To the Graduate Council:

I am submitting herewith a thesis written by James F. Rice, Jr. entitled "Use of a Bioluminescent Reporter to Monitor Exopolysaccharide Production by Environmental Bacteria from Corroded Metal Surfaces." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

Gary S. Sayler, Major Professor

We have read this thesis
and recommend its acceptance:

Accepted for the Council:

Associate Vice Chancellor and
Dean of the Graduate School

USE OF A BIOLUMINESCENT REPORTER TO MONITOR EXOPOLYSACCHARIDE PRODUCTION BY ENVIRONMENTAL BACTERIA FROM CORRODED METAL SURFACES

A Thesis

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ABSTRACT

An alginic acid biosynthesis bioluminescent reporter plasmid, pUTK50, was transconjugated into environmental strains of *Pseudomonas putida*, *Pseudomonas fluorescens*, *Stenotrophomonas maltophilia* and *Pseudomonas syringae.* Bioluminescent transconjugates were selected from each strain for investigation of environmental stress factors promoting alginic acid exopolymer biosynthesis in developing biofilms. Environmental stimuli associated with increased levels of alginate synthesis, in a previously developed *P. aeruginosa* FRD1, were applied to the environmental strains. Increased salt concentrations and higher ratios of nitrate vs. ammonium ions as limited nitrogen source induced bioluminescence in FRD1 and the environmental strains. However, for environmental strains of *P. putida, P. fluorescens, P. syringae* and *S. maltophilia*, polysaccharides were detected with low uronic acids content and different structural components. When tested within a biofilm, *S. maltophilia* O46 demonstrated exceptional adhesive and corrosive properties while alginic acid synthesis was not significantly high. In most of the environmental strains, periods of increased bioluminescence were induced by external stimuli, but exopolysaccharides other than alginic acid were expressed. It is hypothesized that the environmental strains have homologous but non-identical promoter sequences which are responsive to certain environmental stimuli and may control genes necessary for the production of alternative exopolysaccharides.

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CHAPTER I

INTRODUCTION

Prokaryotic microorganisms have developed ingenious survival mechanisms through which they are able to cope with adverse environmental conditions. Many of these protective strategies involve complex regulatory pathways, which may be the result of processes involving the exchange of genetic information between strains, species, and even genera. These processes may have included adaptive and spontaneous mutations, natural conjugation events, horizontal gene transfer, homologous recombination events, and formation and transfer of plasmid DNAs. As a result of these evolutionary processes, mechanisms of regulation may have been streamlined to provide a maximum of efficiency coupled with, in many cases, an apparent benefit for the bacterial cell. One common theme, with increasingly supporting data, is the presence of global regulatory networks which coordinate bacterial responses to environmental conditions. It is now believed by many scientists that production of extracellular polysaccharide by bacterial strains qualifies as one such survival mechanism, and it has been observed experimentally that these polysaccharides seem to be regulated in a manner characteristic of the global regulatory networks [Leigh and Coplin 1992, Huang et al. 1995 and Wozniak and Ohman 1994].

Bacterial exopolysaccharides (EPS) are produced by many bacterial species as a mechanism for coping with various environmental challenges and have been postulated to

be important in processes of bacterial attachment to surfaces and nutrient accumulation [Christensen and Characklis 1990]. Bacterial associations at surfaces facilitate the development of complex bacterial communities, which are commonly referred to as biofilms. These microbial biofilms play an important role for the bacterium by providing a means of protection against desiccation, increases in osmolarity, and access to favorable habitats (e.g. nutrient rich surfaces) [Costerton et al. 1987 and Leigh and Coplin 1992]. Consequentially, biofilms have been problematic for man both in a clinical and in an environmental sense. Exopolymers are often associated with processes of clinical relevance, including bacterial attachment to prosthetic devices, opsonization, pathogenicity, host specificity and adherence to tissues [Costerton et al. 1987]. Specifically, alginate, a heteropolysaccharide commonly produced by *Pseudomonas* RNA homology group I bacteria, is an important virulence factor in the colonization of lung tissue of patients afflicted with the hereditary disease, cystic fibrosis [Deretic et al. 1994]. Environmentally, these biofilms have been associated with the corrosion of metals in water cooling systems [Hernandez-Mena and Friend 1993], fouling of heat exchange surfaces [Uhlinger and White 1983], fouling of ship hulls [Angell et al. 1993], toxicant immobilization [Vandevivere and Baveye 1992], and biocorrosion and dewatering problems in wastewater treatment systems [Pavoni et al. 1972]. The importance of EPS in these processes has stimulated researchers to study bacterial EPS production in detail at both the physiological and the genetic level. However, in order to combat these costly

problems, research is needed in which the mechanism of exopolymer biosynthesis can be studied from an environmentally relevant focus with emphasis on real world problems.

In a previous research project, the bioluminescent reporter plasmid, pUTK50 [Appendix B; Wallace et al. 1994a], was constructed for use in environmental research examining the transcriptional activation of the *alg*D promoter. In this previous research, the *lux*CDABE gene cassette encoding the bioluminescence genes of *Vibrio fischeri* was cloned with a 1.2kb DNA fragment containing the *alg*D promoter from a mucoid cystic fibrosis isolate *Pseudomonas aeruginosa* FRD1 [Wallace et al. 1994a]. This bioluminescent reporter plasmid was constructed in order to examine the effects of environmental stimuli on alginate biosynthesis and to determine the implications in formation and maturation of environmentally relevant microbial biofilms, specifically those related to the costly problems involved in biocorrosion processes.

The objective of this research project was to introduce pUTK50 into environmental bacterial strains isolated from corroded metal surfaces and characterize these reporter strains for studies of EPS production within a developing biofilm. These environmental bacteria were taken from corrosive biofilms found at source water, cooling lines, and reactor surfaces at the TVA Watts Bar nuclear power plant [Wallace et al. 1994b]. Five bacterial isolates were chosen from among twelve bacterial isolates demonstrating homology to at least one of three structural genes (*alg*D, *alg*G, *alg*76) and/or one regulatory gene (*alg*B) involved in the alginic acid biosynthetic pathway of *Pseudomonas aeruginosa* FRD1 [Table 1; Wallace et al. 1994b].

Table 1. Environmental bacterial isolates and their alginate genes similarities, based on DNA probe hybridization.

Bacteria	Source	Hybridization Probes			
		*alg*D	*alg*G	*alg*-76	*alg*B
FRD1	Cystic fibrosis isolate	218[a]	170	55	143
G	Essential electrical cooling water	5	39	28	142
H	Reactor tubercle (interior)	6	19	4	0
I	Reactor vessel (solid debris)	187	1	0	54
L	Water storage tank	74	8	67	29
M	Tubercle attached to weld	128	307	116	124
N	Reactor Vessel (solid debris)	0	0	0	57
O	Raw cooling water header (dark colored)	21	19	7	22
P	Raw cooling water header (light colored)	38	111	2	43
310	Water pipe inlet nodules	20	0	0	7
322	Pipe section with tubercles at site of weld	200	105	15	150
324	Tubercles on carbon steel	123	53	22	17
347	Pipe section (surface)	34	17	3	27

[a] Integrated optical densities obtained by scanning DNA slot blots with a Bioimage Visage 110 (data from Wallace et al. 1994b).

Environmental stimuli associated with enhanced alginate biosynthesis in *Pseudomonas aeruginosa* FRD1 and conditions attributable to surfaces (stainless steel, copper pipes and moldings) supporting biofilm formation were applied to environmental reporter bacteria. It was observed that induction of bioluminescence tended to be strain specific and in some cases followed patterns observed for *P. aeruginosa* FRD1. Generally, increases in osmolarity induced bioluminescence in the environmental strains and in FRD1, and in cases of higher ratios of nitrate vs. ammonium induction, bioluminescence varied among the environmental strains, although FRD1 was induced.

These results seem to indicate that although the environmental bacteria responded to some signals that are linked with enhanced rates of alginic acid accumulation in *P. aeruginosa*, a direct comparison with FRD1 is not necessarily appropriate since nitrogen limitation was not universally observed to induce bioluminescence in all of the environmental strains. This hints at a more complex regulatory network than was previously considered, and in fact recent research papers have hypothesized that alginate synthesis in FRD1 is dependent on the energy status of the cell as well as many other environmental influences [Schlictman et al. 1994]. With this in mind, a creatively broad approach to further research will allow the study of potentially important cellular process involved in the production of EPS and subsequent formation of biofilms, such processes may not be directly related to the effects of known environmental stimuli on alginate biosynthesis in FRD1.

CHAPTER II

LITERATURE REVIEW

Exopolysaccharides (EPS) are important in many bacterial survival strategies including flocculation [Stehr et al. 1995], biofilm development [Costerton et al. 1987] and structural differentiation (in certain bacterial communities) [Denarie et al. 1992]. Complex strategies, such as these, allow bacteria to persist in the presence of adverse environmental conditions, by protecting these prokaryotes from environmental stresses such as dehydration [Lawrence et al. 1994 and Jarman et al. 1978], heavy metal toxicity [Vaisanen et al. 1994, Bitton and Freihofer 1978 and Mittleman and Geesey 1985], increases in osmolarity [Terry et al. 1991], and nutrient depravation [Wrangstadh et al. 1988 and Sokol et al. 1994]. Additionally, EPS have been shown to be essential in both bacteria/plant [Fett et al. 1989, Huang et al. 1995 and Kidambi et al. 1995] and bacteria/animal interactions [Schurr et al. 1994].

The importance of EPS in these bacterial processes has been apparent for many years and researchers have been working towards an understanding of the mechanisms of exopolymer production. One of the primary reasons for this research was to combat the costly problems involved in microbial influenced corrosion (MIC) [Mittleman et al. 1991], treatment of wastewater [Vandevivere and Baveye 1992 and Geddie and Sutherland 1993], reduction of fluid flow in water systems [Walker et al. 1993], increased resistance during heat exchange [Hernandez-Mera et al. 1993 and Nakasono et al. 1993],

plant pathogenesis [Leigh and Coplin 1992], animal pathogenesis [Sutherland 1988], and other industrially/medically related problems [Hernandez and Friend 1993, Read and Costerton 1987 and Williams and Wimpenny 1980]. Unfortunately, results from these studies have been slow coming and there has been disagreement on the exact nature of the mechanisms involved in the regulation and biosynthesis of EPS. Scientists are still trying to figure out exactly what stimuli are responsible for activating EPS synthesis in bacteria.

In recent years, it has become increasingly apparent that the regulation of EPS biosynthesis probably involves complex regulatory networks [Huang et al. 1995 and Vandevivere and Kirchman 1993]. Therefore, in order to develop a full understanding of the molecular biology of EPS synthesis in microorganisms, it is absolutely essential to adequately develop an environmentally relevant model. Such a model must address the roles of exopolymer in bacterial communities, and the multitude of physiological conditions present, such as environmental stress factors which may affect exopolymer production in bacterial cells. Scientists are now working to develop and apply new microbiological tools for the study of the complex mechanisms of EPS biosynthesis in important bacterial species [Davies and Geesey 1994 and Wallace et al. 1994a].

Bacterial exopolysaccharides (overview)

EPS may be simply defined as an extracellular polysaccharide which is either loosely associated with the cell or secreted into the surrounding media [Bitton and Freihofer 1978]. These polysaccharides may be linear or extensively branched and may

contain a wide variety of secondary substituents [Sutherland 1990]. Substituents often include various combinations of acetate, pyruvate ketals, succinyl groups, sulfate, phosphate and amino acid components [Sutherland 1990]. Monosaccharides which are commonly found in EPS include D-glucose, D-galactose and D-mannose in the pyranose forms, as well as L-fucose and L-rhamnose [Sutherland 1988]. Additionally, L-hexoses and the furanose forms of glucose and galactose are found in several rare cases [Sutherland 1990]. Amino substituted monosaccharides, such as N-acetyl-D-glucosamine, N-acetyl-D-galactoseamine, N-acetyl-D-mannoseamine, fucoseamine, and talosamine are present as well. Polyanionic EPS may contain D-glucuronic acid, and D-galacturonic acid, and more rarely D-mannuronic acid and D-guluronic acid [Sutherland 1990]. The hexosaminuronic acid 2-acetoamido-2-deoxy-D-galacturonic acid has also been observed in EPS isolated from *Staphylococcus aureus*. Also, it is not uncommon to find 3-keto-deoxy-D-mannoocotolosonic acid (KDO) in EPS from *Escherichia coli* [Sutherland 1990].

In the most general sense, EPS can be divided into two broad categories, homopolysaccharides and heteropolysaccharides [Mathews and Van Holde 1990]. Homopolysaccharide and heteropolysaccharides are found in a wide range of prokaryotic and eukaryotic organisms. In bacteria, several instances of a particular bacterium producing both the simpler homopolysaccharide (sometimes referred to as a nonspecific EPS) and the more complex heteropolysaccharide (also referred to as a specific polysaccharide) have been demonstrated [Keenleyside et al. 1992, Huang et al. 1995 and

Fett et al. 1989]. However, research indicates that most bacteria do not produce more than one heteropolysaccharide, as a general rule [Fett et al. 1989].

Homopolysaccharides are the simplest EPS containing only one type of saccharide and are predominantly neutral, although there are examples of polyanionic homopolysaccharides as well [Sutherland 1990]. These polymers may contain branches or be wholly linear, and are produced by many bacterial species. Biosynthesis of these biopolymers has been observed to depend on the specific substrates involved [Sutherland 1988], and has been postulated to involve few enzymes beyond those required for nucleotide sugar precursor synthesis [Leigh and Coplin 1992]. Examples of these exopolymers include curdlan [Sutherland 1990], levan [Fett et al. 1989], scleroglucan [Sutherland 1990], pullulan [Sutherland 1990], cellulose [Saxena et al. 1994], dextran [Sutherland 1990], and alginate containing solely D-mannuronic acid residues [Leigh and Coplin 1992]. This list is by no means exhaustive and continues to grow as new molecules are identified. Table 2 lists several common homopolysaccharides produced by various organisms, showing both the composition and the main chain linkage scheme. Many of these polymers contain as the primary constituent β-D-glucose or its anomer α-D-glucose.

Exopolysaccharides having more than a single type of monosaccharide are defined as heteropolysaccharides, and tend to be more complex both in structure and in the mechanisms of exopolymer biosynthesis [Sutherland 1988]. Heteropolysaccharides are distinctly different from homopolysaccharides in that they are believed to be

Table 2. Homopolysaccharides produced by important microorganisms and their composition.

Exopolysaccharide	Constituent	Linkage	Microorganisms	References
Cellulose	D-glucose	β(1,4)	*Acetobacter xylinum, Agrobacterium tumefaciens*	Saxena et al. 1994 and Kamoun et al. 1989
Curdlan	D-glucose	β(1,3)	*Alcaligenes faecalis*	Sutheland et al. 1990
Dextran	D-glucose	α(1,6)	*Leuconostuc mesenteroides*	Sutherland et al. 1990
Elisinan	D-maltotriose	α(1,3)	*Elsinoe leucospila*	Sutherland et al. 1990
Levan	D-fructose	β(2,6)	*Bacillus sp., Streptococcus salivarius, Xanthomonas and Pseudomons sp.*	Leigh and Coplin 1992
Pullulan	Maltotriose	α(1,6)	*Aspergillus niger*	Sutherland et al. 1990
Sialic Acid	Salic acid	α(2,9) and α(2,8)	*Neisseria meningitidis*	Sutherland et al. 1990
Scleroglucan	D-glucose	β(1,3) and β(1,6)	*Sclerotium rulfaii*	Sutherland et al. 1990

synthesized by subunit assembly from nucleotide diphosphate sugar precursors [Leigh and Coplin 1992]. This polymerization occurs on a membrane bound lipid carrier and is followed by secretion into the extracellular milieu. Most of the heteropolysaccharides appear to be polyanionic and are composed of repeating units varying in size from two to eight saccharides [Sutherland 1990]. Side chains are commonly found and are usually one to four sugars in length. Generally, the energy cost for production of these EPS is high and involves multiple genetic loci [Schlictman et al. 1994]. There is often a high carbon demand required for synthesis of these polymers. This has been observed in *P. aeruginosa* where up to 50% of the available carbon is channeled into alginate synthesis [Deretic et al. 1994]. Examples of important heteropolysaccharides include: alginate [Anderson et al. 1987], colanic acid [Keenleyside et al. 1992], marginalan [Osman and Fett 1989], succinoglycan [Rienhold et al. 1994 and Kamoun et al. 1989], and xanthum gum [Ielpi et al. 1993]. Table 3 lists many of the known heteropolysaccharides and gives important information on the constituents and ratios of monosaccharides, and lists the microorganisms in which they are found.

Alginate biosynthesis in *Pseudomonas aeruginosa*

The presence of alginate as a capsular structure provides cells with a mechanism of defense against harsh immunilogical challenges, allowing the bacterium to survive in the lungs of persons afflicted with the hereditary disease cystic fibrosis [Schlictman et al. 1995, Goldberg 1992 and Hassett et al. 1993]. This viscous EPS may also enable cells to

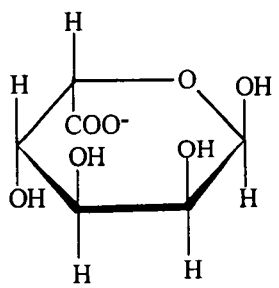
Table 3. Heteropolysaccharides produced by important microorganisms and their composition.

Exopolysaccharide	Constituents	Relative Ratios	Microorganisms	References
Alginate	D-mannuronic Acid, L-guluronic Acid, and O-acetyl (sometimes)	Variable	*Pseudomonas aeruginosa*, other *Pseudomonas* RNA group I isolates, *Azotobacter vinelandii*, and *Azotobacter chronococum* (sp)	Kennedy et al. 1992, Deretic et al. 1994
Amylovoran	D-galactose, D-glucuronic acid, D-glucose	Gal(4):GlcUA(1):Glc(1)	*Erwinia amylovora*	Metzger et al. 1994
Colanic Acid	D-galactose, D-glucuronic acid, L-Fucose and D-glucose	Glc(1):Fuc(2):Gal(2): GlcUA(1)	*Escherichia coli*	Keenleyside et al. 1992
EPS I	N-acetylgalactosamine, 2-N-acetyl-2-deoxy-L-galacturonic acid, and 2-N-acetyl-4-N-(3-hydroxybutanoyl)-2,4,6-trideoxy-D-glucose	1:1:1	*Pseudomonas solanacearum*	Huang et al. 1995
EPSb	galactose, glucose, pyruvate, and acetate	Glc(1):Gal(1):Pyr(1): Ac(1)	*Rhizobium meliloti* SU47	Leigh and Coplin 1992
Gellan	D-glucose, D-glucuronic Acid, L-rhamnose, acetate, L-glycine	Glc(1):GlcUA(1):Rha(1): Ac(1):Gly(1)	*Pseudomonas elodea*	Christensen and Characklis 1990
Marginalan	D-glucose, D-galactose, succinate, and pyruvate	Glc(1):Gal(1):Suc(1): Pyr(1)	*Pseudomonas marginalis*	Osman and Fett 1989
Polysaccharide A	D-glucose, galactose, glucuronic acid, and galacturonic Acid	Glc(1):Gal(0.81):GlcUA (0.42):GalUA(0.32)	*Pseudomonas sp.* NCMB 2021	Christensen et al. 1985A
Polysaccharide B	2-keto-3-deoxy-manno-octulosonic Acid, N-acetylglucosamine, and 6-deoxyhexose	KDO(1):NAcGlcAmine (1): 6-deoxyhexose(1)	*Pseudomonas sp.* NCMB 2021	Christensen et al. 1985A
Sphingan B-35	D-glucuronic acid, D-glucose, L-mannose and L-rhamnose	Glc(2):Man(1):GlcUA(1): Rha(1)	*Sphingomonas sp.*	Pollock et al. 1994
Succinoglycan	glucose, galactose, pyruvate, acetate and succinate	Glc(7):Gal(1):Pyr(1):Ac(1): Suc(1)	*Rhizobium meliloti* SU47, *Agrobacterium and Alcaligenes sp.*	Reinhold et al. 1994
Xanthan Gum	D-glucose, D-mannose, D-gluconic acid, pyruvate and acetate	Glc(2):Man(2):GlcUA(1): Pyr(1):Ac(1)	*Xanthomonas campestris*	Ielpi et al. 1993
Zooglea EPS unknown II	D-glucose, D-galactose, and pyruvate	glc(1):Gal(1):Pyr(1)	*Zooglea ramigera*	Sutheland et al. 1990

better adapt to and persist in other non-clinical environmental niches [Davies et al. 1993]. Research has shown that mucoid cells are more protected against phagocytosis, antibiotics, nutritionally poor environments, and dehydrating conditions such as increased osmolarity [Deretic et. al 1993]. Primarily, most of the research thus far has centered around the environment of the cystic fibrosis lung, but researchers are now beginning to explore the importance of this exopolysaccharide in environmental concerns such as wastewater treatment and corrosion.

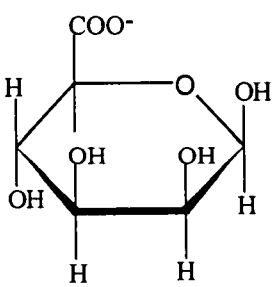
Bacterial alginate is a linear exopolysaccharide (EPS) composed entirely of D-mannuronic acid and L-guluronic acid residues [Figure 1A,B], which are connected by β(1,4) linkages, and is often found in an acetylated form [Boyd and Chackarabarty 1994].

A)



L-Guluronic Acid

B)



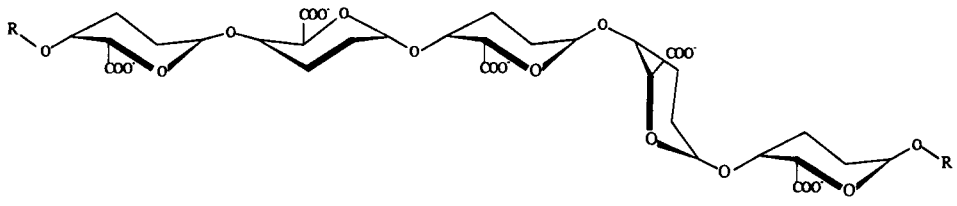
D-Mannuronic Acid

Figure 1. Structures of L-guluronic acid and D-mannuronic acid.

This EPS is secreted into the cellular environment where interactions between alginate, the cell, and neighboring cells associate and can form viscous cellular aggregates. The structural attributes of alginic acid are fundamentally important in fulfilling its roles in these process. Alginate is an extremely large polymer with a very high molecular weight [Sutherland 1977]. Since it is composed of unonic acids, the polymer is polyanionic, which gives it hydroscopic characteristics. The proportions of D-mannuronic acid and L-guluronic acid are important in determining the viscosity of the alginate in solution [Franklin et al. 1994]. It is a well known fact that alginic acid produced by *Azotobacter vinelandii* and alga has the capability of forming solid gels in the presence of calcium ions [Ertesvag et al. 1994]. This property is absent in alginate isolated from *Pseudomonas* species [Franklin et al. 1994]. This is because alginate from *A. vinelandii* contains stretches of poly-L-guluronic acid residues, but in alginate from *Pseudomonads*, L-guluronic acid monomers are always separated by at least one D-mannuronic acid [Figure 2A,B; Ertesvag et al. 1994]. In each of these cases, the more L-guluronic acid residues which are incorporated into the EPS the higher the overall viscosity [Deretic et al. 1994].

In *Pseudomonas aeruginosa*, alginic acid synthesis begins as fructose-6-phosphate is taken from the cellular pool of carbohydrates via the Entner-Doudoroff pathway [Zielinski et al. 1990]. Fructose-6-phosphate is converted to mannose 6-phosphate by the bi-functional enzyme phosphomannose isomerase (AlgA). Mannose-6-phosphate is then converted to mannose-1-phosphate through the action of the enzyme

A)



B)

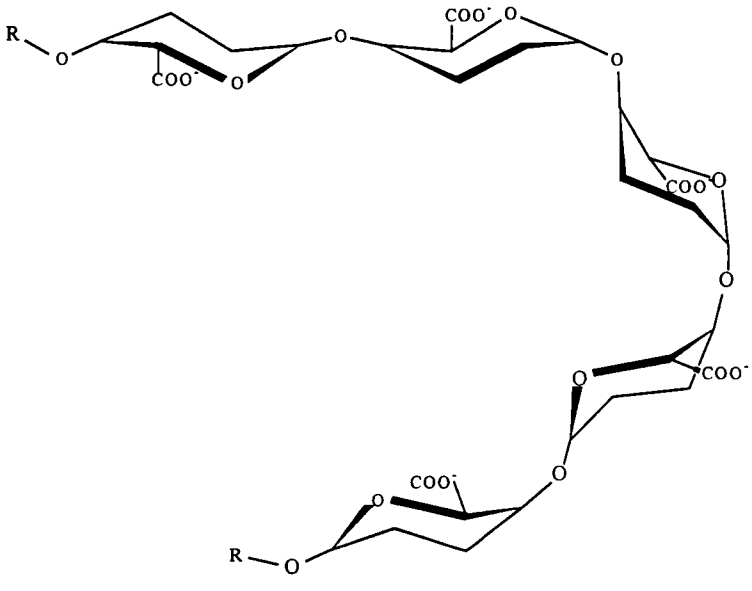


Figure 2. Comparison of hypothetical alginate structures from *Pseudomonas aeruginosa* and *Azotobacter vinelandii:* A) Structure of alginic acid in *P. aeruginosa*, showing the effects of L-guluronic acid in the polymer. B) Alginic acid structure in *A. vinelandii*, showing the effects of poly-L-guluronic acid residues in the biopolymer.

phosphomannomutase (AlgC). AlgA can then use its dual functionality as a GDP-mannose pyrophosphorylase to convert mannose-1-phosphate to GDP-mannose. GDP-mannose may then be converted to GDP-mannuronic acid by the AlgD enzyme (GDP-mannose dehydrogenase), in a reaction which is specific for alginate biosynthesis [Deretic et al. 1994]. After a short series of unknown reactions, mannuronic acid polymers are produced and exported out of the cell via AlgE [Rehm et al. 1994]. It has been hypothesized that AlgE may also be involved in polymerization of the exopolymer, but nothing conclusive has been elucidated [Rehm et al. 1994]. After export and polymerization the AlgF enzyme may acetylate various mannuronic acid residues in response to an unidentified regulatory stimulus [Franklin and Ohman 1993]. Acetylated alginate has been observed to be more resistant to the alginate lyase (AlgL) [Schiller et al. 1994]. Following acetylation, AlgG may epimerize a small percentage of these sugars to L-guluronic acid, and it is believed that the degree of acetylation may affect the extent of the conversion of the D-mannuronic acid residues to L-guluronic acid [Franklin et al. 1994]. Finally, the completed exopolymer is secreted into the cell's surrounding environment.

The genetic machinery for the biosynthesis of alginate can be found universally in *Pseudomonas aeruginosa* [Table 4; Zielinski et al. 1992], in addition to most *Pseudomonas* RNA group I sub-family members [Fialho et al. 1990] and *Azotobacter vinelandii* [Ertesvag et al. 1985]. Currently there are five known regions on the *P. aeruginosa* chromosome (9 min, 10 min, 13 min, 34 min and 68 min) which are involved in the production of alginate [Deretic et al. 1994]. The genes contained within these

Table 4. Essential genetic elements involved in alginate production in *Pseudomonas aeruginosa* FRD1.

Genes	Alternative Designations	Functions of Product	Map Location	Genes	Alternative Designations	Functions of Product	Map Location
*alg*C	*pmm*	Phosphomannomutase	10min	*alg*L		lyase	34min
*alg*D		GDPmannose dehydrogenase	34min	IHF		DNA binding factor	
*alg*A		Phosphomannoisomerase and GDP-mannose pyrophosphorylase	34min	*alg*E		Porin	34min
*alg*G		Epimerase	34min	*alg*B		Transcriptional activating factor	13min
*alg*F		Acetylase	34min	*alg*R1	*alg*R	Transcriptional activating factor	9 min
*alg*U	*alg*T	Sigma factor	68min	*alg*R2	*alg*Q	Kinase	9 min
*muc*A	*alg*S	Regulator	68min	*alg*R3	*alg*P; Hp1	Histone like factor	9 min
*muc*B	*alg*N	Regulator	68min				

regions are tightly regulated in *Pseudomonas sp.* by a complex regulatory network, and respond to a diverse array of stimuli, including the energy status of the cell [Schlictman et al 1995], nitrogen limitation [Terry et al. 1991], and changes in osmolarity in the surrounding environment [Terry et al. 1991].

Possibly the most important regulatory feature for conversion to mucoidy, in *P. aeruginosa*, is the DNA region located at 68 min. Three genetic regions have been identified at this locus, *alg*U, *muc*A and *muc*B [Schurr et al. 1994]. The first gene, *alg*U, encodes a putative sigma factor which is absolutely essential for proper transcription at the *alg*D, *alg*R, and *alg*B promoters [Martin et al. 1994]. The second two encode the regulatory elements, MucA and MucB, which when functional deactivate AlgU, resulting in a non-mucoid phenotype [Schurr et al. 1994 and Wozniak and Ohman 1994]. It appears that there is some method of directed mutation which is utilized by the cell to deactivate MucA. Once mutated, MucB cannot properly interact with MucA, and as a result an active AlgU is present which can then direct transcription at AlgU dependent promoters [Deretic et al. 1994]. This section of the regulatory mechanism has been coined the genetic swith for mucoidy in *P. aeruginosa* [Devries and Ohman 1994]. Recent research by Yu et al. 1995, has shown that AlgU is 66% identical to RpoE from *Escherichia coli* and *Salmonella typhimurium*, and their studies have shown that *rpo*E can complement an *alg*U mutation in *P. aeruginosa*, and that *alg*U is induced by heat shock. They suggest that *alg*U is involved in physiological roles similar to *rpo*E and that alginate production is just a subset of systems regulated by this sigma factor.

Contained within the genetic loci at 9 min are the genes *alg*R1, *alg*R2 and *alg*R3 which encode the regulatory proteins AlgR1, AlgR2 and AlgR3, respectively [Fujiwara et al. 1993]. These genes are organized in a single operon, and are under control of the AlgR1 promoter [Deretic et al. 1989a], which is dependent on AlgU. The AlgR1 regulatory protein has demonstrated homology with other response regulators of bacterial signal transduction systems [Schurr et al. 1994]. Also, the AlgR1 protein can be phosphorylated, in vitro, by typical histidine protein kinases [Schurr et al. 1994]. This suggests that AlgR1 may be acting as a two component regulator to activate transcription at its dependent promoters. Originally, it was thought that AlgR2 was probably the histidine protein kinase for AlgR1 [Fujiwara et al. 1993], but subsequent studies have shown that AlgR2 does not share significant homology with the other two component regulators [Schlictman et al. 1994]. Recently, AlgR2 has been found to function in the TCA cycle to help cells respond to changes in their energy status [Schlictman et al. 1994 and Schlictman et al. 1995]. This is accomplished by AlgR2 affecting the activities of the enzyme succinyl coenzyme A synthetase (Scs) and nucleoside diphosphate kinase (Ndk), which hydrolyze succinyl CoA. This is an important discovery because Scs and Ndk are involved in a substrate level phophorylation of ADP to ATP. This evidence couples AlgR2 to the energy status of the cell [Schlictman et al. 1994]. The exact mechanism by which AlgR2 affects the activities of these enzymes is not completely understood. AlgR3 has been found to be a histone-like DNA binding protein [Wozniak 1994]. Although the exact funcition of this protein is not known, research has suggested an ability to bind the

DNA upstream from the *alg*D promoter, but these experiments have not identified a putative location for this binding [Wozniak 1994].

Most of the genes involved in alginate biosynthesis have been localized to an operon (~18 kb) at 34 min [Zielinski et al. 1990]. These gene include (in order of transcription): *alg*D, *alg*8, *alg*44, *alg*E, *alg*G, *alg*60, *alg*L, *alg*F and *alg*A, and are transcribed, in the order listed, as a polycistronic mRNA under control of the *alg*D promoter [Deretic et al. 1994]. The *alg*D promoter is considered to be a major focal point in alginate synthesis [Maharaj et al. 1992 and Deretic et al. 1989b]. Transcription from this promoter is dependent on direct protein-protein and protein-DNA interactions between AlgR1, IHF, AlgR3 and AlgU [Wozniak 1994]. AlgR1 has been found to bind upstream of the *alg*D transcriptional start site [Figure 3A], and AlgR1 mediated transcription of the *alg*D promoter depends on the formation of a three dimensional folded DNA structure [Wozniak and Ohman 1994]. During this process, AlgR1 binds at the AlgR1 binding sites [Figure 3A], which are defined by the recognition sequence ACCGTTGTC [Deretic et al. 1994]. Studies by Wozniak 1994, have identified two potential binding sites for IHF at -80 bp and +80 bp relative to the *alg*D promoter. Binding of IHF at these sites is hypothesized to facilitate bending of the DNA, in order to promote formation of this structure. Also, it has been hypothesized that AlgR3 interacts directly with the DNA and stabilizes the three dimensional protein-DNA complex [Wozniak 1994].

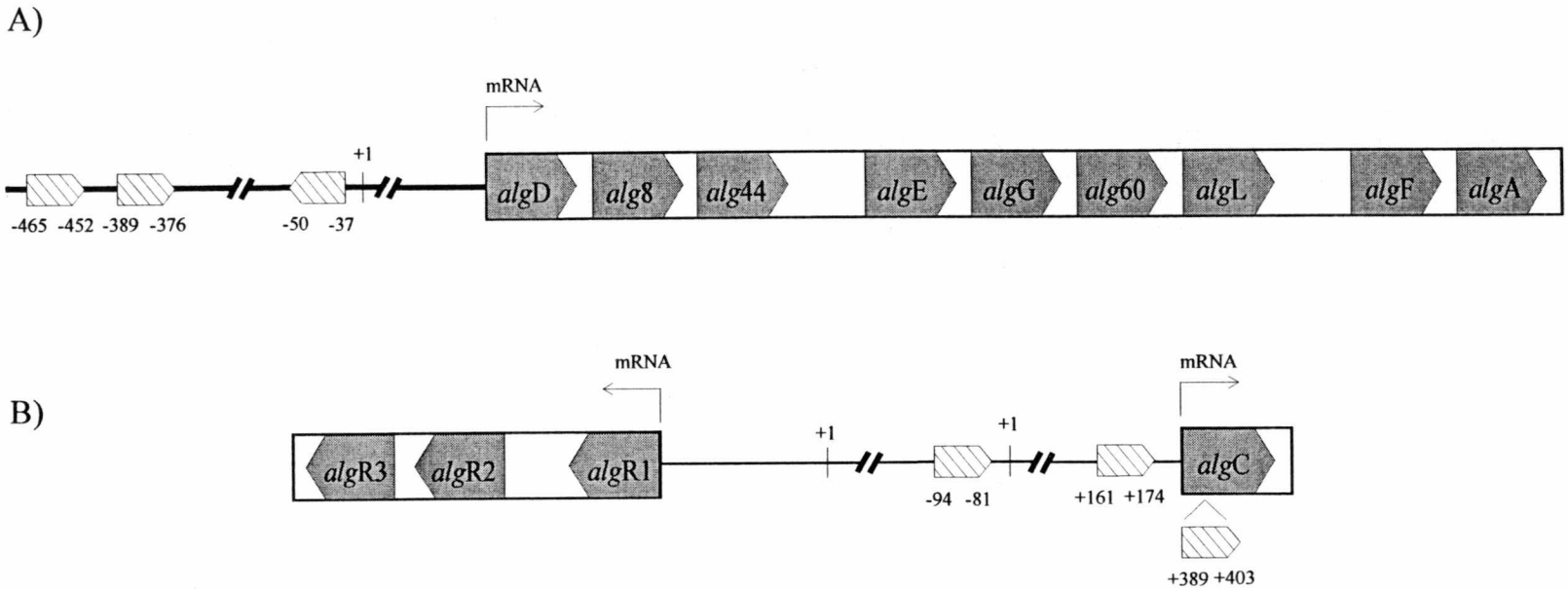


Figure 3. Organization of two major operons involved in the regulation and biosynthesis of alginate in *Pseudomonas aeruginosa* FRD1. (A) Location of the AlgR1 binding sites (▨) and the biosynthetic genes (■) at 34 minuites on the FRD1 chromosome, with respect to each other and the transcription start site. (B) Location of structural genes for three regulatory elements (*alg*R1, *alg*R2, and *alg*R3) and the *alg*C biosynthetic gene. The orientation of each genetic element is designated by the direction of the symbol.

The only alginic acid biosynthetic enzyme not encoded by the major operon at 34 min is *pmm* [Zielinski et al. 1992]. This gene is found at 10 min on the chromosome and has a close proximity to the *alg*R1, *alg*R2 and *alg*R3 operon. This gene produces phosphomannomutase and has recently received the alternate designation *alg*C. Some scientists are opposed to changing the name because of the involvement of this enzyme in lipopolysaccharide biosynthesis [Goldberg et al. 1993]. The *alg*C gene (*pmm*) has been found to be partly constitutive, but is still up-regulated in the presence of AlgR1 (binding sites for AlgR1 are shown in Figure 3B) [Fujiwara et al. 1993]. AlgC transcription is not dependent on AlgU, which may be a necessity for its involvement in other biosynthetic processes. However, AlgU can indirectly enhance *alg*C transcription though its effects on *alg*R1 transcription.

The AlgB regulatory protein is encoded by the region of DNA localized at 13 min [Wozniak and Ohman 1993]. AlgB has shown homology with the response regulators of the superfamily of two component regulators [Wozniak and Ohman 1993], but its exact role in alginate induction is unknown. It is known, however, that the *alg*B gene is induced in response to increasing osmolarity, and that it is essential for full induction of the *alg*D promoter [Deretic et al. 1994].

Biofilm formation and development (overview)

Biofilm formation is an important developmental growth mechanism for many bacteria in oligotrophic habitats [Christensen and Characklis 1990]. Under these nutrient

poor conditions, there is a concentration of nutrients at surfaces which are commonly referred to as conditioning films [Characklis 1990]. These conditioning films are a target for bacterial attachment. Van Loosdrecht et al. 1990 have described three specific mechanisms which may be used by bacterial cells in order to reach a substratum. These include: brownian motion, convective transport, and active movement. Once cells have reached the surface by one of these mechanisms the cells may reversibly/irreversibly associated with the surface. During this initial attachment the physiochemical properties of the surface become very important. These properties include the hydophobicity of the surface [Wieneek and Fleetcher 1995], surface charge, critical surface tension, and Van der Waals electrostatic interactions [Van Loosdrecht et al. 1990 and Characklis 1990]. Primarily, the Van der Waals interactions are critical to the initial attachment process. These electrostatic forces create a primary and secondary maxima which are dependent on the properties of the substratum and the bacterial cell [Van Loosdrecht et al. 1989]. The primary and secondary minima are affected by environmental influences such as medium osmolarity [Characklis 1990]. The rational behind these maxima are that there are certain distances from the surface at which cell-substratum interactions could occur [Characklis 1990]. The secondary maximum would exist some distance from the surface (~5 - 10 nm), and interactions within this region would qualify as reversible adhesion processes. The primary maximum would occur much closer to the surface (<1.0 nm), and cells bound at this maximum would be considered irreversibly bound. Attachment at the primary maximum would have to utilize cellular structures such as flagella, fibriae, pili,

or polymers in order to form strong links (irreversible) with the surface, because the repulsive force, as the cells approached the surface, could increase dramatically [Characklis 1990]. The final step in bacterial colonization of a surface involves the reproduction of bacterial cells, along with secretion of EPS to form a multi-layered biofilm [Davies et al. 1993]. Growth of the biofilm would be accompanied by entrapment of new bacterial cells and nutrients.

The role of EPS in these bacterial biofilms has received enormous attention during that last 20 years. During this time, scientists have postulated about the importance of exopolymers in the initial mechanisms of bacterial associations at surfaces. Previously, some studies have shown that capsules promote the initial attachment to surfaces [Christensen et al. 1985]. However, in these studies, it is hard to elaborate on whether the polymer was produced prior to attachment or as a result of attachment. In one particular study by Christensen et al. 1985, the bacterium *Pseudomonas* NCMB 2021 was shown to produce a discrete capsular material which was postulated to help this bacteium to attach. In this experiment, however, washed cells retained the ability to attach to surfaces and, as such, the study was unable to unequivocally prove that EPS was involved in the adhesion process. Other researchers believe that other structures such as fimbriae and pili are responsible for the initial attachment to surfaces (described above). Wrangstadh et al. 1988 indicated that in their experiments EPS actually decreased absorption, and that EPS was not required for adhesion. Regardless of whether scientists

agree or disagree over the roles of EPS during the initial attachment, it is clear that exopolymers are intimately involved in the growth and maturation of the biofilm.

Methodologies for monitoring biofilm processes

The ability to reproducibly study biofilm processes has, in the past, posed a major problem for scientists wishing to conduct studies, *in situ*, to study bacterial attachment, biofilm formation, and subsequent biofilm development. Primarily, these problems are the result of inadequate technologies for the monitoring of these processes. One reason behind the lagging development of *in vivo* characterization methodologies has probably been the widespread use of liquid monocultures to characterize bacterial processes. These techniques were a requirement for the type of work in which they were being used, but once researchers were past the basic microbiology, there was a need to extrapolate on what they had learned in batch culture and study bacteria as they exist in an environmental sense. Unfortunately, this proved to be a major problem, because the techniques for this kind of study were in their infancy.

Several early techniques for the study of bacterial sorption included adaptations of batch culture techniques. One such experiment by Fletcher 1976 [described by Mittleman et al. 1992] included simple experiments in which glass slides were placed in a simulated natural stream flow system. These slides were then examined using spectrophotometry to measure the optical density (OD) at 590 nm. Prior to the OD readings these glass coverslips were rinsed and fixed using conventional techniques.

Sutherland 1990 describes the use of scanning electron microscopy, transmission electron microscopy, and light microscropy for examination of exopolymeric material associated with biofilm. Vandevivere and Kirchman 1993 have used optical microscopy in order to examine exopolymer production by attached bacteria. Another common technique for the enumeration of cells within a biofilm involves extraction of biofilm members and examination using viable cell counting techniques, such as acridine orange direct counts, and bacterial lipid analyses [Angell et al. 1992]. It is unfortunate that these techniques require that the biofilm be destroyed in order to examine the biofilm bacteria. This makes it difficult to develop an on-line monitoring method based on these techniques.

Recently, several new techniques have evolved for the study of microbial biofilms. These include confocal laser microscopy (CLM) [Costerton et al. 1994 and Lawrence et al. 1994], electrochemical impedance spectroscopy (EIS) [Mittleman et al. 1991], attenuated total reflectance Fourier transform spectroscopy (FTIR) [Mittleman et al. 1991] and computer enhanced microscopy (CEM) [Caldwell et al. 1986]. Although these techniques are adequate at achieving a through study of a biofilm, the necessary equipment is expensive and they are unable to distinguish between viable and nonviable bacterial cells within the biofilm [Mittleman et al. 1991]. One of the best methods, from this assortment for the study of biofilms, is the use of the confocal laser microscope. Lawrence et al. 1994 have successfully monitored diffusion of labeled nutrients in an actively growing biofilm using this method. Dalton et al. 1994 have also used the confocal laser microscopy to examine the infrastructure of the biofilm. Findings from

this research have shed much light on the mechanisms of nutrient transport within biofilm communities.

Since useful techniques are being developed for studying the physiology of the biofilm, scientists are now attempting to create modern molecular microbiological techniques for the study of the genetics within biofilms. One of the most important molecular tools that is being developed is reporter gene technology. Molecular reporters can be used to study the activation of specific genes within biofilm bacteria. Perhaps the most common type of reporter construct for the study of gene activation in biofilms has been the use of LacZ reporters. Davies and Geesey 1994 have developed an *alg*C-*lac*Z transcriptional fusion and utilized this in continuous culture for the study of *alg*C expression. Specifically, they were looking at whether *alg*C in attached bacterial cells was upregulated compared to unattached bacteria. In their experiments they combined the use of reporter gene technology with FTIR, which allowed *in vivo* detection of *alg*C induction. Another type of gene reporter which is being developed for the study of biofilms utilizes the *lux* genes from *Vibrio fischeri* (the use of these genes in modern biofilm studies is discussed in detail below).

Bioluminescence for monitoring promoter activation

Bioluminescent organisms can be found in marine, freshwater, and terrestrial environments [Hastings et al. 1985]. Generally, the most common habitat for these microbes are free living in the ocean as saprophytes growing on dead marine life, as

parasitic organisms in insects, and in symbiotic relationships in teleost fishes [Meighen 1991]. For these microorganisms, the production of light has important roles in their survival [Hastings and Nealson 1977]. Several examples of bacteria which are bioluminescent include *Photobacterium. phosphoreum*, *Vibrio fischeri*, and *Vibrio harveyi* [Hastings et al. 1985]. Scientists have isolated and characterized the proteins and genes involved in the light production processes of these organisms, and have capitalized on their findings and used the essential elements to develop novel microbiological tools for studies of gene expression [Heitzer et. al. 1994].

Bioluminescence as a method of monitoring gene activity provides a rapid non-destructive way in which the induction of specific genes in the bacterial cell may be examined [Arrage et al. 1995 and Engebrecht 1985]. Primarily, one of the most common mechanisms for bioluminescence, in microbiological studies, utilizes the *lux*CDABE operon from *Vibrio fischeri*. This *lux*CDABE gene cassette encodes both the luciferase and the fatty acid reductase enzymes [Meighen 1991]. The genes *lux*C, *lux*D, and *lux*E encode the multi-subunit fatty acid reductase. Specifically, LuxC acts as the reductase, LuxE as the synthetase and LuxD as the transferase in the complete enzyme. The protein products of the *lux*A and *lux*B genes form the heterodimeric luciferase and are involved in the light producing reaction. Scientists have mobilized this gene cassette into useful bacterial vectors, and have strategically removed the regulatory regions located upstream of the *lux* structural genes. This allows a specific promoter and operator region from

some third party regulatory system to be cloned into the *lux* vectors and utilized in studies examining the regulation of this third party regulatory system.

There are many advantages and disadvantages to using this type of reporter system as a method of monitoring gene expression [Meighen 1991]. Perhaps the greatest advantage is the high sensitivity of the luciferase in bacterial cells. It has been observed that other systems require both longer assay times and have a substantially higher detection limit [Meighen 1991]. Another major advantage of the *lux* system is the ability to monitor light *in vivo* without destroying the cells, allowing the monitoring of gene expression over time. Having an assay which does not rely on the destruction of the cell also allows other assays to be coupled with the bioluminescence assays. One example of this is the utilization of mRNA analysis in cultures which contain a bioluminescent reporter plasmid [Wallace et al. 1994a]. There are some disadvantages to using bioluminescence to monitor gene expression, however. The light reactions are more complex than the other gene reporter technologies, and there is always the possibility of another cellular process affecting the light production. This point is further illustrated by the fact that the luciferase reaction is dependent on $FMNH_2$, O_2, and aldehyde concentrations [Hastings and Nealson 1977]. Also, in experiments by Heitzer et al. 1994, it was observed that bioluminescence was affected by various toxic compounds, perhaps through perturbations in the membrane. These differences should not over shadow the usefulness of bioluminescence in experimentation. This is especially true for research in

which the focus is toward an environmentally relevant point of view, and a model test system is required in which no outside disturbances may exist.

Concluding remarks

The coupling of bioluminescent gene reporter technology and studies of biofilm formation and development will allow several current problems to be alleviated in the study of alginic acid biosynthesis in bacterial biofilms. One such problem is that the alginate biosynthetic enzymes have relatively low enzymatic activities [Zielinski et al. 1990]. Therefore, there is a potential problem with conventional reporter systems. Since the bioluminescence genes have a very low detection limit, it is probable that a bioluminescent reporter gene technology will be better suited for examination of the intricate regulator networks which are responsible for alginate production. It is also reasonable to assume that having a very low detection limit will facilitate bioluminescence monitoring within a developing biofilm. Another potential problem for the study of biofilms is the lack of numerous gene reporter technologies for the *in vivo* study of gene expression. One example of the utility of the *lux* system, is the usefulness of bioluminescent reporter strains for examination of the effects of environmental stimuli on gene expression. This is true because once the bacterial population has stabilized numerous environmental stimuli can be displayed to the biofilm bacteria and there responses measured spectrophotometrically. This allows rapid, non destructive, *in situ* monitoring which may be maintained for an indefinite time (theoretically). However, the

literature specifies that there are numerous factors which could effect bioluminescence other than induction of the regulatory region of interest. Therefore, during experimental design, adequate controls must be developed and applied toward any interpretation of data.

CHAPTER III

MATERIALS AND METHODS

Bacterial strains and growth media

Bacterial strains and plasmids used in this study are listed in Table 5 and Table 6, respectively. Maintenance of environmental isolates and taxonomic classification by fatty acid methyl ester (FAME) analysis [Stead 1988] and the Biolog Identification System (Hayward, Calif.) have been described previously [Wallace et al. 1994b]. Frozen stocks of each isolate were cataloged and used to start working cultures on *Pseudomonas* Isolation Agar (PIA, Difco) plates without additional antibiotics. Environmental strains and FRD1 harboring pUTK50 were selected by growth on PIA plates containing either one or both kanamycin sulfate (Sigma) or carbenicillin disodium salt (Sigma) at concentrations of 300-1000 μg/ml of each. *Escherichia coli* strains were stored as frozen stocks and working cultures were grown in Luria Broth (LB; 10 g tryptone, 5 g yeast extract, 5 g NaCl per liter) or on Luria Agar (LA) plates (LB plus 15 g agar per liter) including 30-50 μg/ml of the appropriate antibiotic or combination of antibiotics. *E. coli* harboring pUTK50 was cultured in the presence of both kanamycin and ampicillin, and *E. coli* harboring pRK2013 was cultured in the presence of kanamycin. All incubations were at 30°C.

Liquid culture studies were carried out in a defined alginate-promoting medium

Table 5. Bacterial strains

Bacterial Species	Strain	Description	Source or Reference
E. coli			
	HB101	*pro*A2 *leu*B6 *thi*-1 *hsd*R *hsd*M *rec*A13 *sup*E44 *rps*L20	Wallace et al. 1994a
A. veronii			
	310	Prototrophic, environmental isolate	Wallace et al. 1994b
B. pumilus			
	H	Prototrophic, environmental isolate	Wallace et al 1994b
P. aeruginosa			
	FRD1	Prototrophic, Alg^+ cystic fibrosis isolate	D. Ohman
	FRD1 (pUTK50)	Prototrophic, Alg^+ transconjugate harboring pUTK50 (Kan^R Crb^R), Lux^+	Wallace et al. 1994a
	322	Prototrophic, Alg^+ Kan^R environmental isolate	Wallace et al. 1994b
P. alcaligenes			
	324	Prototrophic, environmental isolate	Wallace et al. 1994b
	347	Prototrophic, environmental isolate	Wallace et al. 1994b
P. aureofaciens			
	G	Prototrophic, environmental isolate	Wallace et al. 1994b
P. fluorescens C			
	M	Prototrophic, Crb^R environmental isolate	Wallace et al. 1994b
	M39	Prototrophic transconjugate harboring pUTK50 (Kan^R Crb^R), Lux^+	This study
P. fluorescens E			
	N	Prototrophic, Crb^R environmental isolate	Wallace et al. 1994b
	N15	Prototrophic transconjugate harboring pUTK50 (Kan^R Crb^R), Lux^+	This study
P. putida			
	I	Prototrophic, environmental isolate	Wallace et al. 1994b
	L	Prototrophic, Crb^R environmental isolate	Wallace et al. 1994b
	L23	Prototrophic transconjugate harboring pUTK50 (Kan^R Crb^R), Lux^+	This study
P. syringae			
	P	Prototrophic, Crb^R environmental isolate	Wallace et al. 1994b
	P4	Prototrophic transconjugate harboring pUTK50 (Kan^R Crb^R), Lux^+	This study
S. maltophilia			
	O	Prototrophic, Crb^R environmental isolate	Wallace et al. 1994b
	O46	Prototrophic transconjugate harboring pUTK50 (Kan^R Crb^R), Lux^+	This study

Abbreviations: Crb, carbenicillin; Kan, kanamycin; Rif, rifampicin; ^{R}, resistant; ^{S}, sensitive.

Table 6. Plasmids

Plasmid	Functional Utility	Relevant Characteristics	Source or Reference
pRK2013	Helper in triparental matings	48kb, ColE1 *mob*$^+$ *tra*$^+$ (RK2) Kan^R Amp^S	Figurski and Helinski, 1979
pDJW126	Template for *alg*D mRNA probe.	*alg*D structural gene in pBluescript	D. Ohman
pUTK50	*alg*D-*lux* bioluminescent reporter	18.7 kb, IncW Kan^R Crb^R (1.2kb *Eco*RI-*Hind*III fragment containing the *alg*D promoter inserted upstream of the *lux*ABCDE cassette in promoterless vector pUCD615)	Wallace et al., 1994a.

Abbreviations: Amp, ampicillin; Kan, kanamycin; ^{R}, resistant; ^{S}, sensitive.

(APM) modified from Mian et al. 1978 and Ohman and Chakrabarty 1981. All reagents used were analytical grade, and were from Sigma, Fisher, or Mallinkrodt. The medium contained 100 mM sodium D-gluconate, 100 mM monosodium glutamate, 10 mM $MgSO_4$, 16.8 mM K_2HPO_4 and 7.5 mM NaH_2PO_4 at pH 7.0 (adjusted with H_3PO_4) and was sterilized by filtration through 0.2 μm cellulose nitrate membranes. APM plates were made by including 15 g per liter of purified agar or agarose and were useful for providing transparent solid media for detection of bioluminescence on plates (below).

For studies on the effects of increased salt concentration, a filter-sterilized stock solution of 5.0 M NaCl was added to yield concentrations of 100 mM, 200 mM, and 300 mM. For studies with limited amounts of NH_4^+ or NO_3^- as nitrogen source, monosodium

glutamate was omitted and filter sterilized stock solutions and mixtures of $(NH_4)_2SO_4$ and/or $NaNO_3$ were added so that the amount of nitrogen in either oxidation state totaled 10 mM; the control in these experiments is designated as that with 10 mM NH_4^+ and 0.0 mM NO_3^-. All liquid cultures included 50 μg/ml or 100 μg/ml kanamycin and 50 μg/ml or 100 μg/ml carbenicillin to maintain selective pressure on pUTK50. All biofilm studies were carried out in a defined medium developed by Ohman and Nivens [Nivens, personal communication] and contained in grams per liter, 0.015 g monosodium glutamate, 0.045 g glycerin, 0.009 g $MgSO_4$, 8.5 g NaCl, 0.0586 g K_2HPO_4, 0.0208 g NaH_2PO_4. Immediately prior to use the media was amended with 50 μg/ml kanamycin to maintain selection for the bioluminescent reporter plasmid pUTK50.

Transfer of pUTK50 into environmental strains

Triparental matings, using helper plasmid pRK2013, were carried out as described previously [Figurski et al. 1979 and Wallace et al. 1994A]. Initial screenings for transconjugates were on PIA with 500 μg/ml kanamycin. All plasmid manipulations were as previously described [Sambrook et al. 1989]. Potential bioluminescent strains were randomly selected (50-100 colonies) and plated in grids on PIA plates with appropriate antibiotics. Colonies acquiring antibiotic resistance were exposed to X-ray film. Non-bioluminescent environmental isolates were challenged further by growth on PIA with 500 μg/ml kanamycin and 500 μg/ml carbenicillin. Screening for transconjugates of *P. putida* L and *P. fluorescens* N was on PIA with both 500 μg/ml

kanamycin and 500 μg/ml carbenicillin. Among the collection of bioluminescent environmental strains generated, one individual from each strain was selected for liquid culture studies: *S. maltophilia* O46, *P. fluorescens* M39, *P. putida* L23, and *P. fluorescens* N15. Plasmid preparations confirmed that these strains harbored pUTK50.

Detection and monitoring of bioluminescence in batch cultures

In order to measure bioluminescence, 2.0 ml aliquots from liquid cultures were placed in scintillation vials and placed in front of a liquid light pipe in a chamber protected from ambient light. The pipe was fed into a photomultiplier tube (model 77340, Oriel, Stratford, CT) and a digital detector (model 7070, Oriel, Stratford, CT). Light output was displayed as an amperometric signal, expressed in nanoamperes (nA), and divided by the sample's absorbance at 600 nm in order to normalize light output to different levels of cell growth. Aliquots were taken at regular intervals in order to maintain a consistent monitoring of *alg*D induction over a one to three day period. Specific care was taken to ensure that periods of increasing bioluminescence were monitored. For comparative analysis, the peak bioluminescence was used to determine the bioluminescence induction factor (BIF). The BIF was derived by dividing the normalized amperometric signal by the appropriate control (eg. no added factors or NH_4^+ only). Thus it became possible to compare results from high-expression systems as in FRD1 to those from lower but significant expression systems in the environmental isolates.

Exopolymer analysis

In order to isolate EPS, aliquots from liquid cultures (0.5 ml) were subjected to centrifugation in a microcentrifuge tube for 15 min at 4°C to pellet cells. The cell free supernatants (0.4 ml) were carefully transferred to a new tube and 5.0 M NaCl was added (0.014 ml) for a final concentration of 175 mM. Ethanol (1.0 ml) was added and the insoluble materials were allowed to precipitate overnight at -20°C [Cote et al. 1988 and Fett et al. 1986]. Samples were subjected to microcentrifugation for 15 min at 4°C. Pelleted material was dried and completely redissolved in 50 mM Tris·HCl pH 8.0, 10 mM EDTA, 175 mM NaCl (0.4 ml). Ethanol (1.0 ml) was added and samples were reprecipitated at -20°C, overnight. Samples were microcentrifuged for 15 min at 4°C, and the pelleted material was dried and rinsed with 70% ethanol (1.0 ml). Rinsed pellets were dried and stored at -20°C until assayed.

Dried exopolymeric material from environmental isolates and FRD1 was redissolved in sterile distilled deionized water (0.4 ml) and examined using the metahydroxybiphenyl/H_2SO_4/borate (MHB) assay, as described by Blumenkrantz and Absoe-Hansen 1973. Aliquots of the dissolved exopolymeric material (0.1 ml) were placed in individual 1.5 ml eppendorf tubes, and 0.6 ml of a H_2SO_4/sodium tetraborate solution (0.0125 M tetraborate dissolved in concentrated sulfuric acid) was added to the eppendorf tubes. These were vigorously vortexed and chilled in an ice bath for 5 min. The chilled solutions were then heated, for 5 min, in a boiling water bath at 100°C. Immediately, heated samples were placed in the ice bath for 5 min. 20µl of a 0.15%

meta-hydroxy-diphenyl solution (prepared in 0.5% NaOH) was then added to the chilled samples, which were then vortexed for 2 min. These mixtures were then allowed to incubate for 30 min, which allowed the hydrolyzed uronic acid residues to react with the meta-hydroxy-diphenyl allowing formation if a chromagen which may be detected at 525 nm. Following incubation periods, the mixtures were analyzed spectrophotometrically at 525 nm, using a Beckman DU-70 Spectrophotometer.

Ethanol precipitated exopolymeric material from the environmental isolates and FRD1 was also examined using ion chromatography (Dionex Chromatography System, Sunnyvale, CA), as previously described [Franklin et al. 1994]. EPS samples were weighed and 2.0 mg of the material was combined with 1.5 ml of 18 MΩ distilled, deionized, and filter sterilized HPLC grade water. These were mixed with 1.5 ml of 4.0 M trifluoroacetic acid (TFA) in order to hydrolyze the high molecular weight exopolymer into its monomeric constituents. Hydrolyzed EPS samples were dried under nitrogen, resuspended in 2.0 ml of HPLC grade water (same as above), passed through a C_{18} column and filtered through a 0.22 μm Millex-GV4 (Millipore) filter. 25 μl of filtered sample was analyzed using a Dionex series 4500 HPLC with a pellicular CarboPac PA1 anion-exchange column and a PA1 guard column. The mobile phase was 100 mM NaOH at a flow rate of 1.0 ml/min. A linear gradient of sodium acetate was applied from 0.0 to 0.1 M, which was applied 20 min after injection. Monomeric sugars were detected by a Dionex pulsed electrochemical detector (PED) operating in the integrated amperomeric mode. Electrode potentials were E_1 0.1 V, E_2 0.6 V, and E_3 0.8 V, and were applied for

300, 120 and 300 ms, respectively. Sodium alginate (Sigma; high viscosity, from seaweed) was used as the standard for the ion chromatography and MHB analyses.

Messenger RNA analysis

To further characterize *alg*D expression in response to changing environmental stimuli, total RNA (tRNA) from FRD1 batch cultures was probed with anti-sense *alg*D mRNA transcripts to insure that induction of bioluminescence correlated with concomitant *alg*D gene expression, as previously described [Figure 4; Wallace et al. 1994a]. Samples for mRNA analysis, consisted of 1.0 ml of liquid culture. These were taken at the same time as samples for bioluminescent measurements and exopolymer assays. Culture aliquots were centrifuged at 11,000 rpm for 10min at 4°C to pellet cells for use in mRNA isolation and quantitation. These cell pellets were immediately washed with ice cold STE and maintained at -80°C until assayed.

Total RNA was obtained using the hot phenol method as described previously [Wallace et al. 1994a]. Cell pellets were resuspended in 500.0 µl lysis buffer (0.05 M sodium acetate, 0.05 M NaCl, 0.003 M EDTA, 1.0% SDS, pH 5.2) which was preheated to 60°C. 250.0 µl each of phenol equilibrated with lysis buffer and chloroform/isoamyl (24:1) was added to the cell suspension, vortexed vigorously for 1.0 min, incubated at 60°C for 5.0 min, and immediately chilled on ice. Suspensions were centrifuged and supernatants reextracted with phenol/chloroform. Nucleic acids were precipitated by addition of 0.3 M sodium acetate and 2.0 volumes of ethanol amended with 1.0 µl

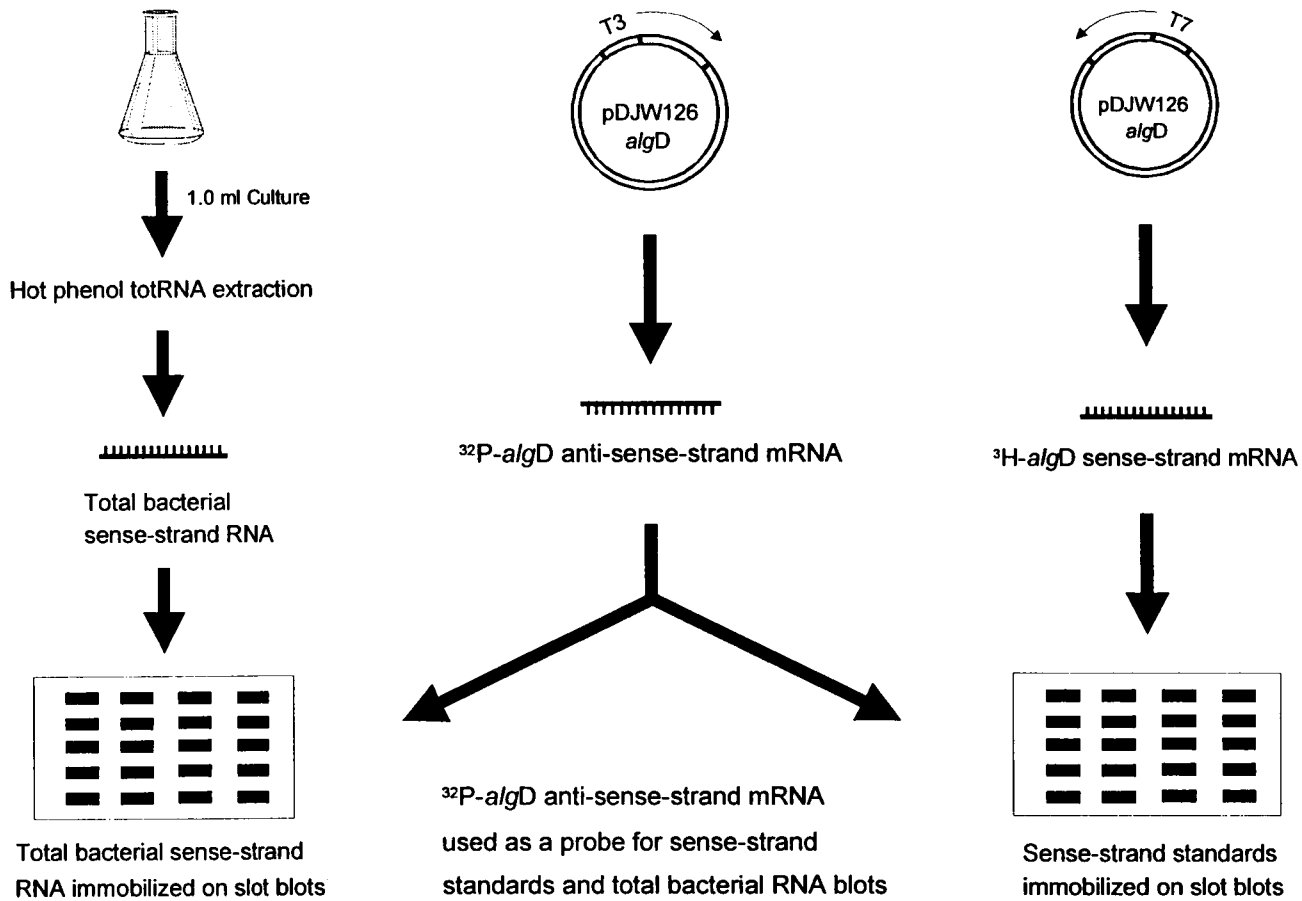


Figure 4. Flow diagram for *algD* mRNA probing studies using in vitro transcription from the T7 and T3 promoters of pDJW126 to generate *algD* sense strand standards and *algD* anti-sense mRNA for probing the *algD* standards and the total bacteial RNA blots

glycogen to aid in precipitation of small quantities of RNA (<10 μg) . The pellets were then washed with 70% ethanol, dried under vacuum, and redissolved in 50 μl Tris-HCl, 1.0 mM EDTA, pH 8.0. RNA preparations were DNase treated, reextracted with phenol/chloroform, and resuspended in diethylpyrocarbonate (DEPC) treated dH_2O. The optical density at 260 nm was determined and these total RNA preparations were stored at -80°C until immobilized onto nylon membranes.

Once RNA samples were spectrophotometrically quantified aliquots of 5.0 μg were denatured in a 1X SSC solution containing 50% formamide, 7% formaldehyde, 0.15M NaCl, 15 mM sodium citrate (pH 7.0) at 68°C for 15 min. These were cooled in an ice bath and two volumes of 20X SSC with 3.0 M NaCl, 0.3 M sodium Citrate, pH 7.0 were added. Denatured samples were vacuum blotted onto 0.2 μm microporous nylon membranes (ICN, Costa Mesa, Calif.) using a Bio-Rad slot blot apparatus (Melville, NY) with a 30.0 cm H_2O vacuum. Slot blot wells were washed twice using 500 μl of a 10X SSC solution containing 1.5 M NaCl, 0.15 M sodium citrate. The membranes with the attached tRNA were air dried, UV irradiated and baked at 80°C for 1.0 hour, in order to affix nucleic acids to the membrane.

The *alg*D mRNA probes and standards were synthesized from pDJW126, which contains a 1.4 kb *Hind*III-*Eco*R1 fragment of the *alg*D structural gene and promoter cloned into the multiple cloning site of pBluescript (KS), allowing either sense or antisense strands to be transcribed using either the T7 or T3 promoters within pDJW126 [Figure 4; Wallace et al. 1994a]. All in vitro transcription reactions were done according

to the manufacturer's recommendations (Stratagene, La Jolla, Calif.). Sense strand *algD* mRNA was quantified by determining the amount of ^{3}H-UTP (ICN, Costa Mesa, Calif.) incorporation into the transcript, allowing a precise standard curve to be generated. Standard *algD* mRNA was vacuum blotted as above. Slot blots containing the tRNA and the standard *algD* mRNA were prehybridized for 2.0 hour with 30.0 ml of 5X SSPE (pH 7.4) containing 50% deionized formamide, 0.0375 M NaCl, 2.5 mM $NaHPO_4$, 0.25 mM EDTA, 0.1% Ficol, 0.1% polyvinylpyrrolidone, 0.1% BSA (5X Denhardt's), and 100.0 μg/ml herring sperm DNA. A ^{32}P-labeled *algD* anti-sense RNA probe was synthesized using the T3 promoter in pDJW126 as described above, added to the prehybridization solution and allowed to incubated overnight at 65°C. Following hybridization, blots were washed twice with 2.0X SSC (pH 7.0) plus 0.3 mM NaCl, 0.03 M sodium citrate, and 0.1% SDS and twice using 0.1X SSC, 0.1% SDS at 65°C for 15 min. Blots were subjected to autioradiography using X-ray film (Eastman Kodak, New Haven, CT, X-Omat RP) with intensifying screens at -80°C for 24 - 48 hours. These autioradiograms were digitized on a Visage 110 computer assisted imager (Millipore, Bedford, Mass.) and compared with the *algD* mRNA standard curve to determine the amount of *algD* message present.

Detection and monitoring of bioluminescence and biomass in bacterial biofilms

Laminar flow biofilm reactors were used to monitor *algD* expression *in situ* within a developing biofilm composed of either FRD1 or an environmental strain O46.

Two types of flow cells were used in this study, which shall be designated as reactor A and reactor B. Both were based on previously developed laminar flow cell systems [Mittleman et al. 1992] and resulted from two different modification strategies.

Reactor A contained a 1 inch by 2 inch stainless steel coupon located within a 1 inch X 5 1/8 inch X 1.0 mm flow channel. This coupon served as the substratum for bacterial attachment and subsequent biofilm growth. This reactor was generated from a joint project involving work by Lisa Kohring, Andrew Arrage, and Peter Angell (unpublished data). It was utilized in preliminary experiments, in order to aid in the development of reactor B, which was specifically designed for characterization of the bioluminescent reporter strains. Reactor A contained two viewing ports, each containing a quartz lens located directly above the stainless steel coupon, and were used for bioluminescence, NADH and tryptophan measurements. These ports had an effective sampling size of 1.0 cm^2.

Reactor B was developed specifically for this research project and did not contain a stainless steel coupon, but maintained the laminar flow channel design developed for reactor A. The housing was constructed of high density polyethylene (same as reactor A). A 2.0 mm thick glass plate was fitted between the top and bottom pieces of the flow cell. This glass plate was necessary in order to block the ultraviolet fluorescence from the bottom flow cell piece, which emits a significant fluorescence signal upon excitation at 295 (used for tryptophan) and at 360 (used for NADH). Reactor B was designed to use a 7/16 inch X 6 inch X 1.0 mm silicon gasket instead of the O-ring used in the previous

design (reactor A). It was hoped that this would help seal the flow channel thereby diminishing the numerous air bubbles commonly introduced into the system during normal operation. This gasket also facilitated use of the glass substratum by requiring less compression force which could have potentially broken the glass plates. The lens was a 1 inch X 2 inch X 1.0 mm quartz plate (GM Associates, Oakland California) and was secured into the flow cell top using silicone sealant (Sears Roebuck and Co., Chicago, IL). The dimensions for reactor B are outlined in Appendix A.

Reactor assembly proceeded as follows: 1) The HDPE body pieces and the glass plate were cleaned in a 1:50 dilution of PCC-54. 2) These pieces were then rinsed in a 0.1N NaOH solution in order to remove previous charge modifications to the HDPE surface. 3) These were thoroughly dried and a black piece of felt was placed on the inner face of the bottom flow cell body component and the glass plate was placed on top of the felt. 4) The top was carefully fitted over the glass plate and was secured using 2 ½ inch bolts. 5) The flow cell was carefully wrapped in cardboard in order to provide some protective support during transportation, and was wrapped using ethylene oxide sterilization paper. 6) The flow cells were then sterilized using ethylene oxide by the University of Tennessee Medical Services. (Note: Reactor A assembly was the same as reactor B assembly, except the glass plate and felt were not necessary because of the metal coupon, and were not used.)

Bioluminescence readings were taken by protecting the flow cell from stray light and taking light readings through the view ports of reactor A and the lens of reactor B,

using a liquid light pipe and digital detector (same as for liquid cultures) to monitor light output as an amperomeric signal. Similarly, NADH and tryptophan estimates were obtained using a Spex Instruments Fluorolog II spectrofluorometer (Edison, NJ) to monitor fluorescence at 460 and 342, respectively, as described previously [Angell et al. 1993].

Sterile biofilm media (described above) was supplied to the previously sterilized flow cell (using ethylene oxide) at a flow rate of 2.5 ml/min. Once the system had become saturated with media the flow was stopped and 10.0 ml of a FRD1 or O46 overnight starter culture, containing 50.0 ml of biofilm media, was introduced into the flow cell, which was then clamped off from the rest of the system, and allowed to incubate so that bacterial cells adapted for attachment could associate with the metal coupon or glass plate. After 1.0 hour, the flow cell was unclamped and flow was resumed at the previous flow rate. Once flow resumed, periodic measurements were taken to monitor bioluminescence, NADH, and tryptophan. In some cases the flow cells were dismantled before the full time course of the experiment in order to sample the biofilm directly for alginate (or other EPSs) and to perform acridine orange direct counts (AODC) and viable cell counts. During these samplings a 1.0 cm^2 area of the biofilm corresponding to the areas monitored by the view port were extracted by clamping a glass extractor sealed with an O-ring onto the coupon, followed by three brief sonications into 1.5 ml of sterile basal salts media. These samples were the source for AODCs,

viable cell counts, and Dionex studies on the supernatant once cells were removed via centrifugation.

CHAPTER IV

RESULTS

Selection of environmental strains for exopolymer studies

In a previous study, 120 environmental isolates were taken from corroded pipes (copper and stainless steel) and reactor debris at a nuclear power plant [Wallace et al. 1994b]. DNA samples isolated from these environmental strains were subjected to slot-blot hybridization using four gene probes specific to sequences from the *P. aeruginosa* FRD1 chromosome encoding regulatory and structural genes in the alginic acid biosynthetic pathway (*alg*D, *alg*G, *alg*-76, and *alg*B). Eleven isolates exhibited sequence homology to at least one of the four probes [Table 1;Wallace et al. 1994b]. Among those strains, the following were shown to contain DNA sequences homologous to all four alginate gene probes: *P. aureofaciens* G, *B. pumilus* H, *S. maltophilia* O, *P. fluorescens* M, *P. syringae* P, *P. aeruginosa* 322, *P. putida* L and *P. alcaligenes* 324 [Table 1; Wallace et al. 1994b]. It has been postulated that several of these bacterial strains may have the potential for alginic acid biosynthesis. Environmental isolates were chosen, for construction of potential bioreporter strains, based partially on these alginate gene similarities.

In order to maximize the possibility for the successful construction of an effective *alg*D reporter bacterium, these potential environmental isolates [Table 5] were screened for uronic acid production using the MHB assay, as outlined above in materials and

methods. The results from these analyses are shown below in Figure 5. For these analyses, cultures were grown in APM without antibiotics, as a general procedure for all strains that were tested. This was done to ensure that conditions were not variable from one strain to another and that the data were as accurate as possible. Also, values were normalized to the culture's optical density in order to correct for varying growth rates between different strains.

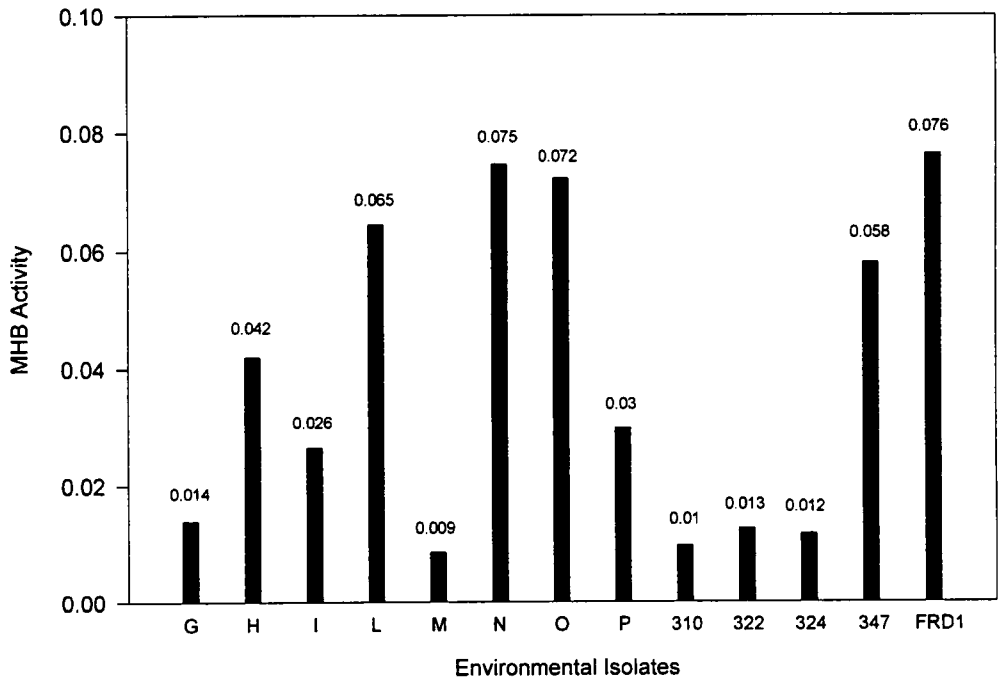


Figure 5. Relative uronic acid production in environmental isolates used in this investigation (Metahydroxybiphenyl/H_2SO_4/borate (MHB) assays for environmental bacterial strains and FRD1 grown in alginate promoting media (APM))

Based on these data, six strains were chosen to be hosts for the bioreporter plasmid pUTK50. These included *P. putida* L, P. *fluorescens* M, *P. fluorescens* N, *S. maltophilia O, P. syringae* P and *P. aeruginosa* 322. 322 and M were picked because of their very high alginate gene similarities [Table 1], which were the highest for any of the environmental strains. However, preliminary MHB analyses did not indicate that these strains produced copious amounts of uronic acids [Figure 5]. This was surprising given the mucoid colony morphology visible on the PIA plates used for the inoculum. L, O, and P were chosen because they demonstrated high alginate gene similarities [Table 1] and because for these bacteria the carbohydrate assays indicated the presence of uronic acids in culture supernatants [Figure 5]. With the exception of L, these bacteria were not as mucoid as 322, FRD1, and M on PIA plates. Lastly, N was chosen based on the apparent presence of uronic acids despite low alginate gene similarities [Figure 5 and Table 1], and it was thought that it would be an interesting subject for comparison to the other environmental strains. Although several other strains had high levels of similarity to the alginate genes and apparently produced uronic acid, they were slow growing in APM and were omitted in subsequent experiments.

Transconjugation of pUTK50 into environmental strains

In this study, triparental transconjugations were performed to transfer the *alg*D-*lux* reporter plasmid pUTK50 [Appendix B; Wallace et al. 1994a] into four environmental strains displaying DNA homology to four of the alginate probes [Wallace

et al. 1994b] and one environmental *P. fluorescens* (N), which demonstrated homology only to the *alg*B gene probe [Wallace et al. 1994b]. Selection of transconjugates using PIA supplemented with 500 μg/ml kanamycin was sufficient for the mobilization of pUTK50 into environmental strains, *P. fluorescens* M, *S. maltophilia* O and *P. syringae* P. Under these conditions, 100% of *P. syringae* P and 2% of *S. maltophilia* O transconjugates demonstrated bioluminescence, but there were no bioluminescent *P. fluorescens* M transconjugates. *P. fluorescens* M, *S. maltophilia* O and *P. syringae* P were challenged further on PIA containing both 500 μg/ml kanamycin and 500 μg/ml carbenicillin. Under these conditions, 26% of *P. fluorescens* M and 6% of *S. maltophilia* O transconjugates were bioluminescent. For environmental strains of *P. fluorescens* N and *P. putida* L, 22% and 12% of transconjugates demonstrated bioluminescence, respectively, when grown on PIA with 500 μg/ml kanamycin and 500 μg/ml carbenicillin.

Unfortunately, these conditions were not suitable for maintaining an appropriate selective pressure for *P. aeruginosa* 322. Although initial studies indicated that this strain was sensitive to kanamycin, attempts to transfer pUTK50 into this bacteria only produced non-bioluminescent, Kan 500μg/ml and Carb 500μg/ml, resistant bacteria which readily grew on PIA plates with the added antibiotics. Plasmid mini-preps indicated that these resistant bacteria did not harbor the plasmid pUTK50. Higher concentrations of the antibiotics were also ineffective in these experiments. Because of these difficulties, *P. aeruginosa* 322 was omitted from future studies using pUTK50.

Effects of increased salt concentration.

P. aeruginosa FRD1 (pUTK50) cultured in the presence of increasing NaCl concentrations elicited peak bioluminescent responses during log phase growth, when alginate production is known to be maximal [Figure 6A-D; Davies et al. 1993]. In these experiments, it was observed that the growth rate of FRD1 under increases in osmolarity was somewhat diminished [Figure 6A-D]. Figure 6A-D also shows a slight shift in the time of maximum bioluminescence for FRD1, which seems to correspond to the shift in the growth curve. Bioluminescence was induced in FRD1 cultures supplemented with 100 mM, 200 mM and 300 mM NaCl [Table 7].

Table 7. Relative induction of bioluminescence by environmental stimuli.

Induction	FRD1	L23	M39	N15	O46
100 mM NaCl	1.4[a]	2.1	10.9	1.8	6.8
200 mM NaCl	1.5	3.3	25.9	2.2	29.6
300 mM NaCl	2.0	3.2	55.4	2.7	107.0
6.75 mM NH^4/3.25 mM NO^3	1.3	1.3	0.6	0.9	0.7
3.25 mM NH^4/6.75 mM NO^3	1.9	1.3	1.5	1.4	1.2
10 mM NO^3	2.7	2.6	1.0	4.5	0.8

[a] Bioluminescence Induction Factors (BIF) were computed by dividing the normalized peak bioluminescence by the appropriate control for each inducing condition.

In order to correlate enhanced bioluminescence with increased transcription of essential alginate biosynthetic genes (e.g. AlgD, GDP-mannose dehydrogenase), total

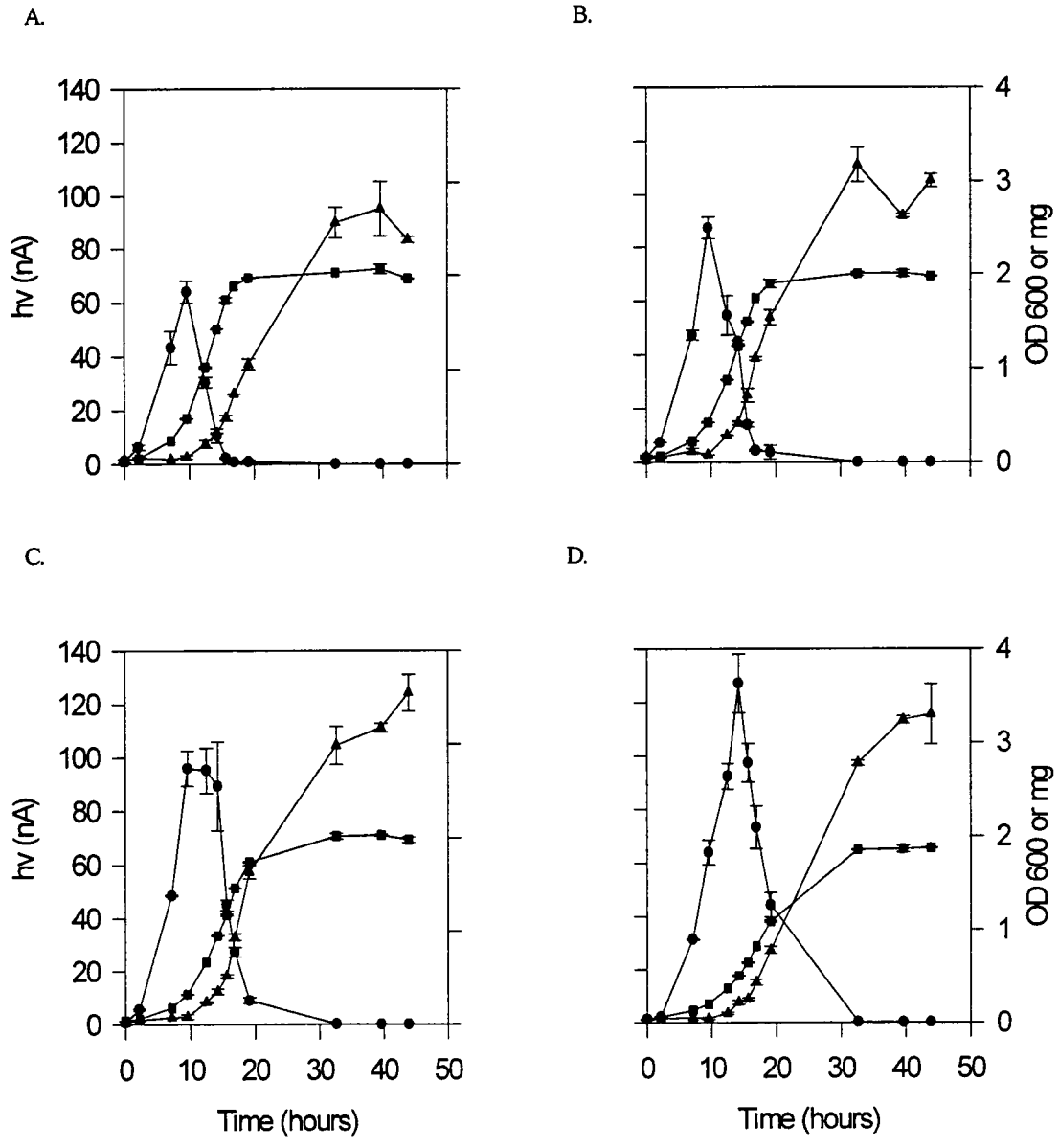


Figure 6. Bioluminescence readings (●), alginate accumulation (▲) and growth (■), during a time course experiment for *Pseudomonas aeruginosa* FRD1 grown in: A) APM; B) APM, 100 mM NaCl; C) APM, 200 mM NaCl; D) APM, 300 mM NaCl. Bioluminescence was normalized by dividing the photomultiplier amperage by the absorbance of the culture at 600 nm. Alginate was analyzed using the metahydroxybiphenyl/H_2SO_4/borate (MHB) assay as described in Material and Methods. Growth was monitored spectrophotometrically at 600 nm. Each point represents the average from duplicate experiments, and error bars represent the standard error of this mean.

RNA transcripts were quantified for these cultures by probing with *alg*D antisense mRNA [Figure 7]. It was observed that, in these cultures, increased bioluminescence correlated well with *alg*D mRNA levels in which the *alg*D mRNA was found to be upregulated in response to increases in medium osmolarity [Figure 7]. In fact, the amount of *alg*D mRNA was approximately twice the control at 19.2 hours for cultures amended with 300 mM NaCl [Figure 7], which correlated well with the induction of bioluminescence [Table 7]. These experiments demonstrated that, for induced cultures, the *alg*D mRNA abundance was prolonged relative to the control cultures, especially in the case of 300 mM NaCl, as illustrated by Figure 7.

To verify that uronic acids were produced at high concentrations in FRD1 cultures, EPS were analyzed using the MHB colorimetric assay and ion chromatography. MHB assays showed that there were substantial concentrations of uronic acids (>2.0 mg/ml) in the culture supernatants [Figure6A-D, Table 8]. Also, ion chromatography

Table 8. Relative induction of uronic acids synthesis in bacterial isolates.

Induction	FRD1	L23	M39	N15	O46
0 mM NaCl	+[a]	++	ND[b]	++	ND
100 mM NaCl	++	+++	ND	+ ½	ND
200 mM NaCl	+++	++	ND	+ ½	ND
300 mM NaCl	+++	+	ND	+	ND

[a] Qualitative comparison of accumulated uronic acids in culture supernatant during time course experiments. Results are not intended to be quantitative but rather represent the relative induction of uronic acids synthesis in bacterial strains. Uronic acids were assayed using the metahydroxybiphenyl /H_2SO_4/borate (MHB) assay as described in materials and methods.

[b] M39 and O46 demonstrated little reactivity to MHB colorimetric assays.

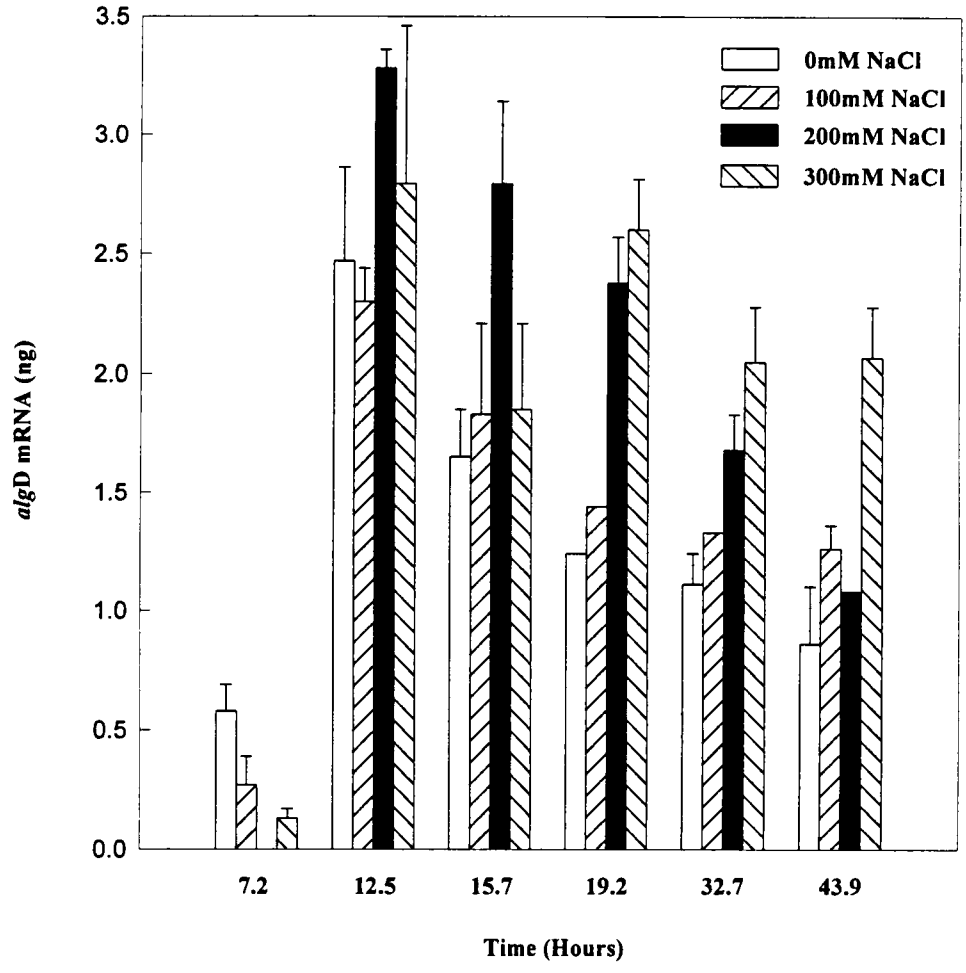


Figure 7. Abundance of *algD* mRNA for *Pseudomonas aeruginosa* FRD1 grown in 50 ml liquid cultures of APM (□); APM, 100 mM NaCl (▨); APM, 200 mM NaCl (■), APM, 300 mM NaCl (▧). Values for mRNA were obtained from quantification of *algD* mRNA slot blots using *algD* sense strand RNA as a standard. Error bars represent the standard error of the mean of two samples.

identified these uronic acids, specifically, as D-mannuronic acid and L-guluronic acid [Figure 8A,B]. However, these studies showed that although there was a slight trend of sustained alginate production for induced cultures, the time of onset and rate of alginic acid accumulation in the culture medium [Figure 6A-D] did not reflect the degree of relative induction as seen in bioluminescent light output [Table 7].

The environmental isolates also exhibited a relative induction in *lux* expression occurring during mid-logarithmic or stationary growth phase, where increasing the osmolarity increased the bioluminescence of liquid cultures [Table 9]. *P. putida* L23 and *P. fluorescens* N15 strains grew somewhat more rapidly than FRD1 on APM medium and demonstrated an increased tolerance to NaCl in so far as their growth was not affected to the degree seen in FRD1 [Figure 9A,C]. The other environmental strains, *S. maltophilia* O46 and *P. fluorescens* M39, grew somewhat slower than N15, or L23 when subjected to NaCl stress [Figure 9B,D], but still grew more rapidly than FRD1 under these conditions. Also, all of the environmental strains grew more rapidly under control conditions compared to FRD1 cultures. It is possible that these results are due to the rich carbon source being used for growth rather than overproduction of alginate, since FRD1 is known to commit up to 50% of its carbon resources for production of copious amounts of this exopolymer [Schlictman et al. 1994].

Induction of bioluminescence during time course experiments for *P. putida* L23 differed from FRD1, in that peak bioluminescence occurred in late logarithmic or stationary phase growth, depending on the inducing condition [Table 9, Figure 9A]. In

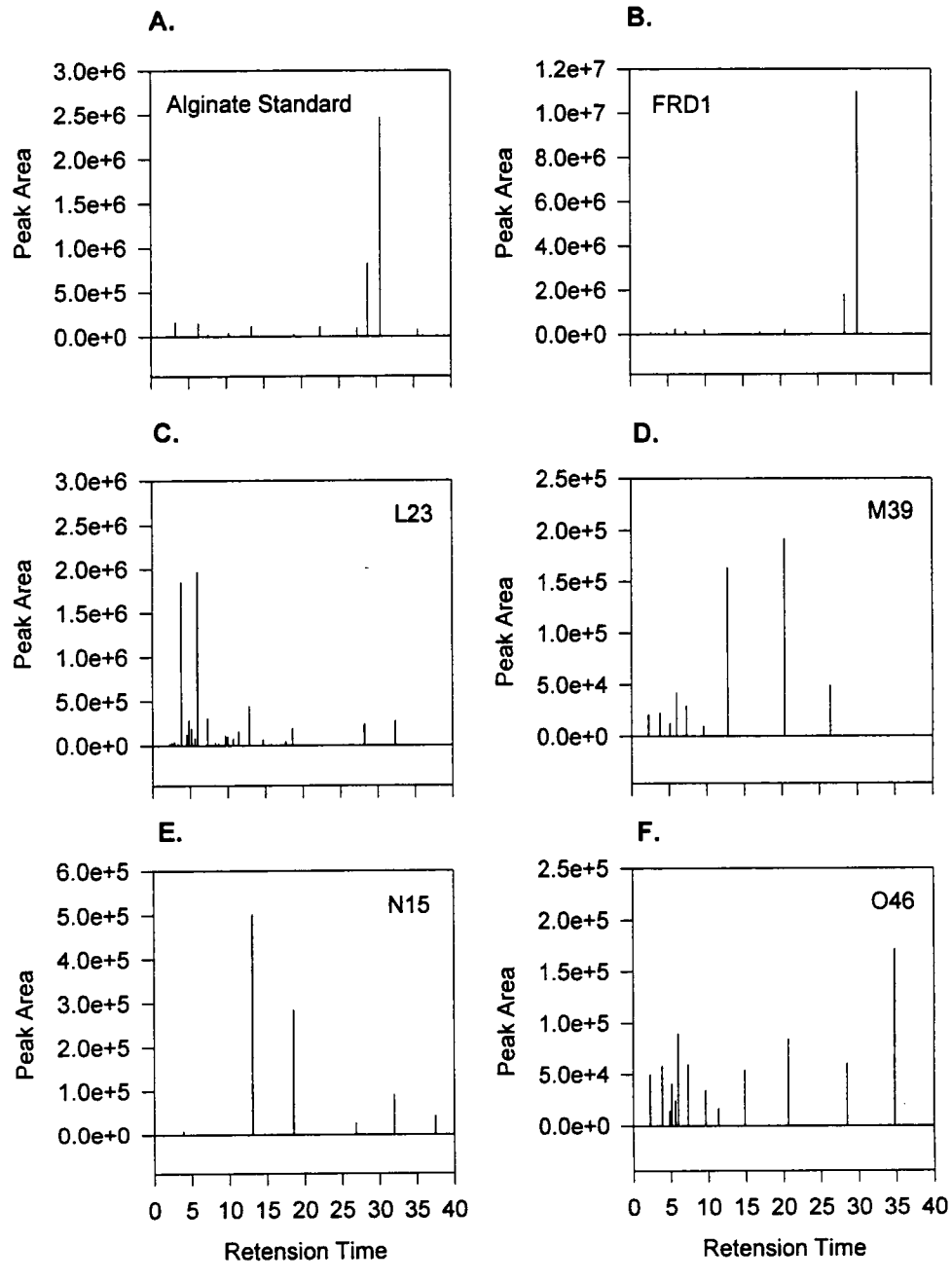


Figure 8. Comparative ion chromatographs for hydrolyzed EPS of environmental isolates and FRD1 containing pUTK50: A) Alginate standard (1 mg/ml); B) EPS (1 mg/ml) from FRD1 grown in APM; C-F) EPS samples from environmental isolates (1 mg/ml), L23, M39, N15, and O46, respectively, grown in APM.

Table 9. Effects of external stimuli on bioluminescence for bacterial isolates harboring pUTK50.

	0.0 mM NaCl	100 mM NaCl	200 mM NaCl	300 mM NaCl	10 mM NH^4	6.75 mM NH^4/ 3.25 mM NO^3	3.25 mM NH^4/ 6.75 mM NO^3	10 mM NO^3
FRD1								
hv	64.1[a]	87.3	96.0	127.0	224.7[c]	281.4	423.6	613.4
Time	9.6[b]	9.6	9.6	14.3	12.0	12.0	12.0	12.0
L23								
hv	0.7	1.4	2.2	2.2	0.2	0.3	0.3	0.5
Time	14.7	12.9	16.8	16.8	6.3	6.3	6.3	6.3
M39								
hv	0.5	6.0	14.2	30.5	1.1	0.7	1.6	1.1
Time	6.0	6.0	7.3	8.3	4.0	4.0	4.0	4.0
N15								
hv	16.6	29.5	36.6	45.2	3.1	2.6	4.4	13.9
Time	7.3	7.25	10.0	13.0	13.0	10.0	10.0	10.0
O46								
hv	0.7	5.0	21.9	79.2	0.7	0.6	0.8	0.9
Time	7.5	7.5	7.5	8.7	10.0	7.0	13.0	7.0

[a] Bioluminescence (hv) in APM cultures under varying environmental stress factors. These data represent the peak maxima of bioluminescence for time course experiments as outlined in materials and methods.

[b] Time of peak bioluminescence (Hours) for time course experiments examining the effects of varying environmental stresses on the *alg*D promoter using the bioluminescent reporter plasmid pUTK50.

[c] For nitrogen source studies, the rich nitrogen source used in APM (100mM monosodium glutamate) was omitted and filter sterilized stock solutions and mixtures of $(NH_4)_2SO_4$ and/or $NaNO_3$ were added so that the amount of nitrogen in either oxidation state totaled 10mM; The control in these experiments is designated as that containing 10 mM NH_4 and 0.0 mM NO_3

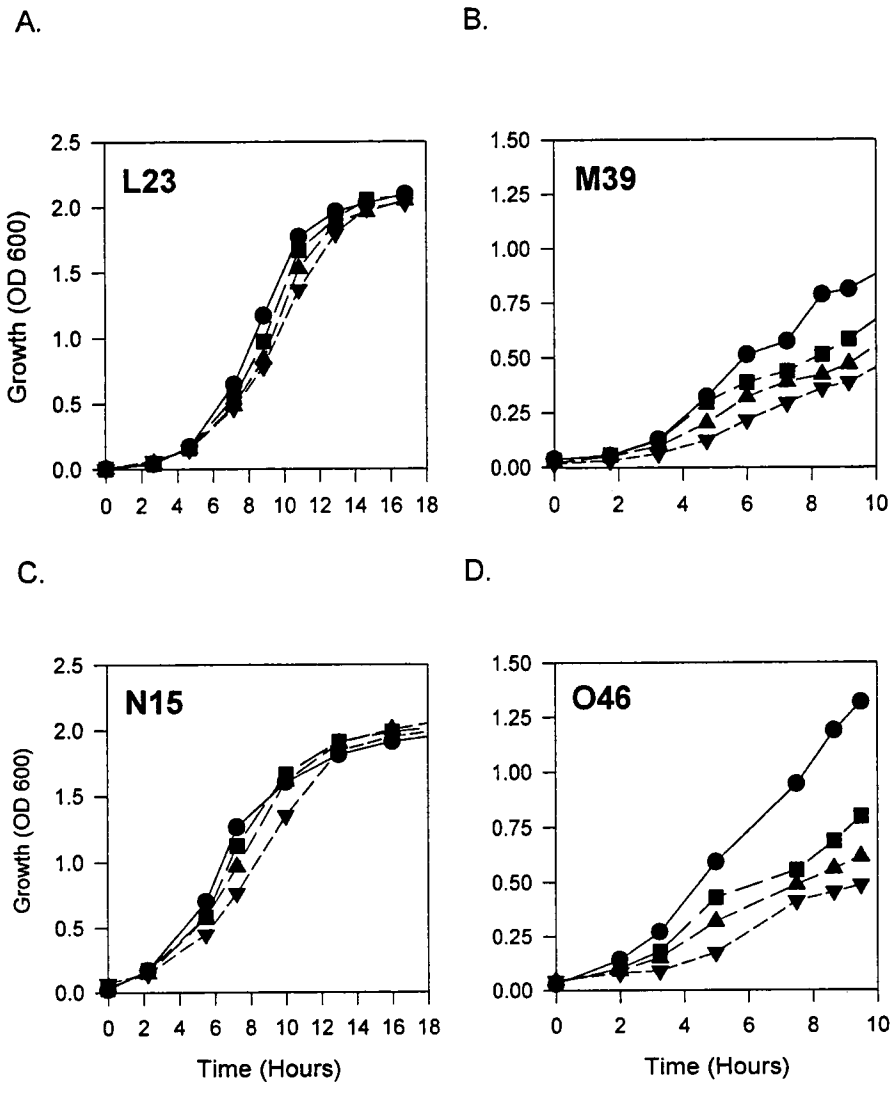


Figure 9. Growth measurements for environmental isolates grown in APM under NaCl induction, 0.0 mM NaCl (●); APM, 100 mM NaCl (■); APM, 200 mM NaCl (▲); APM, 300 mM NaCl (▼). Bacterial growth readings were taken spectrophotometrically at 600 nm. Each point represents data from one experiment.

the case of control cultures containing APM without any additional NaCl, maximum bioluminescence occurred during early stationary phase growth [Table 9, Figure 9A]. A 100 mM increase in NaCl concentration shifted the time of peak bioluminescence to late log phase growth and increased the bioluminescence intensity approximately two fold [Table 7]. Increasing the NaCl concentration to 200 mM or 300 mM induced bioluminescence approximately three fold over the control [Table 7] and shifted bioluminescence into stationary phase growth [Table 9]. The Induction factors for L23 were greater than those observed for FRD1 [Table 7]. Exopolymer from L23 was found to be more reactive in the MHB assay than exopolymer from either M39 or O46 [Table 8]. However, this reactivity did not correlate with bioluminescence, and in cultures with an increased medium osmolarity, there was a decrease in the amount of uronic acids isolated from culture supernatants, as determined using the MHB assay [Table 8].

S. maltophilia O46 and *P. fluorescens* M39 resembled FRD1, in that peak bioluminescence occurred during logarithmic phase growth [Table 9, Figure 9C,D]. The time of maximum bioluminescence was only slightly affected in O46 and M39 cultures amended with 100 mM NaCl, 200 mM NaCl and 300 mM NaCl [Table 8]. Bioluminescence in O46 and M39 control cultures were among the lowest for any of the environmental reporter strains and was highly induced by increasing the medium osmolarity [Table 8]. Attempts to correlate the large induction of bioluminescence with alginate production were complicated by the fact that O46 and M39 showed very little reactivity to the uronic acids (MHB) assay.

In *P. fluorescens* N15 cultures, the times and intensities of bioluminescence were altered by the presence of additional NaCl in the culture media [Table 7, Figure 9C]. Increasing the concentration of NaCl to 100 mM or 200 mM resulted in approximately a two fold increase in bioluminescence [Table 7], and in the case of 200 mM a 2.75 hour shift in the time of bioluminescence [Table 9] was observed. In cultures containing 300 mM NaCl, bioluminescence was induced to an even larger degree [Table 7] and bioluminescence was shifted a total of 5.75 hours compared to control cultures [Table 9]. Ethanol precipitated exopolymer from these cultures was more reactive to MHB assays than EPS isolated from O46 and M39 [Table 8]. However, bioluminescence could not be correlated to this reactivity, and a trend of decreasing MHB reactivity as bioluminescence increased was apparent [Table 8, Table 7].

P. syringae P4 emitted only a weak bioluminescent response, thus the actual bioluminescent measurements were more subject to error. These small numbers also increased the potential for distortions resulting from mathematical operations on the data. Clearly, a salt-dependent induction in bioluminescent gene expression was observed. Because of its low capacity for *lux* expression, P4 was not included in data analysis or subsequent experiments.

Nitrogen source effect

The reference or control culture in these experiments is designated as that with all available nitrogen as NH_4^+ ions. Overall growth was diminished relative to unmodified

APM due to compromised metabolism under conditions of nitrogen limitation. Growth lag was evident for FRD1, in which growth was significantly diminished with decreasing nitrogen availability [Figure 10]. In contrast to FRD1, the environmental strains demonstrated an increased ability to grow in the presence of lower concentrations of available nitrogen [Figure 11A-D]. L23 exhibited a slight growth lag in the presence of reduced levels of NH_4^+ ions and grew well even in cultures containing only 10 mM NO_3^-. Strains M39, N15 and O46 exhibited a slight growth lag in cultures where NH_4^+ ions were reduced and a moderate growth lag in cultures containing 10 mM NO_3^- .

Decreasing the concentration of available nitrogen below 10 mM NH_4^+ resulted in induction of bioluminescence in FRD1 cultures [Table 9]. In these cases, changing the NH_4^+/NO_3^+ ratio to 6.75 mM NH_4^+/3.25 mM NO_3^- resulted in a 1.3 fold increase in bioluminescence [Table 7], which follows current experimental evidence that nitrogen limitation results in induction of alginate biosynthesis [Wallace et al. 1994b and Sutherland 1988]. In cultures containing 3.25 mM NH_4^+/6.75 mM NO_3^- a general trend of increasing bioluminescence in response to decreasing nitrogen availability was apparent, and there was a relative induction of 1.9 times the control culture [Table 7]. Cultures containing NO_3^- ions as the sole nitrogen source demonstrated a maximum relative induction of 2.7 times the bioluminescence of the control culture. The time of peak bioluminescence for FRD1 in these experiments was not variable, as was observed under conditions of increased osmolarity [Table 9].

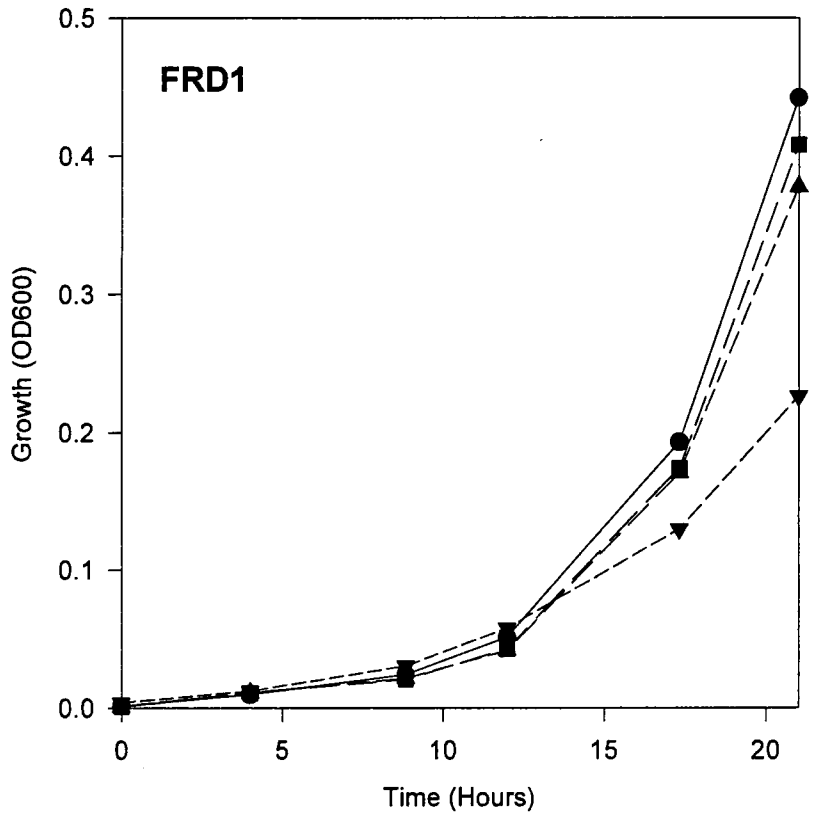


Figure 10. Growth curve for *P. aeruginosa* FRD1 (pUTK50) under conditions of low nitrogen availability growth in APM, 10 mM NH_4^+ (●); APM, 6.75 mM NH_4^+/3.25 mM NO_3^- (■); APM, 3.25 mM NH_4^+/6.75 mM NO_3^- (▲); APM, 10 mM NO_3^- (▼). Bacterial growth readings were taken spectrophotometrically at 600 nm. Each point represents the mean of two independent experiments.

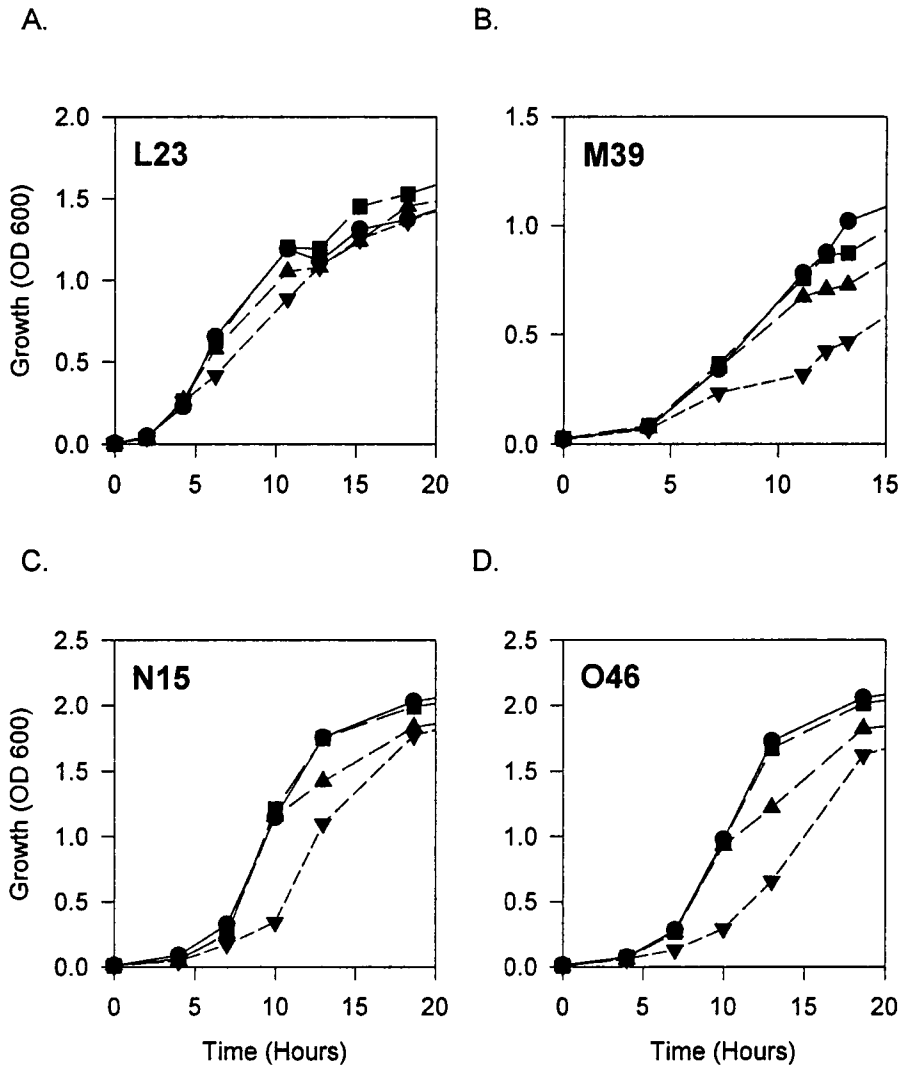


Figure 11. Growth measurements for environmental isolates grown in APM under nitrogen limitation; APM, 10 mM NH_4^+ (●); APM, 6.75 mM NH_4^+/3.25 mM NO_3^- (■); APM, 3.25 mM NH_4^+/6.75 mM NO_3^- (▲); APM, 10 mM NO_3^- (▼). Bacterial growth readings were taken spectrophotometrically at 600 nm. Each point represents data from one experiment

The environmental reporter strains, L23 and N15, demonstrated induction of bioluminescence during late log phase growth, in response to decreases in available nitrogen [Table 9, Figure 11A,C]. For L23, an induction value of 1.3 was observed in response to changes from all ammonium ions to 6.75 mM NH_4^+/3.25 mM NO_3^- and to 3.25 mM NH_4^+/6.75 mM NO_3^- [Table 7]. In L23 cultures containing 10 mM NO_3^-, the bioluminescence was induced 2.6 fold compared to the control cultures [Table 7]. N15 produced the greatest bioluminescence of any environmental isolate in these nitrogen limitation experiments [Table 9], and demonstrated the largest bioluminescence induction factors (BIF) of all the environmental isolates used in the nitrogen experiments [Table 7]. In cultures containing 6.75 mM NH_4^+/3.25 mM NO_3^- no apparent induction was observed, but under more severe nitrogen stress (3.25 mM NH_4^+/6.75 mM NO_3^-) bioluminescence was induced 1.4 fold over control cultures. Bioluminescence induction factors were greatest under conditions of 10 mM NO_3^- and 0.0 mM NH_4^+ ions [Table 7]. The times of maximum bioluminescence in L23 and N15 were not significantly altered under conditions of decreasing nitrogen availability, although N15 demonstrated a slight shift in the times of peak bioluminescence under induced conditions [Table 9].

Induced bioluminescence, in O46 and M39, was observed only in the presence of 3.25 mM NH_4^+/6.75 mM NO_3^-. For these strains, no significant induction was observed in the presence of 6.75 mM NH_4^+/3.25 mM NO_3^- or 10 mM NO_3^- [Table 7]. For M39, the times of peak bioluminescence were not shifted as a result of nitrogen stress, but the times of maximum bioluminescence were somewhat variable for O46 [Table 9].

Preliminary characterization of the exopolymer produced by environmental isolates

A stationary phase culture of O46 in APM was used to isolate high molecular weight EPS components which were analyzed by size exclusion HPLC and colorimetric assays for uronic acids. Size exclusion HPLC data indicated that the O46 EPS had a molecular weight of approximately 500 kD. Additionally, three neutral sugars (rhamnose, glucose and galactose) were identified using GC-MS (unpublished data; Anders Sonesson). No common aminohexoses were detected, but one of the unknown monosaccharides appeared to be an unknown amino sugar. There was no indication that the EPS contained KDO (3-deoxy-*manno*-2-octulosonic acid), fatty acids, or amino acid substituents.

Exopolymers isolated from the environmental isolates were also examined using the Dionex Chromatography System to identify the major components present in alternate exopolysaccharides, and to determine any similarity to the bacterial alginate produced by FRD1. These preliminary studies verified that for FRD1 alginate was present in significant amounts and that this exopolymer was the same as was found in the alginic acid standard [Figure 8a,b]. In addition, it was observed that the environmental isolates had a low but detectable uronic acid content (below 5%) and that for the environmental strains other presumed polysaccharides were present in culture supernatants [Figure 8c-f]. These EPS samples, once hydrolyzed, demonstrated strain specific patterns of monosaccharides, which consisted, in all cases, of one, two or three primary sugars and numerous background monosaccharides. Major peaks, for the environmental strains,

were accepted as those containing a total peak area of over 1E10+5, while all others were considered minor peaks. L23 cultures contained, in addition to several minor peaks, two major peaks eluting at 3.8 and 6.0 minutes [Figure 8c]. These are most probably rhamnose and glucose, respectively. M39 supernatants consisted of primarily two major peaks at 12.8 and 20.4 minutes, which remained unidentified and contained several minor constituents as well [Figure 8d]. Analysis of N15 exopolymer showed two major polysaccharides, which eluted at 13.1 and 18.5 minutes, respectively [Figure 8e]. O46 EPS samples showed only one major peak, which eluted at 35.5 minutes, but two intermediate peaks were present at elution times of 6.0 and 20.7 minutes, and the distinction between these peaks and the minor ones were not as defined as those found in the other strains [Figure 8f].

Biofilm studies

Laminar flow biofilm reactors were used for studies of *S. maltophilia* O46 biofilms. Preliminary experiments were conducted using the reactor A design (described previously in Materials and Methods). These preliminary experiments investigated the ability of O46 to form biofilms which were suitable for bioluminescence, tryptophan, and NADH monitoring. Based on the results from these experiments reactor B was designed and built specifically for these studies.

In the first experiments, reactor A was inoculated with the environmental isolate O46 as described in Materials and Methods. Once the media flow was established,

bacterial biomass within the developing biofilms (estimated using the approximate amount of tryptophan present) began to accumulate [Figure 12]. Biofilm growth occurred gradually over the next 4.5 days, at which time the biofilm growth rate began to decline. During this period of initial growth, the bacterial activity (as measured using NADH estimates) and the bioluminescence closely mirrored the bacterial growth rate. However, once the biofilm growth rate began to diminish, a sharp increase in both the bacterial activity and the bioluminescence was observed at approximately 6 days [Figure 12]. Bacterial activity then leveled off, but the bioluminescence continued to increase demonstrating an oscillating pattern of bioluminescence in which a sharp increase was followed by a corresponding decrease. This pattern continued until a maximum peak of 1.8 nA was reached at 9.6 days [Figure 12]. Once the maximum bioluminescence was reached bacterial bioluminescence decrease rapidly over the next several hours. In these experiments, it was noted that bioluminescence was significantly lower in the down stream viewport. It was hypothesized that this was probably due to limitations in oxygen availability. Furthermore it was believed that this could be caused by a low oxygen tension in the media since no aeration was being used. In order to test this hypothesis it was decided to vigorously aerate the biofilm media in future experiments using reactor B.

A second experiment was conducted in which the environmental reporter O46 was grown in laminar flow biofilm reactors of the reactor B design [Appendix A]. In this experiment, the medium was vigorously aerated for the duration of the experiment. In the beginning, biofilm growth was essentially identical to the initial experiments.

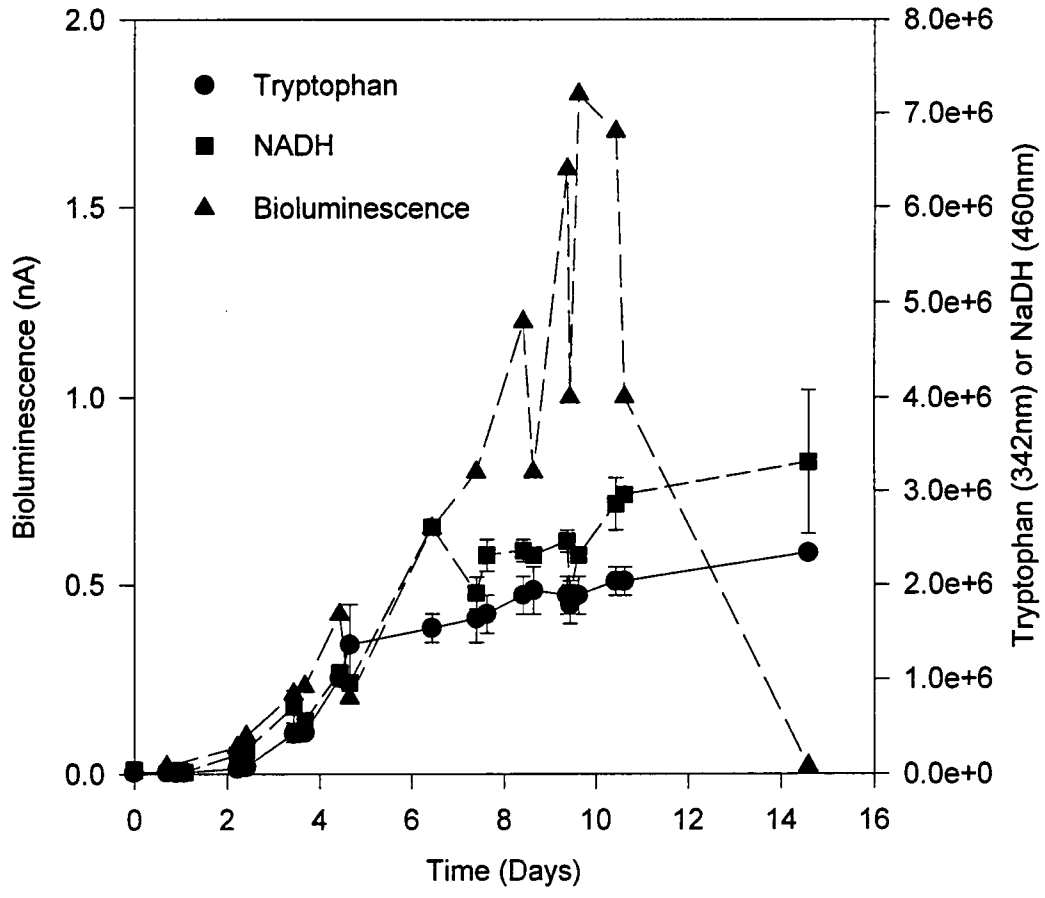


Figure 12. Bioluminescence and biomass determination in a biofilm containing *S. maltophilia* O46 using reactor A. Bioluminescence readings are expressed in nA (▲); fluorescence readings for tryptophan were taken at 342 nm (●) and at 460 nm for NADH estimates (■). Bioluminescence data represents the reading from the upstream port. Fluorescence readings were derived from averaging the readings from both flow cell ports. Error bars represent the standard error of this average.

Accumulation of biomass proceeded over a 4.5 - 5.0 day period and then stabilized [Figure 13]. Unlike the previous experiments NADH readings showed only minimal fluctuations and the magnitude of these numbers were significantly less than in reactor A. However, NADH readings were still correlated with the biomass readings as was observed in prior experiments. Tryptophan readings in reactor B were, like the NADH readings, lower in overall magnitude than in reactor A experiments, but the overall pattern of biomass accumulation was very similar to the first experiments. Bioluminescence closely mirrored the growth of the biofilm until the growth leveled off, and at that point, bioluminescence was greatly induced and achieved a maximum peak of 1.2 nA at about 5.75 days [Figure 13]. This was very different from the patterns of bioluminescence observed in reactor A, in which the bioluminescence gradually peaked at around 10 days and maintained a high level of light output until day 11 [Figure 12]. Reactor B biofilms stopped producing light very quickly reaching 0.0 nA at about 8.0 days. Immediately following the diminished light emission for these biofilms, the NADH and biomass readings began to gradually decrease for the duration of the experiment [Figure 13]. Exopolymer from reactor B biofilms was isolated and analyzed using ion chromatography as outlined for the batch culture studies. Hydrolyzed exopolymer was found to consist primarily of two peaks, a major peak at 6.23 min and a minor peak at 7.67 min [Figure 14]. The peak at 6.23 min, specifically, matched D-glucose standards [Figure 15], but the 7.67 min peak remains unidentified at this time.

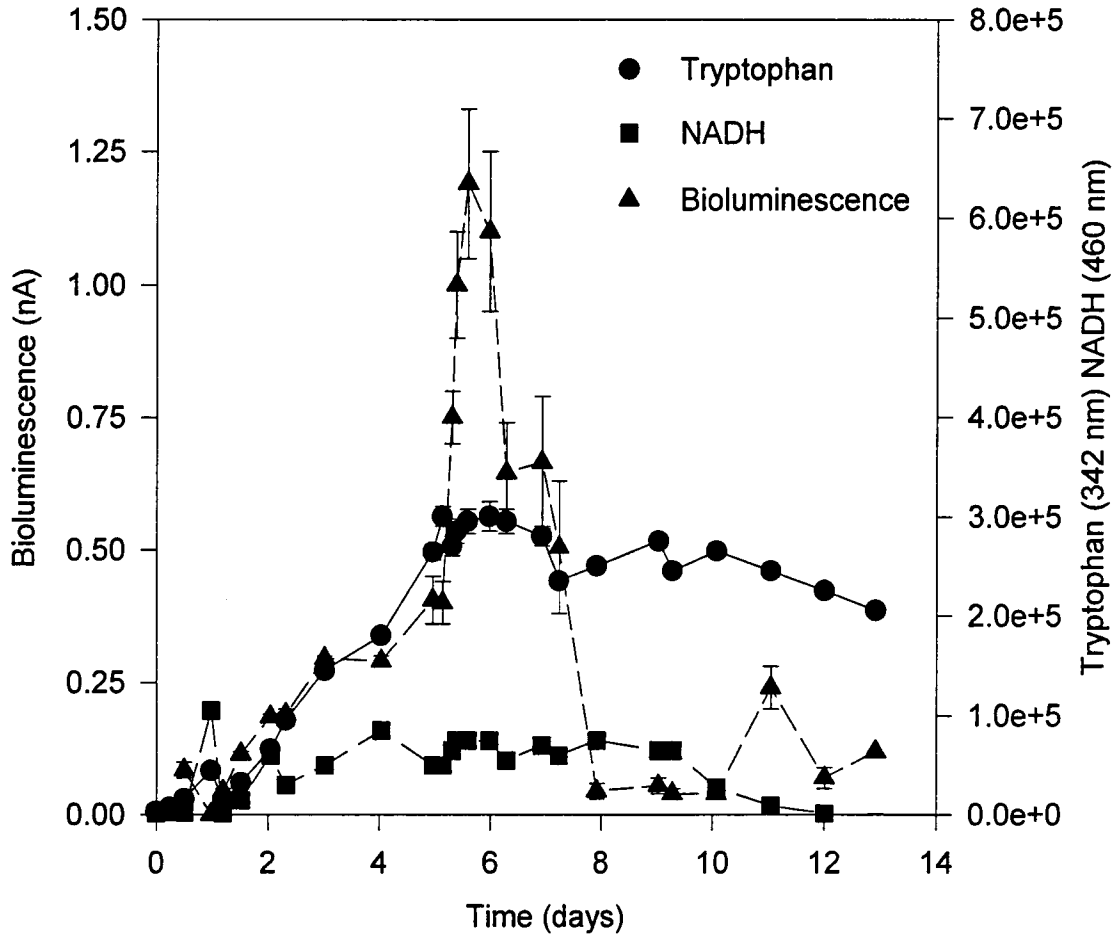


Figure 13. Bioluminescence and biomass determination in a biofilm containing *S. maltophilia* O46 using Reactor B. Bioluminescence readings are expressed in nA (▲); fluorescence readings for tryptophan were taken at 342 nm (●) and at 460 nm for NADH estimates (■). Bioluminescence and fluorescence data were derived from averaging two independent readings, one from the upstream side of the lens and one from the downstream side of the lens.. Error bars represent the standard error of this average

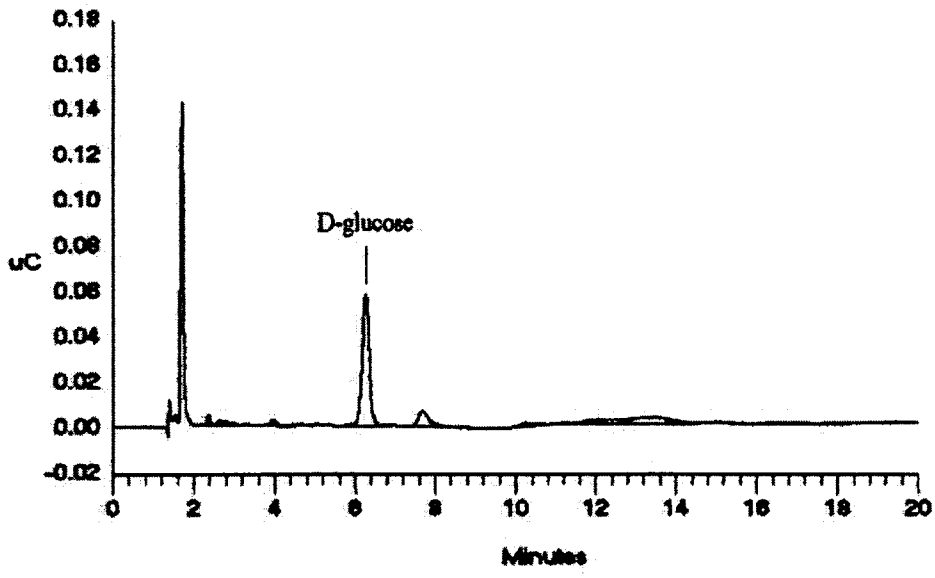


Figure 14. Dionex chromatograph of TFA hydrolyzed exopolymer from a *S. maltophilia* O46 biofilm grown in Reactor B.

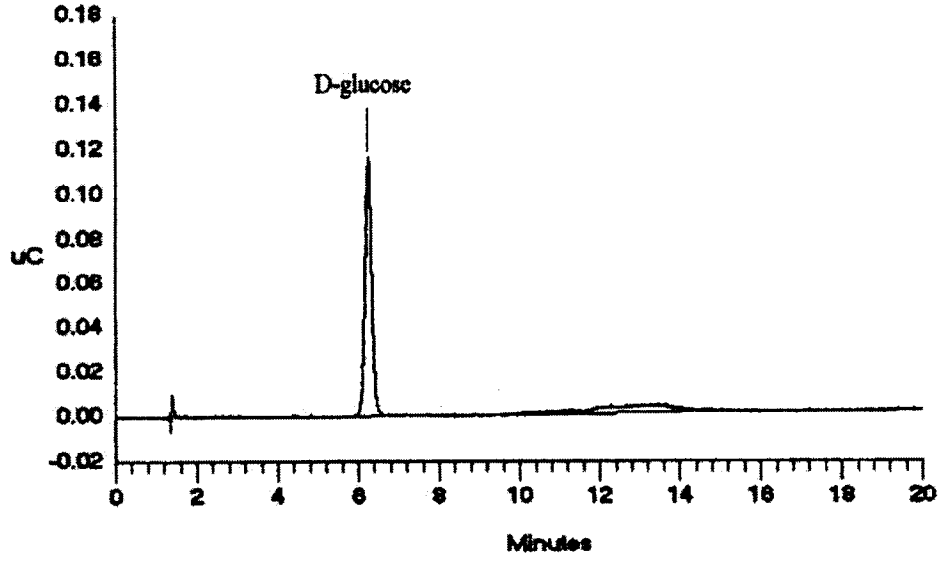


Figure 15. Dionex chromatograph of D-glucose.

In a third series of experiments the effects of increased NaCl concentrations on biofilm formation, development, and bioluminescence were examined. Experimental conditions for this study were identical to the previous reactor B experiment except that the biofilm media was amended with 100 mM NaCl. Biofilms formed very slowly under these conditions with tryptophan readings which were three fold less than found under control conditions (no NaCl, reactor B) after 5 days of growth [Figure 16]. NADH readings for these biofilms were almost immeasurable at the 5th day. Surprisingly, bioluminescence increased dramatically around day 6 and reached a maximum value of 0.37 nA, which then decreased to 0.0 nA by day 8. Therefore, the bioluminescence patterns in the early stages of this experiment were very similar to the bioluminescence patterns observed for the control biofilm. However, unlike the control biofilm, biomass began to increase dramatically from day 8 until day 14. During this time NADH readings began to increase and followed patterns observed in the early stages of biofilm formation under control conditions [Figure 16]. At day 10, bioluminescence increased to 0.18 nA and gradually diminished over the next 3 days. It was also observed that the biomass by day 14 had reached a maximum of about 1.5 fold greater than the maximum biomass observed under control conditions. A comparison of normalized bioluminescence from control and NaCl stressed biofilms indicates that there was no significant induction of light in the stressed biofilms [Figure 17].

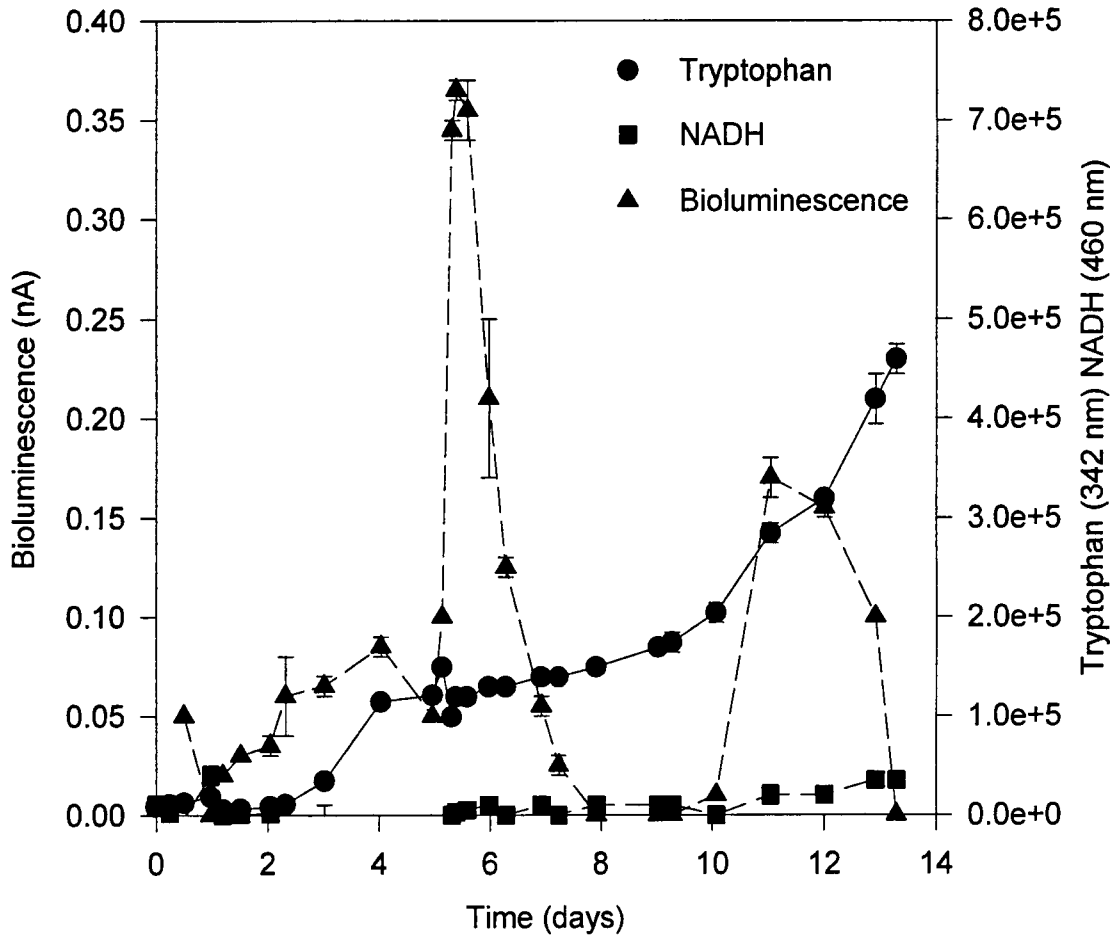


Figure 16. Bioluminescence and biomass determination in a biofilm containing *S. maltophilia* O46 using reactor B under conditions of increased NaCl (+100 mM). Bioluminescence readings are expressed in nA (▲); fluorescence readings for tryptophan were taken at 342 nm (●) and at 460 nm for NADH estimates (■). Bioluminescence and fluorescence data were derived from averaging two independent readings, one from the upstream side of the lens and one from the downstream side of the lens. Error bars represent the standard error of this average.

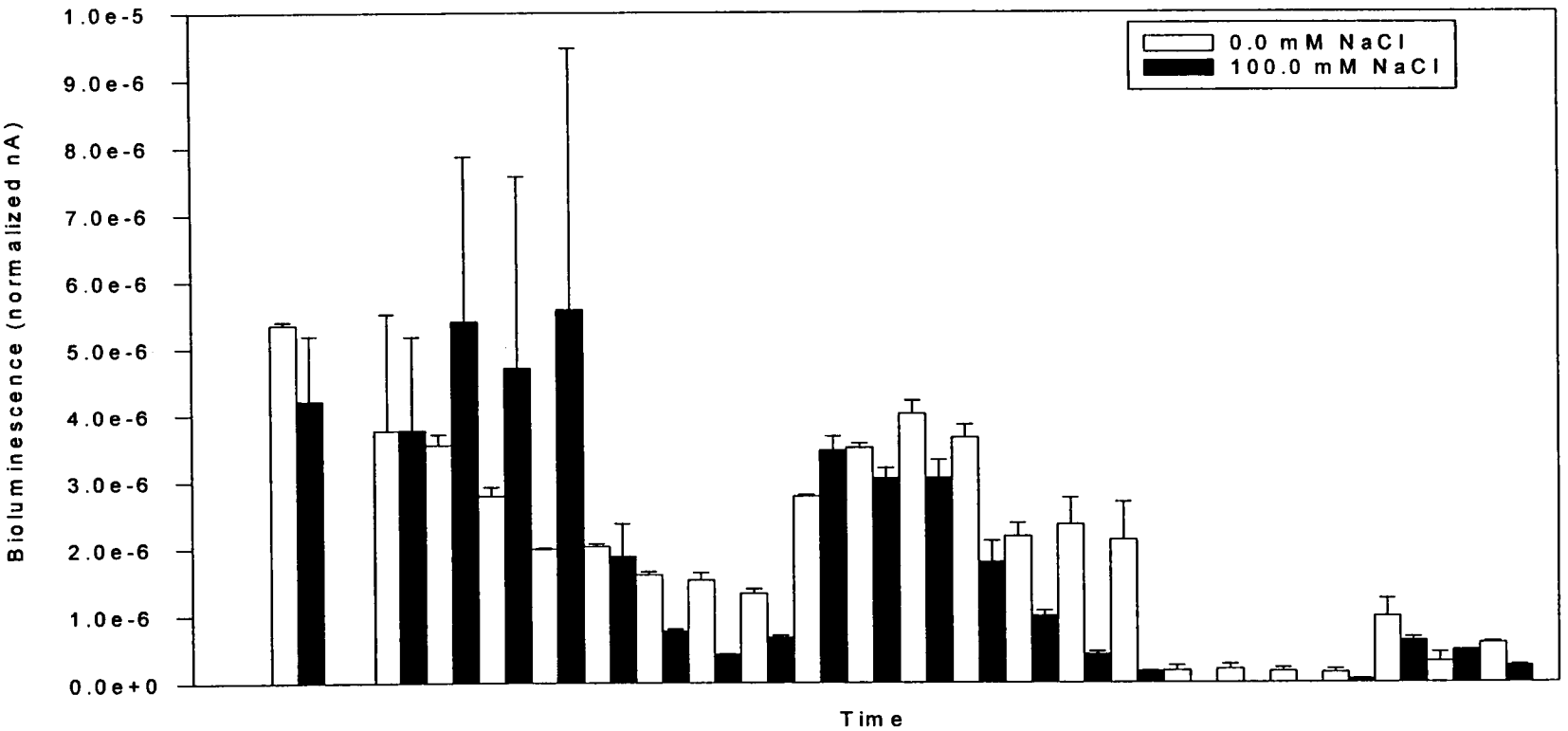


Figure 17. Comparison of normalized bioluminescence between O46 biofilm experiments in reactor B under control conditions and conditions of NaCl induction. Bioluminescence was normalized by dividing the tryptophan readings into the bioluminescence reading for each time point. The time axis corresponds to the experimental data points from Figures 13 and 16. Error bars represent the standard error of the mean for these experiments.

CHAPTER V

DISCUSSION

Initially, liquid culture studies were employed to provide physiologically controlled conditions to make direct comparisons among biofilm strains and FRD1 harboring the *lux* reporter plasmid, pUTK50. Since light production is a function of total biomass, bioluminescence was normalized to optical density for direct comparison of cellular responses to inducing conditions rather than changes in cell numbers. This allowed the data to be expressed in terms of relative induction of *lux* expression referred to as the bioluminescent induction factor (BIF), which permitted direct comparisons of responses differing greatly in terms of absolute bioluminescence. Also, normalizing the data in this way helped to account for the influences of various other factors which are known to affect the bioluminescence reaction, such as aldehyde substrate availability, reducing equivalents, oxygen tension, and the energy status of the cell [Meighen 1991 and Nealson and Hastings 1979], since bioluminescence for each culture is expressed relative to its control. This is particularly important when comparing different strains of bacteria because the same *lux* genes have been demonstrated to bioluminesce differently under similar environmental conditions in experiments with different bacterial species [Meighen 1991].

Liquid culture studies were carried out in a defined medium (APM, Materials and Methods) very rich in a carbon source (100 mM D-gluconate), which can be funneled into

the polysaccharide precursor pathways with a net savings of NAD(H) compared to direct utilization of glucose [Marshall 1992], and very rich in readily assimilable nitrogen (100 mM L-glutamate). Also, the high concentration of gluconate and the zwitterionoic glutamate ions provides an effective buffer against the acidity contributed by acidic EPS secretions. This medium supported very good growth of all reporter strains in both liquid culture and on APM agar plates, but in APM modified for nitrogen source experiments (Materials and Methods) slower growth was observed relative to cultures rich in glutamate, as would be expected because of the limited availability of nitrogen. Growth rates for FRD1, O46, L23, M39 and N15 in shaken flasks were slowed somewhat in cultures with higher salt concentrations, FRD1 showed the most drastic decrease in the growth rate, while the environmental isolates showed only a slight decrease in their growth rates as determined by OD.

In organisms which are known to produce copious amounts of alginate, such as *P. aeruginosa* FRD1, the high concentration of alginate produced was correlated with a high capacity for bioluminescent gene expression. It was found that increased bioluminescence proceeded accumulation of *algD* mRNA transcripts under conditions of NaCl induction, and that in induced cultures the abundance of *algD* mRNA was prolonged relative to uninduced cultures. This may have been due to prolonged stimulation of transcription from the *algD* promoter, but it is also possible that the degradation rates of the mRNA were affected, which allowed accumulation of mRNA transcripts. The time of onset and rate of alginate accumulation in the culture medium

did not reflect the degree of relative induction as seen in the bioluminescent light output, although alginate appeared to persist for a prolonged period of time in induced cultures. These data suggest that the rate of alginate synthesis was not greatly affected in this strain, but rather it was possible for alginate to be synthesized for an extended period of time.

Factors previously associated with increased alginate production, such as high osmolarity or limited nitrogen (as NO_3^-), were applied to environmental reporter strains harboring the *alg*D::*lux* reporter plasmid. Increasing the medium osmolarity resulted in relative induction of bioluminescence in all environmental strains (*P. putida* L23, *P. fluorescens* N15, P. *fluorescens* M39, and *S. maltophilia* O46). Comparison of the BIFs indicates that for the environmental isolates strain specific induction patterns were apparent [Table 7].

Bioluminescent reporter strains, L23 and N15, demonstrated the lowest inducibility among the environmental bacteria. L23 had the lowest overall bioluminescence among the environmental isolates, but this strain also had the highest MHB responses of the the environmental strains. This could be due to the fact that in non-mucoid alginate producers the activity of the alginate biosynthetic enzymes is usually low [Schlictman et al. 1995]. Therefore, L23 may not be able to become fully induced under these experimental conditions. N15 had the lowest alginate gene similarities but was the third highest light producer in these experiments (preceeded by FRD1 and O46). Uronic acids were detected in N15 culture supernatants, but

monosaccharides other than D-mannuronic and L-guluronic acid were observed by Dionex analysis. For this strain, it appears that the bioluminescent reporter plasmid is being induced by some undetermined factor, possibly related to an alternative exopolymer synthesis pathway.

Sodium chloride studies with M39 and O46 showed that these strains were very inducible, as indicated by the minimal bioluminescence of control cultures and the high bioluminescence in induced strains. These bacterial strains were unreactive to MHB assays and ion chromatography did not detect significant quantities of the alginic acid constituents. Additionally, monosaccharides not present in alginate were identified. It may be that, in these reporter strains, the bacteria have lost their ability to synthesize alginate at some point in the biosynthetic pathway, and/or that these bacteria have evolved another mechanism for control of an alternative exopolysaccharide, whose regulatory mechanism is similar to the one found in alginate producers.

Nitrogen limitation induced bioluminescence in the environmental isolates L23 and N15, and there was a definite trend of decreasing nitrogen availability vs. increasing bioluminescence. However, for environmental strains M39 and O46, there was no significant trend in induction of bioluminescence in response to decreased nitrogen availability. Although M39 and O46 exhibited a slight induction of the *alg*D promoter when grown in cultures containing 3.25 mM NH_4^+/6.75 mM NO_3^-, there was no induction in cultures containing only 10 mM NO_3^-. These data show that the bioluminescent

responses vary among the environmental strains and do not necessarily correlate with the patterns of alginic acid synthesis observed in FRD1.

Exopolymers produced by the environmental strains were isolated and characterized as being distinctly different from the FRD1 alginates. These preliminary data do not discount the possibility that alginate can be produced by the strains tested, but it appears that there is a predominance of alternative EPS within culture supernatants under conditions known to induce alginate production. It was observed that each of the bacterial strains produced a unique series of monosaccharides when hydrolyzed exopolymer was examined using ion chromatography. The biopolymer data seems to indicate that there may be more than one exopolysaccharide produced by the environmental bacteria. This has been shown to occur as a result of changes in the nutritional composition of the cellular environment and the cell growth status [Bengtsson 1991, Osman and Fett 1989]. The complex composition of the supernatants from liquid culture studies could be explained by the accumulation of these different EPS in these cultures. In biofilms, the presence of only a β-glucan polymer could be explained, because only the exopolymers which are important in the adherent bacterial community would be retained within the flowcell. The role of these EPS in biofilm development can only be speculative at this point, but it seems likely that alternative EPS are produced instead of alginic acid and that these may be important in biofilm formation and development for the environmental strains.

The evidence presented in this research project does not correlate bioluminescence with alginic acid synthesis in environmental isolates L23, M39, N15, and O46. However, it does propose the possibility that alternate polysaccharides are produced and activators of their synthesis may also induce the *alg*D promoter. It appears that these exopolysaccharides could be transcriptionally regulated in a manner resembling traditional global response systems, such as is observed in other cellular responses such as starvation and nitrogen deprivation [Deretic et al. 1994]. In fact, there is increasing evidence that alginate is regulated in response to changes in the bacteria's energy status [Schlictman et al. 1994] and environmental stimuli previously shown to induce global cellular changes such as the heat shock response [Fett et al. 1989]. In previous studies, the existence of alternate exopolysaccharides has been demonstrated in members of the *Pseudomonas* RNA homology group I sub-family [Fialho et al. 1990], but this study is the first to suggest the possibility that these alternate exopolysaccharides may be controlled by genes which show some homology to the ones responsible for controlling the alginate biosynthetic pathway.

The surface adherent environmental isolate, *S. maltophilia* O46, was chosen for studies using laminar flow biofilm reactors to monitor *in situ* light production and EPS synthesis within an actively growing biofilm. During initial experiments using reactor A, O46 demonstrated a sustained ability to produce light, under conditions necessary for biofilm growth and development. Additionally, this bacterium stabily maintained pUTK50 and allowed visualization of changes in light induction throughout the course of

the experiment. Consequently, these experiments demonstrated the utility of this reporter strain for further studies examining the dynamics of *alg*D induction in response to environmental insults, during *in situ* time course experiments.

In subsequent experimentation, O46 biofilms were grown successfully using laminar flow biofilm reactor B. These experiments examined the effects of an environmental stress factor on bioluminescence in an actively growing biofilm. It was shown in these experiments that bioluminescence was induced once biomass stabilized and that diminished bioluminescence preceded biofilm sloughing in control cultures. It was also shown that increases in NaCl concentrations resulted in reduced rates of biofilm formation and that bioluminescence was not induced in the initial stages of biofilm growth. NaCl stressed biofilms demonstrated abnormal growth in the early stages of the experiments and bioluminescence was shown to be induced in a secondary stage of biofilm growth, unique to the NaCl induced biofilms.

These studies demonstrated that specific periods of EPS induction seem to be important in biofilm processes. In the case of the control biofilm, exopolymer production was essential for maintaining a stable biofilm. Assuming that bioluminescence corresponds to alternate EPS induction, a model can be suggested in which exopolymer accumulation precedes biofilm sloughing, which allows other, possibly nutrient rich areas, to be colonized. In the case of osmotic induction of EPS production, exopolymer accumulation may be essential in order for biofilm bacteria to become salt tolerant, thereby restoring a more normal mode of biofilm growth. These studies illustrate the

versatility and potential utility of this bioluminescent reporter plasmid for studying the role of EPS in biofilm formation and maintenance as well as the effects of other environmental insults on these microbial biofilms. Future experiments should allow the cellular processes involved in MIC to be successfully studied from a molecular point of view.

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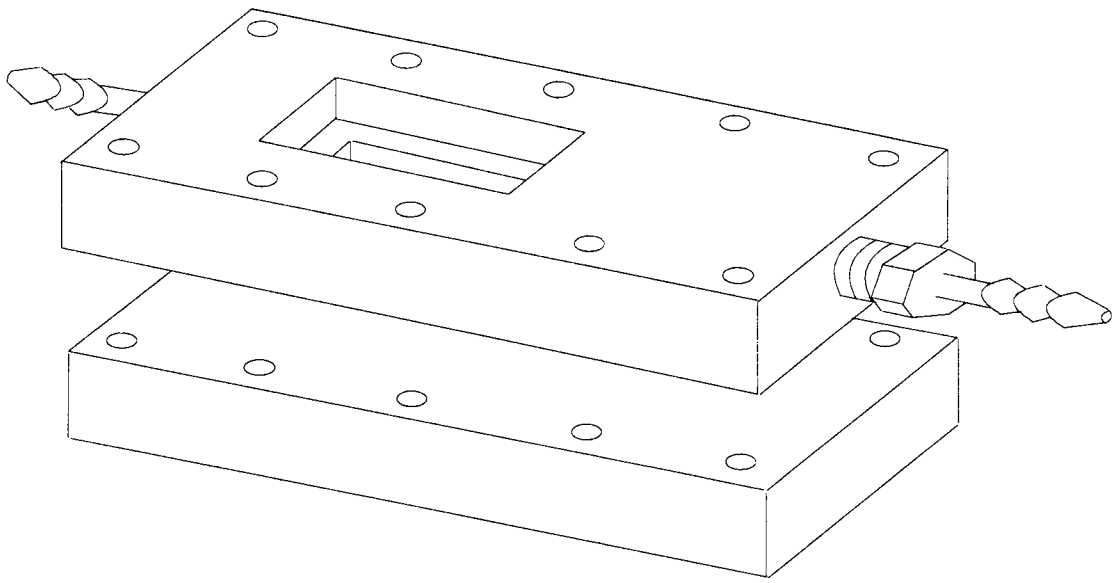
Appendix

Appendix A



Description: Laminar Flow Biofilm Reactor

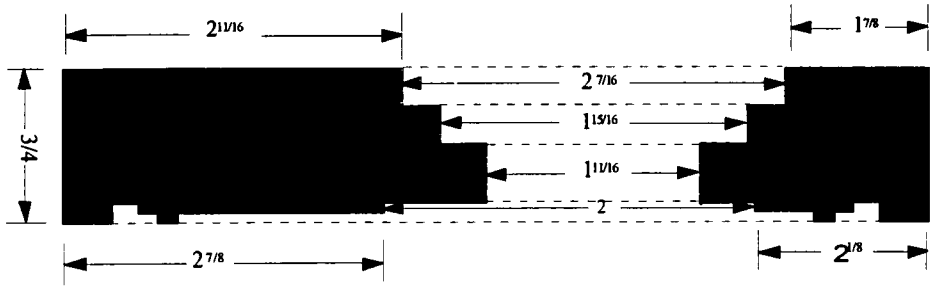
Materials:

Body: UHMW Plastic
Lens: Quartz
Gasket: Silicon
Port screws: Teflon

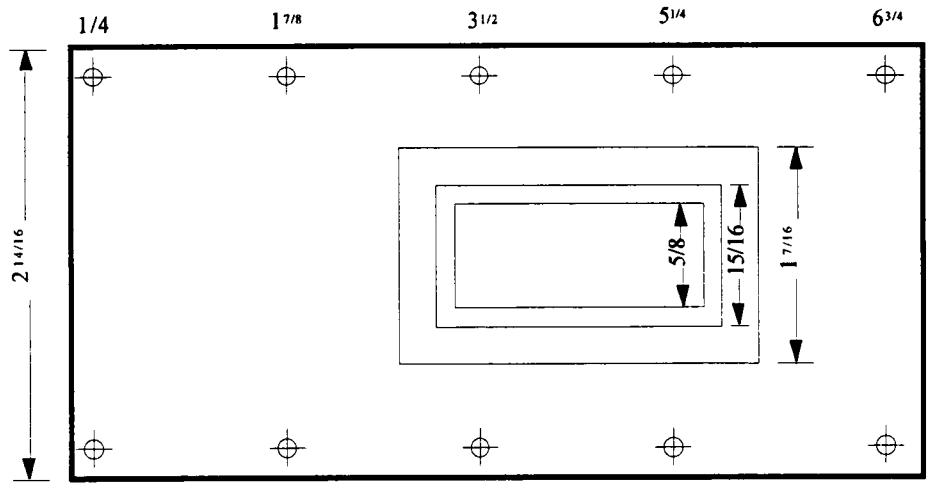
Dimensions:

Body (top): 7 X 2 14/16 X 3/4 inches
Body (bottom): 7 X 2 14/16 X 3/4 inches
Lens: 1 X 2 inches X 1 mm
Gasket: 7/16 X 6 inches X 1 mm
Flow cannel: 1 X 5 1/8 inches X 1 mm
Inlet port: 3/8 inches
Outlet port: 1/8 inches

A.



B.



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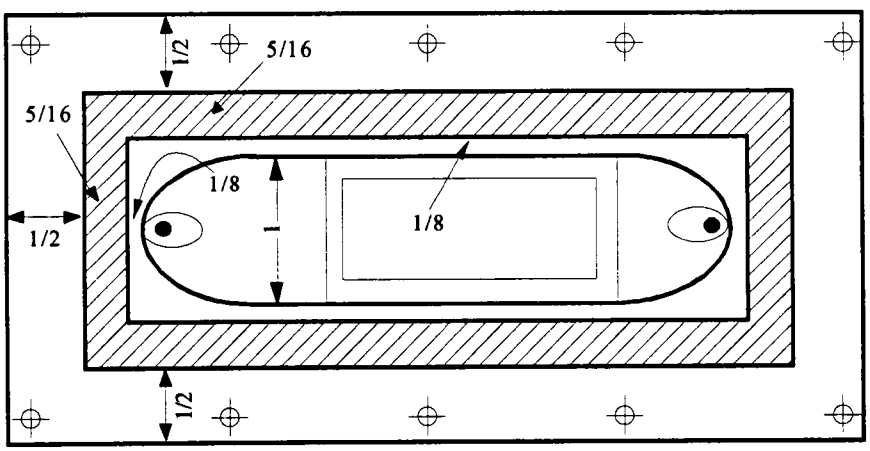
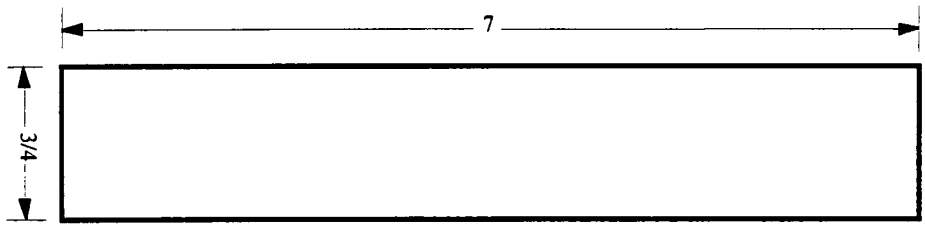


Figure 18. Laminar Flow Biofilm Reactor B schematic, Top A) Cross-sectional view, B) Top view, and C) bottom view of the flow cell top.

A.



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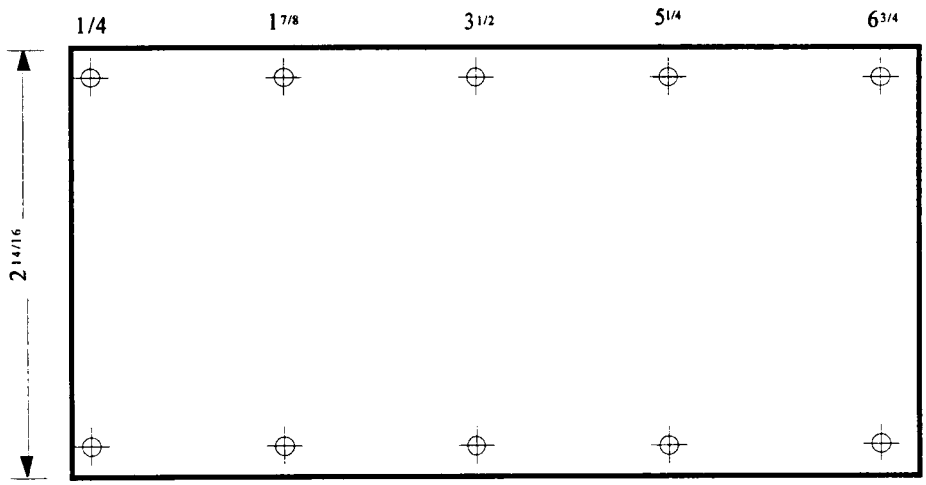

Figure 19. Laminar Flow Biofilm Reactor B schematic, Bottom. A) Cross-sectional view and B) Top View of Bottom flow cell piece.

Appendix B

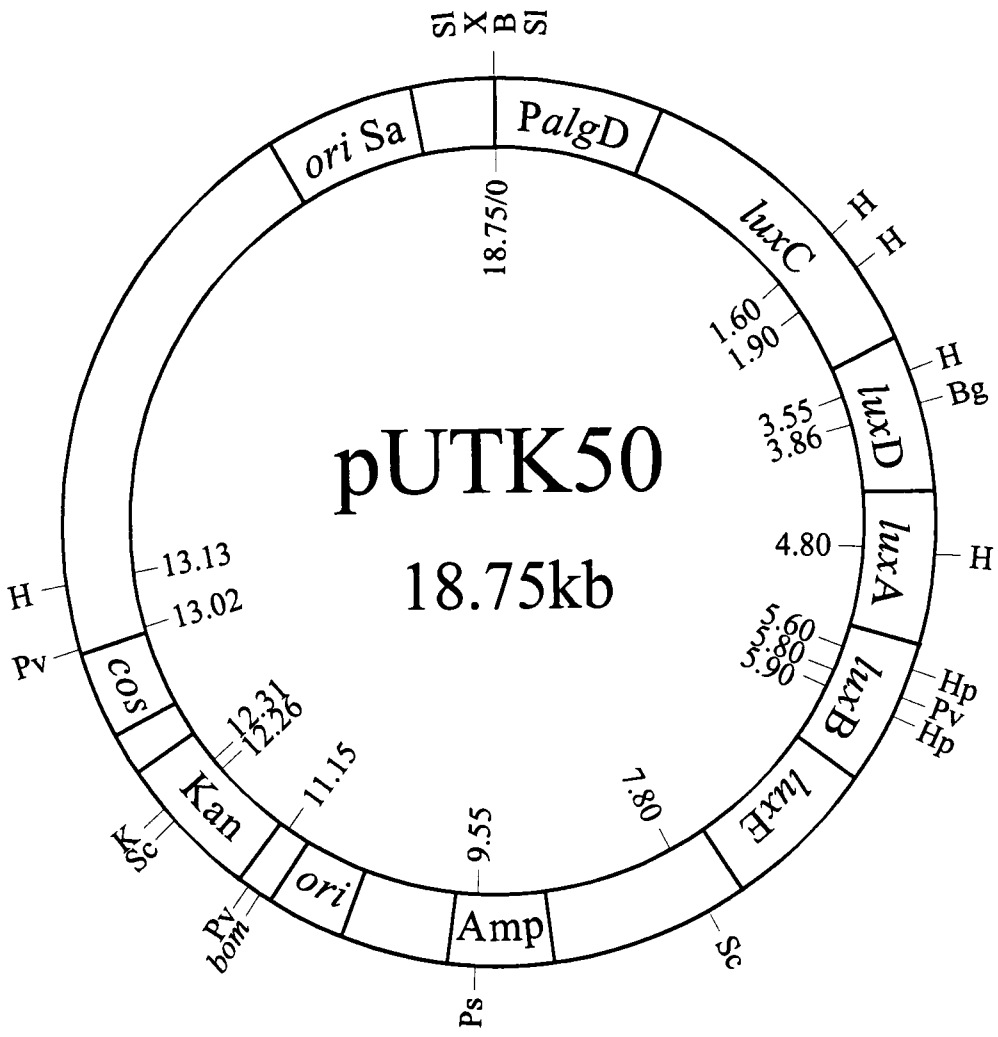

Figure 20. Restriction map of pUTK50. Abbreviations: B, *Bam*H1; Bg, *Bgl*II; H, *Hind*III; Hp, *Hpa*I; K, *Kpn*I; Ps, *Pst*I; Pv, *Pvu*II; Sc, *Sac*I; Sl, *Sal*I; Sm, *Sma*I; X, *Xba*I; *cos*, packaging site of phage lambda; *ori* Sa, origin of replication from plasmid pSa; *ori*, origin of replication from pBR322; *bom*, basis of mobilization. Distances are shown in kilobase pairs (kb). Plasmid map adapted from Wallace et al 1994a.

Vita

James Franklin Rice, Jr. was born in Knoxville, Tennessee on November 12, 1968. He has lived most of his life in the city of Knoxville, and he attended South Knoxville Elementary Grammar School, South Knoxville Middle School, and South Young High School. He began studies at the University of Tennessee, Knoxville, in 1987 in the college of engineering. After several years of study, he discovered an interest in Microbiology through his work as a lab assistant at the Center for Environmental Biotechnology. He switched majors and received his bachelor of arts in Microbiology on May, 23 1993. In the fall of 1993, he enrolled as a graduate student in the department of Microbiology.