EPRI) containing: sodium lactate (0.05 g/l), sodium succinate (0.05 g/l), ammonium nitrate (0.05 g/l), KH_2PO_4 (0.019 g/l), K_2HPO_4 (0.063 g/l), Hutner salts (Cohen-Bazire *et al.*, 1957) (0.01 g/l), dextrose (2.0 g/l) and L-histidine (0.01 g/l). Carbenicillin (350 mg/l for liquid medium and 600 mg/l for solid medium) was filter sterilized and added to autoclaved media in order to maintain plasmids in the cells during culture. YTG medium contained the following components in grams per liter: yeast extract, 5.0; tryptone, 10.0; D-glucose, 2.0; Bacto agar, 1.5.

Determination of growth limiting nutrient for *P. aeruginosa* 8830 in EPRI medium

Growth of *P. aeruginosa* 8830 in EPRI medium was checked to determine the limiting nutrient in the medium. When cells reached stationary phase, they were given additional O_2 , organic carbon (as lactate, succinate and glucose-according to the configuration of EPRI medium) or nitrogen as ammonium nitrate. Any increase in growth as measured by OD_{600} resulting from these additions was considered evidence of that factor as limiting for growth of the organism.

P. aeruginosa 8830 pNZ63 was precultured on solid YTG medium for 24 hour and transferred to EPRI medium supplemented with 350 mg/l carbenicillin and grown for an additional 24 hour. An inoculum of 0.1 ml culture was added to each of nine 1 liter flasks containing EPRI medium supplement with carbenicillin (350 mg/l) and glucose (2.0 g/l). The flask cultures were incubated at 25°C on a rotary shaker at 175 rpm and were sampled for OD_{600} measurements according to progression of growth. After cells

entered stationary phase, oxygen, carbon or nitrogen were added to the cultures and samples were recovered for further OD measurements to check for increase in growth resulting from any of the treatments.

Nutrient supplements were added as follows: Oxygen was added to Three of the nine flasks as filtered air pumped through tubing and driven by a Masterflex pump at maximum speed; organic carbon was added in the same concentration as the original organic carbon: lactate, 0.05 g/l, succinate, 0.05 g/l and glucose, 2.0 g/l; Nitrogen was added as ammonium nitrate, 0.05 g/l.

Biofilm versus planktonic cell expression of alginate synthesis

Cultures of *P. aeruginosa* strain 8830 carrying the plasmid pNZ63 were prepared from stocks maintained at -70°C in skim milk media, by inoculating 0.1 ml into 2 x 50 ml Erlenmeyer starter culture flasks containing 25 ml of culture medium supplemented with carbenicillin. Starter cultures were incubated at room temperature for 48 hours or until cultures became turbid. Cell suspensions of 10 ml were inoculated into 1 liter Erlenmeyer flasks containing 500 ml of culture medium supplemented with carbenicillin. Two duplicate flasks containing sterile medium and a Teflon mesh having a surface area of 332.8 cm² were used to culture the biofilm population. Two additional duplicate flasks containing no mesh were used to culture the planktonic cell population. Flasks were incubated at 24°C for 96 hours on a rotary shaker (175 rpm).

Time course experiment

A time course experiment was run to examine the relationship of *algC* gene expression to the bacterial growth cycle. Starter cultures of *P. aeruginosa* were prepared as above. Three separate flasks each containing 500 ml of sterile medium supplemented with carbenicillin were inoculated with 10 ml of starter culture in late log phase (as determined by turbidity). These flasks were incubated at 24°C on a rotary shaker (175 rpm). Samples from each flask were taken during lag, log and stationary phases of the growth cycle and immediately assayed for turbidity, total cell counts and β -galactosidase activity. Sub-samples of 10 ml were transferred to chromic acid washed scintillation vials and stored at -70°C for subsequent determination of protein and uronic acids.

At the end of the stationary phase, 10 ml of culture from one flask was transferred to 500 ml of sterile culture medium supplemented with carbenicillin to examine the effects of fresh medium on alginate regulation. Cells were analyzed for β -galactosidase activity and cell densities were determined by optical density at 600 nm. In addition, 10 ml of late stationary phase culture were transferred from one of the Three original flasks to each of 7 flasks containing 250 ml sterile culture medium supplemented with carbenicillin and containing 3.5 g of Teflon mesh having a surface area of 20.7 cm².

The cells which were attached to the Teflon mesh were harvested at intervals from different flasks over a period of 48 hours. Cells were immediately analyzed for β -galactosidase activity and were enumerated by total cell counts according to the method of Hobbie, et al. (1977).

Cell counts

Cells were enumerated by the spread plate method using R2A agar (American Public Health Association, 1989). A cell suspension of 10 ml was removed from each resuspended washed population. These samples were homogenized using a Tekmar Tissue-Mizer at maximum speed for 30 seconds at 4°C. A subsample of the homogenized cell suspension was analyzed by phase contrast microscopy to determine that cells did not display clumping. Cells were enumerated in triplicate and reported as CFU per ml.

Cell harvesting

Teflon mesh was aseptically removed from culture flasks and gently rinsed with sterile medium to remove loosely adherent cells. The remaining attached cells (biofilm population) were aseptically recovered by scraping the cells from the mesh with a sterile brush into 250 ml chilled sterile medium. Both the biofilm population and the planktonic population were washed by centrifugation (250 ml in sterile acid washed centrifuge tubes) at 16,274 x g for 20 minutes at 4°C and resuspended in sterile chilled culture medium. Resuspended cultures were assayed for viable cell numbers on R2A agar immediately following centrifugation. Sub-samples were frozen at -70°C for subsequent protein analysis, β -galactosidase activity, uronic acids, and Fourier transform infrared spectroscopy (FTIR).

Portions of scraped and unscraped mesh were immersed in 2% formaldehyde, stained with acridine orange and examined by fluorescence microscopy to check culture

purity and cell removal. No cells were observed on the surface of the scraped mesh samples.

β -Galactosidase assay

β -galactosidase is an enzyme which hydrolyzes β -D-galactosides. It can easily be measured with chromogenic substrates, colorless substrates which are hydrolyzed to yield colored products. An example is *o*-nitrophenyl- β -D-galactoside. This compound is colorless, but in the presence of β -galactosidase it is converted to galactose and *o*-nitrophenol. The *o*-nitrophenyl is yellow and can be measured by its absorption at 420 nm. If the *o*-nitrophenyl- β -D-galactoside (ONPG) concentration is high enough, the amount of *o*-nitrophenol produced is proportional to the amount of enzyme present and to the time the enzyme reacts with the ONPG. In order for the assay to be linear, the ONPG must be in excess. The reaction is stopped by adding a concentrated Na_2CO_3 solution which shifts the pH to 11. At this pH, β -galactosidase is inactive (Miller, 1972).

Enzyme assays for β -galactosidase specific activity were performed by taking aliquots of 0.6 ml thawed cell suspensions (previously frozen to -70°C) which were mixed with 0.4 ml Z-buffer (Miller, 1972) and lysed using 1 drop of chloroform and 2 drops of 0.1% SDS per ml cell suspension. Lysed cell suspensions were incubated for 5 hours with 0.2 mg ONPG and analyzed using a Varian DMS 90 Spectrophotometer following the procedure of Miller, (1972). β -galactosidase specific activity was determined by comparing results with a standard solution of ONP following subtraction of blank samples and background light scattering (OD 550 nm). Results are reported as

micromoles of ONP produced per minute per mg of protein or per cell at 25°C, pH 7.0.

Lowry protein assay

The Lowry protein assay was performed on thawed samples using a Varian DMS 90 Spectrophotometer following the procedure as modified by Peterson, (1977). Briefly, a 4% sodium carbonate solution is prepared in 0.2 N sodium hydroxide. This solution is combined with 2% copper sulfate in distilled water and 4% sodium tartrate in distilled water, in a 100:1:1 proportion. This final solution is mixed 1:1 with sample and vortexed. After 10 minutes incubation at room temperature, 0.2 ml of Folin's Reagent (1:1 with distilled water) was added for each 1 ml of sample used and the mixtures were vortexed. The samples were then allowed to react for 30 minutes until a blue color developed. Samples were then measured for optical absorbance at 550 nm. Test samples were measured against control tubes to which no Folin's Reagent had been added and to tubes to which no sample had been added. Results were compared with freshly performed standard curves derived from measurements of bovine serum albumin (BSA). Protein is reported as mg protein per ml resuspended cell culture.

Collection of infrared spectra

Total carbohydrate associated with washed, resuspended biofilm cells recovered from the Teflon mesh and planktonic cells was determined by attenuated total reflectance Fourier transform infrared spectroscopy. Cell suspensions were transferred to an open boat Micro Circle cell (Spectra Tech, Stamford, CT) containing a germanium internal reflection element (IRE). Samples were dried on the IRE and then positioned in the front

and back beams on the optical bench of a Perkin Elmer Model 1800 spectrometer equipped with a KBr beam splitter and a liquid N₂-cooled, medium range mercury-cadmium-telluride detector (5000-580 cm⁻¹). Spectra were collected from each circle cell using the single beam mode over the range of 2000-700 cm⁻¹ and ratioed against spectra collected from each cell prior to introduction of the cell suspensions. Spectra of cell suspensions dried on the IRE were compared with a spectrum of sodium alginate (Sigma Chemical Co. St. Louis Mo) to verify the peak assignment. The amounts of total carbohydrate and protein associated with the dried cells were obtained from the areas of the absorbance peaks centered at 1050 cm⁻¹ for complex sugar rings and 1534 cm⁻¹ for amide II respectively (Naumann *et al.*, 1982).

Uronic acids assay

Total uronic acids were measured in thawed samples of washed cell suspensions following the method of Kintner and Van Buren, (1982) using a Varian DMS 90 Spectrophotometer. Alginate is a polymer of D-mannuronic and L-guluronic acids. A concentrated solution of H₂SO₄ containing 0.0125 M sodium tetraborate is used to hydrolyze the alginate polymer to its individual uronic acid residues. Meta-hydroxydiphenyl (0.15%) in 0.5% NaOH is used to react with uronic acid polymers to yield a color yellow/orange color with maximum absorbance at 520 nm. Borate is used in the assay to increase the color intensity of the reaction, and heating is used to help with degradation of the alginate polymer as well as reduce interference by other compounds (Knutson and Jeanes, 1968; Blumenkrantz and Asboe-Hansen, 1973). Glucose is known

to interfere with the reaction. When samples are heated to 100°C, the contribution by glucose to the absorbance is reduced to less than 1%.

The experimental protocol use was to prepare stock solutions in an ice bath, using chromic acid washed glassware. An aliquot of sample (0.2 ml) was added to each of ten test tubes in triplicate. To each test tube, 1.2 ml of sulfuric acid/tetraborate solution was added. The contents were mixed by vortex action and placed for 5 minutes in a water bath at 100°C. The tubes were cooled in an ice bath and 20 µl of meta-hydroxydiphenyl reagent was added to each tube and mixed by vortex action. Within 5 minutes, absorbance at 520 nm was measured for each sample using a spectrophotometer. Due to interference by exocellular carbohydrates, control samples containing 0.5 M NaOH instead of meta-hydroxydiphenyl reagent were assayed for absorbance at 520 nm. One control sample was assayed for each test sample analyzed. Results were compared with a standard curve prepared by using non-acetylated alginate obtained from *Macrocystis pyrifera* (high viscosity, Sigma).

ELISA test for presence of alginate

An antibody to L-guluronic acid residues was developed by Randall Irvin in 1984. The monoclonal cell line *Ps* 53 produced an immunoglobulin M which reacts with *Pseudomonas aeruginosa* alginate and produces immunofluorescent staining of the glycocalyx with an apparent specificity for L-guluronic acid residues in ELISA (Irvin and Ceri, 1985).

In order to determine whether alginate was present in samples of *P. aeruginosa* 8830 pNZ63 grown on Teflon mesh and in liquid medium, an independent measurement based on antibody binding was performed. Using mouse anti-alginate developed by Irvin *et al.*, samples containing cells with loose and bound polymer were examined for the presence of alginate. An ELISA test was done using Mu chain specific goat anti-mouse peroxidase conjugate. Attached and suspended whole cell samples grown in EPRI medium supplemented with 2 g/l glucose and 350 mg/l carbenicillin were compared with alginate produced by *P. aeruginosa* grown on the surface of YTG agar and phosphate-citrate buffer as a control.

Authentic *P. aeruginosa* alginate was prepared according to Darzins and Chakrabarty, (1984) and was used as a positive control. Sterile culture medium supplemented with carbenicillin (350 mg/l) was used as a negative control. Wells in a polystyrene microtiter plate were coated with sample for 24 hours at 4°C. Four rows of samples were used: Row A, Buffer; Row B, Authentic Alginate; Row C, Attached 8830 pNZ63; Row D, Suspended 8830 pNZ63. BSA (0.1% in deionized water) was added to each well and allowed to coat for 2 hours. Mouse anti-alginate (1:1000 dilution) was added and allowed to react for 2 hours. An aliquot of 0.4 mg/ml anti-mouse IgM peroxidase conjugate was added to each well and incubated for 2 hours. Each well was washed Three times by adding 0.05 ml phosphate-citrate buffer which was aspirated by pipet and discarded. O-phenylenediamine dihydrochloride was prepared in phosphate-citrate buffer to 0.4 mg/ml and added to each well to react with horseradish peroxidase. Reactions were terminated after 2 hours, using 3M H₂SO₄. All reactions were carried out

at room temperature. Wells were inspected visually for the presence of the indicated color change.

algC expression by biofilm cells in continuous culture

A continuous culture apparatus was used to monitor *in situ* reporter gene activity of biofilm cells by epi-fluorescence microscopy. A glass capillary having a rectangular cross section with internal dimensions of 0.3 mm depth, 6.0 mm width, and 2.0 cm length was used as a substratum for growth of biofilm cells. The capillary was fitted to a customized holder which could be mounted atop a microscope stage. Medium was pumped from a sterile reservoir via surgical tubing through the glass capillary to a waste reservoir. An inoculation port was positioned upstream from the capillary. The continuous culture apparatus was closed to the outside environment but maintained in equilibrium with atmospheric pressure by use of gas permeable filters. Defined culture medium used in these experiments was amended with 2.0 g/l glucose and methylumbelliferyl β -D-galactoside. Two continuous culture systems were operated in parallel. The first was inoculated with *P. aeruginosa* strain 8830 harboring the pNZ63 plasmid. The second system was used as a control and was inoculated with a *P. aeruginosa* strain 8830 not carrying the *lacZ* gene. This was done to insure that any observed fluorescence was due to the presence of converted substrate and not some other fluorescent phenomenon. The strain used in the control line was not carbenicillin resistant and, therefore, the medium for that organism did not contain an antibiotic. No fluorescence was detected in the control system. Both systems were run at a flow rate of

0.19 ml min⁻¹ producing laminar flow within the capillary with a Reynolds number of 1.0. Residence time in the capillary was 18.9 min; less than the doubling time of the organism in suspension, allowing only biofilm organisms to be retained within the capillary.

Confocal laser microscopy

Imaging of *in vivo* expression of β -galactosidase gene expression was performed using an Olympus BH-2 microscope connected to a Bio-Rad MRC 600 Confocal Laser Scanning attachment. Biofilm cells attached to the walls of a glass capillary were observed with the microscope using reflected light to illuminate all cells. The same field was then scanned using laser light at an excitation wavelength of 488 nm with an emission wavelength of 510 nm to observe fluorescence due to β -galactosidase conversion of the methylumbelliferyl substrate. Images were recorded for bacteria containing the pNZ63 plasmid containing *lacZ* under the control of the *algC* promoter and for bacteria not containing the *lacZ* gene.

Results

Determination of growth limiting nutrient for *P. aeruginosa* 8830 in EPRI medium

For many years, it has been known that limitations in phosphorous or nitrogen can sometimes lead to the overproduction of extracellular polysaccharides (DeVault *et al.*, 1989; Deretic *et al.*, 1990). In order to ensure that growth in EPRI medium was balanced, and to ensure that alginate synthesis would not be the result of nutrient limitation, an experiment was run to determine whether oxygen, nitrogen or organic carbon were

limiting for growth of *P. aeruginosa* 8830. Figure 2.1 shows growth of the study organism under these conditions. At around 60 hour, a plateau in growth is observed. This is possibly due to exhaustion of one or more of the Three carbon sources (e.g., succinate and lactate) before growth is resumed on another carbon source (glucose). This would result in diauxic growth (known to exist in the enterobacteriaceae). Following this period of diauxic growth, log phase growth is followed by a second stationary phase. At this time, the individual supplemental nutrients were added. No increase in growth was detected up to 84 hour for either oxygen or organic carbon supplements. However, addition of nitrogen resulted in an immediate increase in optical density indicating rapid cell growth. This indicated that nitrogen is limiting for *P. aeruginosa* 8830 or grown in EPRI medium. Following this experiment, all subsequent experiments using EPRI medium were performed with a carbon to nitrogen ratio of 7: 1.

Biofilm versus planktonic cell expression of alginate synthesis

β -galactosidase was used as a reporter of *algC* promoter activation to determine whether attachment to a Teflon surface influences the regulation of alginate biosynthesis in *P. aeruginosa*. When the activity of biofilm cells grown on Teflon mesh was compared with that of planktonic cells grown in suspension, the results demonstrated a greater than Three-fold increase in reporter gene activity per mg protein for the biofilm population (Figure 2.2).

FTIR spectroscopy of attached and suspended cell preparations

These studies revealed that total carbohydrate when normalized to amide II

protein was 2.14 fold higher in the biofilm population compared to the planktonic cell population (Figure 2.3).

Uronic acids analysis of biofilm versus planktonic cell cultures

When normalized to protein, biofilm cells exhibited a greater than 2 fold higher uronic acid content after 96 hours incubation than was found in planktonic cells when normalized to protein (Figure 2.4).

ELISA assay for L-guluronic acid

Verification that the carbohydrate detected by FTIR and uronic acids detected by the colorimetric assay were contributed by alginate was obtained by an ELISA assay specific for L-guluronic acid. Both biofilm and planktonic cells produced a positive reaction which was not observed in controls not containing alginate. No effort was made to use this assay to detect quantitative differences in alginate content of the 2 cell populations.

Time course characterization of alginate synthesis

Up-expression of *algC* was evaluated at different stages of the cell growth cycle in order to observe whether differences in the rate of alginate biosynthesis might manifest themselves during different growth phases. Planktonic cell cultures of *P. aeruginosa* were sampled over the course of the growth curve (48 hour) and assayed for reporter gene (*lacZ*) activity normalized to protein and for cell number. Maximum *algC* promoter activity was observed during the early log phase of growth. All Three experimental

populations showed a large spike in reporter gene specific activity at around 10 hour. This spike in activity was followed by a decrease, detected during mid and late log phase. A subsequent increase in activity was noted as cells entered stationary phase and again as they entered the death phase (Figure 2.4). When cells from one of the replicate cultures were re-inoculated into fresh medium, *algC* activity decreased initially and then increased during early log phase as observed previously, displaying minimum activity at stationary phase (Figure 1.4). The degree of maximum up-expression of *algC* following reinoculation into fresh medium was less than that observed during the previous growth cycle.

In a separate experiment, *algC* activity of the biofilm cell population was measured over the same time course as the planktonic cell culture (Figure 2.5). Maximum expression of the *algC* promoter was observed at approximately 24 hours for attached cells, possibly the result of attaining a starvation state. With the exception of the initial 2 hour period following inoculation, the biofilm cell population showed a higher rate of activity when normalized to cell number than did the planktonic cell population. The maximum activity of the biofilm cells was approximately 85 times higher than the maximum activity of the planktonic cells.

Continuous culture experiment

A flow cell mounted on a microscope stage was used to evaluate the relationship between *algC* promoter activity and surface attachment of individual cells. When methylumbelliferyl β -D-galactoside was present in the medium flowing through the

rectangular glass capillary which had been inoculated with cells carrying the pNZ63 plasmid (which contained the β -galactosidase gene under the control of the *algC* promoter), 30% of the cells adhering to the flow cell exhibited fluorescence 48 hours after inoculation (Figure 2.6). Cells not carrying the *lacZ* gene showed no fluorescence in the presence of the methylumbelliferyl substrate. This observation indicated that fluorescence in the pNZ63 plasmid carrying population was due solely to reporter gene (and, therefore, *algC* gene) activation. These observations signify activation of the alginate genes during adherence to a surface for at least 30% of the biofilm population.

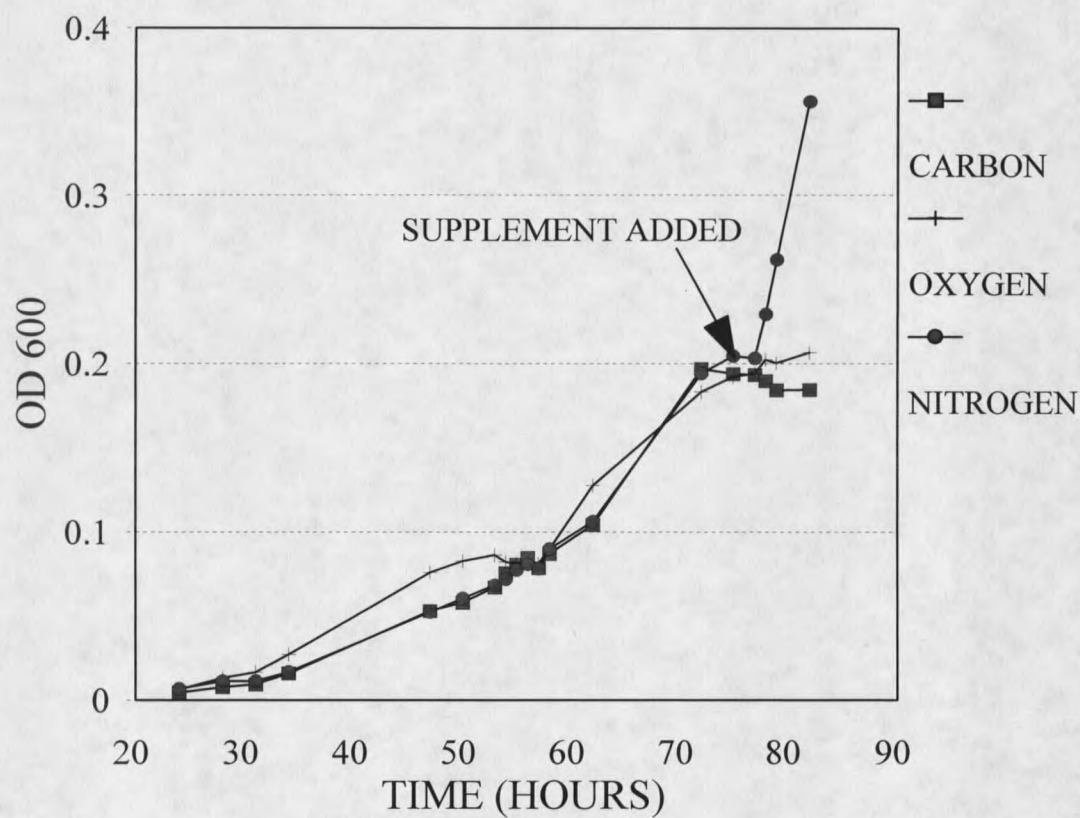


Figure 2.1. Limitations to batch culture growth of *P. aeruginosa* 8830 in EPRI medium.

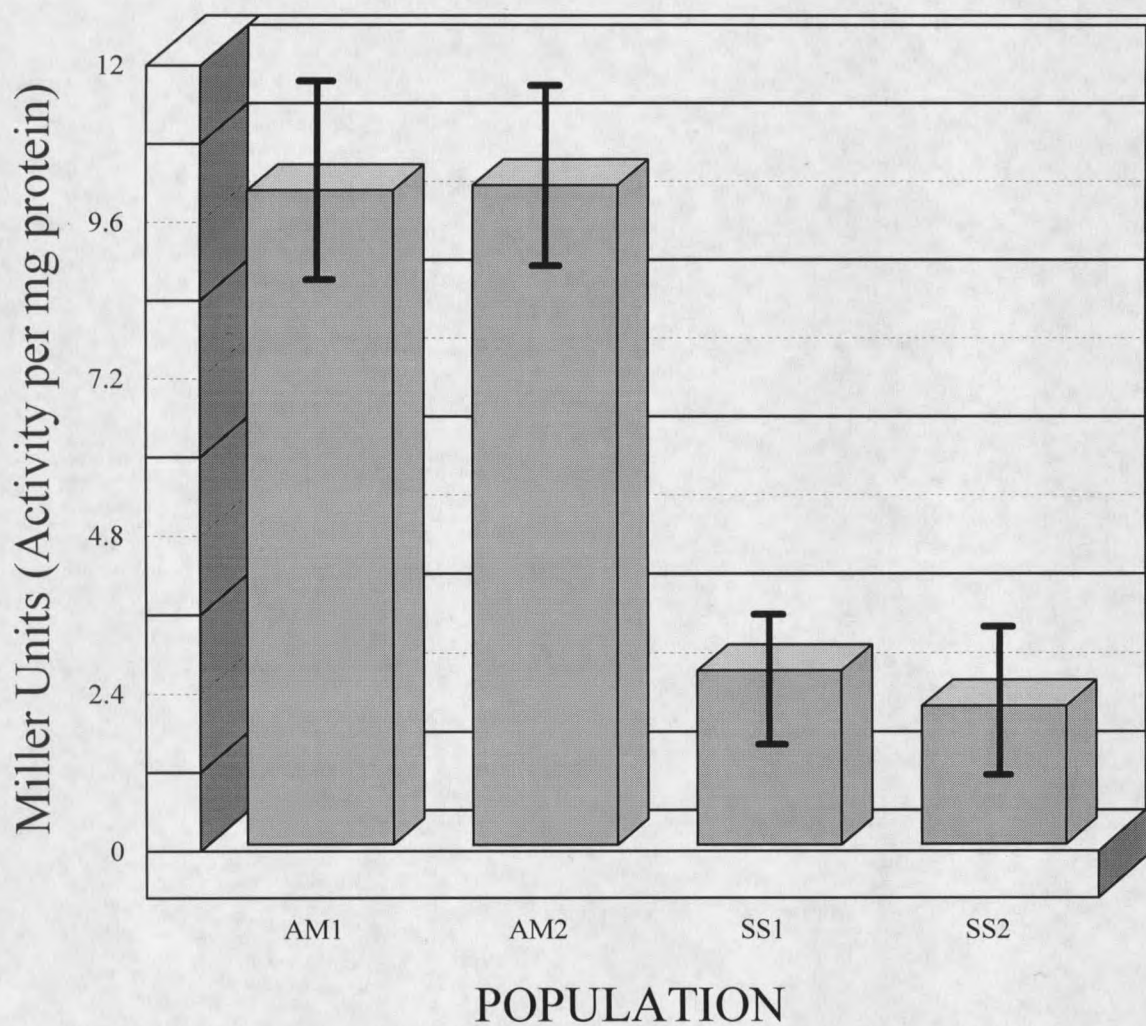


Figure 2.2. Specific activity of the reporter for *algC* in *P. aeruginosa* 8830 grown attached to Teflon mesh and in suspension in EPRI medium in batch culture. Attached populations are represented as AM1 and AM2. Suspended cell populations are represented as SS1 and SS2. Error bars represent 95% confidence limits for Three separate samples.

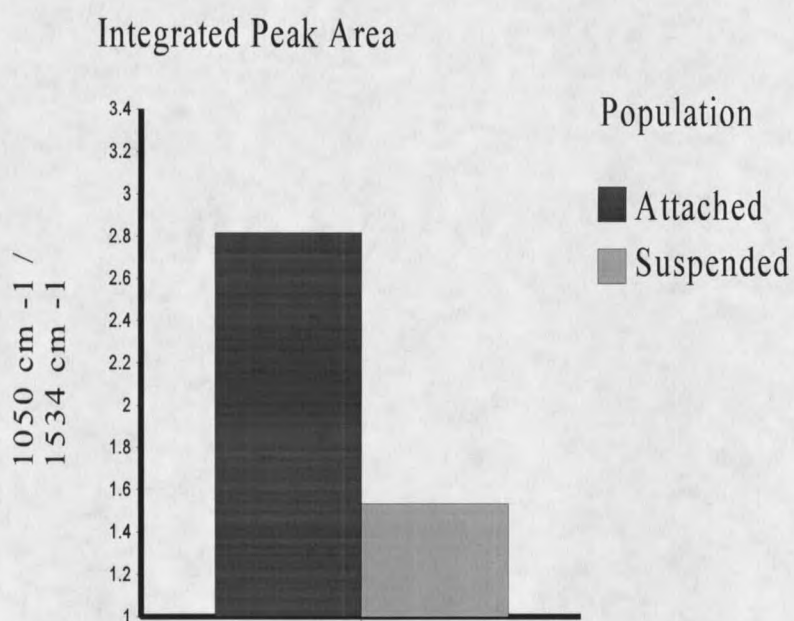


Figure 2.3. Integrated area of carbohydrate peaks produced by *P. aeruginosa* 8830 pNZ63 grown in continuous culture on germanium crystal in EPRI medium.

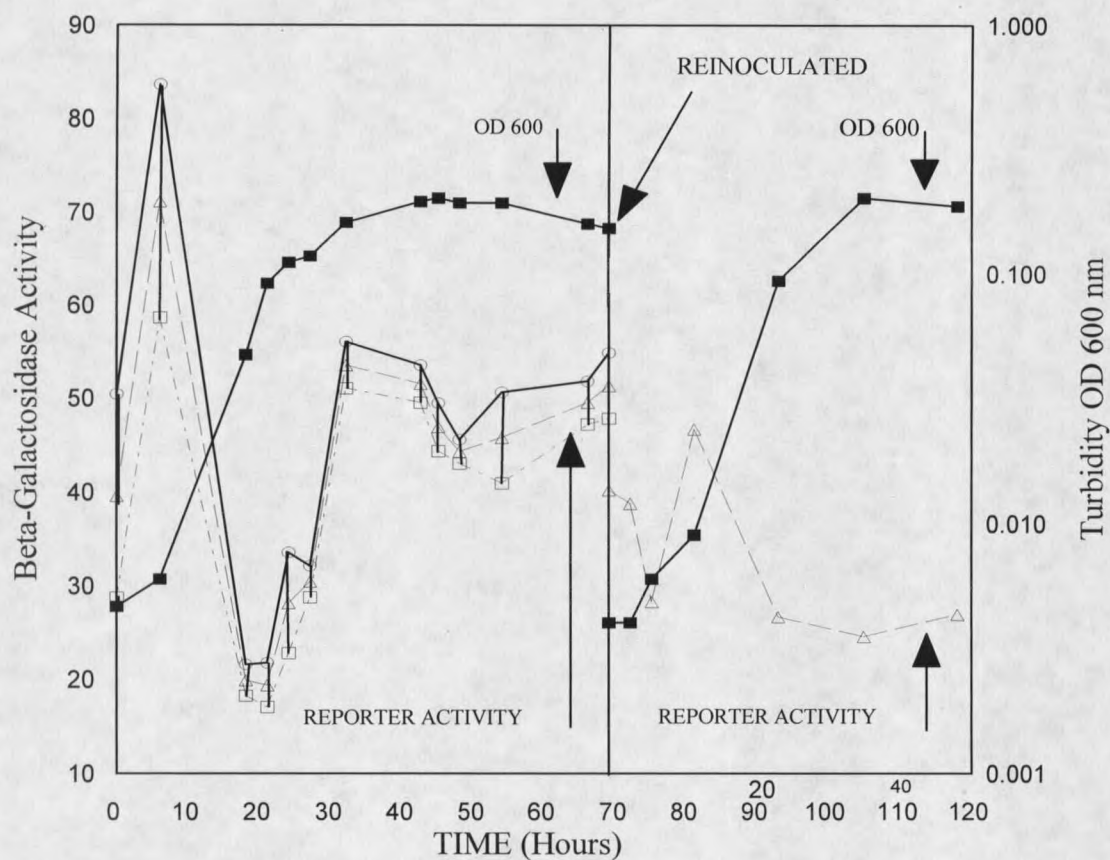


Figure 2.4. Relationship of β -galactosidase specific activity (micromoles of ONPG converted milliliter⁻¹ minute⁻¹ optical density at 600 nm $[\text{OD}_{600}]^{-1}$) to cell growth cycle (OD_{600}) of planktonic population in batch culture. Solid line with black squares represents average OD_{600} measurements for thourree replicates; open squares, triangles and circles depict β -galactosidase specific activity measurements for thourree replicates. Vertical bars represent ± 1 standard deviation.

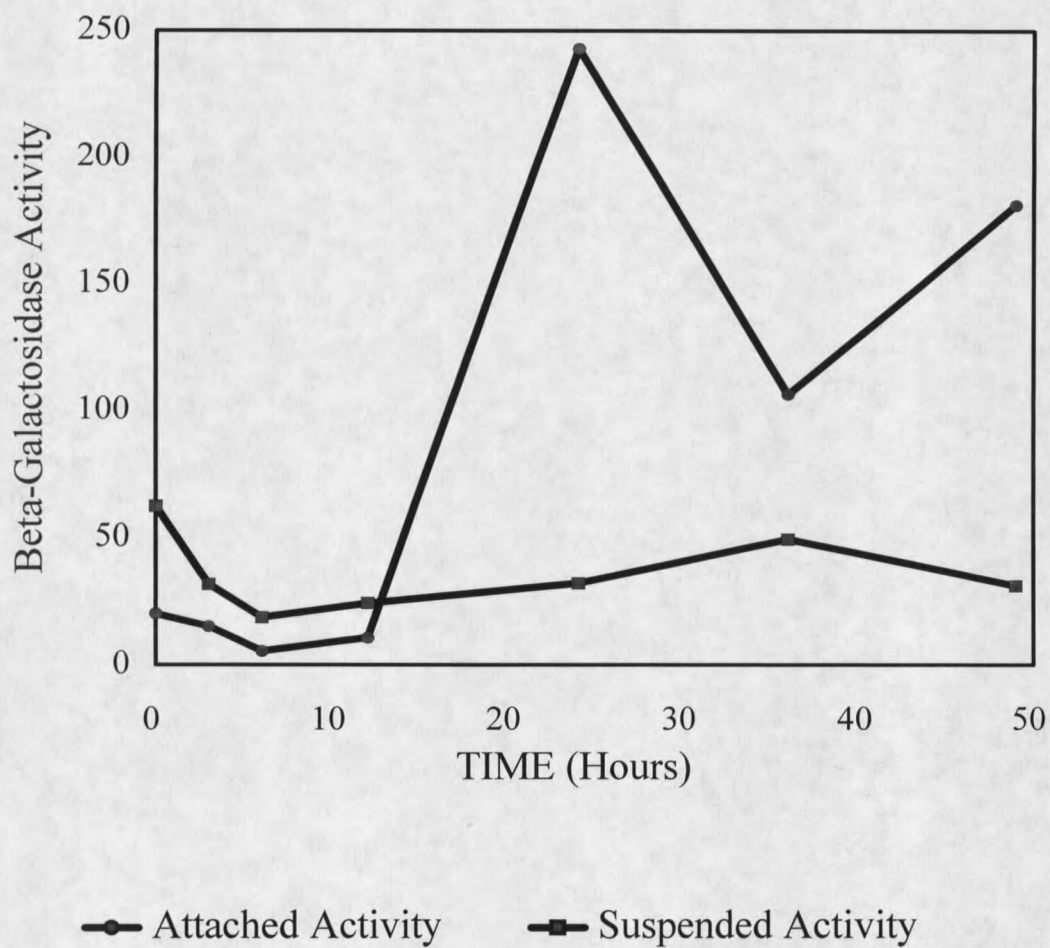


Figure 2.5. Comparison of β -galactosidase specific activity (nanomoles of ONPG converted milliliter⁻¹ minute⁻¹ cell number⁻¹) in biofilm (attached) and planktonic (suspended) cell populations in batch culture.

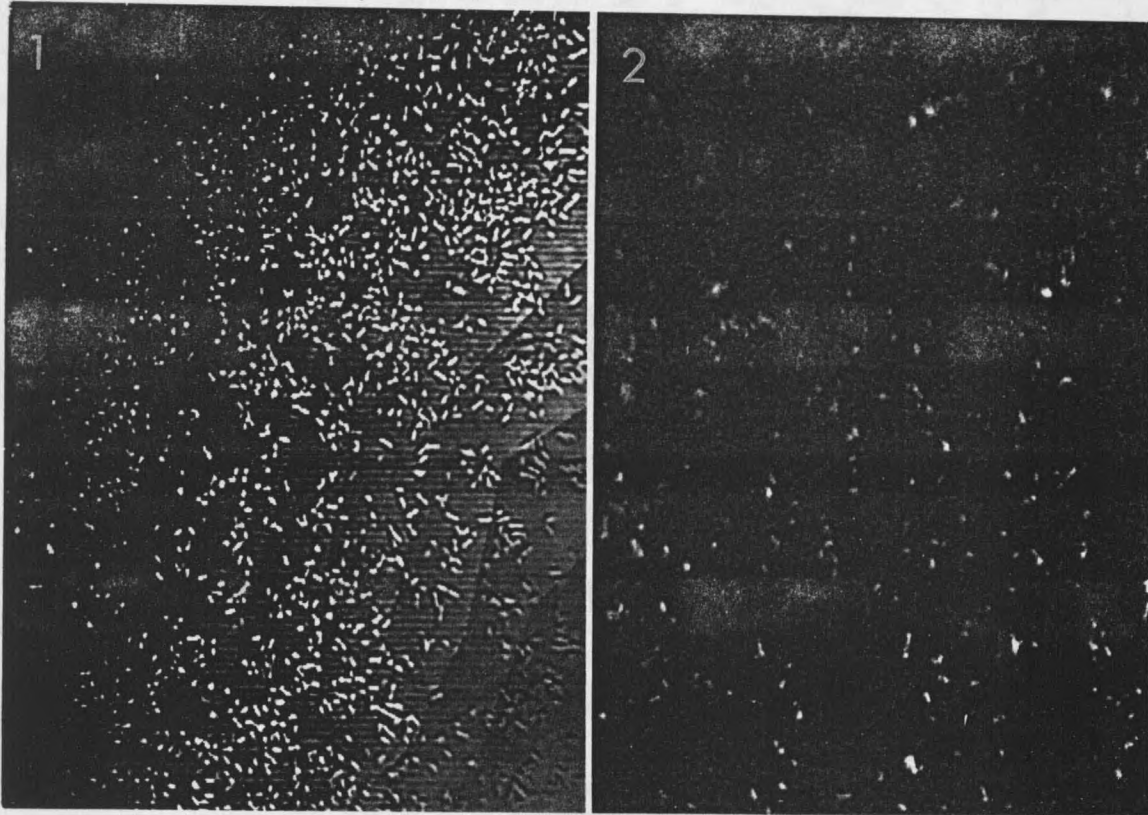


Figure 2.6. Scanning confocal laser micrograph of *P. aeruginosa* attached to the inner surface of a glass flow cell. (1) Cells illuminated with reflected white light, showing all bacteria in the field. (2) Fluorescing cells excited by laser light at a wavelength of 488 nm, showing cells which are producing β -galactosidase, indicating *algC* activity.

Discussion

The results described in this chapter demonstrate activation of the *algC* promoter in *P. aeruginosa* strain 8830 which is necessary for the production of the exopolysaccharide alginate. This activation resulted from association of the bacteria with a Teflon or glass substratum. Previous work has demonstrated exopolymer production by this strain when growing on the surface of agar plates containing a high organic carbon concentration (Darzins and Chakrabarty, 1984). However, using a more dilute medium, significant *algC* activity was detected only when the organisms were grown attached to a solid surface. While *P. aeruginosa* is known to form biofilms on natural surfaces and CF lung epithelial cells (Christensen and Characklis, 1990; Costerton *et al.*, 1987), very little is known on the role of adherence to the triggering of exopolysaccharide formation. This is, to our knowledge, the first report on the activation of an alginate gene promoter as a consequence of the cell's attachment to a solid surface.

Surface activation of bacterial genes has been reported previously by Dagostino, *et al.* (1991) in *Pseudomonas* S9 growing on polystyrene microtiter plates. In that study transposon mutagenesis was used to insert promoterless *lacZ* genes into recipient organisms, giving some the ability to display β -galactosidase activity at a surface but not in liquid or on agar media. The authors, however, did not identify the specific target genes controlling *lacZ* activation. In a separate study by Belas, *et al.* (1986) the *laf* gene was activated in *Vibrio parahaemolyticus* on agar medium but not in liquid. Activation

was, however, shown to occur in a highly viscous liquid. In that study the authors concluded that gene activation was a consequence of medium viscosity.

In cystic fibrosis lung infections, a succession from nonmucoid to mucoid cells (Dogget *et al.*, 1966) is thought to reflect an adaptation by *P. aeruginosa* to an environment deficient in water and high in K^+ , Na^+ , HCO_3^- and Cl^- (Knowles *et al.*, 1983). Laboratory studies have shown that high osmolarity activates *P. aeruginosa* transcription of *algD* (Berry *et al.*, 1989) and *algC* (Zielinski, 1991) leading to enhanced alginate production. Ethanol has likewise been shown to enhance alginate synthesis by that organism on solid and in liquid media (DeVault *et al.*, 1990). The effects of ethanol and salt are believed to result in a response leading to membrane perturbations similar to what is found in the environment of the CF lung. In a separate study, Roberson and Firestone, (1992) showed that desiccation of *P. aeruginosa* growing in a sand matrix resulted in more exopolymer than when cells were grown at high water potential.

Activation of *algC* in cells grown on a surface, as demonstrated in the present work, may be the result of decreasing water or nutrient levels at the point of contact with the substratum. The control of production of alginate containing biofilms known to be produced by *P. aeruginosa* on substrata in aquatic environments may, therefore, be related to the control mechanisms of alginate production by these organisms in the lungs of cystic fibrosis patients.

Production of alginate has been shown to vary throughout the cell growth cycle. Annison and Couperwhite, (1987) observed the production of alginate almost exclusively during the exponential phase of growth with *P. aeruginosa* as did Deavin, *et al.* (1977)

with *Azotobacter vinelandii*. In a separate series of experiments, Piggot, *et al.* (1982) found exopolysaccharide production did not commence in *P. aeruginosa* until late in the exponential phase and continued maximally during the stationary phase of growth. In our studies, we found maximum *algC* expression to occur in the late lag and early exponential phases of the growth cycle for planktonic cells in batch culture. During stationary phase, we saw a second increase in exopolymer production paralleling the data of Piggot, *et al.* (1982). Attached cells also display variation in *algC* expression. However, overall, expression is greater than with suspended cells for the duration of the growth cycle.

There is a possibility that the differences in promoter activity observed in the two populations resulted from different average plasmid copy numbers in the respective populations. This is unlikely, however, in view of the fact that the cells of each population were derived from the same stock, precultured under the same conditions and subsequently exposed to identical media during growth.

In aquatic environments and industrial process systems where continuous or semi-continuous flow predominates, surface associated organisms represent the principal microbial component. It is valuable, therefore, when examining the production of polymers, to take into account the behavior of the organisms in the attached state in continuous culture. Observations made *in situ* of exopolymer gene activation in continuous culture are possible when using a reporter gene system that is detectable at the level of the single cell. Using a fluorescent substrate to evaluate *in vivo* β -galactosidase activity, we demonstrated an accumulation of fluorescent product within the cells. This

product can be detected microscopically in real time, so that alginate promoter activity in attached cells can be evaluated without destructive interference. Using this technique it was possible to detect *algC* activity in cells attached to the inner surfaces of a glass capillary having a rectangular cross section.

Fluorescence by attached bacteria carrying the pNZ63 plasmid demonstrated that the cells were actively producing the *algC* gene product as had been observed in our batch culture experiments. Interestingly, not all cells were fluorescent even though in continuous culture. This implies that differences in exopolysaccharide production exist within the population. This may be due to the age, nutritional status or the degree/nature of adherence of the cells to the solid surface. Furthermore, this result demonstrates that active production of alginate is not necessary for cells to remain attached to the substratum.

In this paper, we have reported the activation of a specific gene for the production of bacterial exopolysaccharide as the result of attachment to a surface. This activation can be detected and quantified in individual cells attached to a substratum as well as in whole cultures. The method of activation and the range of surfaces upon which such activation occurs is not known at present. Further work is required to examine these unknown aspects of polymer production by *P. aeruginosa*.

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CHAPTER 3

REGULATION OF THE ALGINATE BIOSYNTHETIC
GENE *algC* IN *Pseudomonas aeruginosa* DURING BIOFILM
DEVELOPMENT IN CONTINUOUS CULTUREIntroduction

Pseudomonas aeruginosa has been shown to preferentially assume a biofilm mode of growth (Costerton, 1984), embedding itself in a gelatinous organic polymer matrix composed primarily of alginate (Costerton *et al.*, 1987). Biofilms produced by these organisms have been shown to be resistant to antibiotic therapy (Anwar *et al.*, 1989) resulting in chronic infections of the urinary tract (McFeters *et al.*, 1986) and lung epithelia of cystic fibrosis (CF) patients (Chartrand and Marks, 1983; Govan and Harris, 1986). *P. aeruginosa* biofilms have also been shown to be important as fouling agents in industrial process systems (Christensen and Characklis, 1990), and to form at surfaces in the natural environment (Geesey *et al.*, 1978). Control of these biofilms continues to be problematic due in part to a lack of understanding of the *in situ* regulation of biofilm matrix polymer formation.

Previous work has shown alginate production to be up-regulated in *P. aeruginosa* during nitrogen limitation (DeVault *et al.*, 1989), membrane perturbation induced by ethanol, when cells were exposed to media of high osmolarity (DeVault *et al.*, 1990) and

under conditions of high oxygen tension (Bayer *et al.*, 1990). Recently, we have reported that a specific gene (*algC*) involved in alginate biosynthesis by this organism is up-regulated as the result of attachment to a surface (Davies *et al.*, 1993). Little is known, however, of the regulation of alginate by this organism in biofilms in continuous culture.

Investigations into the regulation of alginate in continuous culture have been largely performed on total extracellular polymeric substance (EPS) or on exopolysaccharides which in the case of *P. aeruginosa* are believed to include alginate. Bakke, *et al.* (1984) examined the production of EPS by *P. aeruginosa* biofilms, showing that EPS biosynthesis was not directly proportional to substrate flux. This work did not, however, examine the regulation of EPS production. Robinson *et al.* (Robinson *et al.*, 1984) found EPS formation was inversely related to the growth rate of *P. aeruginosa* in suspension in a chemostat, however, these results were not duplicated in biofilms. Vandevivere and Kirchman, (1993) found higher rates of exopolysaccharide synthesis in a sub-surface bacterium attached to sand under conditions of continuous flow when compared with unattached bacteria.

In the present work, the behavior of a principal gene in the alginate biosynthetic pathway of *P. aeruginosa* was examined in axenic biofilms at the population level as well as at the single cell level. The gene *algC*, encoding the enzyme phosphomannomutase (PMM), was studied because it is an essential enzyme for biosynthesis of alginate and a key point of regulation in the alginate pathway. The gene product of *algC* acts as a bifunctional enzyme, having phosphomannomutase activity, converting mannose 6-phosphate to mannose 1-phosphate, as well as phosphoglucomutase activity,

interconverting glucose 6-phosphate and glucose 1-phosphate (Ye *et al.*, 1994). In its role as phosphomannomutase, the *algC* gene product is responsible for the formation of alginate and in its role as phosphoglucomutase, the same gene product is responsible for the production of lipopolysaccharide (Coyne *et al.*, 1994). The promoter sequence for *algC* along with 1.0 kb of upstream DNA was fused to a promoterless *lacZ* reporter gene to form the plasmid pNZ63 (Zielinski *et al.*, 1991). The plasmid was introduced into the stable mucoid *P. aeruginosa* strain 8830. Strain 8830 was used in a previous study to demonstrate the up-regulation of *algC* by attachment to glass and Teflon substrata.

Materials and Methods

Bacteria and media

The bacterium used in the study of *algC* regulation and biofilm development was *P. aeruginosa* 8830 containing the *algC-lacZ* transcriptional fusion plasmid pNZ63. This reporter plasmid and its characteristics have been described by Zielinski *et al.* (Zielinski *et al.*, 1991; Zielinski *et al.*, 1992). In experiments run to investigate the relationship between lipopolysaccharide (LPS) regulation and attachment to glass, Three variants of the strain *P. aeruginosa* PAO1 were used. The first variant was the wild-type, a clinical nonmucoid isolate from a cystic fibrosis patient. The second variant was the mutant AK0401, which was O antigen-deficient. The third variant was the mutant AK1012, which was deficient in LPS A-band. The characteristics of these strains has been

described previously (Jarrell and Kropinski, 1977, Lightfoot and Lam, 1991; Goldberg *et al.*, 1993).

All batch and continuous culture experiments were conducted using a defined culture medium (modified EPRI) containing: sodium lactate (0.05 g/l), sodium succinate (0.05 g/l), ammonium nitrate (0.381 g/l), KH_2PO_4 (0.19 g/l), K_2HPO_4 (0.63 g/l), Hutner salts (Cohen and Bazire, 1957) (0.01 g/l), dextrose (1.0 g/l), and L-histidine (0.01 g/l). Solid YTG medium was used for the enumeration of bacteria recovered from batch and continuous culture experiments. When grown on this medium, *P. aeruginosa* 8830 produces mucoid colonies which show up-expressed *algC* activity. YTG medium contained the following components in grams per liter: yeast extract, 5.0; tryptone, 10.0; glucose, 2.0; Bacto agar, 1.5. YTG medium amended with carbenicillin was prepared with 350 mg/l of the filter-sterilized antibiotic added to the medium following autoclave sterilization. YTG medium amended with 5-bromo-4-chloro-3-indolyl β -D-galactoside (X-gal) was prepared by adding 0.04 g/l of the β -galactoside in N,N-dimethyl formamide. Phosphate buffer was prepared by adding 7.0 gram K_2HPO_4 and 3.0 gram KH_2PO_4 to one liter of ultra pure water, pH, 7.0.

Plasmid retention studies

Activity of the constitutive marker for carbenicillin resistance on the pNZ63 fusion plasmid was examined to determine whether the presence of antibiotic pressure or cell attachment would influence plasmid copy number. The study organism was examined under Three separate growth conditions: planktonic cells grown in batch in

defined culture medium; planktonic cells grown in batch in defined culture medium amended with 350 mg/l carbenicillin; biofilm cells attached to Teflon mesh during growth in batch in defined culture medium amended with 350 mg/l carbenicillin. Cell cultures of *P. aeruginosa* were recovered from frozen skim milk stock and grown until turbid in preculture flasks of defined culture medium supplemented with 350 mg/l carbenicillin. An inoculum of 0.1 ml preculture was added to each of 3 sets of quintuplicate 250 ml flasks containing 100 ml defined culture medium. Each set corresponded to one of the Three populations listed above. Cells were grown at 25°C on a rotary shaker at 175 rpm until cultures reached stationary phase. Cells were centrifuged at 16,274 x g for 30 min and washed Three times in PO₄ buffer and frozen at -40°C. Cells attached to Teflon mesh were removed by scraping with a sterile brush, resuspended in defined culture medium, centrifuged and washed as above. All samples were resuspended in 2 ml PO₄ buffer, pH 7.0. The washing procedure was intended to both concentrate the sample and to remove carbenicillin from the preparation.

β-lactamase assay

β-lactamase activity was determined using a procedure based upon that described by Cohenford *et al.* (1987). Whole cell samples were thawed and lysed at 0°C, by cavitation for 2 x 30 sec at high setting using a Bronwill Biosonik IV, ultra sonic cell disrupter. Aliquots of 0.5 ml lysed cell suspensions were mixed with 1.0 ml β-lactam substrate (100 ug/l carbenicillin), vortexed and allowed to stand for 10 min at 25°C. The reaction was terminated by the addition of 1.5 ml neocuproine-copper reagent. Samples

were vortexed and incubated at 25°C for an additional 30 min to allow color development. The β -lactam acid generated by the enzyme when reacted with the neocuproine-copper solution yielded an orange color with a maximum absorbance at 454.5 nm. All samples were analyzed at the above wavelength using a Milton Roy Spectronic 601 spectrophotometer.

Continuous culture studies

P. aeruginosa was grown in continuous culture to determine the ability of biofilm bacteria to retain the pNZ63 plasmid in the absence of antibiotic pressure, and to evaluate *algC* expression in biofilm cells. A continuous culture vessel having a liquid volume of 375 ml and containing defined culture medium was used without supplemented carbenicillin to grow biofilms for up to 22 days. The culture vessel was inoculated with 10 ml late log phase starter culture in defined culture medium containing 350 mg/l carbenicillin. Thus, the carbenicillin concentration in the vessel was 0.93 mg/l prior to the initiation of flowing conditions. The bacteria were allowed to colonize the surfaces within the culture vessel over a period of 24 h in batch mode. Fluid samples were collected from the culture vessel at the end of the batch mode period during two experimental runs to represent the planktonic cell population. Following the initial batch period, flow was turned on and adjusted to achieve washout conditions, resulting in a dilution rate in the culture vessel of $D = 3.53 \text{ h}^{-1}$. Previous experiments in our lab have shown *P. aeruginosa* 8830 to have a growth rate $\mu = 0.69 \text{ h}^{-1}$ when grown in defined culture medium. Therefore, cells collected in the effluent after the onset of flowing

conditions would have been shed from the growing biofilm on the culture vessel surfaces where they were immune from washout.

Three separate continuous culture experiments were run using Teflon mesh having a surface area of 137.2 cm² as a substratum for biofilm growth within the continuous culture vessel. Teflon was used in this study because it has previously been shown to promote biofilm growth and *algC* up-expression in *P. aeruginosa* 8830 (Davies *et al.*, 1993). Bacteria shed from the biofilm under flowing conditions were collected each day from the effluent of the continuous culture vessel to determine whether these cells would maintain their plasmid conferred antibiotic resistance in the absence of antibiotic pressure. These bacteria were enumerated on solid YTG medium containing 350 mg/l carbenicillin to recover CFU containing a functional β -lactamase marker and on solid YTG medium to determine total bacterial numbers. Efficiency of plasmid retention was determined as the fraction of carbenicillin resistant CFU recovered relative to the total CFU recovered. Following growth on YTG, colonies were replica plated onto YTG amended with 350 mg/l carbenicillin to determine whether injury was responsible for decreased resistance to carbenicillin. For a discussion of injury in bacteria, see LeChevallier and McFeters, (1984), and McFeters, *et al.* (1986). The number of CFU recovered on carbenicillin containing plates was compared with the number of CFU recovered on YTG plates containing X-gal, to ensure that the retention of antibiotic resistance was coincidental with retention of the β -galactosidase gene.

In the effort to evaluate *algC* expression during biofilm development in continuous culture, biofilms were grown in the culture vessel until the point at which

bacteria shed into the effluent were shown to maintain a constant density as determined by standard plate count technique using YTG medium. After several days of maintenance of relatively constant effluent cell densities, Teflon mesh substrata containing biofilms were recovered from two continuous culture experiments. The biofilm bacteria were aseptically removed from the mesh by scraping using a sterile brush. These were suspended in sterile culture medium and frozen for subsequent analysis. These samples were analyzed for uronic acid accumulation, protein content, β -lactamase specific activity, and β -galactosidase specific activity. Scraped biofilm samples were subjected to the same analyses as planktonic samples recovered after the 24 h batch culture.

During the period of relatively stable effluent cell densities, samples were collected daily from the reactor effluent and enumerated on YTG medium. Subsamples were frozen at -40°C and subsequently analyzed for β -galactosidase specific activity and protein concentration to evaluate *algC* expression.

β -Galactosidase assay

The β -galactosidase assay was performed on thawed samples with a Milton Roy Spectronic 601 spectrophotometer following the procedure of Miller, (1972) as described previously (Davies *et al.*, 1993). β -Galactosidase specific activity is reported as nanomoles of ONP produced per minute per milligram of protein at 25°C , pH 7.0. For a full description of the β -Galactosidase assay, see Chapter 2.

Lowry protein assay

Proteins were assayed by the Lowry Protein Assay which was performed on

thawed samples as described previously (Davies *et al.*, 1993) and analyzed with a Milton Roy Spectronic 601 spectrophotometer. For a full description of the Lowry protein assay, refer to Chapter 2.

Uronic acid assay

Total uronic acids were measured in thawed samples of scraped biofilm and whole culture following the method of Kintner and Van Buren, (1982) using a Milton Roy Spectronic 601 spectrophotometer. For a full description of uronic acid analysis, refer to Chapter 2.

Effect of fluid velocity on attachment of *P. aeruginosa* 8830

Initial experiments conducted to study the attachment of *P. aeruginosa* 8830 pDAD6 and pNZ63, indicated that variations in fluid velocity within the flow cell could influence the ability of the bacteria to attach to the glass substratum. An experiment was run to observe how differences in fluid velocity influence the number of bacteria that would attach to the glass coverslip as well as how long the bacteria would remain attached to a specific location. The flow cell was configured as described above, with the exception that medium from culture flasks containing bacteria was used for the influent. The flow rate during the experiments was maintained at a velocity of $0.0 \mu\text{m sec}^{-1}$, $20.0 \mu\text{m sec}^{-1}$, $30.3 \mu\text{m sec}^{-1}$, and $143 \mu\text{m sec}^{-1}$. At the beginning of each experimental run, log phase bacteria (corrected to an optical density of 0.200 OD, Abs.. 600 nm) were allowed to enter the flow cell at each of the velocities indicated above. Prior to addition of the bacteria, the surface of the glass coverslip had been cleaned with soap and water,

acetone and ethanol. The bacteria were added to the flow cell as a slug dose of 10 ml culture. Following the addition of the bacteria, sterile EPRI medium was flowed through the reactor for the remainder of the experiment (with the exception of the no flow example). The number of bacteria attaching to an individual field was examined over a period of 180 minutes and recorded at approximately 15 minutes intervals for each of the fluid velocities listed above. Field size at the magnification used for these experiments was $100\ \mu\text{m}^2$. Flow velocities were determined *in situ* by recording the rate of flow of introduced $1.0\ \mu\text{m}$ diameter polystyrene beads as they passed through the field of view immediately below the surface of the glass substratum.

Microscopic analysis of *algC* activity in continuous culture

In a previous paper, we reported the use of a fluorogenic galactoside which when hydrolyzed by the pNZ63 reporter gene product (β -galactosidase) can be detected by epifluorescence microscopy within individual cells of *P. aeruginosa* (Davies *et al.*, 1993). This fluorogenic compound was used in the present study as the substrate for reporter enzyme activity to evaluate *algC* expression in individual bacteria in continuous culture. In order to determine the effect of the presence of the methylumbelliferyl β -D-galactopyranoside on the viability of the study bacteria, a test was performed in which the bacteria were grown for 52 hour in batch culture in the presence and absence of 0.01 g/l of the fluorogenic substrate in defined culture medium. Bacteria were sampled throughout the incubation period and their ability to be cultured on solid YTG medium was evaluated.

A continuous-culture apparatus was developed to monitor *in situ* reporter gene expression of individual bacteria during initial stages of biofilm development (Figure 3.1). The apparatus was configured as a once through flow cell system. The influent defined culture medium supplemented with 350 mg/l carbenicillin was retained in a four liter glass reservoir. Methylumbelliferyl β -D-galactopyranoside dissolved in 5.0 ml N,N-dimethyl formamide was added to the defined culture medium to a final concentration of 0.01 g/l. Medium from the influent reservoir was pumped through silicone tubing via a Masterflex pump to an aeration flask sparged with filtered air. The aerated medium was pumped to a flat plate flow cell using a Masterflex 8 roller-head peristaltic pump at a flow rate of 0.13 ml min⁻¹. The flow cell was constructed of polycarbonate having a depth of 0.1 mm, a width of 1.4 cm, and a length of 4.0 cm, the upper face was capped with a glass coverslip. The glass coverslip was used as a substratum for bacterial attachment and biofilm development because it is a relatively inert material, is transparent, and has been shown to result in *algC* activity similar to what has been observed for Teflon mesh (unpublished data). Flow through the cell was laminar, having a Reynolds number of 0.17, with a fluid residence time of 0.43 minutes. The flow cell was sealed to prevent contamination and affixed to the stage of an Olympus BH2 microscope containing a BH2-RFL fluorescent attachment. Medium leaving the flow cell was pumped to an effluent reservoir via silicone tubing. The entire system was closed to the outside environment but maintained in equilibrium with atmospheric pressure by a 0.2 μ m pore size gas permeable filter fitted to each flask.

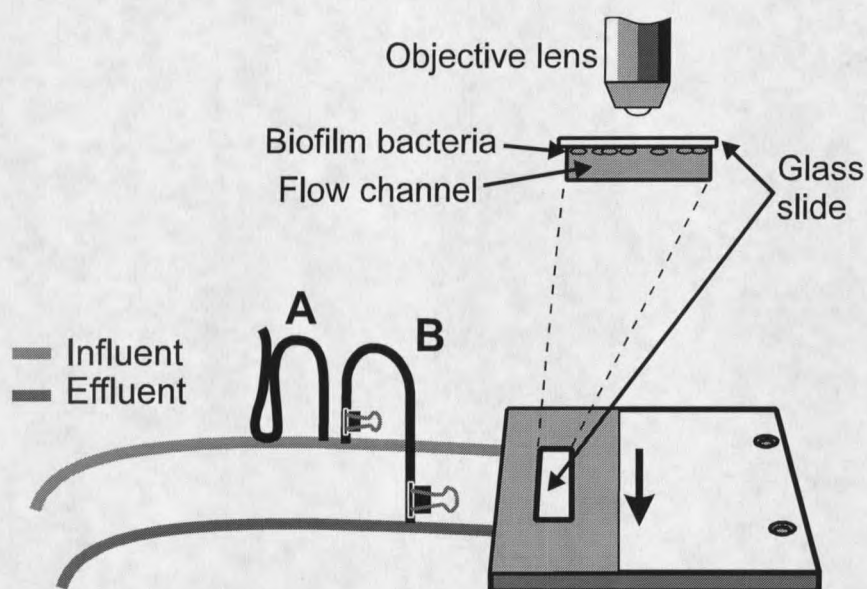


Figure 3.1. Schematic representation of continuous culture flow cell.

Log phase *P. aeruginosa* were inoculated through a septum approximately 1 cm upstream from the flow cell while flow was maintained. Bacteria were allowed to attach to and grow on the surfaces of the system downstream from the site of inoculation over a period of 24 h. Flow through the system was then increased to remove any bacteria attached to the inside surface of the coverslip (as determined by microscopy). Bacteria shed from biofilm upstream from the flow cell were then allowed to recolonize the surfaces of the flow cell under conditions of normal flow. After 2 h, cells attached to the inner surface of the glass coverslip were viewed by transmitted light using phase contrast at 1025 x magnification to detect total cells. The same field was then observed under UV excitation using a UG1 blue Olympus filter block to detect the conversion of the methylumbelliferyl substrate. Fluorescent and phase contrast images were recorded using an American Innovations image analysis system. Each image was taken using an

integration of 50 scans in order to increase the amount of detectable fluorescence and to collect data on only those bacteria which were attached to the coverslip. Planktonic bacteria flowing beneath the coverslip were not detectable during the integration period due to their motion. Information on number and location of attached bacteria within a single field was collected at approximately 15 minute intervals for a period of 165 minutes. All images were stored as separate files for subsequent retrieval and analysis.

Analysis of microscopic images

Stored digital images of bacteria were retrieved and displayed on a computer screen. Clear plastic overlays placed on the screen were used to mark the time and position of cells attached to the inner surface of the glass coverslip. Three distinct subpopulations of bacteria were identified: 1) bacteria that were already attached to the coverslip in the previous observation period, 2) bacteria that had attached to the coverslip since the previous observation period, 3) bacteria that had detached from the coverslip since the previous observation period. Bacteria within each of the Three subpopulations were recorded as being fluorescent or nonfluorescent indicating whether they manifested *algC* up-expression.

Attachment of *Pseudomonas aeruginosa* PAO1 mutants to a glass surface

The regulation of *algC* is known to be involved in the production of lipopolysaccharide as well as alginate in strains 8830 and PAO1, both clinical isolates of *P. aeruginosa*. In order to investigate whether changes in LPS may affect attachment of *P. aeruginosa*, an experiment was performed to look at the ability of *P. aeruginosa* PAO1

to attach to a glass coverslip affixed to a flow cell as described above. Three strains of *P. aeruginosa* PAO1 were used in this study: wild type, strain AK1401 and AK1012. The wild type, had complete LPS core and both A and B band polysaccharides. Strain AK1401 is an O antigen-deficient mutant, originally isolated by its resistance to bacteriophage E79 (Berry and Kropinski, 1986). Strain AK1012 is an LPS rough mutant derived from strain PAO1 by its resistance to lysis by the O-side chain-specific bacteriophage E79 (Jarrell and Kropinski, 1977). Chemical analysis determined that the block in LPS synthesis in strain AK1012 was in the attachment of glucose residues to the galactosamine in the LPS core. In addition, strain AK1012 does not incorporate A band polysaccharide antigen onto the LPS (Lightfoot and Lam, 1991). The *algC* gene product phosphomannomutase/phosphoglucomutase has been shown by Goldberg *et al.* (1993) to be essential for the smooth phenotype in *P. aeruginosa* PAO1 and has been used to complement the LPS defect in strain AK1012. This work indicated that the defect in strain AK1012 is a deficiency in PMM activity.

The Three strains of PAO1 were cultured in EPRI medium supplemented with 2.0 g/l glucose in triplicate 250 ml flasks. These cultures were grown to late log phase. Prior to the attachment studies, cultures were normalized to an optical density of 0.200 absorbance at OD₆₀₀ nm (apx. 10⁷ CFU/ml) by the addition of sterile EPRI medium. The cultures were then connected to a Masterflex pump and passed through the flow cell at a constant flow rate of 0.13 ml/minutes. The glass coverslips used during these attachment assays were cleaned with soap and water, acetone and 95% ethanol prior to each use. Bacterial attachment was monitored by recording the number of cells attached to the glass

slide within one microscope field, over the course of 30 minutes. Bacterial cell numbers at the surface of the coverslip were recorded approximately every two minutes over the interval of the attachment assay.

Results

Plasmid retention studies

The transcriptional fusion plasmid, pNZ63 contains an inducible β -galactosidase gene controlled by the promoter region of the *algC* gene of *P. aeruginosa*. Studies of reporter gene activity using such a plasmid are only valid when the copy number variation from cell to cell does not influence the interpretation of reporter gene activity measurements. In order to evaluate the stability of the pNZ63 plasmid in *P. aeruginosa* strain 8830, we decided to examine the activity of the β -lactamase marker which is constitutively expressed and not under *algC* promoter control. When β -lactamase specific activity is compared between planktonic cells in the presence and absence of β -lactam antibiotic pressure in batch culture, no significant difference is observed (Table 3.1). This indicates that the presence of antibiotic does not significantly influence the average copy number of plasmid within the population. When β -lactamase specific activity of biofilm bacteria is compared with that of planktonic cells when both are grown in the presence of antibiotic in batch culture, the average specific activity of the biofilm population is approximately 1.5 times of that of the planktonic population (Table 3.1.). This result indicates that plasmid copy number may be 50% higher in the biofilm population than the planktonic population.

Table 3.1. *P. aeruginosa* 8830 plasmid-borne β -lactamase specific activity

Population	Antibiotic Presence ^a	β -lactamase specific activity ^b (mean $\pm$ SD)
Batch ^c		
Biofilm	+	55.1 $\pm$ 7.1
Planktonic	+	37.9 $\pm$ 7.6
Planktonic	-	38.5 $\pm$ 6.4
Continuous Culture ^d		
Biofilm	-	51.6 $\pm$ 1.9

^aCarbenicillin, 350 mg/l.

^bNanomoles of carbenicillin converted per milligram of protein per minute.

^cMean of five separate experiments.

^dMean of two separate experiments.

The biofilm bacterial population which developed in the absence of antibiotic in continuous culture over a period of 18 and 22 days showed no significant difference in β -lactamase specific activity when compared with the population of bacteria growing as a biofilm in batch culture in the presence of antibiotic (Table 3.1.). These results indicated that carbenicillin resistance was not lost by the vast majority of bacterial cells within the biofilm population during growth in continuous culture in the absence of antibiotic pressure over a period of at least 18 days.

To determine whether subpopulations of *P. aeruginosa* 8830 containing the pNZ63 plasmid lose carbenicillin resistance after removal of antibiotic pressure, individual cells that had detached from growing biofilm were recovered from the effluent of the continuous culture vessel operated under washout conditions and screened for their

ability to grow in the presence of antibiotic. Samples were removed from the continuous culture vessel and plated on selective media on a daily basis to examine the resistance of individual colony forming units to antibiotic. Microscopic analysis showed that colony forming units were comprised of single cells. Results from a representative experiment using Teflon mesh as a substratum for biofilm growth in the absence of antibiotic are shown in Figure 3.2. Over a period of 360 hour, no overall decrease in carbenicillin resistance was observed for cells shed from the biofilm into the effluent. These results indicated that the population of bacteria shed from the biofilm showed no net loss of plasmid borne β -lactamase activity during the time-course of the experiment.

The data point labeled "Scraped Biofilm" in Figure 3.2, indicates the population of total biofilm cells scraped from the mesh at the end of the experiment which were culturable in the presence and absence of antibiotic. The percent of scraped biofilm cells resistant to carbenicillin was not significantly different from shed cells, indicating that the shed cells did not represent a subpopulation of the total biofilm population with respect to plasmid copy number. No overall loss of carbenicillin resistance with time was demonstrated during any of the continuous culture experiments. Recovery of resistant cells was on average, lower than 100 percent of the total recoverable cells in all experiments. To check the discrepancy between total and resistant CFU recovered, replica plating was performed by transferring cells from each colony of planktonic, shed biofilm and biofilm bacteria recovered on non-selective medium to carbenicillin containing medium. Of those colonies transferred, 100 percent were shown to be carbenicillin resistant. No statistical difference was observed between CFU having

antibiotic resistance and CFU having β -galactosidase activity (data not shown). These results indicated that those bacteria which were not recovered on antibiotic containing medium when plated directly from the continuous culture effluent were a population of injured cells. Injured bacteria are those which are not recoverable on selective medium unless they are resuscitated first on a non-selective medium; see LeChevallier and McFeters, (1984) and McFeters, *et al.* (1986).

Effect of fluid velocity on attachment of *P. aeruginosa* 8830

The effect of fluid velocity on the ability of *P. aeruginosa* 8830 to attach to the glass substratum of the flow cell is shown in figure 3.3. At the highest flow velocity ($143 \mu\text{m sec}^{-1}$), almost no bacteria were observed to attach to the glass surface. This indicated that the ability of these bacteria to attach to the surface was overcome by the fluid shear within the flow cell. Previous experiments (data not shown) have demonstrated that when *P. aeruginosa* 8830 become established on a glass surface, they can not be removed by increasing the flow to the maximum rate obtainable using this system (i.e., $143 \mu\text{m sec}^{-1}$). Under conditions of no flow ($0.0 \mu\text{m sec}^{-1}$), the number of bacteria on the surface rapidly increased to a maximum of 145 cells per field. The bacteria were motile, and were observed to swim to the glass surface where they would attach. After 150 minutes, however, almost none of the bacteria were left on the surface of the glass coverslip. Since no fluid shear could account for this detachment phenomenon, it is assumed that the bacteria were able to leave the surface via an active desorption process. At the two intermediate flow rates ($20.0 \mu\text{m sec}^{-1}$, and $30.3 \mu\text{m sec}^{-1}$), the bacteria are able to attach

to the glass substratum (although at a lower rate than was observed with the no flow example) and remain attached throughout the experiment. Following a period of higher initial attachment, the bacteria appear to reach an almost steady state number of around 28-40 cells per field. This indicated that the conditions which were causing detachment of the cells in the no flow experiment were not present to the same degree in the low and medium flow experiments. Furthermore, shear during the two intermediate flow experiments was not sufficient to overcome the binding affinity of the attached bacteria, as it apparently had been in the high flow example. The flow rates used for studying the activity of both *algC* and *algD* (Chapter 4 and Chapter 5) activity were 0.13 ml min^{-1} , which resulted in a flow velocity of $30.3 \text{ } \mu\text{m sec}^{-1}$.

Continuous culture mature biofilm experiment

Once it had been established that pNZ63 did not dilute out of the population of biofilm cells in the absence of antibiotic pressure, it became feasible to run experiments to examine the regulation of the alginate *algC* gene over time in a mature biofilm using a plasmid borne reporter. Three independent chemostat experiments were run using Teflon mesh as a substratum for biofilm growth; two for a period of 432 hours and the other for 576 hours. After establishing an attached population of cells by operating the reactor in batch mode for 24 h, flow of culture medium was initiated and adjusted to achieve washout conditions. After several days, the density of viable cells shed from the biofilm in the reactor effluent stabilized to a number fluctuating around a mean of 5.08×10^6 CFU/ml (standard deviation = $\pm 2.37 \times 10^6$) for the Three separate experiments. Figure

3.4 shows results from the chemostat experiment which was run for 576 h. With the exception of a low cell density obtained at $t=336$ hour, shed cell densities appeared to plateau between 2×10^6 and 6×10^6 colony forming units (CFU)/ml after 288 h (Figure 3.3). This range of shed cell densities was typical of that exhibited by cells shed from biofilms in previous experiments. Uncontrollable episodic detachment of bacteria from the biofilm may have accounted for observed variations in effluent cell densities.

Alginate accumulation based on uronic acid concentrations and expression of *algC* based on reporter gene product specific activity were determined in the planktonic cell population at the end of the 24 h batch culture period and in the scraped biofilm population at the end of the continuous culture period for two separate chemostat experiments (Table 3.2). Expression of *algC* was nearly 20 fold higher while alginate levels were over 2-fold higher in the biofilm population than in the suspended cell population, respectively (Table 3.2).

Expression of *algC* was also evaluated in the population of bacteria shed from the biofilm which were recovered from the chemostat effluent of the experiment run for 576 h. Fluctuations were observed in reporter gene product specific activity as the biofilm developed throughout the course of the experiment (Figure 3.4). The average β -galactosidase specific activity for the shed cells collected in the effluent from 288 hour through 576 hour is shown in Table 3.2. This averaged activity had a value which fell between those derived for scraped biofilm cells and planktonic cells indicating an intermediate stage in *algC* activity (Table 3.2).

Evaluation of *algC* expression at the single cell level

A novel microscopic technique was employed for observing single cell *algC* expression during the initial phases of biofilm development. Using a once through flow cell reactor system, gene expression was monitored in bacteria which had detached from

Table 3.2. *P. aeruginosa* 8830 continuous-culture biofilm activity versus planktonic cell activity.

Population	Uronic Acids (ng produced/ μ g of protein)	β -Galactosidase specific activity (nmol/min/mg of protein)
Biofilm ^a	80.0 $\pm$ 0.14	19.35 $\pm$ 8.8
Planktonic ^a	36.5 $\pm$ 0.70	1.06 $\pm$ 0.05
Shed Cell ^b	ND ^c	6.21 $\pm$ 4.3

^aMean of two separate experiments $\pm$ 1 standard deviation.

^bMean of nine samples from one chemostat experiment.

^cND, not done.

an upstream biofilm and subsequently reattached to a glass coverslip where they could be observed by fluorescence and transmitted white light microscopy. In order to determine that the fluorogenic galactoside used in these experiments was not toxic to the cells, an experiment was run to evaluate the influence of methylumbelliferyl β -D-galactoside on cell growth. Results from the experiment showed no significant difference between the growth curves of cells recovered from flasks containing the fluorogenic compound and those recovered from flasks without the substrate (data not shown).

Figure 3.5 depicts a series of photomicrographs taken via image analysis during the continuous culture microscopy experiment which tracks the attachment and

subsequent up-expression of *algC* in a bacterial cell. At $t=0$, some bacteria have already attached to the surface (Figure 3.5, A) and a fraction of these demonstrate *algC* up-expression as indicated by whole cell fluorescence (Figure 3.5, B). After 16 min, a new bacterium has attached to a previously uncolonized area of the substratum (Figure 3.5, C; arrow). This bacterium does not demonstrate fluorescence at $t=16$ min (Figure 3.5, D; arrow) or at $t=31$ min (Figure 3.5, E and 3.5, F; arrow). At $t=45$, the bacterium is still present at its attachment site (Figure 3.5, G; arrow), and has begun to fluoresce (Figure 3.5, H; arrow).

Combining the approach described above and image analysis, Three distinct populations of bacteria were followed over a period of 165 minutes. These were: 1) bacteria that were already attached to the coverslip in the previous observation period, 2) bacteria that had attached to the coverslip since the previous observation period, and, 3) bacteria that had detached from the coverslip since the previous observation period. For each of these Three groups, it is possible also to observe bacteria that are fluorescent in the presence of methylumbelliferyl β -D-galactoside, representing those that have detectable *algC* promoter activity (i.e., those that are producing PMM/PGM), and those that are not fluorescent, representing bacteria that do not have detectable *algC* promoter activity. From this information, it is possible to determine 4 populations of bacteria in these experiments each having members either that display *algC* reporter gene activity or do not display *algC* reporter gene activity. These are: 1) total bacteria at the surface, 2) bacteria attaching to the surface, 3) bacteria detaching from the surface, and, 4) bacteria that change their *algC* regulation while they are attached to the surface.

Figure 3.6 shows the total bacteria attached to the surface of the glass coverslip recorded at roughly 15 min intervals. At the beginning of observation, 63% of cells already attached to the surface were nonfluorescent. As the experiment progressed, the number of nonfluorescent cells at the substratum decreased from 45 to 6 in number while fluorescent bacteria at the substratum increased in number from 26 to 50. At the end of the observation period, 89% of the cells at the surface were shown to be fluorescent.

Figure 3.7 depicts numbers of bacteria from the bulk aqueous phase that had attached to the coverslip at roughly 15 min intervals over the 165 minute observation period. Each time point indicates the number of fluorescent or nonfluorescent bacteria that have attached to some location within the field of view since the previous observation period. As can be seen, the majority of cells (over 93%) were not fluorescent at the time of attachment to the substratum. Of those cells that had attached to the substratum, many eventually detached and entered the bulk phase. Of the total that detached from the substratum, over 70% were nonfluorescent, indicating that they were not actively expressing the *algC* gene.

Figure 3.8 compares the fluorescent and nonfluorescent bacteria which had detached from the substratum since the previous observation period as a percent, respectively, of the total fluorescent or nonfluorescent population present at the surface. Those bacteria that were not up-expressed for *algC* transcription (the nonfluorescent population) showed a higher propensity to detach from the surface than those bacteria which were up-expressed for *algC* transcription (the fluorescent population).

Finally, Figure 3.9 shows bacteria attached to the substratum which had switched from nonfluorescent to fluorescent and visa versa demonstrating that these cells had changed regulation of *algC* transcription while attached to the substratum. The majority of attached cells switched from nonfluorescent to fluorescent, with only a small fraction losing fluorescence during the time they were attached to the substratum.

Attachment of *Pseudomonas aeruginosa* PAO1 mutants to a glass surface

The influence of differences in LPS on attachment to a glass coverslip in the flow cell reactor was examined in *Pseudomonas aeruginosa* PAO1. Figure 3.10 shows the results of this study. The wild type strain showed essentially no ability to attach to the coverslip under the conditions of the experiment. The wild type bacteria have a complete LPS core and smooth phenotype. These cells have a functional *algC* and, therefore, represent bacteria in which that gene is turned "on". The other two strains represent bacteria in which the *algC* gene is turned "off". In strain AK1012, the amount of attachment was significantly greater than that which was observed for the wild type. This indicated that a complete core may interfere in some way with cell attachment to a surface. The strain AK1401 showed the greatest ability to attach to the glass surface averaging about 5 times the average number of AK1012 cells at the glass surface. These results indicated that when AlgC is involved primarily in the biosynthesis of LPS (in *Pseudomonas aeruginosa* PAO1), attachment to glass is decreased relative to when AlgC is devoted to alginate biosynthesis. The amount of wild type PAO1 cells attaching during these assays as comparable to what has been seen with strain 8830 previously.

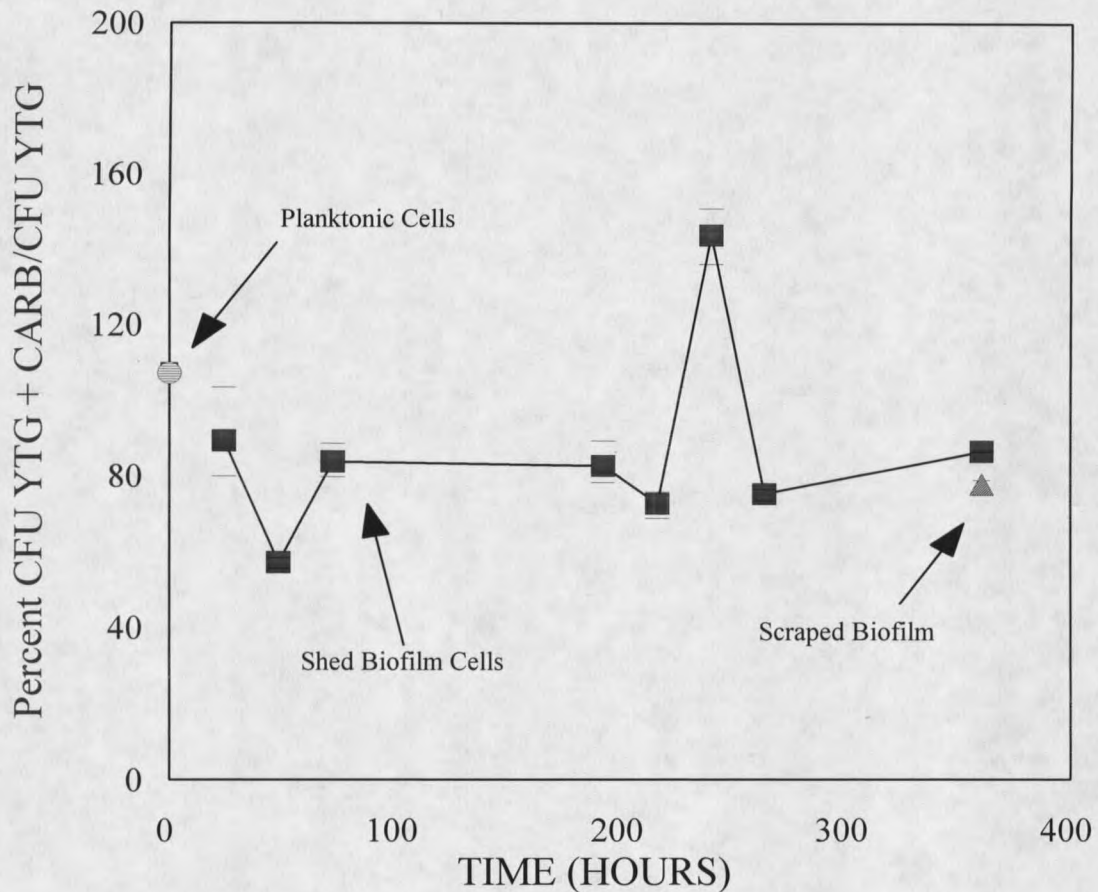


Figure 3.2. Cells grown in continuous culture that display carbenicillin resistance, expressed as a percentage of total cells. Planktonic cells were collected from the reactor before onset of continuous-flow operation; shed cells were collected from the reactor effluent when running under washout conditions; biofilm cells were scraped from Teflon mesh from the reactor at the end of the experiment. Error bars represent ± 1 standard deviation of the mean value of triplicate samples.

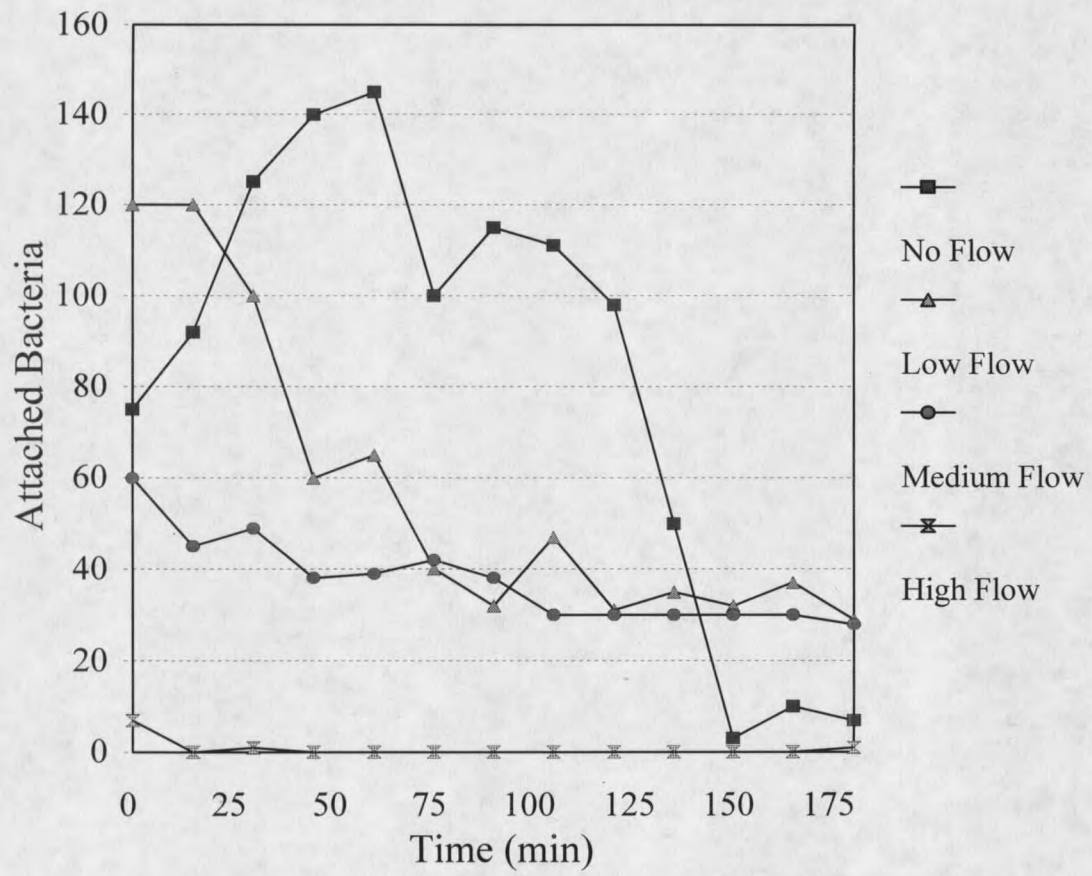


Figure 3.3. Effect of fluid velocity on attachment of *P. aeruginosa* 8830.

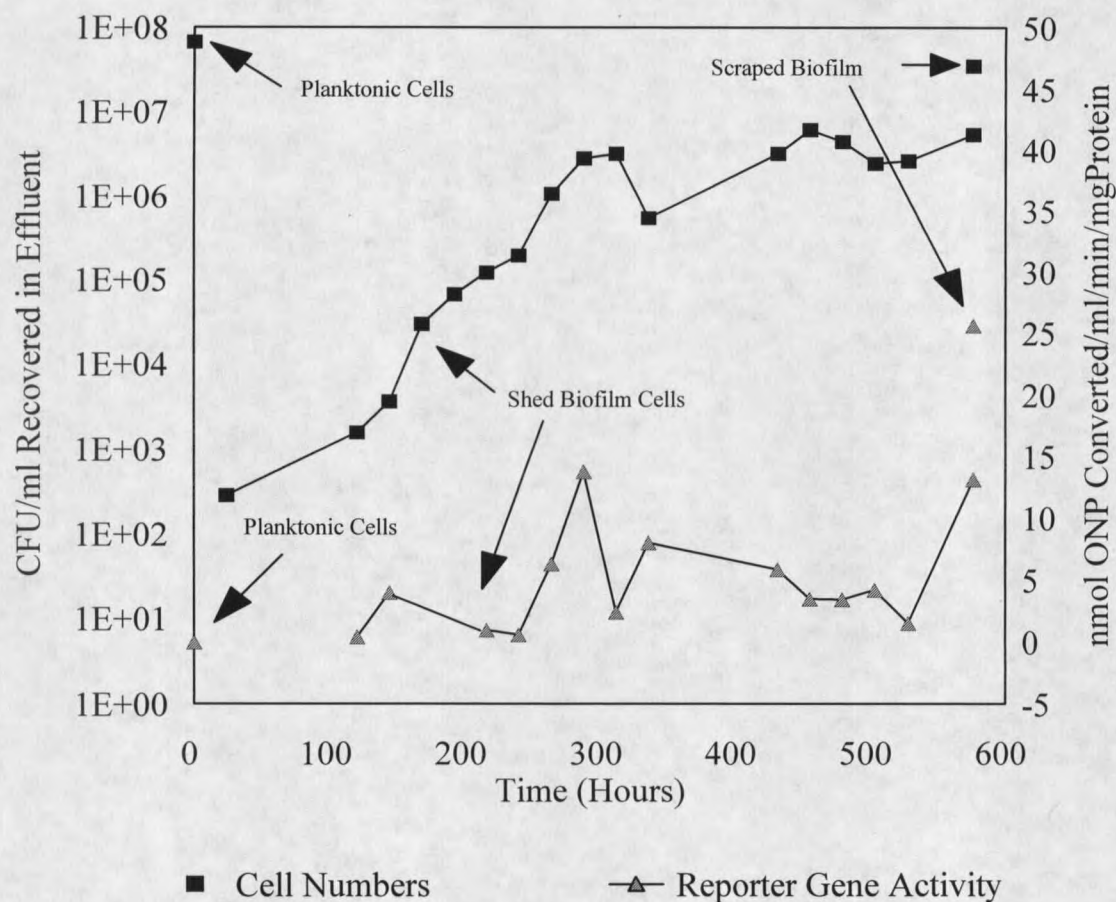


Figure 3.4. Specific activity of *algC* reporter gene (hatched triangles) and viable cell numbers (closed squares) for *P. aeruginosa* 8830 from continuous culture vessel effluent. Planktonic cells, cells shed from the biofilm into the effluent and total biofilm cells scraped from Teflon mesh are represented.

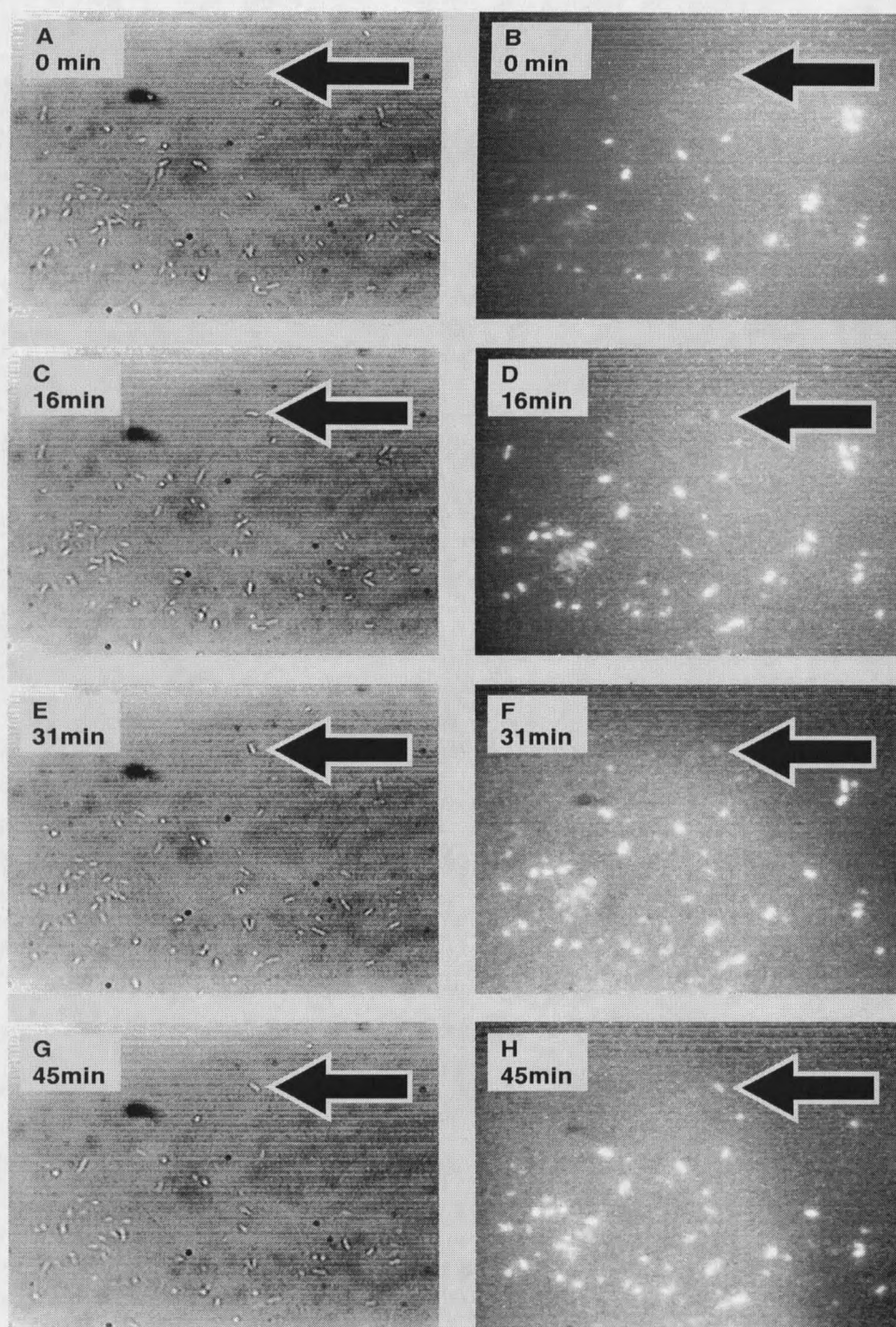


Figure 3.5. Photomicrograph of *P. aeruginosa* 8830 cells attached to a glass substratum. Magnification, x 1,250. Phase contrast image of total cells (left) and fluorescent cells displaying β -galactosidase activity (right). The arrow denotes a cell attaching to the surface, with subsequent *algC* reporter gene up-expression.

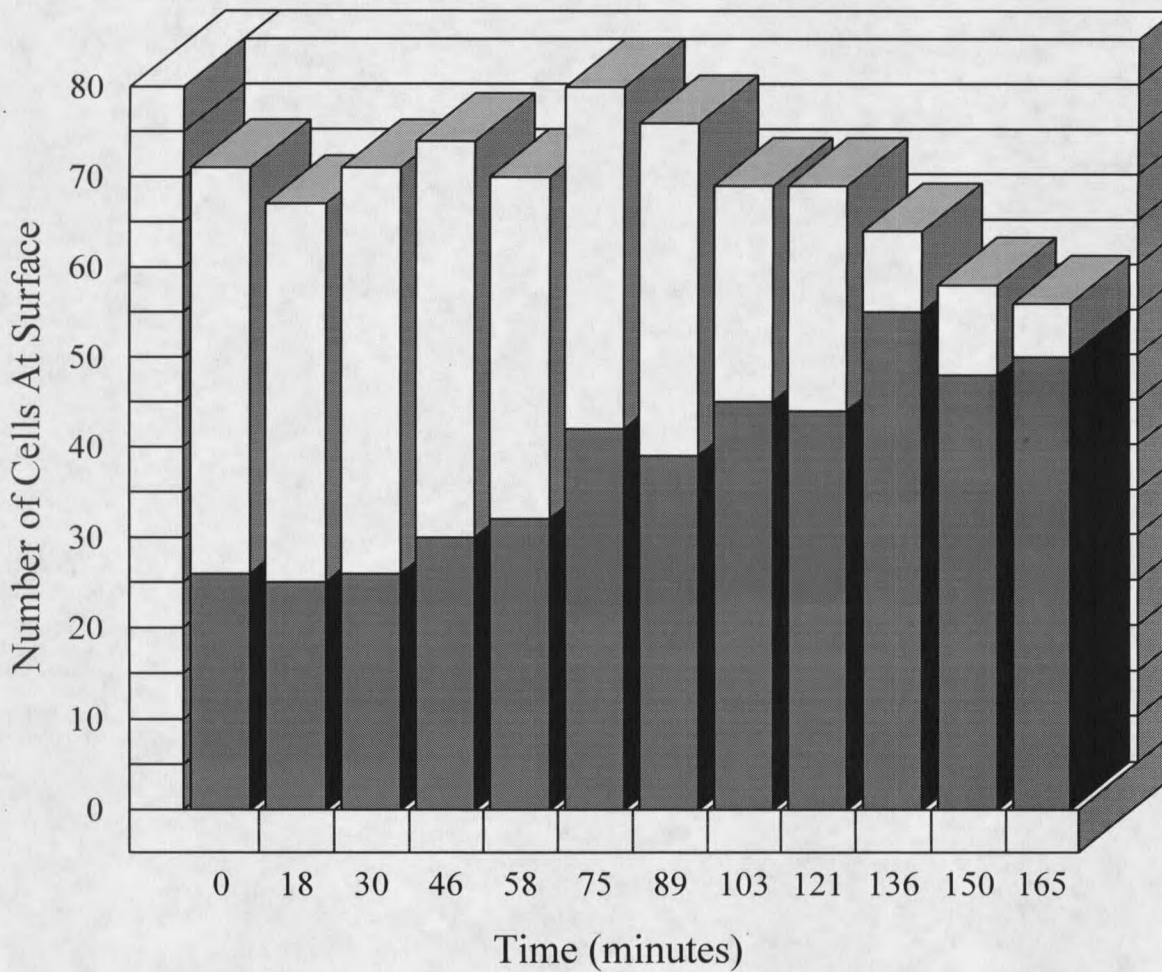


Figure 3.6. Bacteria attached to the inner surface of the glass coverslip in a flow cell culture chamber. Abscissa represents time points at which bacteria were observed during 165 min of colonization from the bulk liquid phase. Dark shaded bars represent fluorescent cells; light bars represent nonfluorescent cells.

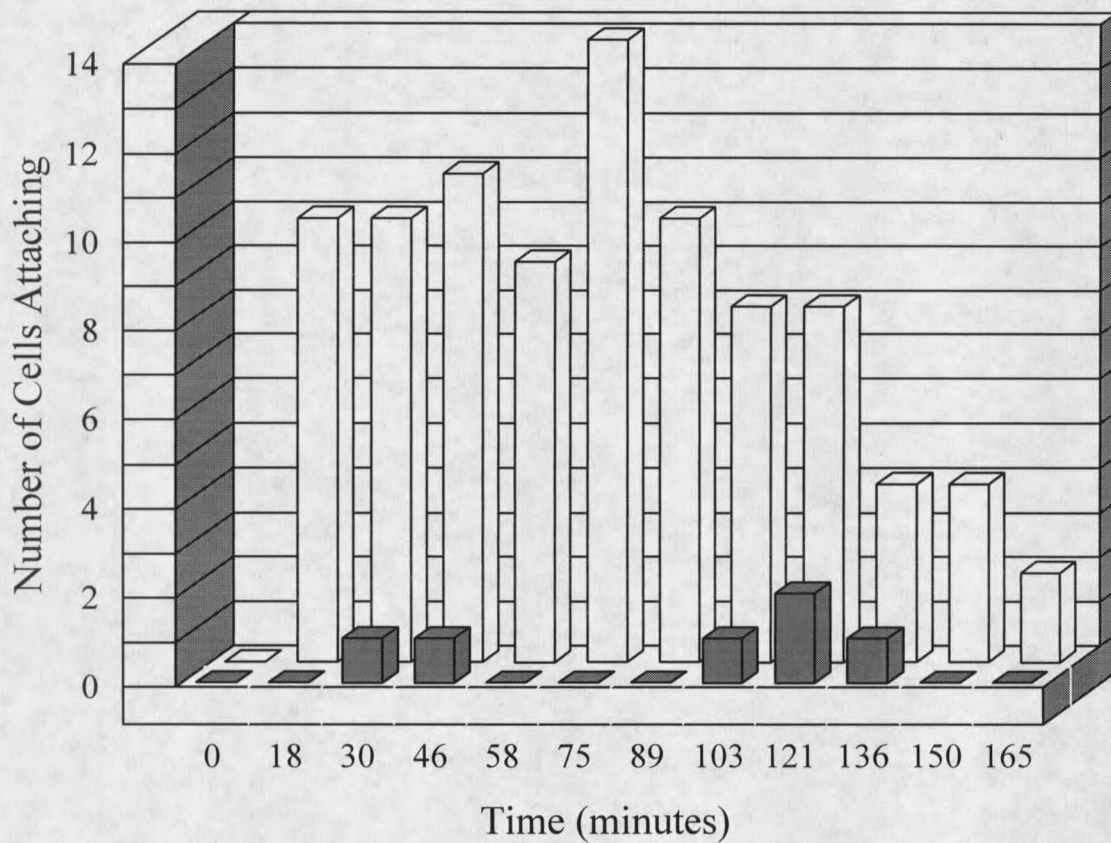


Figure 3.7. Attachment of bacteria to the inner surface of the glass coverslip in a flow cell culture chamber. Each bar represents the number of bacteria which have attached to the substratum since the previous time point indicated on the abscissa. Dark shaded bars represent fluorescent cells; light shaded bars represent nonfluorescent cells.

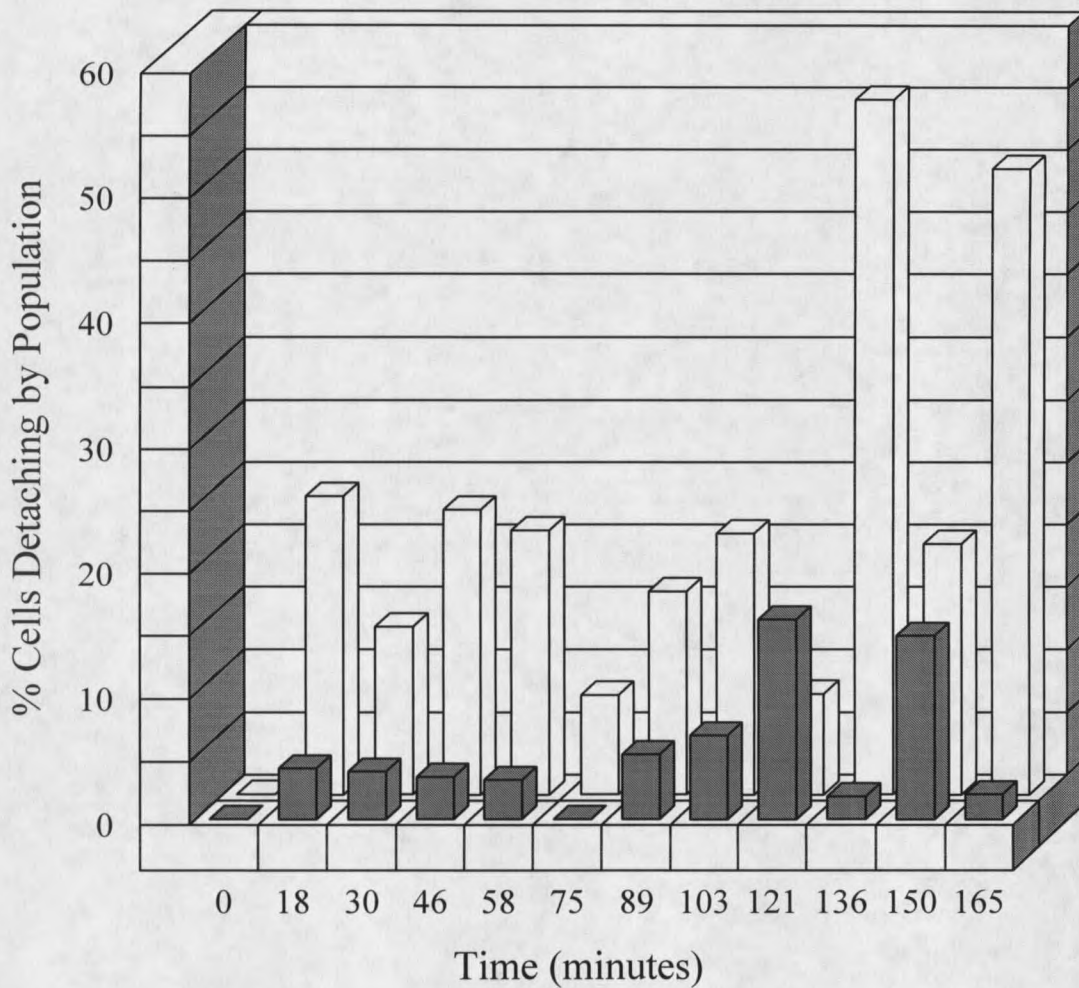


Figure 3.8. Bacteria detaching from the inner surface of the glass coverslip in a flow cell culture chamber. Each bar represents the percentage of attached fluorescent or nonfluorescent bacteria that have detached from the substratum and entered the bulk liquid since the previous time point indicated on the abscissa. Dark shaded bars represent fluorescent cells; light shaded bars represent nonfluorescent cells.

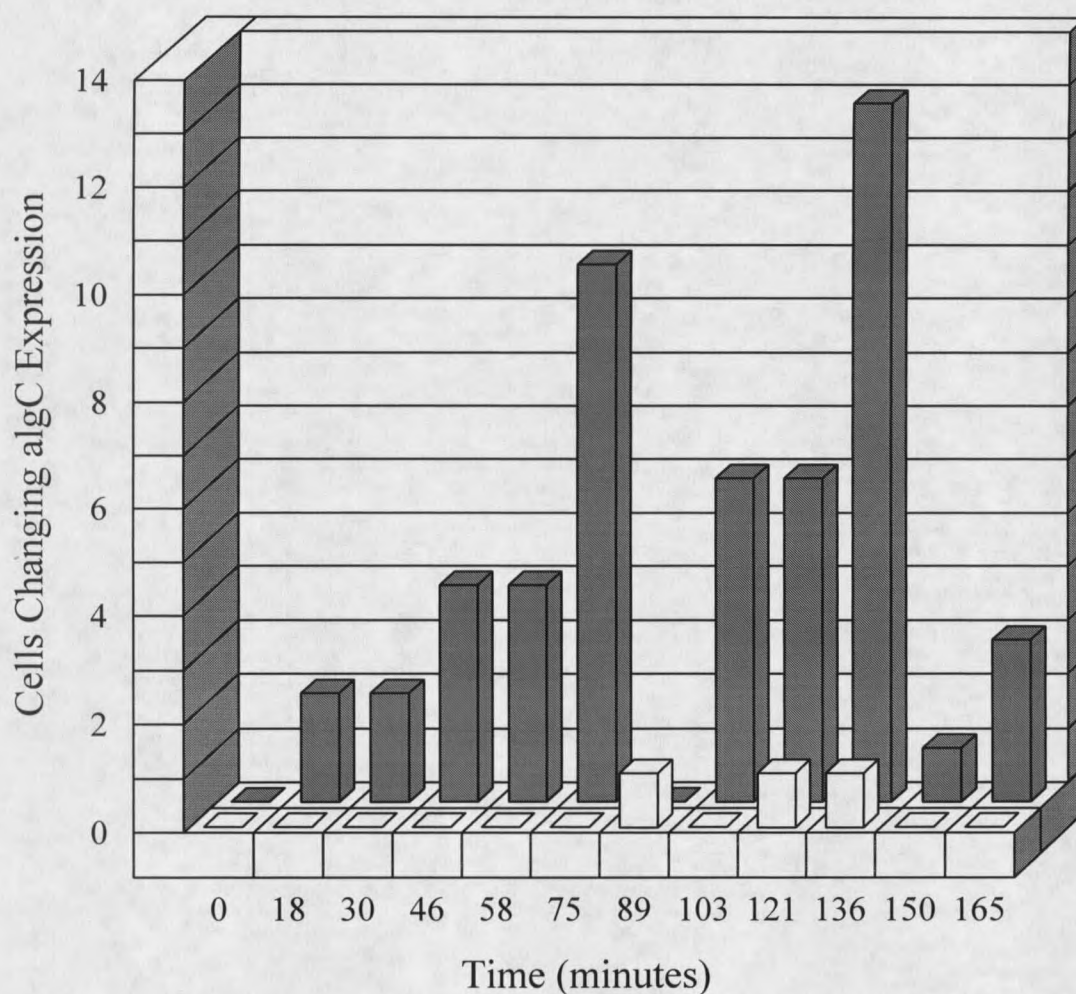


Figure 3.9. Change in regulation of *algC* activity by attached bacteria. Dark shaded bars represent attached nonfluorescent cells that have become fluorescent following attachment to the substratum. Light shaded bars represent attached fluorescent cells that have become nonfluorescent following attachment to the substratum.

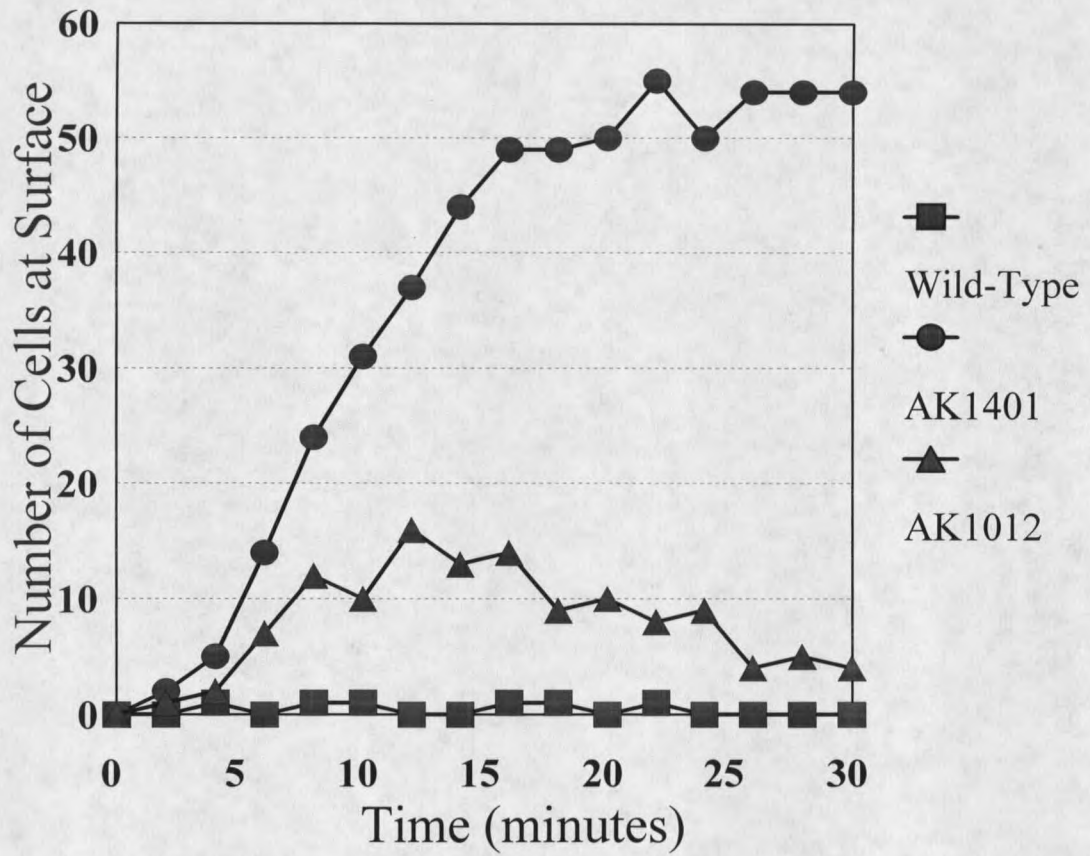


Figure 3.10. Attachment of *Pseudomonas aeruginosa* PAO1 LPS mutants to a glass surface.

Discussion

In the current study, we have found that the transcriptional fusion plasmid pNZ63 which carries the reporter construct for *algC* promoter activity was not lost from *P. aeruginosa* 8830 during growth in batch and continuous culture in the absence of antibiotic pressure. This result was important, because it provided assurance that differences in expression of the plasmid borne reporter would not be due to the influence of changing copy number in the biofilm population. Differences in antibiotic resistance which were observed between bacteria recovered on antibiotic containing medium and non-selective media (Figure 3.2) could all be accounted for by injury detected by replica plating. It may be argued that all or part of the plasmid had integrated into the host chromosome during the course of our experiments. If this were the case, the integration would have to occur throughout the population and in four separate experiments. We feel this to be an unlikely possibility. Our studies have shown, however, that if integration had occurred, both the antibiotic resistance marker and the *algC-lacZ* transcriptional fusion remained intact and functional. Differences in β -lactamase activity were observed between the biofilm and planktonic cell populations. The physiological basis of these differences is not currently known.

Initial experiments with *P. aeruginosa* 8830 were performed to investigate the influence of fluid velocity on attachment. It was found that no significant attachment occurred when the flow rate was $143 \mu\text{m sec}^{-1}$. It is postulated that this was because the fluid shear was too great to be overcome by the attachment mechanism of these bacteria

to the glass surface. Escher (1986) demonstrated that there is an exponential influence of shear stress on *Pseudomonas aeruginosa* attaching to a stainless steel surface, with cellular accumulation decreasing with increasing shear.

At present, the initial attachment mechanism to glass is unknown. Previous observations have indicated that flagella may play a role in initial attachment. Korber (1989) observed that *Pseudomonas fluorescens* which lacked flagella had a recolonization rate on a surface that was four times lower than the same organism with flagella. Mueller (1990) observed a similar trend for *P. fluorescens*, with a two fold increase in attachment rates for flagellated organisms on stainless steel, glass and polycarbonate. In the present work, it has been observed that when *P. aeruginosa* 8830 interacts with a glass surface, it appears to initiate contact with the surface via its polar flagella. Observations discussed in Chapter 3, also implicate LPS as a factor involved in initial attachment.

When *P. aeruginosa* 8830 was allowed to attach to the glass substratum in the absence of flow, the initial colonization was higher than was observed under any of the flowing conditions. Attachment under these conditions was only transient, with almost no bacteria left at the surface after 150 minutes. That maximum attachment occurred under conditions of no flow is probably best explained by the lack of shear to remove bacteria once they have interacted with the glass surface. To explain why almost all of the attached bacteria eventually left the surface, is more difficult. With no flow in the reactor, fluid shear can not have been responsible for detaching the bacteria. Changes in the chemistry of the fluid environment due to stagnant conditions, and/or changes in bacterial physiology most probably accounted for detachment under conditions of no

flow. In the next chapter, the mechanisms of detachment of these bacteria will be discussed in greater depth.

Under intermediate flow conditions, ($20.0 \mu\text{m sec}^{-1}$ and $30.3 \mu\text{m sec}^{-1}$) bacteria attached to the glass surface and remained attached throughout the observation period. It is presumed that these intermediate flow rates are low enough to not shear the bacteria from the surface, while being high enough to prevent changes in the flow cell environment that are caused by stagnation and result in detachment. The flow rate used during all continuous culture experiments described in this work, was 0.13 ml sec^{-1} , which represents a flow velocity of $30.3 \mu\text{m sec}^{-1}$ within the flow cell. This flow rate had previously been chosen empirically, because it appeared to result in the greatest amount of biofilm formation within the flow cell.

Using a continuous culture apparatus, we demonstrated that *algC* reporter gene product specific activity in biofilm bacteria was more than 19 times higher than in planktonic bacteria. This result was consistent with previous studies in our lab where we demonstrated higher levels of *algC* expression in biofilm bacteria grown in batch culture when compared with planktonic cells in batch culture (Davies *et al.*, 1993). Uronic acids accumulation was also higher in the continuous culture biofilm when compared with planktonic bacteria, although the difference was only slightly more than two fold between the two populations. The discrepancy between *algC* reporter activity and uronic acids accumulation may reflect a difference in the end product resulting from phosphomannomutase activity. According to Goldberg, *et al.* (1993) *P. aeruginosa* strain PAO1 requires the enzyme encoded by *algC* for the synthesis of lipopolysaccharide (LPS)

O-side chains. The up-expression of *algC* may therefore reflect either the production of LPS or alginate. Since mannose residues within LPS would not be detected by the uronic acids assay performed in this study, LPS production may explain the difference observed between *algC* up-expression and uronic acids accumulation. Other factors would possibly affect the relationship between the level of reporter gene expression and accumulation of the end product of *algC* transcription. Uronic acids accumulation and *lacZ* (reporter) enzyme activity do not necessarily have to vary proportionally to one another. The differences observed between *algC* activity and uronic acids accumulation may result from differences in rates of formation and activity of those enzymes required for processing of mannose-1-phosphate into the end product, alginate. Furthermore, exopolymers such as alginate may be shed into the bulk liquid by the biofilm growing in continuous culture, thus, reducing the net accumulation of uronic acids within the biofilm.

The population of bacteria shed from the biofilm into the effluent of the continuous culture vessel was shown to have a β -galactosidase specific activity that was intermediate between the biofilm population and the planktonic cell population. This result indicates that as bacteria are shed from the biofilm, the *algC* gene is down-regulated. The level of down regulation is expected to continue until the level observed for planktonic bacteria is achieved. It should be noted that it is also possible that bacteria shed from the biofilm into the effluent were a subpopulation of biofilm cells which had a lower level of *algC* activity than the average of the total biofilm population. Such a population may be more likely to detach from the biofilm due to diminished levels of

alginate biosynthesis as was observed during our microscopic analysis of *algC* activity.

In this study we have introduced a novel approach to detect and record *in situ* gene activity within single bacterial cells attached to a surface. With this approach, we are, to the best of our knowledge, the first to report *in vivo* regulation of a specific bacterial gene at the level of the single cell. Results from these studies indicated that the glass substratum promoted up-expression of *algC* in these bacteria. As biofilms developed, *algC* expression was correlated with the ability of bacteria to remain at the glass substratum. Furthermore, *algC* up-expression was not shown to be a prerequisite to surface attachment, indicating that alginate biosynthesis is not necessary for attachment to the glass surface. However, down-expression of *algC* appears to be associated with subsequent detachment of the bacteria from the surface.

The microscopic analyses of *algC* expression showed that substratum-associated cells can lose fluorescence after a period of time (usually not less than 30 min) although the β -galactoside substrate is not limiting. Furthermore, some of these cells that had lost fluorescence were shown to regain fluorescence after some period of time. These observations indicated that loss of fluorescence was not due to irreversible inactivation of the cells. While a change in permeability to the substrate may account for the above phenomenon, it is more likely due to a loss of β -galactosidase activity and, hence reduced expression of the *algC* gene within the cells. Such a conclusion is consistent with the time frame involved in the switch from a fluorescent cell, to a nonfluorescent one. Furthermore, we observed that when cells lost their fluorescence, they did so entirely. If

the shut-down of a transport mechanism were responsible, some leakiness would be expected, resulting in diminished fluorescence.

The mechanism of the regulation reported above is not known at present. It is possible that activation of *algC* transcription is affected by a member of the homoserine lactone family of autoinducers. The *P. aeruginosa* gene products *lasI* and *LasR* are known to be activators of extracellular virulence factors regulating the production of proteases and exotoxins (Fuqua *et al.*, 1994; Passador *et al.*, 1993; Pearson *et al.*, 1994). These compounds are involved in bacterial quorum sensing and are known to cause induction of gene expression only in situations of relatively high population density. While alginate is an extracellular virulence factor of *P. aeruginosa*, we do not find evidence for quorum sensing in the microscopic analyses we have performed with this organism. Those bacteria which were up-expressed for *algC* activity at the surface of the glass coverslip during our experiments were often found juxtaposed to bacteria which showed no detectable *algC* activity. Furthermore, activation of *algC* when observed at the single cell level was never observed to occur as a group or population phenomenon.

Sensory transduction involving some surface characteristic is possibly responsible for the up-expression of the gene shortly after interaction with the substratum. If this is the case, both glass and Teflon surfaces (or the conditioning films that adhere to those surfaces in liquid medium) promote similar outcomes with respect to regulation. Activation of genes in bacteria following association with different surfaces has been described by a number of authors in recent years. Dagostino, *et al.* (1991) reported activation of an unknown gene in *Pseudomonas* sp. strain S9 on polystyrene, Belas, *et al.*

(1986) demonstrated activation of the *laf* gene in *Vibrio parahaemolyticus* when grown on agar medium, Vandevivere and Kirchman, (1993) demonstrated surface activation of exopolysaccharide biosynthesis by a subsurface bacterium, and in work described in chapter 2, the activation of the *algC* gene in *P. aeruginosa* after attachment to Teflon and glass has been reported (Davies *et al.*, 1993).

The *algC* gene has been shown to have bifunctional activity, being involved in the biosynthesis of both alginate and lipopolysaccharide (Ye *et al.*, 1994). The production of the LPS O-side chains in *P. aeruginosa* PAO1 have been shown to require PMM activity (Goldberg *et al.* 1993). This requirement is also true for *P. aeruginosa* 8830 (Ye *et al.*, 1994). The regulation of *algC*, therefore, affects more than just alginate biosynthesis. We examined two strains of *P. aeruginosa* PAO1 which were mutants for *algC* activity. In the first, AK1401 there is no O antigen. The second, AK1012 is deficient in the O-side chain, which can be complemented back with the addition of PMM, the *algC* gene product, derived from *P. aeruginosa* 8830 (Ye *et al.*, 1994; Currie *et al.*, 1995).

Following attachment assays with these bacteria, it was found that the wild type PAO1 did not attach well to the glass surface, while both of the LPS mutants did attach well. This result indicated that *algC* gene product activity, when shunted to LPS biosynthesis results in diminished ability to attach to a glass surface. In experiments performed with *P. aeruginosa* 8830, initial attachment to glass is very poor. However, once *algC* transcription is shown to be up regulated, the bacteria have been shown to be better able to remain at the glass surface. It is possible, under these conditions, that the activity of the *algC* gene product (PMM/PGM) is dedicated to the synthesis of alginate.

As new LPS is produced in cells growing at the glass surface, a shunting of AlgC to alginate would result in O antigen deficient LPS. This in turn could contribute to the observed increase in ability to remain attached to the glass coverslip. Previous research in our lab (chapter 2) has shown that alginate biosynthesis is activated when *P. aeruginosa* 8830 attaches either to glass or Teflon. The up-expression of *algC* is, therefore, believed to be the result of the need for PMM in alginate biosynthesis and not LPS.

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CHAPTER 4

REGULATION OF THE ALGINATE BIOSYNTHETIC
GENE *algD* IN *Pseudomonas aeruginosa* DURING BIOFILM
DEVELOPMENT IN CONTINUOUS CULTURE

Introduction

In the previous study, the behavior of one of the principal genes in the alginate biosynthesis pathway of *P. aeruginosa* 8830 was examined at the population level as well as at the single cell level (Davies and Geesey, 1995). The gene *algC*, encoding the enzyme phosphomannomutase (PMM), was investigated because it is essential for biosynthesis of alginate and is a key point of regulation in the alginate pathway, possessing its own promoter. The gene product of *algC* has been shown to be a bifunctional enzyme, having phosphomannomutase activity, converting mannose 6-phosphate to mannose 1-phosphate, as well as phosphoglucomutase activity, interconverting glucose 6-phosphate and glucose 1-phosphate (Ye *et al.*, 1994). In its role as phosphomannomutase, the *algC* gene product is responsible for the formation of alginate and also, possibly lipopolysaccharide O-antigen subunits (see Chapter 3). In its role as phosphoglucomutase, the *algC* gene product is responsible for the production of a complete LPS core (Coyne *et al.*, 1994). Due to its role as a bifunctional enzyme, *algC* might be transcribed without producing alginate as a final product, although, this has not been experimentally established.

It has been shown that A-band LPS contributes to inflammation in *Pseudomonas* infections, and it is believed to be a factor contributing to persistence in cystic fibrosis infections (Currie *et al.*, 1995). Since AlgC is required for A-band LPS synthesis, and cystic fibrosis lung infections are known to involve alginate producing *P. aeruginosa*, it may be possible that *algC* transcriptional activity can lead to both alginate and LPS at the same time (refer to Chapter 3).

In order to isolate alginate biosynthesis from LPS biosynthesis, it was necessary to follow the activity of a gene whose product is used only in the synthesis of alginate. The first gene in the alginate biosynthesis operon that is dedicated to alginate production is *algD* which encodes the enzyme GDP-mannose dehydrogenase. Furthermore, it has been shown that whenever *algD* transcription occurs in *P. aeruginosa* 8821 or 8830, alginate production also takes place (Deretic *et al.*, 1987a; Deretic *et al.*, 1987b; DeVault *et al.*, 1990; Wozniak, 1994).

GDP-mannose dehydrogenase catalyzes the oxidation of GDP-mannose into GDP-mannuronic acid, a direct precursor of alginate (Deretic *et al.*, 1987c). The *algD* promoter has become a benchmark for monitoring molecular events which govern expression of the alginate system (Mohr *et al.*, 1992). This is because transcription of *algD* is the first committed step in the biosynthesis of alginate, resulting in a functional enzyme which catalyzes a four electron-transfer in an apparently irreversible step (Roychoudhury *et al.*, 1989). The *algD* gene has been shown to be transcriptionally activated by the DNA binding protein AlgR1 (DeVault *et al.*, 1989). The AlgR1 protein has been shown to possess significant amino acid homology to a class of proteins

responsive to environmental stimuli (Deretic *et al.*, 1989), and has been shown to be functionally interchangeable with the OmpR DNA binding protein of *E. coli* which can be used to activate *algD* transcription in *E. coli* in response to medium osmolarity (Berry *et al.*, 1989).

In tests to determine whether *algD* is itself an environmentally responsive gene, Berry *et al.* (1989), and DeVault *et al.* (1990), investigated the influence of medium osmolarity and ethanol on alginate biosynthesis. These researchers showed that when either salt or ethanol was added to growing cultures of *Pseudomonas aeruginosa* wild type 8821 or mucoid 8830, *algD* transcription and alginate biosynthesis were significantly increased as a result of enhanced *algD* promoter activity in liquid medium and increased alginate production after prolonged exposure on solid medium.

The sequence of the *algD* gene and its promoter have been determined by Deretic *et al.* (1987c). The promoter has a novel secondary structure, possessing multiple direct and inverted repeats throughout the -50 to -110 region, as well as in the -35 and -10 regions (Berry *et al.*, 1989; Mohr *et al.*, 1992).

In the present study, the activity of the *algD* promoter in *P. aeruginosa* 8830 was examined during axenic biofilm development in continuous culture. Using the flow cell construct described in Chapter 3, initial events and subsequent cell cluster development were followed over time, and *in situ* activity of the *algD* gene was monitored. The results of these experiments are then compared with those obtained from previous work on the *algC* promoter.

Materials and Methods

Bacteria and media

The bacteria used in this study were *P. aeruginosa* 8830 (Darzins and Chakrabarty, 1984), *E. coli* JM109 (Messing *et al.*, 1981), and *E. coli* pRK2013 (Figurski and Helinski, 1979).

All batch and continuous culture experiments were conducted using a defined culture medium (modified EPRI) containing: sodium lactate (0.05 g/l), sodium succinate (0.05 g/l), ammonium nitrate (0.381 g/l), KH_2PO_4 (0.19 g/l), K_2HPO_4 (0.63 g/l), Hutner salts (Cohen-Bazire *et al.*, 1957) (0.01 g/l), dextrose (1.0 g/l), and L-histidine (0.01 g/l). Solid YTG medium was used for the enumeration of bacteria recovered from batch and continuous culture experiments. When grown on this medium, *P. aeruginosa* 8830 produces mucoid colonies which show up-expressed alginate biosynthetic activity. YTG medium contained the following components in grams per liter: yeast extract, 5.0; tryptone, 10.0; glucose, 2.0; Bacto agar, 1.5. YTG medium amended with carbenicillin was prepared with 350 mg/l of the filter-sterilized antibiotic added to the medium following autoclave sterilization. YTG medium amended with 5-bromo-4-chloro-3-indolyl β -D-galactoside (X-gal) was prepared by adding 0.04 g/l of the β -galactoside in N,N-dimethyl formamide. Phosphate buffer was prepared by adding 7.0 gram K_2HPO_4 and 3.0 gram KH_2PO_4 to one liter of ultra pure water, pH, 7.0.

Triparental Matings

In order to produce a *P. aeruginosa* 8830 strain containing a reporter gene construct for *algD*, it was necessary to first develop an *algD/lacZ* transcriptional fusion plasmid, and then to mate that plasmid into the recipient strain. Construction of the plasmid was carried out in the laboratory of Dr. A. M. Chakrabarty by Dr. Rick W. Ye. The plasmid was produced in an *E. coli* JM109 vector and was shown to possess β -galactosidase by development of blue colonies on X-gal plates and by ONPG hydrolysis by cell extracts.

Mating was carried out using a donor, helper and recipient for the new plasmid construct using a modification of the method described by Figurski and Helinski (1979). The donor strain *E. coli* JM109 carried the *algD/lacZ* transcriptional fusion plasmid designated pDAD6. The plasmid contains a constitutive marker for carbenicillin resistance (350 mg/l), and a marker for ampicillin resistance (100 mg/l). The helper strain, *E. coli* pRK2013 carries a plasmid containing a set of RP4 transfer genes along with kanamycin resistance (50 mg/l).

The Donor and helper strains were grown on R2A medium containing 100 mg/l ampicillin and 50 mg/l kanamycin respectively. Selective culture was carried out on these strains in order to ensure that the bacteria used during the matings all contained at least one plasmid per cell. The *P. aeruginosa* 8830 recipient strain was grown on PIA medium without antibiotic. All cultures were transferred twice using the T-streak for isolation in order to ensure culture purity and restreaked to form a confluent lawn.

Following growth for 24 hours at 30° C, each plate was scraped using an inoculating loop and the bacteria were transferred to separate presterilized centrifuge tubes containing 20 ml sterile phosphate buffered water (PBS), K_2HPO_4 , 7.0 g/l; KH_2PO_4 , 3.0 g/l. Each strain was washed twice in PBS by centrifugation at 10,000 x G for 10 minutes. This step was performed in order to remove any antibiotic that may have been transferred from the culture plates when removing the bacteria. The Three strains were combined in one test tube, vortexed, spun down in the centrifuge at 10,000 x G for 10 min and resuspended in 1.0 ml PBS. The bacteria were subsequently planted as seven 0.02 ml drops onto Three plates containing R2A medium. R2A medium was used because it has been observed that *P. aeruginosa* 8830 will not produce mucoid colonies on this medium. Previous work in our lab had shown that when grown on YTG medium as mucoid colonies, mating efficiency was extremely poor. These plates were then placed into a 30°C incubator during which time mating was allowed to occur. After 24 hours, all colonies formed on the plates were picked and diluted in PBS using 5 x 10 fold serial dilutions. Aliquots of 0.1 ml were next plated on selective PIA medium containing 350 mg/l carbenicillin and 43 mg/l X-gal. Following overnight growth, all colonies which showed development of blue color were replated on identical selective medium and grown to check for blue colonies and mucoidy. These bacteria were also checked on R2A medium to check to ensure a decrease in both alginate development and X-gal conversion. Stocks of these organisms were prepared and stored at -70°C for future experimental work. Activity of the *algD* promoter was checked using different media and growth conditions, to ensure proper behavior of the plasmid construct.

β -galactosidase specific activity was monitored in test tubes containing either EPRI medium amended with carbenicillin (350 mg/l) and glucose (2.0 g/l) or YTG broth. Cells were lysed and β -galactosidase was assayed using ONPG as a substrate. Stock cultures of these bacteria were prepared using 2% glycerol and 20% peptone in DI water. These were frozen at -70°C and were used for all subsequent experiments for *algD* activity.

β -Galactosidase assay

The β -galactosidase assay was performed on thawed samples with a Milton Roy Spectronic 601 spectrophotometer following the procedure of Miller, (1972) as described previously (Davies *et al.*, 1993). β -Galactosidase specific activity is reported as nanomoles of ONP produced per minute per milligram of protein at 25°C, pH 7.0. For a full description of the β -galactosidase assay, see Chapter 2.

Lowry protein assay

The Lowry protein assay was performed on thawed samples as described previously (Davies *et al.*, 1993) and analyzed with a Milton Roy Spectronic 601 spectrophotometer. For a full description of the lowry protein assay, refer to Chapter 2.

Uronic acid assay

Total uronic acids were measured in thawed samples of scraped biofilm and whole culture following the method of Kintner and Van Buren, (1982) using a Milton Roy Spectronic 601 spectrophotometer. For a full description of uronic acid analysis, refer to Chapter 2.

Microscopic analysis of *algD* activity in continuous culture

In Chapter 2, we reported the use of a fluorogenic galactoside which when hydrolyzed by the pNZ63 reporter gene product (β -galactosidase) can be detected by epifluorescence microscopy within individual cells of *P. aeruginosa* (Davies *et al.*, 1993). This fluorogenic compound was used in the present study as the substrate for reporter enzyme activity to evaluate *algD* expression in individual bacteria in continuous culture.

A continuous-culture apparatus was developed to monitor *in situ* reporter gene expression of individual bacteria during initial stages of biofilm development (Figure 3.1). The apparatus was configured as a once through flow cell system. The influent defined culture medium supplemented with 350 mg/l carbenicillin was retained in a four liter glass reservoir. Methylumbelliferyl β -D-galactopyranoside dissolved in 5.0 ml N,N-dimethyl formamide was added to the defined culture medium to a final concentration of 0.01 g/l. Medium from the influent reservoir was pumped through silicone tubing via a Masterflex pump to an aeration flask sparged with filtered air. The aerated medium was pumped to a flat plate flow cell using a Masterflex 8 roller-head peristaltic pump at a flow rate of 0.13 ml min⁻¹. The flow cell was constructed of polycarbonate having a depth of 0.1 mm, a width of 1.4 cm, and a length of 4.0 cm, the upper face was capped with a glass coverslip. The glass coverslip was used as a substratum for bacterial attachment and biofilm development because it is a relatively inert material, is transparent, and has been shown to result in *algC* activity similar to what has been observed for Teflon mesh (unpublished data). Flow through the cell was laminar, having a Reynolds number of 0.17, with a fluid residence time of 0.43 minutes. The flow cell was sealed to prevent

contamination and affixed to the stage of an Olympus BH2 microscope containing a BH2-RFL fluorescent attachment. Medium leaving the flow cell was pumped to an effluent reservoir via silicone tubing. The entire system was closed to the outside environment but maintained in equilibrium with atmospheric pressure by a 0.2 μm pore size gas permeable filter fitted to each flask.

Log phase *P. aeruginosa* were inoculated through a septum approximately 1 cm upstream from the flow cell while flow was maintained. Bacteria were allowed to attach to and grow on the surfaces of the system downstream from the site of inoculation over a period of 24 h. Flow through the system was then increased to remove any bacteria attached to the inside surface of the coverslip (as determined by microscopy). Bacteria shed from biofilm upstream from the flow cell were then allowed to recolonize the surfaces of the flow cell under conditions of normal flow. After 2 h, cells attached to the inner surface of the glass coverslip were viewed by transmitted light using phase contrast at 1025 x magnification to detect total cells. The same field was then observed under UV excitation using a UG1 blue Olympus filter block to detect the conversion of the methylumbelliferyl substrate. Fluorescent and phase contrast images were recorded using an American Innovisions image analysis system. Each image was taken using an integration of 50 scans in order to increase the amount of detectable fluorescence and to collect data on only those bacteria which were attached to the coverslip. Planktonic bacteria flowing beneath the coverslip were not detectable during the integration period due to their motion. Information on number and location of attached bacteria within a

single field was collected at approximately 15 minute intervals for a period of 150 and 345 minutes. All images were stored as separate files for subsequent retrieval and analysis.

Analysis of microscopic images

Stored digital images of bacteria were retrieved and displayed on a computer screen. Clear plastic overlays placed on the screen were used to mark the time and position of cells attached to the inner surface of the glass coverslip. Three distinct subpopulations of bacteria were identified: 1) bacteria that were already attached to the coverslip in the previous observation period, 2) bacteria that had attached to the coverslip since the previous observation period, 3) bacteria that had detached from the coverslip since the previous observation period. Bacteria within each of the Three subpopulations were recorded as being fluorescent or nonfluorescent indicating whether they manifested *algD* up-expression. Results are reported from a representative experiment run for a period of 345 minutes. Five different aspects of biofilm formation are reported: 1) Activity of the *algD* promoter for total bacteria attached to the glass slide at each observation time; 2) activity of the *algD* promoter for bacteria attaching to the glass slide at each time point; 3) activity of the *algD* promoter for bacteria detaching from the glass slide at each time point; 4) change in activity of the *algD* promoter for bacteria attached to the glass slide at each time point; 5) activity of the *algD* promoter for bacteria which remain attached to the glass slide from one observation period to the next.

Results are also reported from a separate experiment of 150 minutes duration. In this experiment, individual bacteria were followed over the course of the experiment. The activity of the *algD* promoter is reported for each bacterium from the time of attachment to the glass slide until it detached from the substratum or until the end of the experiment, whichever happened first. Thus the relationship of alginate production and the fate of individual bacteria can be tracked over the course of the entire experiment.

Results

Triparental matings

Triparental mating was used to transfer the *algD/lacZ* plasmid, designated pDAD6, from *E. coli* JM109 to *P. aeruginosa* 8830. Following successful mating, bacteria were screened for activity and transferred Three times on selective media. These bacteria were mucoid on YTG solid medium, and were able to grow in the presence of 350 mg/l carbenicillin. Measurements of β -galactosidase activity demonstrated the presence of a functional *lacZ* gene and gene product. Uronic acids production in YTG broth and the formation of mucoid colonies on YTG medium indicated that alginate biosynthetic activity was also functional. No uronic acid production and no β -galactosidase activity were detected when these bacteria were grown in EPRI medium.

Microscopic analysis of *algD* activity in continuous culture

The activity of the *algD* promoter was evaluated during initial events of biofilm development by *P. aeruginosa* 8830 using a reporter construct, pDAD6 containing an

algD/lacZ transcriptional fusion (see above). Using a once through flow cell reactor system, gene expression was monitored in bacteria which had detached from an upstream biofilm and subsequently reattached to a glass coverslip where they could be observed by fluorescence and transmitted light microscopy. The fluorogenic galactoside used in these experiments was methylumbelliferyl β -D-galactopyranoside.

Using this system (for more information, refer to Chapter 3), it was possible to discern Three distinct populations of bacteria which could be followed as a group or individually throughout the course of the experiments. These were: 1) bacteria viewed at one observation period that were already attached to the coverslip in the previous observation period, 2) bacteria viewed at one observation period that had attached to the coverslip since the previous observation period, and, 3) bacteria viewed at one observation period that had detached from the coverslip since the previous observation period. For each of these Three groups, it is possible to observe bacteria that are fluorescent in the presence of methylumbelliferyl β -D-galactoside, representing those that have detectable and thus enhanced *algD* promoter activity (i.e., alginate biosynthetic activity), and those that are not fluorescent, representing bacteria that maintain only a basal level of *algD* promoter activity. From this information, it is possible to determine 5 populations of bacteria in these experiments. These are: 1) total bacteria at the surface, 2) bacteria attaching to the surface, 3) bacteria detaching from the surface, 4) bacteria that change their *algD* regulation while they are attached to the surface, and 5) bacteria that are able to remain attached to the surface for more than one observation period. Each of

these populations contained subpopulations which indicated the presence or absence of *algD* reporter gene expression.

Figure 4.1 shows the total bacteria attached to the surface of the glass coverslip recorded at roughly 15 minutes intervals. During the course of this experiment, the number of bacteria expressing *algD* transcriptional activity as a function of the total number of bacteria attached to the surface remains relatively constant at about 2/3. There is no evident trend toward increase or decrease in *algD* activity with the progression of time. The total number of bacteria at the surface (both active and non-active for *algD* transcription) increases only marginally from the beginning of the experiment to the end. The bulk of increase in bacteria (from approximately 300 to a maximum of approximately 375) is made up of nonfluorescent individuals. At 165 minutes, there was a sloughing event which resulted in the removal of over 62% of the total population of bacteria at the glass surface. Interestingly, of the remaining bacteria left at the surface, none displayed detectable fluorescence. This was not because all of the fluorescent bacteria had left the surface. In point of fact, essentially all of the bacteria that remained following the sloughing event had been fluorescent prior to that event. Unfortunately, it was impossible to track the bacteria individually during this period. This was due to the movement in the direction of flow by all the bacteria (attached cells included) during the event that followed the 135 minutes observation. No bacteria were observed to attach during the sloughing period. Movement by the surface associated cells may have been the result of motility, or possibly, the result of fluid shear acting on the bacteria following a disruption of their attachment mechanism(s). It was, therefore, impossible to relocate the previous

positions of all 123 remaining bacteria at the 165 minutes time point. At 345 minutes, the observation area is shown to be in the process of recolonization. The increase in fluorescent bacteria at that time point is due almost entirely to bacteria that had remained attached at the 165 minutes observation point, indicating that these bacteria were starting to renew production of *algD* transcripts following the sloughing event.

Figure 4.2 shows the number of bacteria that exhibit enhanced *algD* activity upon attachment to the glass substratum. At all time points, the bacteria that attach to the glass coverslip are predominantly nonfluorescent, indicating that they are not actively producing alginate above basal constitutive levels. The implication of such an observation is that *algD* transcriptional activity is not necessary for attachment to a glass substratum. While it is very difficult to determine quantitatively, the bacteria in the bulk liquid have a far lower level of *algD* activity than is found at the surface. Estimates of around 10 to 20 percent of the total bacteria in the bulk liquid are observed to be fluorescent. When bacteria are grown in bulk liquid in EPRI medium, no *algD* activity is detectable when ONPG is used as a substrate. Of the total bacteria that attached to the glass slide in the experiment (shown in Figure 4.2), only 11% were shown to be fluorescent, while 89% did not display fluorescence. In a separate experiment, run for 150 minutes, only 5% of the bacteria that attached to the glass substratum were shown to be fluorescent, while, 95% did not display *algD* activity.

Figure 4.3 shows the number of bacteria that detach from the glass slide and enter the bulk liquid. Throughout the experiment, the majority of bacteria that detached from the glass substratum did not demonstrate *algD* transcriptional activity. Overall, only 14%

of the bacteria that detached from the surface had detectable fluorescence, while 86% of the bacteria which detached were nonfluorescent. In a separate experiment, 16% of the bacteria that detached over a period of 150 minutes were fluorescent, while 84% were not. These observations indicated that *algD* transcriptional activity is correlated with the ability to remain attached to the glass surface. It may be inferred that alginate biosynthesis helps prevent detachment from the surface of a glass substratum.

Figure 4.4 depicts the number of bacteria that are able to remain attached the surface of the glass substratum, neither detaching nor attaching since the previous observation period. This figure demonstrates that the number of fluorescent bacteria that are able to remain attached varied from 85% to 95% of the total number at the surface during the previous time period. Of those bacteria that were not fluorescent, those that were able to remain attached varied from a low of 30% to a high of 95%. The average percent of fluorescent bacteria that remained at the surface for a minimum of two observation periods was 92.5%, while the average number of nonfluorescent bacteria that remained at the surface for a minimum of two observation periods was only 64.9%. These results indicate that fluorescent is correlated with the ability of individual bacteria to remain attached to the surface of the glass substratum for a minimum of two observation periods. It should be noted, however, that transcriptional activity at the *algD* promoter was not a requirement for maintaining attachment at the glass surface. The lack of observable *algD* activity does not indicate that no alginate biosynthesis is occurring, or that alginate has not been produced by an organism in the past.

Results discussed in Chapter 3, demonstrated that *algC* transcriptional activity could change while a bacterium was attached to the glass substratum. This change in regulation was also examined for transcription of the *algD* operon. Figure 4.5 shows the change in regulation of the *algD* promoter for attached bacteria over the course of the 135 minutes experiment described above. Over the course of the experiment, 56% of the bacteria that changed regulation, went from "off" to "on" for detectable fluorescence (*algD* transcription), while 44% were observed to change from "on" to "off". In a separate experiment, 80% of the attached bacteria were observed to switch from "off" to "on", with only 20% changing from "on" to "off". These results indicated that when bacteria are at a surface, there is a greater chance that they will up-regulate rather than down regulate alginate biosynthetic activity, but the proportion fluctuates from one experiment to another.

In an attempt to more closely examine the behavior of individual cells at a surface during initial events in biofilm development, single bacteria were tracked over the course of a 150 minutes flow cell experiment. As in previous experiments, the medium flow rate was run at wash-out to ensure that bacterial growth did not occur in the bulk phase in the system, and that the physiology of bacteria in the bulk liquid was of cells that had previously been attached to and subsequently detached from system surfaces. Each bacterium that attached during the course of 150 minutes was followed until it detached or until the end of the experiment. The activity of the *algD* promoter was determined by detection of fluorescence (as above). Table 4.1 shows the results of this experiment. Individual bacteria are denoted by numbers across the top row of the table. Observation

time points are listed in the first column. A total of 40 bacteria were followed during the course of the experiment (this number represents the total number of bacteria in the observation field). Unlike the previously described experiments, no bacteria were attached to the glass slide 15 minutes prior to time 0. Boxes marked (+), denote a bacterium that is fluorescent and, therefore, up-expressed for *algD* transcription. Boxes marked (-) denote a bacterium that is not fluorescent and, therefore is not up-expressed for *algD* transcription. At the time of attachment, only 7 out of 40 bacteria were fluorescent. Of the total number of bacteria that attached (40), 25 did not remain attached for more than 15 minutes. These bacteria came to the surface, attached only transiently and reentered the bulk liquid. While at the surface, most of these bacteria were observed to change position slightly, indicating that they were not firmly fixed at the substratum. Of those bacteria that remained attached for 45 minutes or longer, 9 out of 11 showed *algD* activity. Of those same 11 bacteria, none were observed to leave the surface unless they became down-regulated for *algD* promoter activity. This same phenomenon was observed in the previous experiment (run for 165 minutes) where the majority of bacteria that detached from the surface following prolonged attachment (at least 1 hour) were observed first to down regulate *algD*. Those bacteria that remained at the surface for more than 45 minutes did not change position on the surface, except in the case of bacteria that eventually detached from the surface. In such cases, a position change was detected only immediately prior to detachment. Again, this phenomenon was observed during the previous experiment. When bacteria remained at the surface for one hour or longer (11 individuals), all but three remained at the surface until the end of the

experiment. Only two bacteria were observed to leave the surface after a minimum attachment period of 1 hour 15 minutes, and no bacteria that were attached for 1 hour 30 minutes or longer were observed to detach from the surface. The observations based on the behavior of a limited number of individual cells indicates that the longer an organism remains attached to the surface of the coverslip during the experiment, the greater the probability that organism will not detach for the remainder of the experiment.

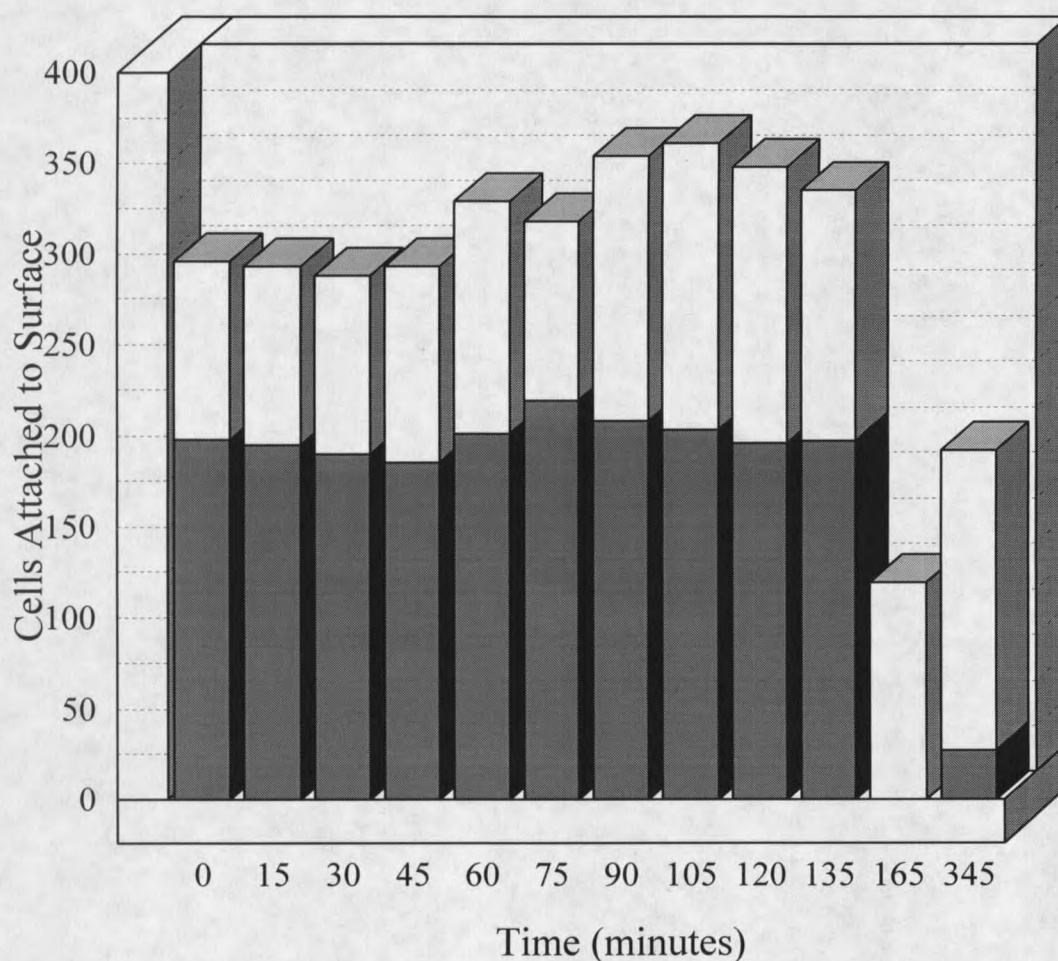


Figure 4.1. *P. aeruginosa* 8830 attached to inner surface of glass coverslip in flow-through reactor. Abscissa represents time points at which bacteria were observed during 345 min of colonization from the bulk phase. The time point at 165 min indicates a sloughing event. Dark shaded bars represent fluorescent bacteria, indicating *algD* transcriptional activity. Light bars represent nonfluorescent bacteria, indicating no detectable *algD* activity.

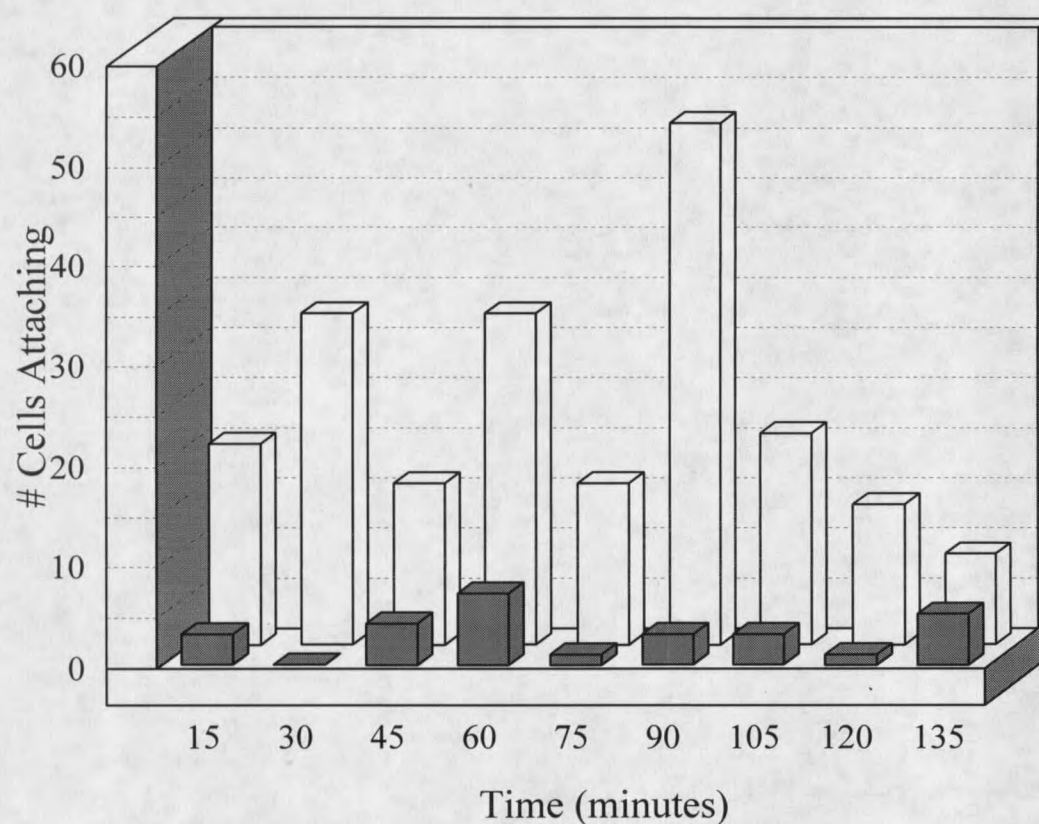


Figure 4.2. Attachment of bacteria to inner surface of glass coverslip in flow cell reactor. Each bar represents the number of bacteria which have attached to the substratum since the previous time point on the abscissa. Dark shaded bars represent fluorescent bacteria, indicating cells having detectable *algD* transcriptional activity. Light bars represent nonfluorescent bacteria, indicating cells not having detectable *algD* transcriptional activity.

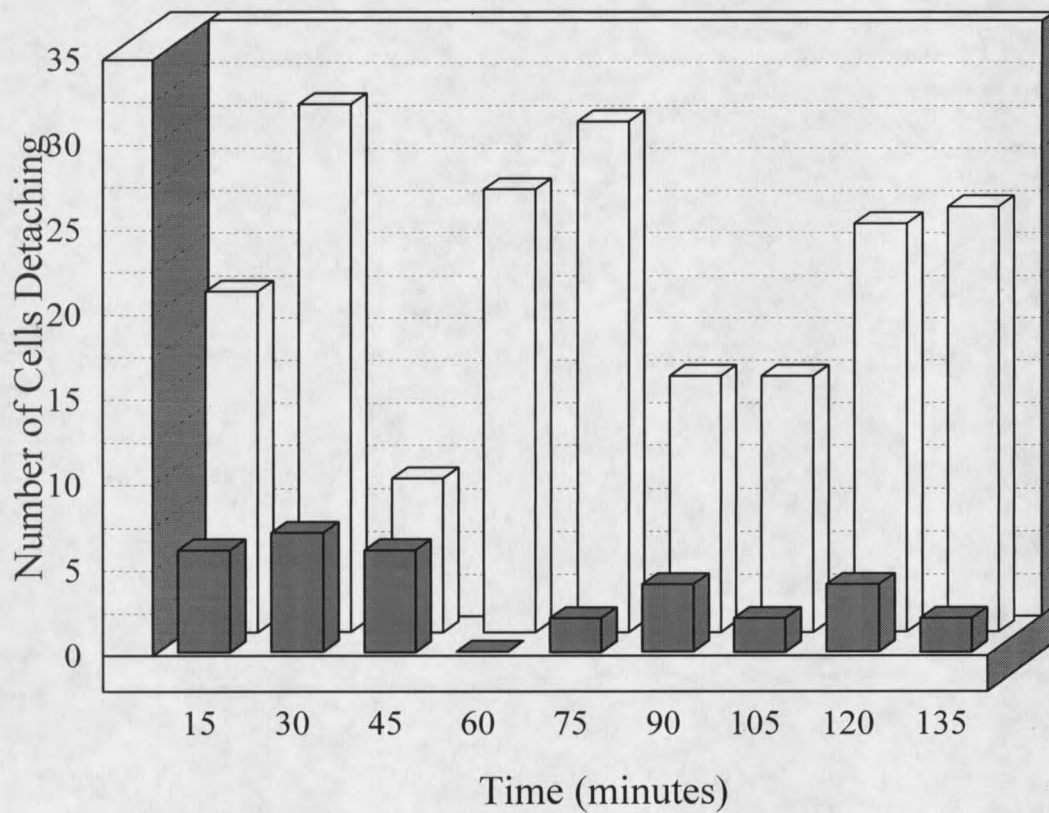


Figure 4.3. Bacteria detaching from inner surface of glass coverslip in flow cell reactor. Each bar represents the number of cells from the previous observation period that have detached from the substratum and entered the bulk liquid prior to the time point indicated on the abscissa. Dark shaded bars represent fluorescent bacteria, indicating *algD* transcriptional activity. Light bars represent nonfluorescent bacteria, indicating no detectable *algD* transcriptional activity.

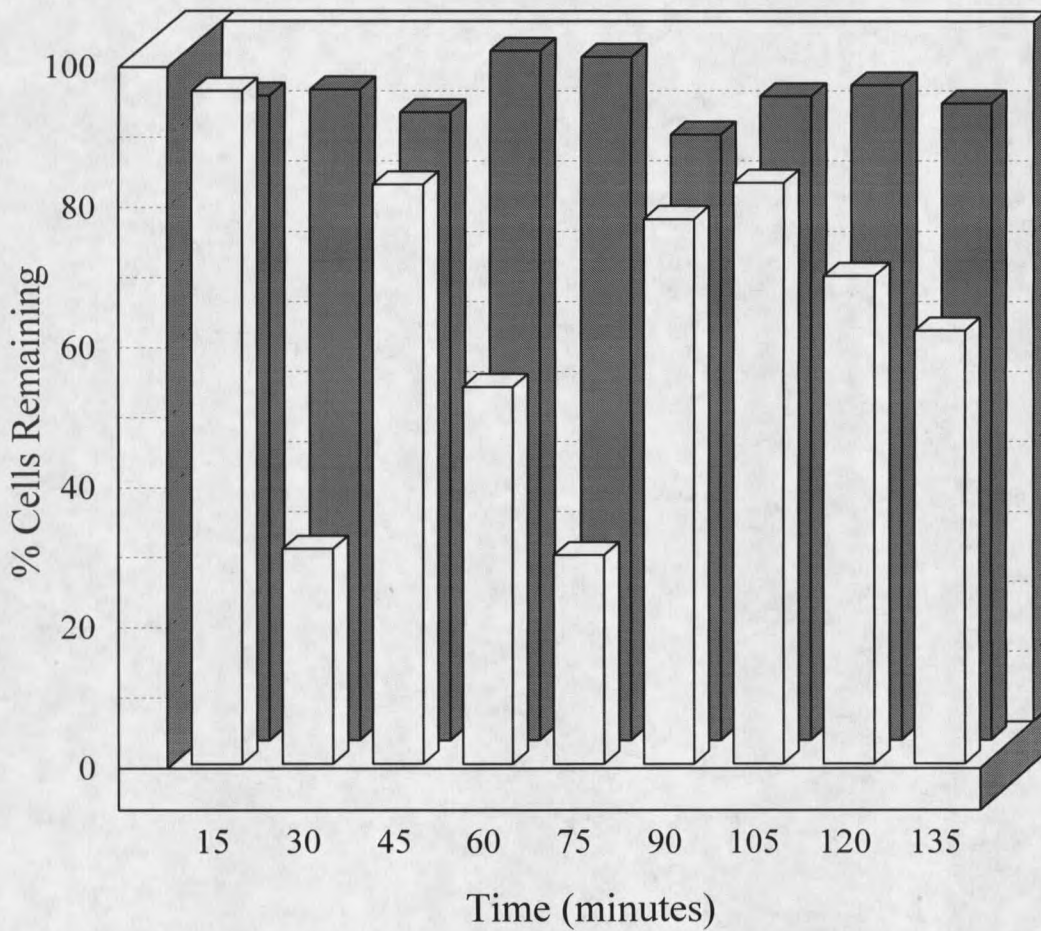


Figure 4.4. Bacteria remaining attached to the inner surface of glass coverslip in flow cell reactor. Each bar represents the number of bacteria attached to the coverslip which have remained at the same location since the previous observation time indicated on the abscissa. Dark shaded bars represent fluorescent bacteria, indicating cells that have detectable *algD* transcriptional activity. Light bars represent nonfluorescent bacteria, indicating cells that have no detectable *algD* activity.

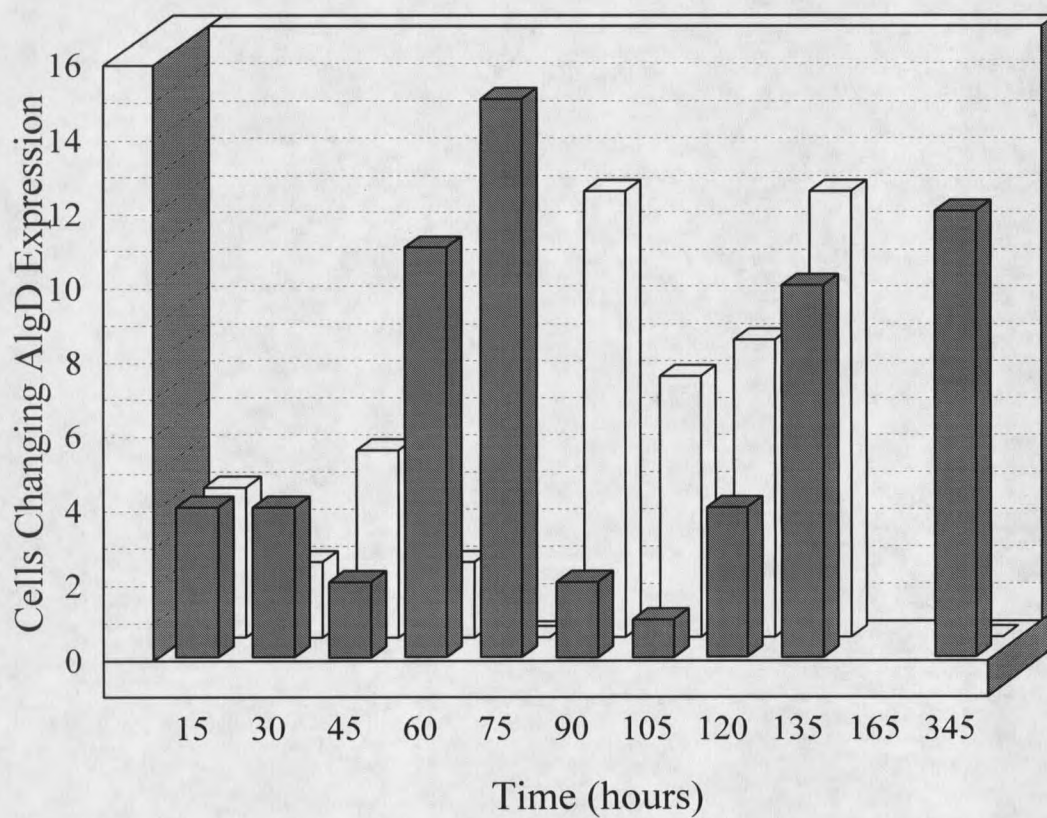


Figure 4.5. Change in regulation of *algD* transcriptional activity by attached bacteria. Dark shaded bars represent attached nonfluorescent bacteria that have become fluorescent following attachment to the substratum. Light bars represent fluorescent bacteria that have become nonfluorescent following attachment to the substratum.

Table 4.1. History of *P. aeruginosa* 8830 attached to a glass substratum grown in EPRI medium in continuous culture. Boxes containing a plus sign represent individual attached bacteria expressing *algD* transcriptional activity. Boxes containing a dash represent individual attached bacteria not expressing *algD* transcriptional activity.

T/x	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
0	-	-	+	+	-	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-
15	-	+	+		+	+	+		+	+			+							
30	-	+			+		+		+	+			+							
45	-	+			-		+		+	+			+							
60	-	-					+		+	+			+							
75							+		+	+			+							
90							+		+	+			+							
105							+		+	+			+							
120							+		+	+			-							
135							+		+	+			-							
150							+		+	+			-							

T/x	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
0																				
15	-	+	-																	
30		-		-	-	-	-	-	-											
45										-										
60																				
75																				
90											-	-								
105											-	-	+	-	-	-	-			
120											-	-	+	+				-		
135											-	-	+	+				-		
150											-	+	+	+				-	-	-

x = bacterial identification number.

Discussion

In the present study, activity of the *algD* promoter was followed in continuous culture biofilms of *P. aeruginosa* 8830. Construction of an *algD/lacZ* fusion plasmid was performed in the laboratory of Dr. Ananda Chakrabarty. Following successful mating of this plasmid into *P. aeruginosa* 8830, the activity of alginate biosynthesis could be followed using the flow cell system described in Chapter 3. The *algD/lacZ* plasmid (pDAD6) and the *algC/lacZ* plasmid (pNZ63) were both constructed from a pKRZ-1 plasmid, a broad host-range promoterless *lacZ* structural vector based on the pSA origin of replication (Zeilinski *et al.* 1991; Sà-Correia *et al.* 1987). These two plasmids are, therefore, related and can be assumed to act similarly in the *P. aeruginosa* 8830 background, with the exception of differences in the *algC* and *algD* promoter regions. Nevertheless, no strict comparisons of *algD* specific activity and *algC* specific activity will be made in this report. No important differences were noted in the behavior in continuous culture of *P. aeruginosa* 8830 carrying these different plasmids.

In experiments performed to examine the regulation of the *algD* promoter on biofilm development, it was observed that the total number of bacteria at the glass surface remained essentially steady and that there was not a relative increase in the number of bacteria that were up-regulated for *algD* transcription as was observed under similar conditions for the *algC* gene. This difference between *algD* and *algC* might indicate a difference in regulation between these two operons. As the population of bacteria in the *algC* experiment continued at the surface, many of the down regulated bacteria "switched

on *algC* transcription. This was also the case with *algD*. In the first experiment described, 56% of the bacteria that changed regulation of *algD*, did so by turning on alginate biosynthesis. In another experiment, 80% of the bacteria that changed regulation did so by turning on alginate biosynthesis. The trend toward up-regulating a gene following attachment is, therefore, duplicated in the case of *algD*. However, the percent of bacteria that change regulation following attachment to a surface differs. With *algC*, almost all of the bacteria on a surface have been shown to be up-regulated for PMM/PGM biosynthesis. Up-expression of *algD* is observed for only approximately 60% of the bacteria in the experiment depicted in figure 4.2, and this number actually decreases as the experiment progresses. The trend is misleading, however, because the majority of nonfluorescent bacteria at the surface are cells that have attached for a period of only 15 minutes or less. Such an example can be seen with the bacteria represented in Table 4.1. Here, when the bacteria that are resident on the surface for less than 30 minutes are subtracted from the total, the behavior of *algD* begins to look more like what has been observed previously for *algC*. With the background removed, those bacteria in Figure 4.1 that have been attached for more than one observation period would account for over 90% of the total population. Of these bacteria, almost all (between 90% and 100%) were positive for *algD* up-expression.

Differences between the *algC* promoter and the *algD* promoter are found primarily in the fact that a higher percentage of bacteria were observed to turn *algC* transcription on after they have attached to the surface of the glass coverslip. The number of bacteria that up-regulated *algC* transcription while attached to the surface of the flow

cell coverslip as a percent of the total number of bacteria to attach to the surface over the course of the experiment was 57%. The number of bacteria that up-expressed *algD* transcription as a percent of the total bacteria that attached to the surface was only 31%. It may be presumed that there is a preference for attachment of bacteria that are up-regulated for alginate biosynthesis. This is not believed to be the case, since a strain not able to produce alginate (*P. aeruginosa* 8822) has been shown to colonize the glass surface at a higher rate than the mucoid strain *P. aeruginosa* 8830 (observations made previously in our laboratory). The behavior of the *algD* promoter is, therefore, similar to what was observed for the *algC* promoter. Both promoters showed a trend to become up-regulated following attachment to the flow cell glass surface. Both promoters were active in the majority of the attached populations and, when attaching or detaching, both promoters tend to be down expressed. In Chapter 2, it was demonstrated that attachment to either glass or Teflon resulted in up-expression of *algC* as well as an increase in the production of alginate. The data here and in Chapter 3, derived from the microscopic investigations of both *algC* and *algD* further support the observation that alginate biosynthesis is up-regulated following attachment to a surface.

The results of the present work examining the activity of the *algD* promoter demonstrate a pattern of regulation of alginate biosynthesis in *P. aeruginosa* 8830 during early biofilm development in continuous culture. Using novel techniques combining reporter gene technology with *in situ* measurements of the behavior of individual cells, it has been possible to construct a working model describing exopolymer regulation during biofilm formation, through development on the surface and ultimately, detachment from

the substratum. The results of such an investigation are only valid for one particular organism under study and the system within which behavior of that organism was observed. The results of such work do, however, provided us with a working model of how regulation of biofilm matrix polymer may progress during biofilm formation in a flowing system.

The working model indicated by the results described in this chapter is one in which the bacterium *P. aeruginosa* 8830 goes through a life cycle involving both biofilm and free-living modes of existence.. Initially, bacteria that have detached from a surface enter the bulk liquid. When in the bulk liquid the majority of cells are not up-regulated for the production of alginate. If these bacteria subsequently interact with a (glass) substratum, they may attach rather tenuously to that surface. The majority of bacteria that do so, are washed off the surface and continue to be carried downstream. Of those bacteria that are not washed downstream, most ultimately up-regulate *algD* and begin to produce alginate. This production of alginate is continued as long as the bacteria remain at the surface. If and when there is a change in the environment, the bacteria respond by down-regulating *algD* and ultimately detach from the surface and are carried further downstream where they may repeat this cycle.

It is important to note that the organism whose behavior is described in these studies is a clinical isolate that has been chemically mutagenized to overproduce alginate. The behavior of strains of *Pseudomonas aeruginosa* isolated from other environments may be different. Unfortunately, alginate production in environmental isolates of *P. aeruginosa* is not generally detectable (May *et al.*, 1991). Furthermore, the number of

genetic constructs and mutants of such bacteria do not compare with what is available of *P. aeruginosa* 8821 and its derivatives. It is for these reasons that *P. aeruginosa* 8830 has remained the focus of this study.

In nature, biofilms are generally composed of multiple species, unless they are found in extreme environments. Furthermore, there is little information on the variety of matrix polymers produced in nature by bacterial assemblages, or on the prevalence of alginate as a matrix polymer. One notable exception is found in work performed by Wallace *et al.* (1994), in which 11 out of 120 bacterial isolates from biofilms growing in a nuclear power plant cooling water system were shown to have alginate biosynthesis genes.

It is hoped that in the future, more such studies will be performed to examine the distribution of exopolysaccharides in the environment, and that studies of the regulation of matrix polymer material in bacteria will extend to include environmental isolates in both pure and mixed cultures.

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CHAPTER 5

CHARACTERIZATION OF THE EFFECTS OF ALGINATE LYASE

ON *Pseudomonas aeruginosa* BIOFILMSIntroduction

The control and removal of biofilm material from pipe and conduit surfaces has historically been carried out by the addition of corrosive chemicals such as chlorine or strong alkali solutions or through mechanical means. Such treatments are generally harsh to both the plumbing system and the environment, and have been necessary due to the recalcitrant nature of biofilms within those systems. The resistance to treatment by biocides has been due in large measure to the protective character of intact biofilm matrix polymers (Srinivasan *et al.*, 1995; Stewart, 1994; Tashiro *et al.*, 1991). In medicine, the use of elevated doses of antibiotics has been necessary in treatment when biofilms are believed to be involved. This is due at least in part to the enhanced protection of biofilm bacteria by the exopolysaccharide matrix material outside the cells (Costerton *et al.*, 1989; Nichols *et al.*, 1989; Anwar *et al.*, 1989).

The control of biofilm development and persistence could be enhanced if the matrix polymers which protect biofilms could be degraded. The availability of a matrix degrading enzyme is therefore of considerable interest. Genetic studies of *P. aeruginosa* have shown that the *algD* operon encodes a gene for alginate lyase, an enzyme that is capable of cleaving the linkage between the uronic acid moieties which make up the alginate polymer. This enzyme is, therefore, capable of breaking down the alginate polymer into small subunits.

It has been shown by Boyd *et al.* 1995, that when alginate lyase is induced artificially in these bacteria when grown on agar based medium, the bacteria growing on the surface of that medium are more easily removed by gentle rinsing when compared with uninduced bacteria. It is believed that cleavage of the chemical bonds between the uronic acid residues of the alginate polymers results in a breakdown of the alginate matrix allowing dispersal of the bacteria.

With the indication that alginate lyase may be involved in breaking down alginate polymer matrices, it became of interest to investigate the possibility that the enzyme may be involved in the regulation of biofilm stability as a key factor in causing sloughing of biological films. If alginate lyase could be implicated in mediating sloughing of biofilms, then the possibility of either using lyase induced in bacteria within biofilms or through the artificial addition of lyase to biofilms it should be possible to control biofilm development in industry and in medical situations.

Alginate is produced by *Pseudomonas aeruginosa* through a complex pathway beginning with glucose (Figure 1.3). The finished product is a long polymer with a molecular weight averaging 1.8×10^6 Daltons which is composed of approximately 95% mannuronic acid residues (Abrahamson *et al.* 1995). During the production of alginate, polymerization and export from the cell membrane are also required. The location of epimerization, acetylation and polymerization is believed to be the periplasmic space within the bacterial cell envelope (May *et al.*, 1991).

In addition to the production of alginate, at least one of the genes in the pathway (*algC*) is involved in the synthesis of lipopolysaccharide (LPS). This gene has been shown to be expressed when *P. aeruginosa* attaches to a glass or Teflon surface (Davies *et al.* 1993). It is postulated

that changes in the structure of LPS may occur as an initial phase of biofilm development, and that LPS participates in the attachment process. This may be followed by the production of the alginate matrix polymer which acts as a structural support for the development of the biofilm away from the substratum.

A map of the genes known to be involved in the synthesis of alginate is shown in Figure 1.4. These genes are located on five separate segments of the bacterial chromosome. Each segment is an individual operon controlled by its own promoter which regulates transcription. When the promoter of any of these operons is activated, all of its downstream genes are transcribed.

Each of the five alginate operons contains genes having functional relatedness. The *algD* group contains genes which are dedicated to the synthesis of alginate, the *algC* segment contains a single structural gene whose product is involved in both alginate and LPS synthesis, and the remainder contain regulatory genes which control transcription of the *algD* and *algC* operons (Berry *et al.*, 1988; Chitnis and Ohman, 1990; May *et al.*, 1991).

It is interesting that the gene, *algL* which encodes the information for production of alginate lyase, is nested within the *algD* gene cluster (Boyd *et al.*, 1993; Schiller *et al.*, 1993). This would suggest that *algL* is transcribed along with the biosynthesis genes required for alginate production. Such positioning is unexpected, since the activity of alginate lyase is known to result in the cleavage of the chemical linkage between uronic acid residues in alginate polymers (Boyd *et al.* 1995). Such activity should lead to the complete or partial destruction of synthesized alginate polymer strands. The activity of alginate lyase has been characterized as an endolytic enzyme which cleaves alginate polymer strands by the β -elimination of the 4-O-

glycosidic bond to yield two shorter saccharide polymers (Gacesa, 1987). An unsaturated sugar residue (4-deoxy-L-erythro-hex-4-ene-pyranosyluronate) is created on the non-reducing end of the 5' product at the site of cleavage (Preiss and Ashwell, 1962a). Continued enzymatic activity results in the conversion of alginate to 2-keto-3-deoxy-D-gluconate (Preiss and Ashwell, 1962b).

It has been postulated that alginate lyase is used by the bacterium as a processing enzyme, necessary for production of alginate polymers in their final form prior to export from the cell envelope (Boyd and Chakrabarty, 1995). There is, however, no evidence of lyase activity during alginate synthesis (Boyd *et al.* 1993). It has been shown previously and in this study, that alginate can be produced and biofilm formation can occur in bacteria from which alginate lyase can be recovered and detected. It is postulated, therefore, that the lyase produced by these bacteria is somehow prevented from acting on alginate polymers outside of the cell. This could be achieved by regulating the transport of alginate lyase across the cell envelope or by processing of the enzyme after it is produced. When isolated from whole cell extracts, lyase has been shown to contain a leader sequence at the N-terminus (Boyd *et al.* 1995). This leader may prevent transport of the enzyme outside the cell envelope and/or it may affect the ability of the enzyme to act upon the substrate.

The above information suggests that alginate lyase is produced while alginate is being synthesized but, through some form of regulation, is prevented from interfering with polymerization of the acid polysaccharide. Previous work in our laboratory has shown that *P. aeruginosa* biofilms undergo unpredictable sloughing events with large sections of biofilm becoming detached from either the matrix or the substratum. It is believed that these events may

be triggered by the release of active alginate lyase into the external milieu where alginate strands are broken apart releasing fragments of biofilm from the overall matrix.

The current understanding of the activity and regulation of alginate lyase indicates that it may play an important role in mediating detachment or sloughing in *P. aeruginosa* biofilms. In the present effort, we endeavored to further examine the role of this important enzyme in biofilm regulation and to determine whether continued research in this area might lead to practical applications in biofilm control.

In the present investigation, strains of *P. aeruginosa* were constructed which contained plasmids with the structural gene for alginate lyase under the control of an inducible *tac* promoter. These bacteria could then be grown in a biofilm and subsequently induced by the addition of IPTG to stimulate lyase production. Under such conditions lyase would accumulate within the cells in the biofilm and after a period of time should leak through the cell envelope and enter the outside environment.

Materials and Methods

Bacterial strains and media

The bacterium used in this study was *Pseudomonas aeruginosa* 8830, a *his*⁻, stable mucoid strain derived from a bacterium isolated from the lung of a cystic fibrosis (CF) patient. This bacterium has been shown to produce alginate in large quantities compared to environmental isolates and to be capable of up and down regulation of alginate synthesis. During the study, strain 8830 was engineered to contain plasmids which encoded either a *tac/lacZ* or a *tac/algL* gene sequence. Both plasmids contained a constitutive resistance marker for

carbenicillin, used for plasmid selection and retention. The *tac/lacZ* construct could be induced using isopropyl- β -D-thiogalactopyranoside (IPTG) to produce the enzyme β -D-galactosidase and the *tac/algL* construct could be induced by IPTG to produce alginate lyase.

All continuous culture biofilm experiments were performed using EPRI medium containing the following constituents per liter: sodium lactate (0.05 g), sodium succinate (0.05 g), ammonium nitrate (3.81 g), KH_2PO_4 (0.19 g), K_2HPO_4 (0.63 g), Hutner salts (0.01 g), glucose (1.0 g), and L-histidine (0.01 g) (Davies *et al.*, 1993). Solid YTG medium was used for the enumeration of bacteria recovered from batch and continuous culture experiments and to check plasmid retention. YTG medium was used for the enumeration of bacteria recovered from batch and continuous cultures and to perform measurements of lyase specific activity and lyase induction studies. YTG medium contained the following components per liter: yeast extract, (5.0 g); tryptone, (10.0 g); and glucose, (2.0 g). Solid YTG medium was amended with 15.0 g/l Bacto Agar. Both continuous culture and batch culture media were amended with 350 $\mu\text{g/ml}$ carbenicillin where indicated.

β -Galactosidase assay

The β -galactosidase assay was performed on thawed samples with a Milton Roy Spectronic 601 spectrophotometer following the procedure of Miller, (1972) as described previously (Davies *et al.*, 1993). β -Galactosidase specific activity is reported as nanomoles of ONP produced per minute per milligram of protein at 25°C, pH 7.0. For a full description of the β -galactosidase assay, see Chapter 2.

Lowry protein assay

The Lowry protein assay was performed on thawed samples as described previously (Davies *et al.*, 1993) and analyzed with a Milton Roy Spectronic 601 spectrophotometer. For a full description of the lowry protein assay, refer to Chapter 2.

Alginate lyase activity measurements

Alginate lyase activity was determined by measuring the increase in absorbance at 232 nm of unsaturated oligosaccharide products generated by enzymatic breakdown of the polymer (Boyd and Turvey, 1977). Alginate used in this assay was obtained from *Macrocystis pyrifera*, and is non-acetylated (high viscosity, Sigma). The standard reaction contained 1.0 g/l alginate, 50 mM Piperazine-N,N'-bis[2-ethanesulfonic acid] (PIPES, pH 7.0) and 400 mM NaCl and was carried out at 20°C. Enzyme specific activity is expressed as change in optical density per gram of alginate per minute at 20°C, pH = 7.0.

Gel Electrophoresis

A 5% nondenaturing polyacrylamide gel was used to examine alginate degradation. The gel consisted of 0.4 M Tris-HCl (pH 8.8) and 5% acrylamide-bisacrylamide (Bucke, 1974; Buckmire, 1984). Each sample contained approximately 20 µl of alginate in 25 µl of 1.5 M Tris-HCl (pH 8.8), and 5 µl of 0.05% bromophenol blue. The gel was run in a buffer of 0.025 M Tris (pH 9.0) and 0.19 M glycine at 15 mAmp for the first 40 minutes and then at 25 mAmp until the dye reached the bottom of the gel. The alginate was visualized by being stained with 0.08% toluidine blue-O in 7% glacial acetic acid for 45 minutes and then destained with water. The gel presented in this section was prepared by A. Boyd.

Continuous culture studies

A continuous-culture apparatus was used to monitor *in situ* biofilm development along with reporter gene expression for alginate synthesis and sloughing of bacteria (Figure 3.1). The apparatus was configured as a once-through flow cell system. The influent defined culture medium supplemented with carbenicillin was retained in a 4-liter glass reservoir. Methylumbelliferyl- β -D-galactopyranoside dissolved in 5.0 ml of N,N-dimethyl formamide was added to the defined culture medium to a final concentration of 0.01g/l. Medium from the influent reservoir was pumped through silicone tubing via a Masterflex pump to an aeration flask sparged with filtered air. The aerated medium was pumped to a flat plate flow cell by using a Masterflex 8 roller-head peristaltic pump at a flow rate of 0.13 ml min^{-1} .

The flow cell was constructed of polycarbonate and was 0.1 mm deep, 1.4 cm wide, and 4.0 cm long; the upper face was capped with a glass coverslip. The glass coverslip was precleaned with detergent, acetone, ethanol and ultrapure water prior to insertion into the flow cell. The flow cell was sealed to prevent contamination and affixed to the stage of an Olympus BH2 microscope containing a BH2-RFL fluorescent attachment. Medium leaving the flow cell was pumped to an effluent reservoir via silicone tubing. Samples for uronic acid and alginate lyase analyses were taken from the effluent line while chilling the collection vials on ice.

Log phase *P. aeruginosa* cells were inoculated through a septum approximately 10 cm upstream from the flow cell while flow was maintained. Bacteria were allowed to attach to and grow on the surfaces of the system downstream from the site of inoculation. Growth on the inner surface of the glass coverslip was allowed to continue until cell clusters developed to a thickness of at least 20 μm . Clusters were viewed by transmitted light using phase contrast at 1,250 x

magnification. The same field was then observed under UV excitation with a UG1 blue Olympus filter block to detect the conversion of the methylumbeliferyl substrate, which indicated *algD* activity and, therefore, active alginate synthesis within the cluster. Stored digital images of cell clusters were generated using a CCD color camera and software by American Innovisions, version 150.

Investigation of biofilm architecture of IPTG induced and non-induced cell clusters

Cell clusters were grown in the flow-cell under a variety of conditions to investigate the qualitative effects of alginate lyase biosynthesis on biofilm formation and development. Cells were also examined for their ability to produce alginate. The first biofilms examined were developed using *P. aeruginosa* strain 8830 carrying *algD/lacZ*. These bacteria did not produce elevated levels of alginate lyase and *algD* activity was followed to determine when alginate biosynthetic activity took place. The second biofilms were developed using vector control bacteria carrying *tac/lacZ*. Such bacteria induced with IPTG would produce β -galactosidase, but not elevated levels of alginate lyase. These bacteria were used as an IPTG control. The third group of biofilms was developed using the test organism carrying the *tac/algL* construct, along with the *algD/lacZ* construct. This strain would produce elevated levels of alginate lyase following addition of IPTG, and would report on the activity of alginate biosynthesis.

Induction with IPTG was carried out by adding the chemical at a final concentration of 10 mM to the mixing flask upstream from the flow-cell. IPTG was added to biofilm cultures for a period of three hours, and cell clusters were observed at a minimum of thirty sites both before

and after induction. From the time of IPTG entry into the flow-cell, observations were made at one minute intervals for three hours, followed by observations at 6 hr intervals for 48 hours

No flow experiment

It had been noted previously in our laboratory, that the suspension of flow in our flow-cell reactors would result in sloughing of biofilm material after a period of several hours to a few days. In order to more closely examine this phenomenon, *P. aeruginosa* were grown in the flow-cell reactor for a period of several days until clusters formed varying in thickness from 20 - 30 μm . Flow was then turned off for a period of 72-73 hours, the cell clusters were examined for changes in architecture at 1 minutes intervals and flow was resumed to investigate any changes that might be influenced by shear stress.

Salt and SDS addition to IPTG induced and non-induced cell clusters

Both NaCl and sodium dodecyl sulfate (SDS) were used to increase the permeability of cell membranes of IPTG induced and non-induced bacteria within the flow-cell. NaCl at 0.3 M and SDS at 0.2% were added to 10 ml of medium in the inoculation port after biofilm clusters of greater than 20 μm had developed. Induced bacteria were allowed to interact with 10 mM IPTG for a period of three hours prior to addition of salt or SDS to the mixing flask. Following addition of salt or SDS, flow was maintained until these amendments reached the flow-cell. Flow was turned off for a period of 30 minutes with observations of cell clusters made at 1 minutes intervals. Flow was then reinitiated for a period of 24 hours to investigate any changes that might be influenced by shear stress.

Addition of purified lyase to developed cell clusters

The effects of lyase released from bacteria following *tac* promoter induction of *algL* were compared with the effects of purified lyase added externally to developed cell clusters.

Differences in the consequences of both types of experiment were expected to yield insight into the effects of lyase on the cell cluster matrix polymer. *P. aeruginosa* 8830 pDAD6 was grown in the flow cell in EPRI medium for a period of several days until cell clusters had developed which varied in thickness from 20 - 100 μm . Flow in the reactor was stopped and purified alginate lyase was added at a concentration of 10 mM. Lyase was first examined for its activity in phosphate buffer and subsequently in solution containing 400 mM NaCl. This was done to investigate the influence of salt on lyase activity. It had been noted elsewhere that lyase activity was enhanced in the presence of NaCl (personal communication, A. Boyd). The enzyme in solution was allowed to interact with the cell clusters for a period of several hours. Multiple cell clusters were examined and images of these were stored digitally.

Purified alginate lyase was prepared by A. Boyd, in the Laboratory of A. M. Chakrabarty. The complete procedure for overexpression and purification of the enzyme is described elsewhere (Boyd *et al.*, 1995). Briefly, *P. aeruginosa* 8830 *tac/algL* was grown in LB broth and induced with IPTG. The cells were washed and disrupted by sonication to produce a cell-free extract. The cell-free extract was loaded on a hydroxylapatite column and fractionated. The active fractions were then passed through a Phenyl-Superose column and fractionated a second time. The active fractions were then concentrated by ultrafiltration and applied to a Superdex-75 column and fractionated a third time. Activity of the fractions was determined by alginate lyase activity (see above). Purified alginate lyase was stored in 20% glycerol, 300 mM K-Phos buffer,

250 mM ammonium sulfate, 0.5 mM dithiothreitol. Purity was determined to be from 80% to 85%.

Results

Biofilm development by IPTG induced and non-induced bacteria

P. aeruginosa was grown in a flow-cell reactor to examine the effects of up-expression of alginate lyase on biofilm development. *P. aeruginosa* 8830 carrying a plasmid containing the reporter gene construct *algD/lacZ* was used to investigate the formation of cell clusters by bacteria that were not induced for up-expression of alginate lyase. Cluster formation by these bacteria is shown in Figure 5.1. The cell cluster in the photomicrograph has a thickness of 40 μm and is approximately 100 μm in diameter. The fluorescent image pair indicates those cells which are producing alginate. Figure 5.2, shows a similar cell cluster formed by cells carrying the *tac/algL* construct. These bacteria were exposed to IPTG continuously during growth of the cell cluster. The cell cluster has a diameter of 100 μm with a thickness of approximately 40 μm . The fluorescent image pair in Figure 5.2, indicates that alginate is synthesized by these organisms in the presence of elevated biosynthesis of alginate lyase. The overall structure of cell position, cluster size and shape does not differ qualitatively between the control cell cluster in Figure 5.1, and the cell cluster producing alginate lyase.

Sloughing induced by termination of flow

As a positive control for bacterial sloughing in the flow-cell reactor, *P. aeruginosa* was grown as a biofilm and then allowed to slough under controlled reactor operating conditions. It

had been observed previously in our laboratory that when flow is turned off for an extended period of time, bacteria will slough from a biofilm. We reproduced this by turning off flow in the reactor for a period of 73 hours. Figure 5.3 shows two clusters of bacteria and additional individual cells attached to the glass coverslip of the flow-cell reactor at the time flow was stopped (Figure 5.3, panel A). After 73 hr without flow, the number of bacteria in the cell clusters had been reduced dramatically and many of the bacteria attached to the glass were no longer present, indicating sloughing and detachment (Figure 5.3, panel B). Since no shear could have been responsible for the loss of bacteria (due to lack of flow), it is evident that the cells are capable of active detachment or sloughing. Furthermore, when these bacteria slough/detach as a result of turning off flow, they leave from the cell clusters where they are attached to other cells, as well as directly from the substratum. Flow was reinstated in the reactor to remove any additional bacteria that were not firmly attached to the substratum. After 1 minute following reinitiation of flow, several of the bacteria from Figure 5.3, panel B are seen to have detached, indicating a loose association with the surface which can be overcome with the addition of fluid shear. Flow in the reactor was maintained constant for a period of several hours. Over the first 35 minutes a few of the remaining bacteria were shown to detach from the substratum (Figure 5.3, panel D). After 35 minutes no bacteria were observed to leave the surface for the next 24 hr when the experiment was terminated.

Alginate lyase activity of suspended cell cultures and biofilm populations

The activity of alginate lyase was measured in induced and non-induced strains of *P. aeruginosa* 8830 grown in test tubes. Lyase activity was not detectable in the control strains not

carrying the *tac/algL* plasmid in either the cell fraction or the cell-free medium of suspended cultures grown in test tubes. In bacteria carrying the *tac/lacZ* construct, we were able to detect the leakiness of the *tac* promoter. In these bacteria, the *tac* promoter was shown to be very leaky (ie: transcription through the *tac* promoter in the absence of inducer) and to account for up to 1/3 the activity of induced strains. In other words, the presence of the *tac* promoter on the plasmid accounted for a significant increase in transcription of the plasmid even in the absence of inducer. The activity of alginate lyase was then measured in the strain carrying the *tac/algL* construct. These experiments were carried out in broth culture in both YTG and EPRI medium. Lyase activity in induced strains grown in YTG medium was approximately 2-fold higher than the activity measured for non-induced bacteria in the same medium. No alginate lyase activity was detected for either induced or non-induced bacteria grown in EPRI medium.

Alginate lyase activity measurements were also performed on samples grown as biofilms in the flow-cell reactor. These samples showed a 10 fold increase in alginate lyase present in the cell fraction of biofilm removed from the continuous culture system containing EPRI medium 30 minutes after induction with 10 mM IPTG. No detectable alginate lyase was recovered from the menstruum. These results indicated that IPTG was able to induce cell-associated lyase biosynthetic activity in biofilm bacteria, but not in free-living cells grown in EPRI medium. No enzyme activity was detectable in the cell-free menstruum from the reactor system.

Sloughing in biofilm bacteria induced by IPTG

The ability of bacteria in biofilm cell clusters to slough as a result of induction of alginate lyase was investigated. After several days of growth in the flow-cell reactor, biofilm cell clusters

had formed which were 30-40 μm in thickness. Inducer was then added to the flow-cell for a period of 24 hours. During this time, no sloughing or change in the cell clusters in the reactor was observed (data not shown). These observations demonstrated that alginate lyase induction was insufficient by itself to cause sloughing in biofilm bacteria in the flow-cell reactor.

Salt and SDS addition to IPTG induced and non-induced cell clusters

As has been noted above, the amount of lyase in the cell-free medium following IPTG induction in test tubes was not detectable, whereas, lyase in the cell extract was detectable. An attempt was, thus, made to release the lyase from the cells by changing the osmolarity of the medium or by fluidization of the cytoplasmic membrane. In the first experiment, NaCl was added to the medium to a final concentration of 0.3 M after cell clusters had developed in the reactor. Figure 5.4, panel A shows the study cell cluster prior to salt addition after 2 days growth under continuous induction with IPTG during conditions of continuous flow. After salt addition, the cell cluster began to rapidly break apart, with individual cells and small groups of 10-20 cells leaving the cell cluster. After 5 minutes following salt addition (Figure 5.4, panel B) the majority of bacteria in the cell cluster had sloughed and traveled out of the field of view. Six minutes following the addition of salt much of the remainder of the cells in the cluster had sloughed, however, none of the bacteria at the substratum were shown to detach (Figure 5.4, panel C). Continued sloughing of bacteria after 6 minutes was insubstantial (Figure 5.4, panel D), with a few cells detaching from the surface, however, the rate of detachment was not higher than has been noted in previous experiments where lyase was not induced (data not shown).

A control was run to determine whether salt alone was responsible for the observed sloughing. Biofilm cell clusters were grown in the flow-cell as above and salt was added to the reactor. Several cell clusters were followed over a period of up to 24 hours. In these experiments no sloughing was observed to occur.

An additional set of experiments was run in order to evaluate whether NaCl was responsible for release of lyase from the cells or, whether the observed sloughing resulted from a specific interaction between NaCl and alginate. During these experiments SDS rather than NaCl was used to enhance cell envelope permeability to alginate lyase. The former compound should not interact with the alginate polymers in the same manner as would NaCl.

Initially, a control was run to examine the effect of SDS alone on the integrity of cell clusters in the flow-cell reactor. The experimental setup was identical to that described above for NaCl with no IPTG added. Following the addition of SDS, several cell clusters were followed for a period of 24 hours. During the observation interval, no sloughing or change in the nature of the cell clusters was observed (data not shown). The presence of SDS alone was, therefore, not sufficient to induce sloughing of bacteria.

Next, an experiment was run to examine the effect of SDS on IPTG induced bacteria. Figure 5.5 shows a series of photomicrographs taken of a single cell cluster grown for 3 days in the presence of IPTG. Prior to addition of 0.2% SDS, Figure 5.5, panel A shows the cell cluster to be well developed with a thickness of approximately 40 μm and a diameter of over 100 μm . At the instant SDS was added to the flow-cell (Figure 5.5, panel B) some changes in the cell cluster begin to manifest themselves as a separation of the cells from one another within the cluster. After 5 minutes most of the cell cluster has broken apart with individual cells separated

from one another (Figure 5.5, panel C). This breakdown of the matrix of the cell cluster continued for another minute (Figure 5.5, panel D) until the majority of the cell cluster had dispersed. Following the sloughing event during the initial 7 minutes after addition of SDS, flow was reinstated within the reactor, washing away most of the disintegrated cell cluster (Figure 5.5, panel E). Little additional breakdown of the cell cluster occurred after reinstating flow, as can be seen at 35 minutes (Figure 5.5, panel F).

Addition of purified lyase to developed cell clusters

Sloughing of IPTG induced bacteria in the presence of either salt or SDS indicated that alginate lyase was at least in part responsible for the destruction of cell clusters in the previous experiments. In order to investigate the effect of lyase alone on cell cluster integrity, it was decided to introduce purified lyase via the bulk liquid to developed cell clusters within the reactor. In the first such experiment, alginate lyase purified from IPTG induced *P. aeruginosa* 8830 pABL-1 was added suspended in phosphate buffer to cell clusters that had developed over a period of several days. The cell clusters were monitored over a period of 24 hours and no detachment of cells or observable changes in the cell clusters was noted (data not shown).

Next, lyase in combination with 400 mM NaCl was added to the flow cell after cell cluster development had occurred. During this experiment three cell clusters of different size were monitored since the activity of the enzyme might differ depending on mass transport limitations. The small cell cluster is shown in Figure 5.6. This cluster was 12 μm thick and 50 μm in diameter at the beginning of the experiment. Figure 5.6, panel A, shows the cell cluster before addition of the alginate lyase. After 5 minutes, (Figure 5.6, panel B) the cell cluster has

demonstrated some shrinkage in diameter, but is still 12 μm thick. As the experiment progressed, images were recorded at 30 minutes (Figure 5.6, panel C), 60 minutes (Figure 5.6, panel D), 120 minutes and 20 hours (Figure 5.6 panel E). It is apparent that the type of sloughing/detachment observed when lyase was induced in cell cluster-bacteria using IPTG was not evident when lyase was added externally. With progression of the experiment, it seems that there is no bacterial detachment from the surface, and only minimal loss of cells from the cell cluster. At 20 hours, the cell cluster has, however, been reduced in thickness from 12 μm to 6 μm , and some loss of bacteria is evident.

The progression of alginate lyase activity on a cell cluster of approximately 110 μm diameter and 50 μm thick is depicted in Figure 5.7. The time of each recorded image is the same as in Figure 5.6. As the alginate lyase is allowed to interact with the cluster, very little gross change was observed. It can be noted that bacteria along the margin of the cluster appear to be either eroded from the cluster, or, perhaps some rheological change has taken place within the matrix supporting the cluster. At the end of 20 hours, the cluster was measured to be only 20 μm thick.

A third cell cluster treated with alginate lyase is shown in Figure 5.8. This cluster was 80 μm thick and 200 μm in diameter. Changes occurring within this cell cluster were more evident than had been observed with the two smaller clusters. With progression of the experiment, the cell cluster appeared more and more granular, indicating areas where cells had disappeared from the cluster, leaving pits behind. This is particularly obvious in panel D, and panel E. The most stark difference is the disappearance of the cluster at 20 hours (panel F).

An SDS-PAGE gel was run on samples taken from this and another similar experiment in order to examine the alginate fraction. Activity of alginate lyase on the alginate polymers would result in multiple bands of varying lengths that would run out on the gel as a smear. If the alginate lyase had failed to cleave the alginate in all but one or two locations, then the gel would have a band only at the top. The gel depicted in Figure 5.9 has five lanes to which four samples were added. The first lane was the whole cell fraction scraped from the flow cell reactor after termination of the experiment. It can be seen that a band of alginate can be seen running to the bottom of the gel. This indicates significant cleavage of the polymer. Lane 2, shows the alginate banding pattern derived from induced bacteria in which 0.4 M NaCl was added. Again, there is considerable destruction of the alginate, caused by lyase released from the bacteria. Lane 3, is a control lane to which no sample had been added. Lane 4, shows the banding pattern from the medium fraction of the lyase treated sample. There appears to be a weak band at the top, and a weak smear running the length of the gel. This indicates that alginate in the medium was acted upon by the lyase and that the concentration of alginate in the medium was less than in the biofilm sample. The banding pattern in lane 5 shows a prominent band near the top of the gel, with a weak smear similar to that seen in lane 4. The prominent band at the top of the gel represents alginate that is of high molecular weight and has not been acted upon extensively by alginate lyase. This band probably indicates that lyase released from the bacteria was not active in the medium.

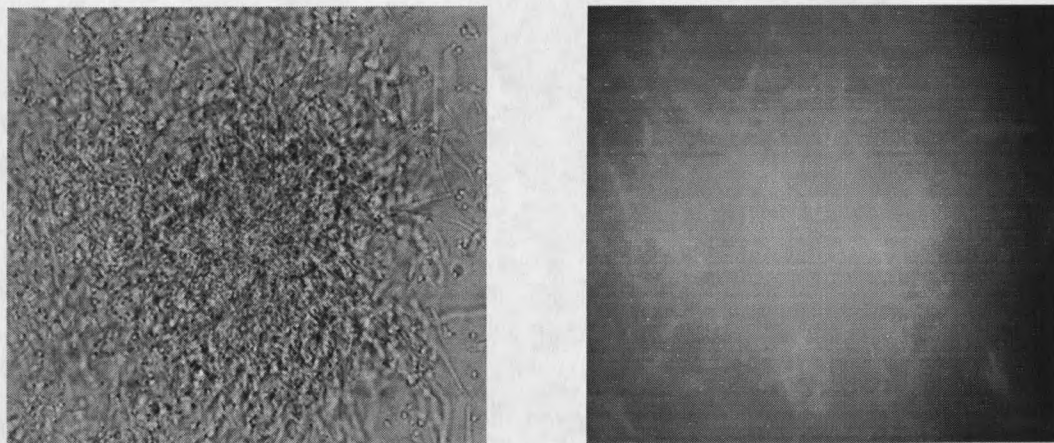


Figure 5.1. Cell cluster of *P. aeruginosa algD/lacZ, tac/algL* grown under continuous IPTG induction. Left panel, phase contrast image showing total cells. Right panel, fluorescent image showing area of alginate synthesis. Magnification = 1,250 x.

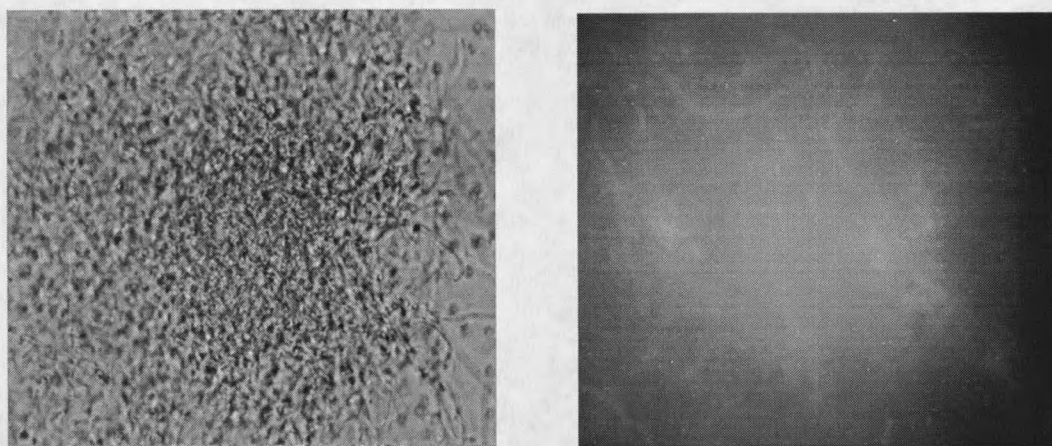


Figure 5.2. Cell cluster of *P. aeruginosa algD/lacZ*. Left panel, transmitted image showing total cells. Right panel, fluorescent image showing area of alginate synthesis. Magnification = 1,250 x.

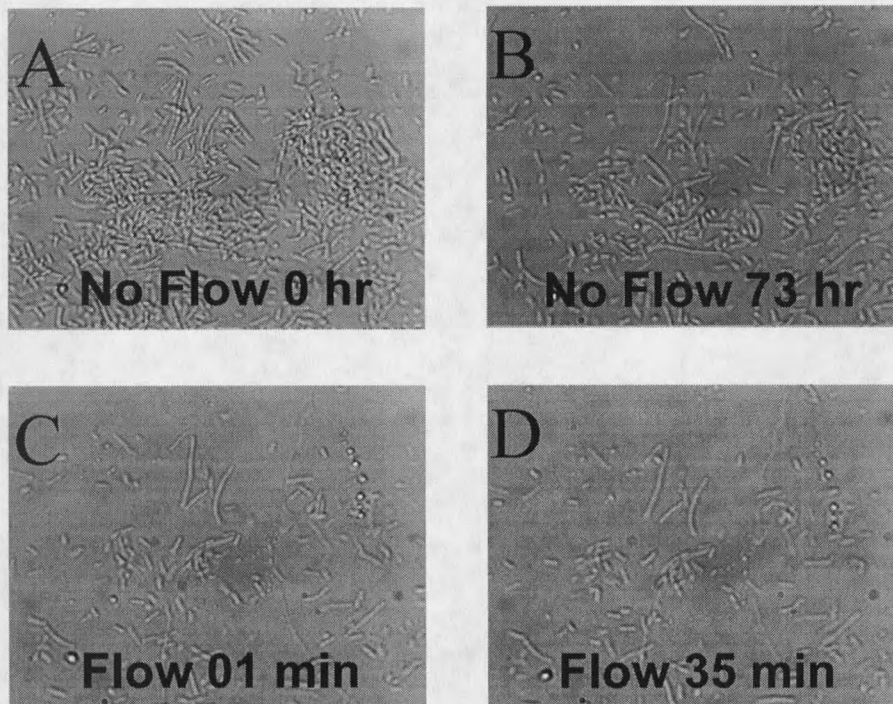


Figure 5.3. Sloughing event following termination of flow. Panel A, developed cell clusters at time of termination of flow. Panel B, cell clusters following stagnant conditions for 73 hr. Panel C, flow reinitiated for 1 min. Panel D, flow initiated for 35 min. Magnification = 1,250 x.

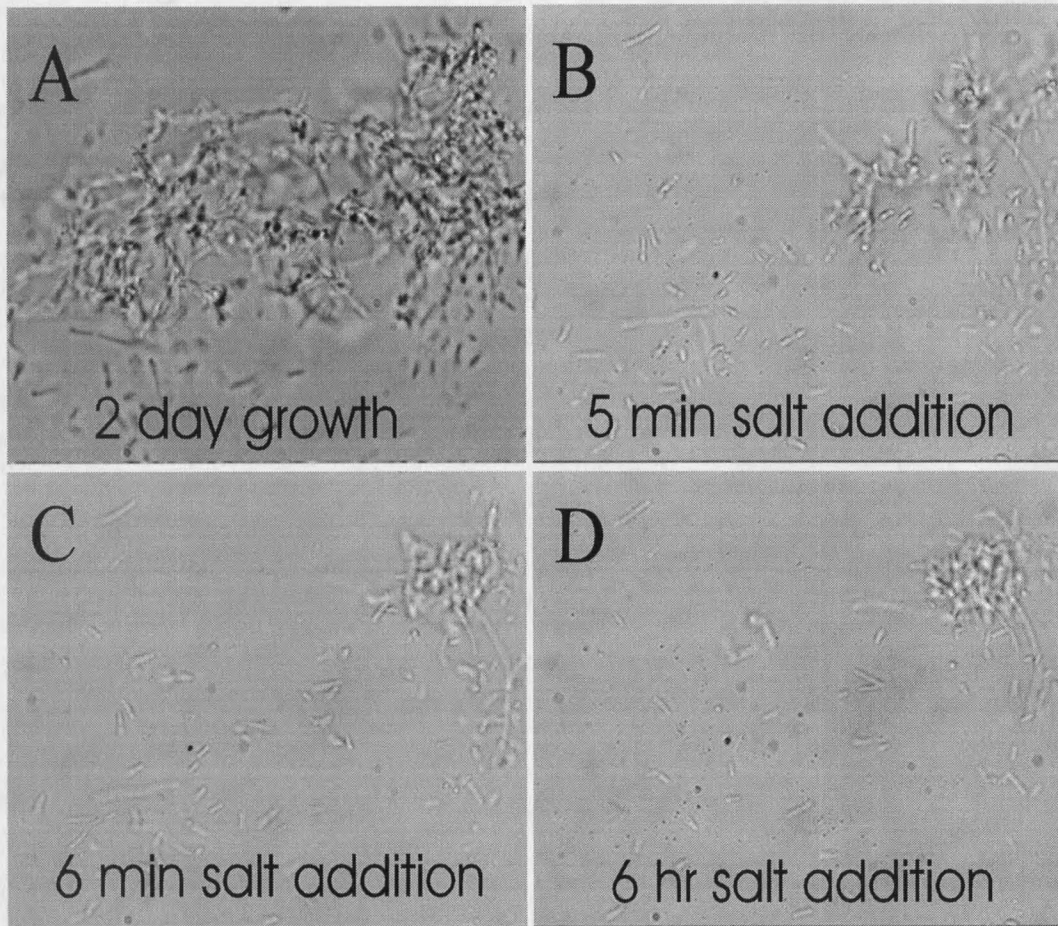


Figure 5.4. Addition of 0.3 mM NaCl to *P. aeruginosa tac/algL* cell cluster under conditions of continuous flow. Panel A, developed cell cluster after 24 hr of growth followed by IPTG induction for 24 hr. Panel B, cell cluster 5 min after addition of salt. Panel C, cell cluster 6 minutes after salt addition. Panel D, cell cluster 6 hr after salt addition.

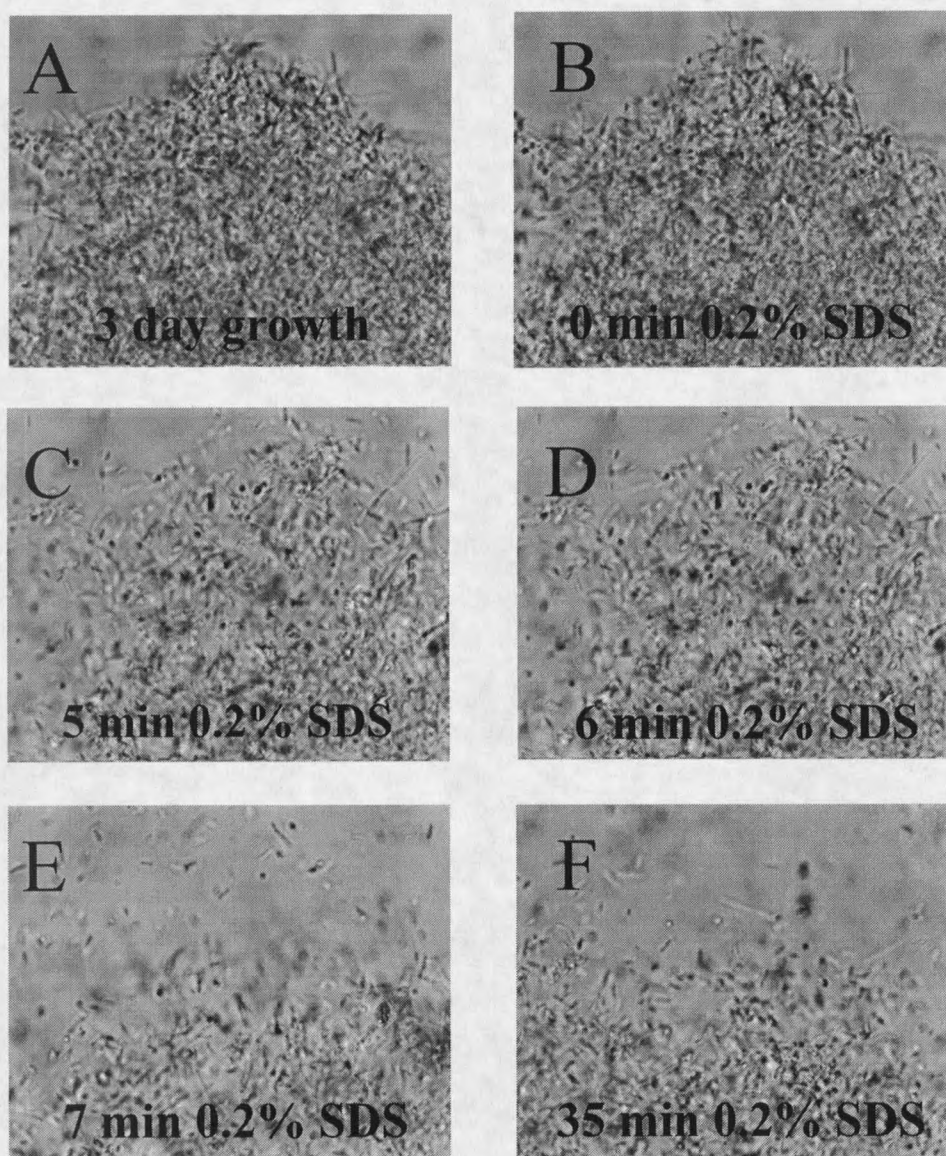


Figure 5.5. Addition of SDS to cell cluster grown for 24 hr in presence of IPTG. Magnification = 1,250 x.

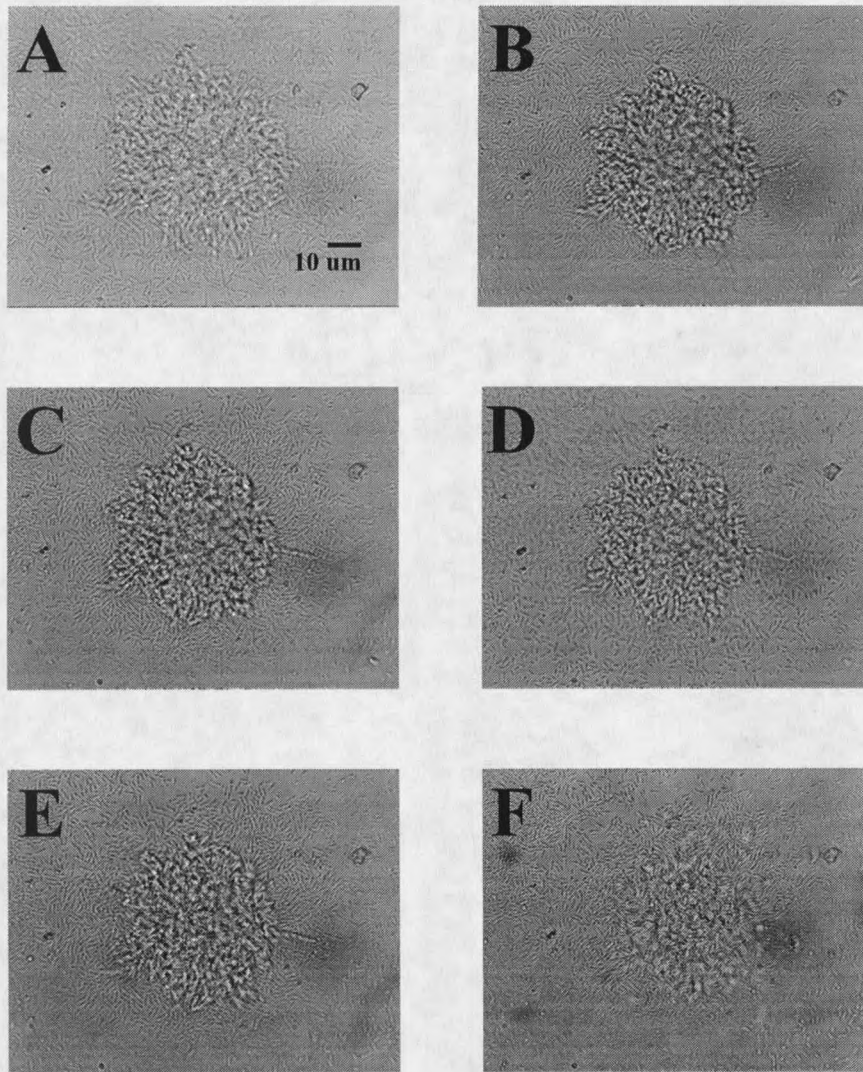


Figure 5.6. Small cell cluster to which alginate lyase has been added. A, cell cluster before addition of lyase; B, 5 min. after lyase addition; C, 30 min. after lyase addition; D, 60 min. after addition of lyase; E, 120 min. after addition of lyase; F, 20 hr. after addition of lyase.

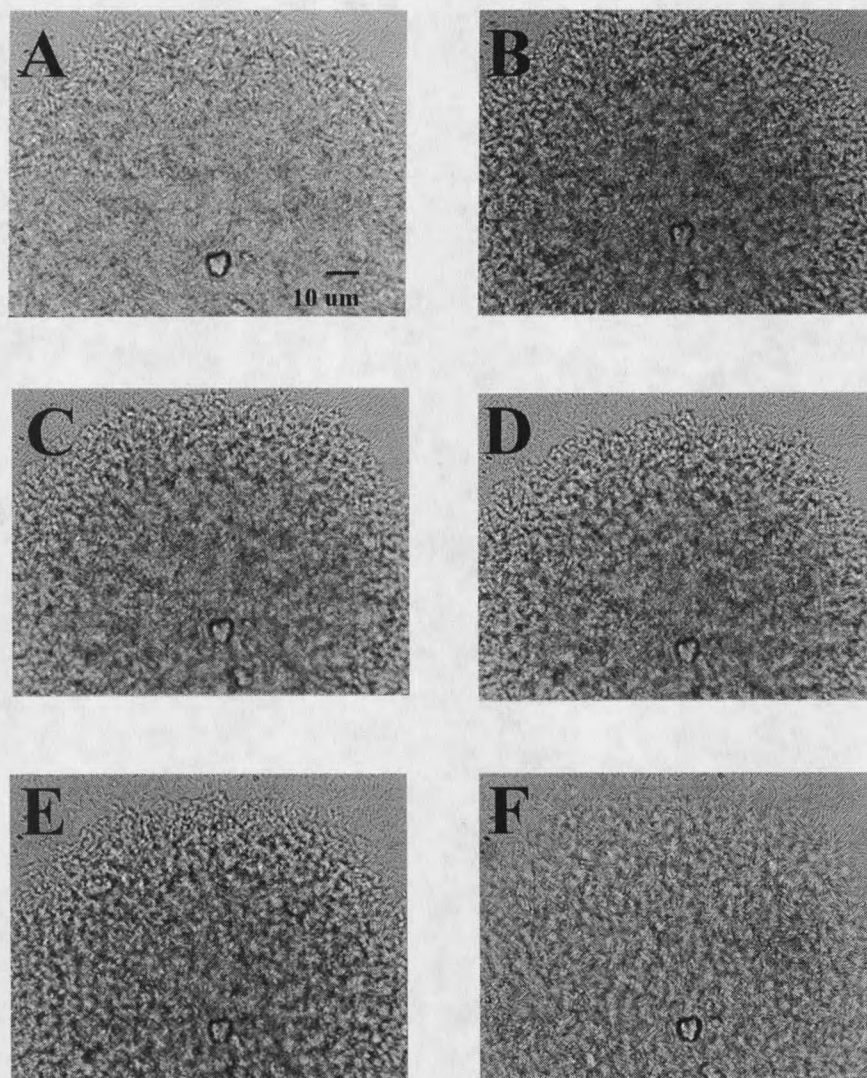


Figure 5.7. Medium cell cluster to which alginate lyase has been added. A, cell cluster before addition of lyase; B, 5 min. after lyase addition; C, 30 min. after lyase addition; D, 60 min. after addition of lyase; E, 120 min. after addition of lyase; F, 20 hr. after addition of lyase.

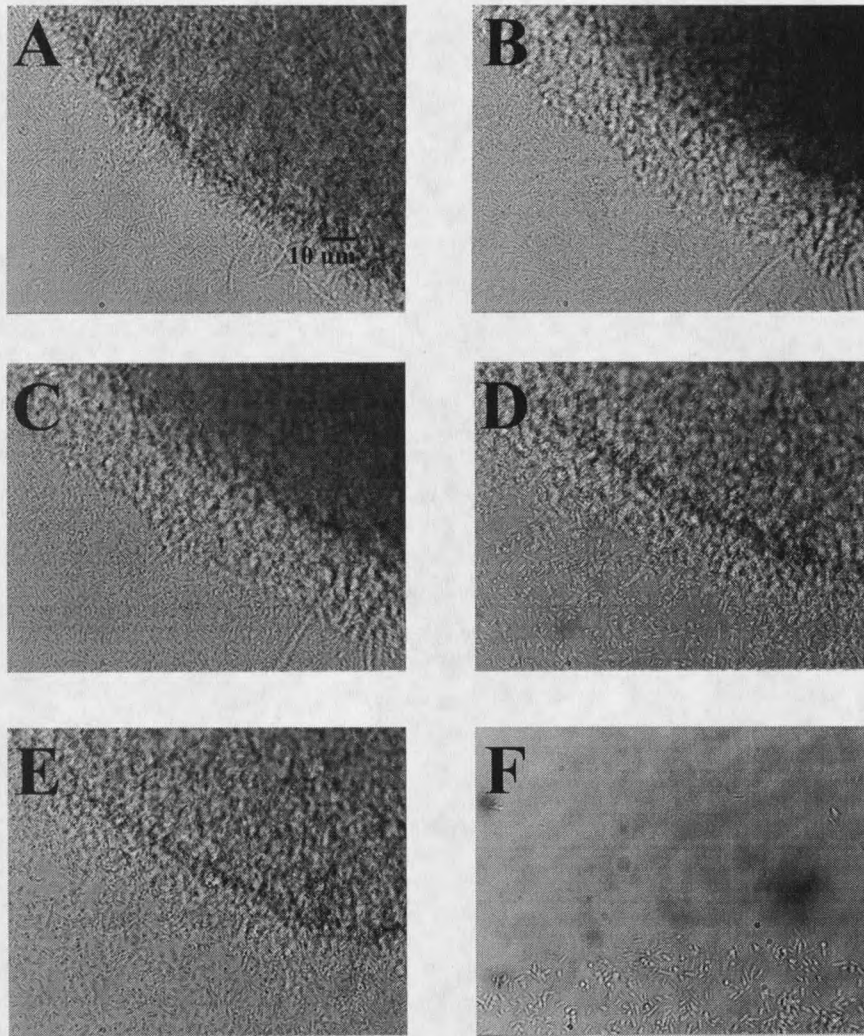


Figure 5.8. Large cell cluster to which alginate lyase has been added. A, cell cluster before addition of lyase; B, 5 min. after lyase addition; C, 30 min. after lyase addition; D, 60 min. after addition of lyase; E, 120 min. after addition of lyase; F, 20 hr. after addition of lyase.

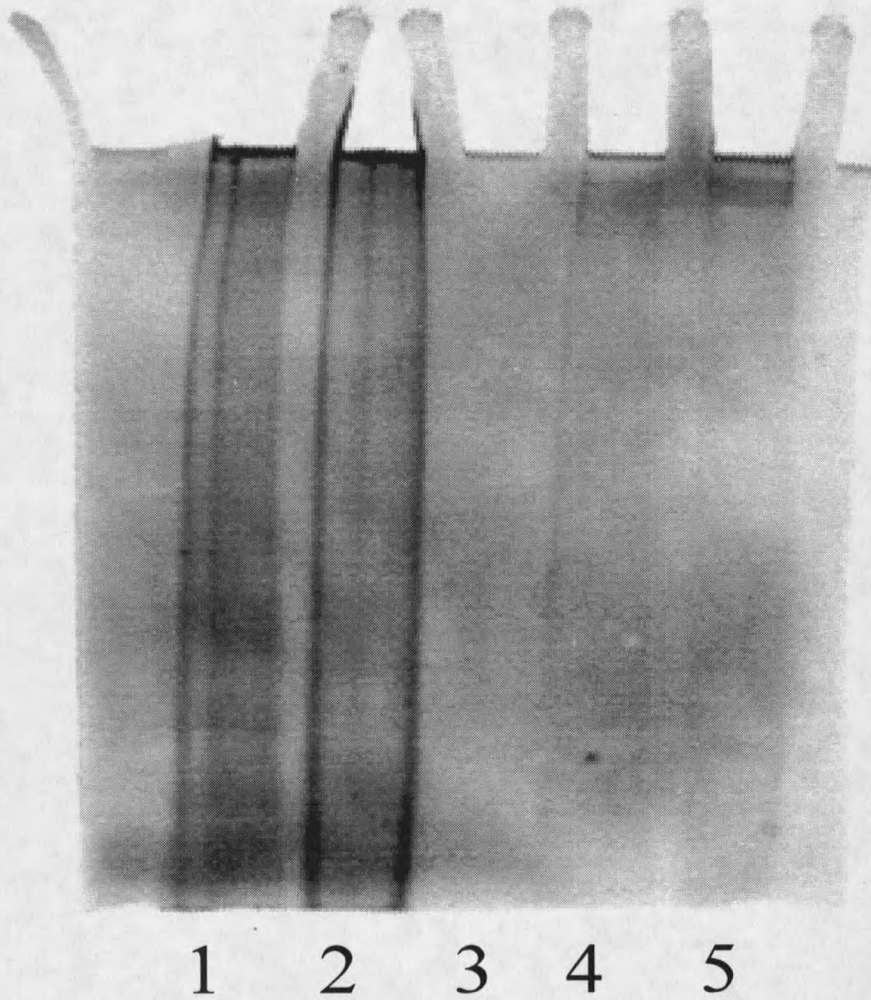


Figure 5.9. Sodium dodecyl sulfate polyacrylamide gel (5%) showing purified lyase activity on alginate produced in continuous culture by *P. aeruginosa* 8830. Lane 1, whole biofilm sample treated with purified alginate lyase and 4.0 mM NaCl. Lane 2, whole biofilm sample treated with 4.0 mM NaCl in PBS. Lane 3, control (no alginate). Lane 4, biofilm supernatant treated with alginate lyase and 4.0 mM NaCl. Lane 5, biofilm supernatant treated with 4.0 mM NaCl. Gel, courtesy of A. Boyd.

Discussion

The over-production of alginate lyase by *Pseudomonas aeruginosa* strain 8830 in this study appeared to have no effect on the ability of these bacteria to develop biofilms. This observation indicated that alginate lyase does not sufficiently interfere with alginate production to result in an observable change in the matrix composition of biofilm cell clusters. Such an observation implies that intracellular processing of alginate by alginate lyase does not result in significant destruction of those alginate polymer strands which are responsible for maintenance of cell cluster integrity.

This conclusion is relevant in terms of understanding the role of lyase on alginate production and biofilm development. It has been observed previously in our laboratory that sloughing events are preceded by down regulation of alginate biosynthesis. During this study, however, it has been shown that production of alginate lyase will not result in down regulation of the *algD* promoter. We may conclude from this that alginate lyase, by itself, does not regulate alginate biosynthesis and that separate or additional factors are involved in down-regulating alginate production. Furthermore, the positioning of *algL* within the *algD* biosynthetic cluster can be reconciled since alginate biosynthesis has been shown to take place even in the presence of very high levels of AlgL, as was demonstrated in Figure 5, above.

In sloughing events induced by turning off flow within the continuous culture system, bacteria in the cell clusters shown in Figure 6 were shown to break apart from one another and to disperse without the assistance of flowing conditions. This demonstrated primarily, that sloughing by these bacteria is not a strictly hydrodynamic process, and secondarily that following

a sloughing event bacteria actively disperse away from the site of the cell cluster. During the sloughing event, cells from the cluster were able to actively swim away from one another and could be imaged afterward as individual cells present in the bulk medium. These observations suggest that the polymer matrix responsible for maintaining the integrity of the cell clusters (biofilm) in the flow-cell had been broken down to the extent that motility of individual bacteria did not appear to be impaired, and cells were no longer connected to one another via polymer strand binding. The implication is that in a sloughing event induced by medium stagnation, the biofilm matrix polymer is significantly degraded.

In addition to the breaking apart of cell clusters, stagnation of the medium resulted in the detachment of bacteria directly from the substratum. It is believed that attachment to the substratum is not mediated by alginate, therefore, in addition to alginate lyase, other enzymes may be involved in a sloughing event induced by stagnation. When sloughing was artificially induced by the over-production of alginate lyase along with either salt or SDS addition, those bacteria directly attached to the substratum did not appear to be affected. The ability to eliminate biofilm bacteria from a system by inducing sloughing will require further investigation into the mechanism by which cell detachment from the substratum is mediated and regulated.

The extensive breakdown of the matrix polymer following medium stagnation may have been due to the release of alginate lyase by the biofilm bacteria into the surrounding medium. During the period of alginate synthesis (at all times during development of the biofilm), one may assume from the configuration of the *algD* gene cluster that alginate lyase is continuously produced. Therefore, there should be a reservoir of the enzyme (in either active or inactive form) within the bacteria which after significant accumulation within the cell, may be released into the

surrounding environment. As a triggering mechanism mediating such a release, two possibilities are proposed. In the first, stagnant conditions within the flow-cell may result in a build-up of waste products. Waste products may themselves act as a trigger, such as through interaction of cell receptors with a specific accumulated compound in the medium, or they may result in secondary effects, such as may result from the accumulation of CO₂ and, or, organic acids which may depress the local pH. Alternatively the bacteria may be able to respond to a depletion of nutrients from the environment, such as loss of the organic carbon source, or the loss of O₂.

The loss of flowing conditions itself might be responsible for triggering the bacterial sloughing observed within the flow-cell. If this were the case, one would expect cells to slough shortly after the cessation of flow, yet, in all cases, bacterial sloughing has only been observed after at least 48 hr of stagnant conditions. Such a time frame would be unnecessary if the enzyme to be released is accumulated within the cell prior to turning off flow. The precise mechanism(s) by which an environmental signal may trigger a sloughing event is not understood at present. Further investigation into the signaling which leads to sloughing of biofilm material would contribute greatly to our ability to control biofilm development and persistence.

In experiments run to examine the activity of inducible lyase production, no detectable levels of the enzyme were found in the control samples. This finding indicated very low background production of alginate lyase under conditions of growth in broth cultures. This observation was consistent with previous observations in our laboratory that *algD* activity is low in bacteria grown in batch culture. Since the production of alginate lyase is believed to be tied to *algD* promoter activity, this observation was expected. In strains carrying the *tac/algL* construct, induction of alginate lyase synthesis was demonstrated following the addition of IPTG. The

amount of enzyme recovered was significantly greater in the cell extract fraction than was recovered from the cell-free medium. This implied that while production of large amounts of lyase could be induced in these bacteria, the majority of the enzyme was not released into the surrounding medium. The lack of transport was presumably due to only *algL* being induced in these cultures and not any of the potential processing or export proteins that may be required for release of active enzyme into the medium. The enzyme recovered during these experiments was shown to be capable of degradation of non-acetylated alginate.

Induction of lyase by biofilm cultures was expected to result in significant leakage of the enzyme into the surrounding medium. However, when biofilms were allowed to develop, the structure of the cell clusters did not appear different from those produced by bacteria not carrying the *tac/algL* construct. This indicated that leakiness of the *tac* promoter did not result in significant degradation of the matrix polymer responsible for cell cluster integrity.

When bacteria in the flow-cell were induced to over-produce alginate lyase, no change was observed in the appearance of the cell clusters examined. This result was reproducible even when cell clusters were grown from single bacteria under continuous IPTG induction. The lack of noticeable effects following over-production of lyase was unexpected. Previous work by Boyd and Chakrabarty (1994), showed that when these bacteria were grown on agar medium, cells over-producing lyase were more easily removed from the surface than were control bacteria. This result is believed to be the consequence of lyase degradation of the alginate strands which enmesh bacteria in colonies on the agar surface.

In the laboratory of Dr. A. M. Chakrabarty it had been noted that enhanced enzyme specific activity resulted when purified alginate lyase was combined with 0.4 M NaCl to alginate

in the absence of bacteria. The addition of salt could influence lyase activity by direct interaction with the enzyme and/or through interaction with the alginate. Direct interaction with the enzyme could result in a conformational change which would enhance the affinity of the lyase for the substrate binding site. Interaction of salt with alginate could result in displacement of multivalent cations with Na^+ . It has been shown that alginate binding of divalent cations results in cross linking of individual strands principally through the interaction of two carboxyl groups (each from a separate alginate polymer strand) and the two positive charges of the cation (Jang et al. 1990). When a monovalent cation, such as sodium, is added in sufficient excess, the divalent cations will be displaced by two monovalent cations which can only bind to one polymer strand at a time. The result is a separation of polymer strands. This separation could enhance the ability of alginate lyase to interact with the substrate binding site, or alternatively, could complement the enzyme activity resulting in a more rapid breakdown of the overall polymer matrix.

In an effort to investigate why alginate lyase production alone would not induce sloughing in biofilm cultures, salt (NaCl) was added to developed cell clusters following addition of IPTG. As has been noted above, this treatment did induce sloughing of the bacteria. The addition of salt alone was not sufficient to cause a similar response. This addition, while expected to enhance degradation of the alginate polymers, was done principally to release enzyme from the bacteria. The fact that no sloughing was observed as the result of salt addition alone, indicated that displacement of divalent cations by sodium did not occur to the extent necessary to disrupt the alginate polymer matrix. As a check on the role played by salt in the study, an attempt was made to reproduce the same effect by the addition of SDS. A detergent, SDS is able to interact with the cytoplasmic membrane to make bacteria leaky, thereby releasing

enzyme into the outside medium. Salt is believed to cause a similar release of enzyme through osmotic shock. When SDS was added to cell clusters following up-expression of lyase production, sloughing also occurred. The interactions of the detergent with the alginate polymers and or enzyme may be similar to those described above for NaCl, since sodium should dissociate from both compounds in a similar manner. Further investigation into the role played by salt and SDS is needed if the mechanism of sloughing in *P. aeruginosa* biofilms is to be completely understood.

The addition of purified lyase to cell clusters of *P. aeruginosa* 8830 did not result in the same degree of sloughing or cell detachment that was observed when bacteria were induced with IPTG and then treated with either salt or SDS. When alginate lyase was added to cell clusters without NaCl, no change in the cell clusters was observed. This observation is believed to be the result of a requirement of alginate lyase for salt. Earlier, it was shown that salt alone could not cause sloughing of the cell clusters. This indicates that salt and alginate work in a synergistic way to bring about the destruction of biofilm matrix polymer. When lyase was added to the cell clusters in the presence of salt, the results were less than dramatic. While indications of alginate cleavage were present, wholesale sloughing was not observed. The discrepancy between the significant and rapid sloughing observed with induced cells and the negligible sloughing observed when lyase was added in pure form, may be the consequence of the location from which the enzyme is able to attack the polymer. The results shown in Figure 5.9 indicated that both IPTG induced lyase and purified lyase are capable of breaking apart alginate. The differences lay not in the degree to which these different applications are able to break down the polymer, but rather, the location at which the polymer is broken down. Lyase added externally to

a biofilm culture has to nonspecifically work its way from the outside of the cell clusters toward the interior. Lyase originating from the bacteria is able to work from inside the cell cluster out. Cleavage of attachment points between alginate polymer strands and bacteria shortly after the release of the lyase may account for this enhanced ability to induce sloughing when compared to lyase added externally.

Purified alginate lyase recovered from *P. aeruginosa* 8830 has been shown to be very unstable, reacting only for a few minutes *in vitro* before losing activity (personal communication, A. Boyd). The banding pattern observed in Figure 5.9, lanes 4 and 5, may suggest this. The large molecular weight alginate found at the top of lane 5, may represent long polymer strands that have been cleaved near the surface of a bacterium. The lack of significant large molecular weight alginate present in lane 4, may indicate that the enzyme if added from outside of a cell cluster is required to chew its way in, thereby releasing predominantly low molecular weight alginate fragments.

The present study has demonstrated that sloughing of cells in biofilms of *P. aeruginosa* can be induced artificially under controlled laboratory conditions. The mechanism by which this sloughing is mediated is currently believed to be through the destruction of alginate polymers via the activity of alginate lyase with a possible requirement for salt. If we are to take advantage of the natural phenomenon of microbially induced sloughing, further investigation will be needed. The exact role and activity of alginate lyase will require additional experimentation, the contribution of salt and SDS to the process is not resolved; the signal responsible for mediating sloughing under "natural" conditions not being currently known, the regulation of detachment from the substratum is unclear, and the activity of sloughing in multi species biofilms has not yet

been addressed. Continuation of research into the cellular regulation of sloughing in *P. aeruginosa* should resolve these issues in the future.

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CHAPTER 6

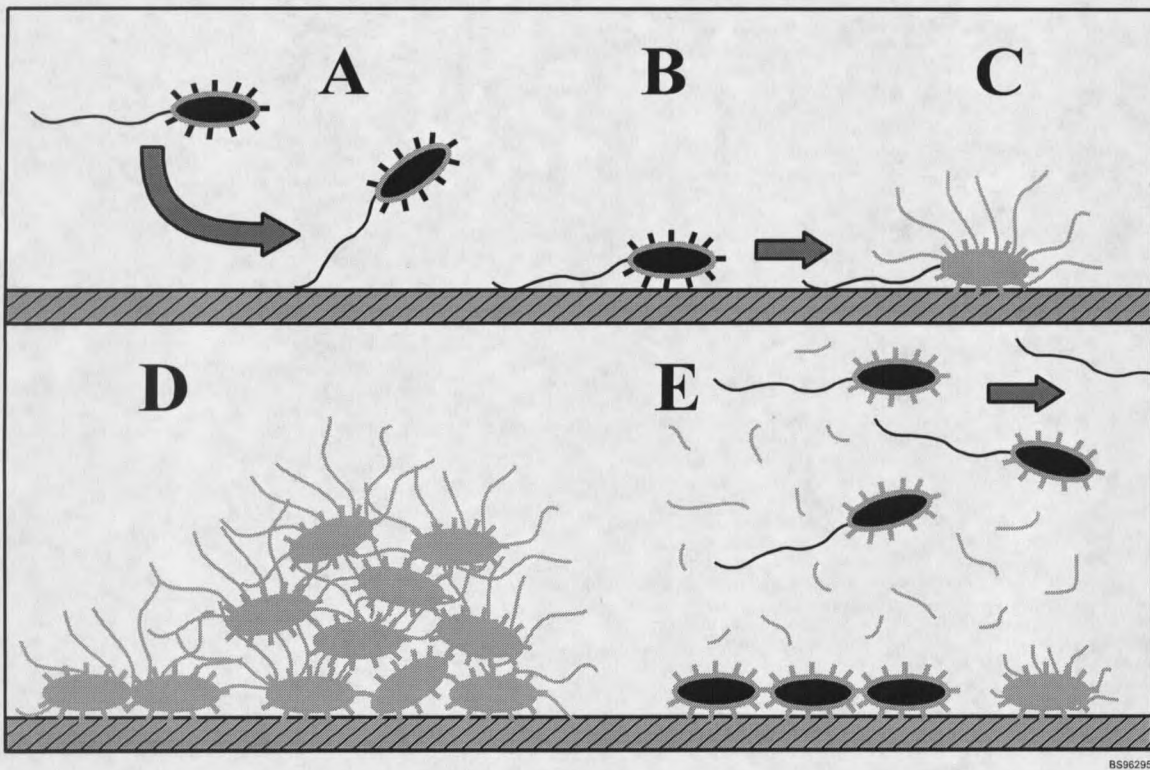
DISCUSSION

This research has described the behavior of three significant genetic elements in *P. aeruginosa* 8830, as they relate to alginate biosynthesis and processing. In Chapter 2, the up-regulation of alginate biosynthesis was observed when bacteria attached to a glass or Teflon surface. The increase in alginate production was demonstrated by uronic acids analysis, by FTIR analysis, by ELISA, and by reporter gene activity for the *algC* gene. In Chapter 3, the activity of the *algC* promoter was examined in more detail. It was found that the *algC* promoter is down-regulated in individual cells (but never in the entire population) in the bulk liquid, becomes up-regulated following attachment to a glass surface, and becomes down regulated before detaching from the surface. The possible role of regulating LPS was also suggested, indicating that *algC* may influence attachment, the initial step in biofilm formation. In Chapter 4, the activity of the *algD* promoter was examined. It was found that *algD* is predominantly down-regulated in bacteria in the bulk liquid, and that up-regulation occurs for most bacteria found attached or in a cell cluster. Prior to attachment, all of the bacteria at a surface were shown to down-regulate *algD* promoter activity. So, *algC* and *algD* were shown to have similar regulatory behavior, however, *algC* was shown to be more responsive to attachment (implicating its role in initial events in biofilm formation) and *algD* was shown to be more responsive to detachment (implicating its role in regulating only alginate biosynthesis). In Chapter 5, the activity of alginate lyase was studied, showing that it was capable of influencing sloughing in biofilms,

whether added externally, or produced as the result of IPTG induction. Regulation of alginate lyase appears to be at the transcriptional level as a member of the *algD* operon, and post-transcriptionally, being activated and exported as a result of changes in the environment.

One of the consequences of this study has been the development of a working hypothesis which describes the life cycle of *P. aeruginosa* 8830 as it progresses through the stages of planktonic growth, biofilm development and ultimately biofilm destruction following changes in the regulation of the promoters for the *algC* and *algD* operons, and regulation of AlgL. Results from the current work demonstrated that *P. aeruginosa* 8830 is able to respond to environmental stimuli which act as signals to the bacteria, influencing their mode of growth and physiology. Following attachment to a surface, at least two genes were found to be turned on, which enhanced the formation of a biofilm. Change in the regulation of a gene in response to attachment was first described by Dagostino, (1991). In her work, a marine bacterium was shown to turn on an unidentified gene as the result of attachment to polystyrene. In the present work, the up-regulation of a specific gene of known function has been described. The ability to examine specific gene regulation while at the same time observe the behavior of a bacterium under different environmental conditions has been a unique aspect of the work presented here.

Figure 6.1 diagrams the progression of regulation of *algC*, *algD*, and AlgL as biofilm formation, development and breakdown progresses. Bacteria represented schematically, are depicted as existing in a continuous or semi-continuous flowing system. Such environments are known to exist in streams (Geesey *et al.*, 1978), in industrial systems (Grobe *et al.*, 1995), and in pulmonary infections involving *Pseudomonas aeruginosa* (DeVault *et al.*, 1989). The model presented here for a biofilm life cycle is, therefore, applicable to any of these diverse situations.



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Figure 6.1. Schematic representation of *P. aeruginosa* 8830 proposed life cycle as a biofilm. A, planktonic bacteria in the bulk liquid interact with the substratum and initiate attachment. B, cell surface molecules interact with the substratum resulting in reversible binding. C, *algC* and *algD* regulation changes, resulting in alteration of LPS and production of alginate. D, cell cluster development progresses as alginate is produced. E, changes in environmental conditions result in the down regulation of *algD* and *algC* and the activation of alginate lyase, resulting in destruction of cell cluster matrix polymer, releasing bacteria into the bulk liquid.

In the bulk phase (Figure 6.1, A) the bacteria are motile and are shown to swim in the menstroom. Occasionally a bacterium will interact with the substratum via one or more of its cell surface components. If the shear is not too high (Chapter, 4), the bacteria may attach to the surface. During this period, the *algC* and *algD* promoters are down-regulated in the majority of the population (Chapter 2, Chapter 3 and Chapter 4). Such a situation should result in production of a shortened LPS, lacking A-band polysaccharide (Lightfoot and Lam, 1991). This condition

has been shown to enhance the ability of *P. aeruginosa* PAO1 to attach to a glass substratum (Chapter 3). In addition to depression of LPS A-band synthesis, alginate production has been shown to be down regulated in these bacteria (Chapter 2 and Chapter 4). The presence of alginate has been demonstrated by Allison and Sutherland (1987), and in our lab, to inhibit attachment of bacteria to a glass substratum. In Figure 6.1, B, bacteria are shown to lie down on the substratum and exhibit a more intimate association with the surface. The majority of bacteria that stick in this way remain at the surface only for a brief period, (Chapter 3 and Chapter 4). If the bacterium remains at the surface and is not washed downstream, it has been shown in the majority of cases to up-regulate both *algC* and *algD* (Chapter 3 and Chapter 4). These bacteria then begin to produce alginate and presumably enhance their ability to remain attached to the substratum (Chapter 4). It has been noted in our lab and in the laboratory of Dr. Gary Sayler, that *P. aeruginosa* 8822 (which does not produce alginate) sticks better to a glass surface than does *P. aeruginosa* 8830. However, strain 8822 is not capable of forming biofilm matrix polymer and only develops as a monolayer of cells at the glass surface. In Figure 6.1, D, alginate production leads to the formation of cell clusters, with bacteria enmeshed in the alginate matrix (Chapter 5). The condition of these cells continues until some change occurs in their environment (Chapter 5). When this occurs, both *algC* and *algD* promoter activity have been seen to down-regulate (Chapter 3 and Chapter 4). In addition to the reduction in alginate biosynthesis, it appears that AlgL is activated and exported from the bacteria (Chapter 5). The release of alginate lyase by biofilm bacteria results in the cleavage of alginate polymer strands and bacteria in cell clusters are released into the bulk liquid (Chapter 5).

This complex regulation of surface attachment, biofilm formation and detachment/sloughing is strongly suggested by the research results presented in this work. The exact mechanisms of environmental signaling, the possibility of cell-cell communication and the post-transcriptional regulation of genes described in this report have yet to be elucidated. The proposed model of biofilm development presented here, is only presumed to apply to *P. aeruginosa* 8830 growing in axenic biofilms. In nature, bacterial biofilms are complex assemblages composed of multiple species and quite probably multiple types of matrix polymer. The ability of biofilms to slough in the environment has, however, been noted and implies that common response mechanisms to environmental stimuli may exist. The author hopes that this work will be of some use to those who in the future will try to further our understanding of the regulation of biofilms by bacteria in environmental, industrial and medical ecosystems.

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