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Effects of external stimuli on environmental bacterial strains harboring an *algD-lux* bioluminescent reporter plasmid for the study of corrosive biofilms

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An alginic acid biosynthesis bioluminescent reporter plasmid, pUTK50, was transconjugated into environmental strains of *Pseudomonas putida*, *Pseudomonas fluorescens*, and *Stenotrophomonas maltophilia*. Bioluminescent transconjugates were selected from each strain for investigation of environmental stress factors that promote alginic acid exopolymer biosynthesis in developing biofilms. Environmental stimuli associated with increased levels of alginate synthesis, in a previously developed organism, *P. aeruginosa* FRD1, were applied to the environmental strains. Increased salt concentrations and higher ratios of nitrate vs ammonium ions as the limiting nitrogen source induced bioluminescence in FRD1 and the environmental strains. However, for environmental strains of *P. putida*, *P. fluorescens* 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 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 nonidentical promoter sequences which are responsive to certain environmental stimuli and may control genes necessary for the production of alternative exopolysaccharides.

Keywords: alginate; biofilms; bioluminescence; bioreporter; exopolysaccharides; microbially induced corrosion

Introduction

Bacterial exopolysaccharides (EPS) are believed to play an important role in the environment by promoting survival strategies such as bacterial attachment to surfaces and nutrient trapping, which facilitate processes of biofilm formation and development [5]. These microbial biofilms have been implicated in corrosion of metals, bacterial attachment to prosthetic devices, fouling of heat exchange surfaces, toxicant immobilization, and fouling of ship hulls [3,6,19]. Clinically, alginate, a heteropolysaccharide commonly found in *Pseudomonas* RNA homology group 1 bacteria, is an important virulence factor involved in colonization of lung tissue in patients afflicted with the hereditary disease cystic fibrosis [11]. 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.

Previously, a bioluminescent reporter plasmid was developed to study the process of EPS synthesis in the formation and maturation of environmentally relevant microbial biofilms. This reporter plasmid, pUTK50, contains the regulatory region of the *alg*D biosynthetic operon (alginic acid synthesis) fused with the promotorless *lux* gene cassette of pUCD615 [30]. The plasmid was used to demonstrate bioluminescence in response to conditions known to affect alginate synthesis in the cystic fibrosis isolate *Pseudomonas aeruginosa* FRD1 [30].

The present study describes the conjugal transfer of the *alg*D-*lux* plasmid construct into several bacterial isolates taken from corrosive biofilms found at source water, cooling lines, and reactor surfaces at a nuclear power plant, and the characterization of these reporter strains for studies of EPS production *in situ* within a developing biofilm. The strains chosen were among a group shown to contain sequences homologous to at least one of three structural genes (*alg*D, *alg*G, and *alg*-76) or one regulatory gene (*alg*B) of the alginic acid synthesis pathway of FRD1 [31]. Under the influence of environmental conditions associated with enhanced alginate production in FRD1 and conditions attributable to the surfaces (stainless steel, copper pipes, welds) supporting biofilm formation, distinct changes were observed in bioluminescent light output relative to control cultures without the environmental influences.

Materials and methods

Bacterial strains and growth media

Bacterial strains and plasmids used in this study are listed in Table 1. Maintenance of environmental isolates and taxonomic classification by fatty acid methyl ester (FAME) analysis [27] and the Biolog Identification System (Hayward, CA, USA) were described previously [31]. Frozen stocks of each isolate were cataloged and used to start working cultures of Pseudomonas Isolation Agar (PIA, Difco Laboratories, Detroit, MI, USA) plates without additional antibiotics. Environmental strains and FRD1

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Table 1 Bacterial strains and plasmids

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	[30]
P. aeruginosa	FRD1	Prototrophic, Alg^+ cystic fibrosis isolate	Ohman DE, University of Tennessee, Memphis, TN, USA
	FRD1 (pUTK50)	Prototrophic, Alg^+ transconjugate harboring pUTK50 (Kan^R Crb^R), Lux^+	[30]
P. fluorescens C	M	Prototrophic, Crb^R environmental isolate	[31]
	M39	Prototrophic transconjugate harboring pUTK50 (Kan^R Crb^R), Lux^+	This study
P. fluorescens E	N	Prototrophic, Crb^R environmental isolate	[31]
	N15	Prototrophic transconjugate harboring pUTK50 (Kan^R Crb^R), Lux^+	This study
P. putida	L	Prototrophic, Crb^R environmental isolate	[31]
	L23	Prototrophic transconjugate harboring pUTK50 (Kan^R Crb^R), Lux^+	This study
S. maltophilia	O	Prototrophic, Crb^R environmental isolate	[31]
	O46	Prototrophic transconjugate harboring pUTK50 (Kan^R Crb^R), Lux^+	This study

Plasmid	Functional utility	Relevant characteristics	Source or reference
pRK2013	Helper in triparental matings	48 kb, ColE1 mob^+ tra^+ (RK2) Kan^R Amp^S	[14]
pUTK50	*alg*D-lux bioluminescent reporter	18.7 kb, IncW Kan^R Crb^R (1.2 kb *Eco*RI-*Hind*III fragment containing the *alg*D promoter inserted upstream of the *lux*ABCDE cassette in promoterless vector pUCD615)	[31]

Abbreviations: Amp, ampicillin; Crb, carbenicillin; Kan, kanamycin; Rif, rifampicin; R, resistant; S, sensitive

harboring pUTK50 were selected by growth on PIA plates containing either kanamycin sulfate (Sigma Chemical Co, St Louis, MO, USA) or carbenicillin disodium salt (Sigma) or both at concentrations of 300–1000 $\mu g\ ml^{-1}$ of each. *Escherichia coli* strains were stored as frozen stocks and working cultures were grown in Luria Broth (10 g tryptone, 5 g yeast extract, 5 g NaCl per liter) or on Luria Agar (Luria Broth plus 15 g agar per liter) including 30–50 $\mu g\ ml^{-1}$ 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.

Studies involving liquid cultures were carried out in a defined alginate-promoting medium (APM) modified from Mian *et al* [22] and Ohman and Chakrabarty [25]. Reagents used were the best grade available from Sigma, Fisher (Fisher Scientific, Pittsburgh, PA, USA), or Mallinckrodt (Mallinckrodt Chemical Inc, Chesterfield, MO, USA). 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 pore size 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.

For studies on the effects of increased salt concentration, a filter-sterilized stock solution of 5 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 a 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 total nitrogen in either oxidation state totaled 10 mM; the control in these experiments contained 10 mM NH_4^+ and no NO_3^-. All liquid cultures included 50 $\mu g\ ml^{-1}$ or 100 $\mu g\ ml^{-1}$ kanamycin and 50 $\mu g\ ml^{-1}$ or 100 $\mu g\ ml^{-1}$ carbenicillin to maintain selective pressure on pUTK50. All studies of biofilms were carried out in a defined medium development by Ohman and Nivens (Nivens DE, personal communication, Microbial Insights Inc, Knoxville, TN) and contained in grams per liter, 0.015 g monosodium glutamate, 0.045 g glycerin, 0.009 g $MgSO_4$, 8.5 g NaCl, 0.059 g K_2HPO_4 and 0.021 g NaH_2O_4. Immediately prior to use the medium was amended with 50 $\mu g\ ml^{-1}$ 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 [14,30]. Initial screenings for transconjugates were on PIA with 500 $\mu g\ ml^{-1}$ kanamycin. Potentially bioluminescent strains were randomly selected (50–100 individuals) and plated in grids on PIA plates with appropriate antibiotics. Colonies of organisms that had acquired antibiotic resistance were exposed to X-ray film. Non-bioluminescent environmental isolates were challenged further by growth on PIA with 500 $\mu g\ ml^{-1}$ kanamycin and 500 $\mu g\ ml^{-1}$ carbenicillin. Screening for transconjugates of *P. putida* L and *P. fluorescens* N was on PIA with both 500 $\mu g\ ml^{-1}$ kanamycin and 500 $\mu g\ ml^{-1}$ carbenicillin. Among the banks 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, 0.2-ml aliquots from liquid cultures were placed in scintillation vials positioned in front of a fiber optic cable in a chamber protected from stray light. The cable was fed into a photomultiplier tube (model 77340, Oriel, Stratford, CT, USA) and a digital detector (model 7070, Oriel). 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. Samples were taken at regular intervals in order to maintain a consistent monitoring of *alg*D induction over a 1- to 3-day period. Specific care was taken to ensure that periods of increasing bioluminescence were monitored.

Exopolymer analysis

In order to isolate EPS, 0.5-ml aliquots from liquid cultures were spun in a microcentrifuge tube for 15 min at 4° C to pellet cells. Cell-free supernatant fluid (0.4 ml) was carefully transfered to a new tube and 0.014 ml of 5 M NaCl was added 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 [7,13]. Samples were spun in a microcentrifuge 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 again added and samples were reprecipitated at −20° C, overnight. Samples were spun in a microcentrifuge and the pelleted material was dried and rinsed with 1.0 ml of 70% ethanol. Rinsed pellets were dried and stored at −20° C until assayed. Dried exopolymeric material from environmental isolates and from FRD1 was redissolved in 0.4 ml of sterile distilled deionized water and examined using the metahydroxybiphenyl/

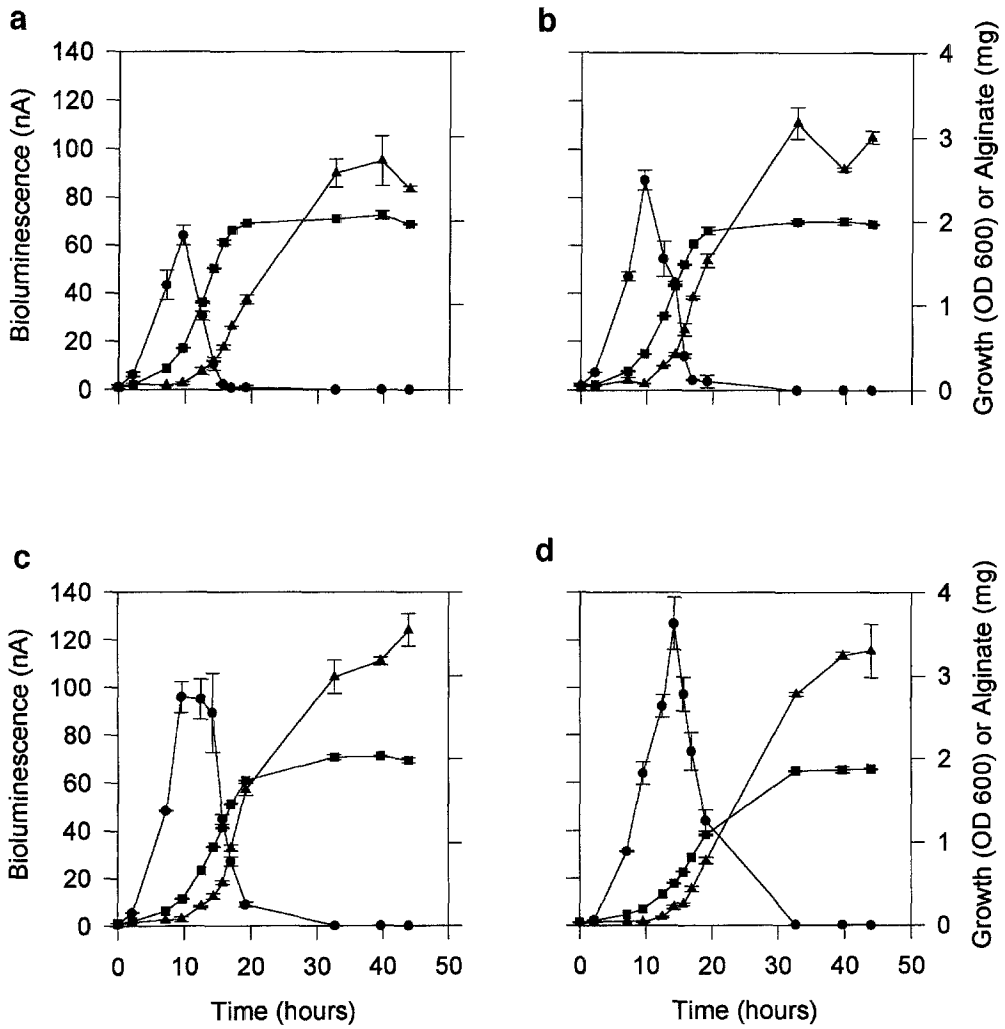

Figure 1 Bioluminescence readings (●), alginate accumulation (▲) and growth (■), during a time course experiment for *Pseudomonas aeruginosa* FRD1 grown in: (a) APM; (b) APM + 100 mM NaCl; (c) APM + 200 mM NaCl; (d) APM + 300 mM NaCl. Bioluminescence was normalized by dividing the photomultiplier amperage 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

Table 2 Relative induction of bioluminescence by environmental stimuli

Induction	Bioluminescence in strains				
	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

Table 3 Relative induction of uronic acids synthesis in bacterial isolates

Induction	Bioluminescence in strains				
	FRD1	L23	M39	N15	O46
0 mM NaCl	+[a]	++	ND[b]	++	ND
100 mM NaCl	++	+++	ND	+ 1/2	ND
200 mM NaCl	+++	++	ND	+ 1/2	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] Strains M39 and O46 demonstrated little reactivity to MHB colorimetric assays

H_2SO_4/borate (MHB) assay, as described by Blumenkrantz and Absoe-Hansen [4] and the Dionex Chromatography System (Sunnyvale, CA, USA), as previously described [15]. Sodium alginate (Sigma; high viscosity, from seaweed) was used as the standard in each case.

Detection and monitoring of bioluminescence and biomass in bacterial biofilms

Laminar flow biofilm reactors were used to monitor *alg*D expression *in situ* within a developing biofilm composed of either *P. aeruginosa* FRD1 or environmental strain, O46, of *S. maltophilia*. The flow cells used resulted from modifications of a previously described laminar flow cell [23]. These reactors contained a 2.5 × 5.0 cm stainless steel coupon located within a 2.5 cm × 13 cm × 1 mm flow channel and served as the substratum for bacterial attachment and subsequent biofilm development. Two viewing ports, each containing a quartz lens, were located directly above this metal coupon, and were used for bioluminescence, NADH and tryptophan measurements. These ports had an effective sampling size of 1 cm^2. Bioluminescence readings were taken by protecting the flow cell from stray light and taking light readings through the view ports, using a fiber optic cable and digital detector (as for liquid cultures) to monitor light output as an amperometric signal. Similarly, NADH and trytophan estimates were obtained by using a Spex Instruments Fluorolog II spectrofluorometer (Edison, NJ, USA) to monitor fluorescence at 460 and 342 nm, respectively, as described previously [2].

Sterile biofilm medium (described above) was supplied to the flow cell (previously sterilized using ethylene oxide) at a flow rate of 2.5 ml min^{-1}. Once the system had become saturated with medium the flow was stopped and 10 ml of a *P. aeruginosa* FRD1 or *S. maltophilia* O46 overnight starter culture, containing 50 ml of biofilm medium, 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. After 1 h, 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

Table 4 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
P. aeruginosa FRD1								
hv	64.1[a]	87.3	96.0	127.0	224.7	281.4	423.6	613.4
time	9.6[b]	9.6	9.6	14.3	12.0	12.0	12.0	12.0
P. putida 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
P. fluorescens C 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
P. fluorescens E 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
S. maltophilia 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 (nA) 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

alginate (or other EPSs) and to take acridine orange direct counts (AODC) and viable cell counts. During these samplings, a 1 cm^2 area of the biofilm corresponding to the areas monitored by the view port was 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 medium. These samples were the source for AODCs, viable cell counts, and MHB assays on the supernatant medium once cells were removed via centrifugation.

Results

Transconjugation of pUTK50 into environmental strains

In a previous study, 120 environmental isolates were taken from corroded copper and stainless steel pipes and reactor debris at a nuclear power plant. 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 [31]. Among those strains, the following contained DNA sequences homologous to all four alginate gene probes (Table 1): *S. maltophilia* O, *P. syringae* M and *P. putida* L.

In this study, triparental transconjugations were performed to transfer the *alg*D-*lux* reporter plasmid pUTK50 [30] into three environmental strains displaying DNA homology to four of the alginate probes [31] and one environmental strain, *P. fluorescens* N, which demonstrated homology only to the *alg*B gene probe [31]. Selection of transconjugates using PIA supplemented with 500 μg ml^{-1} kanamycin was sufficient for the mobilization of pUTK50 into environmental strains *P. fluorescens* M and *S. maltophilia* O. Under these conditions, 2% of *S. maltophilia* O transconjugates demonstrated bioluminescence, but there were no bioluminescent *P. fluorescens* M transconjugates. *P. fluorescens* M and *S. maltophilia* O were challenged further on PIA containing both 500 μg ml^{-1} kanamycin and 500 μg ml^{-1} carbenicillin. Under these conditions, 26% of *P. fluorescens* M and 6% of *S. maltophilia* O transconjugates were bioluminescent. For the environmental strains *P. fluorescens* N and *P. putida* L, 22% and 12% of transconjugates demonstrated bioluminescence, respectively, when grown on PIA with 500 μg ml^{-1} kanamycin and 500 μg ml^{-1} carbenicillin.

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 maximal (Figure 1a–d; [8]). Bioluminescence was induced in *P. aeruginosa* FRD1 cultures supplemented with 100 mM, 200 mM and 300 mM NaCl (Table 2). Uronic acid assays performed on these cultures verified that uronic acids were produced at high concentrations (Table 3), and ion chromatography identified these uronic acids as D-mannuronic acid and L-guluronic acid (Figure 2a,b). 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 1a–d) did not reflect the degree of relative induction as seen in bioluminescent light output (Table 2).

P. putida L23 differed from FRD1 in that peak bioluminescence occurred in late logarithmic or stationary phase growth (Table 4, Figure 3a). In the case of APM without additional NaCl, maximum bioluminescence occurred at early stationary phase growth (Table 4, Figure 3a). A 100 mM increase in NaCl concentration shifted the time of peak bioluminescence to late log phase growth and increased bioluminescence intensity approximately two-fold (Tables 2 and 4). Increasing the NaCl concentration to 200 mM or 300 mM resulted in bioluminescence approximately three-fold over the control (Table 2) and shifted bioluminescence into stationary phase growth (Table 4). Exopolymer from L23 was more reactive to the MHB assay than exopolymer from either *P. fluorescens* M39 or *S. maltophilia* O46 (Table 3). 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 3).

S. maltophilia O46 and *P. fluorescens* M39 resembled *P. aeruginosa* FRD1, in that peak bioluminescence occurred in logarithmic phase growth (Table 4, Figure 3b, d). The time of bioluminescence was only slightly affected in *S. maltophilia* O46 and *P. fluorescens* M39 cultures amended with 100 mM NaCl, 200 mM NaCl and 300 mM NaCl (Table 4). Bioluminescence in control cultures of these two organisms was the lowest for any of the environmental reporter strains (Table 4) and was highly induced by increasing the medium osmolarity (Table 2). Attempts to correlate the large induction of bioluminescence with alginate production were complicated by the fact that the two organisms showed very little reactivity in the uronic acids (MHB) assay.

In *P. fluorescens* N15 cultures, the time and intensity of bioluminescence were affected by the presence of additional NaCl (Table 4, Figure 3c). Increasing the concentration of NaCl to 100 mM or 200 mM resulted in approximately a two-fold increase in bioluminescence (Table 2), and in the case of 200 mM at 2.75-h shift in the time of bioluminescence (Table 4) was observed. In cultures containing 300 mM NaCl, bioluminescence was induced to an even larger degree (Table 2) and bioluminescence was shifted a total of 5.75 h compared to control cultures (Table 4). Ethanol-precipitated exopolymer from these cultures was more reactive in MHB assays than EPS isolated from *S. maltophilia* O46 and *P. fluorescens* M39. However, bioluminescence could not be correlated to this reactivity, and a trend of decreasing MHB reactivity as bioluminescence increased was apparent (Tables 3 and 4).

Effects of ammonium ion versus nitrate ion as limited nitrogen source

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 under conditions of nitrogen limitation (Figures 4 and 5a–d). Growth lag was especially evident for *P. aeruginosa*

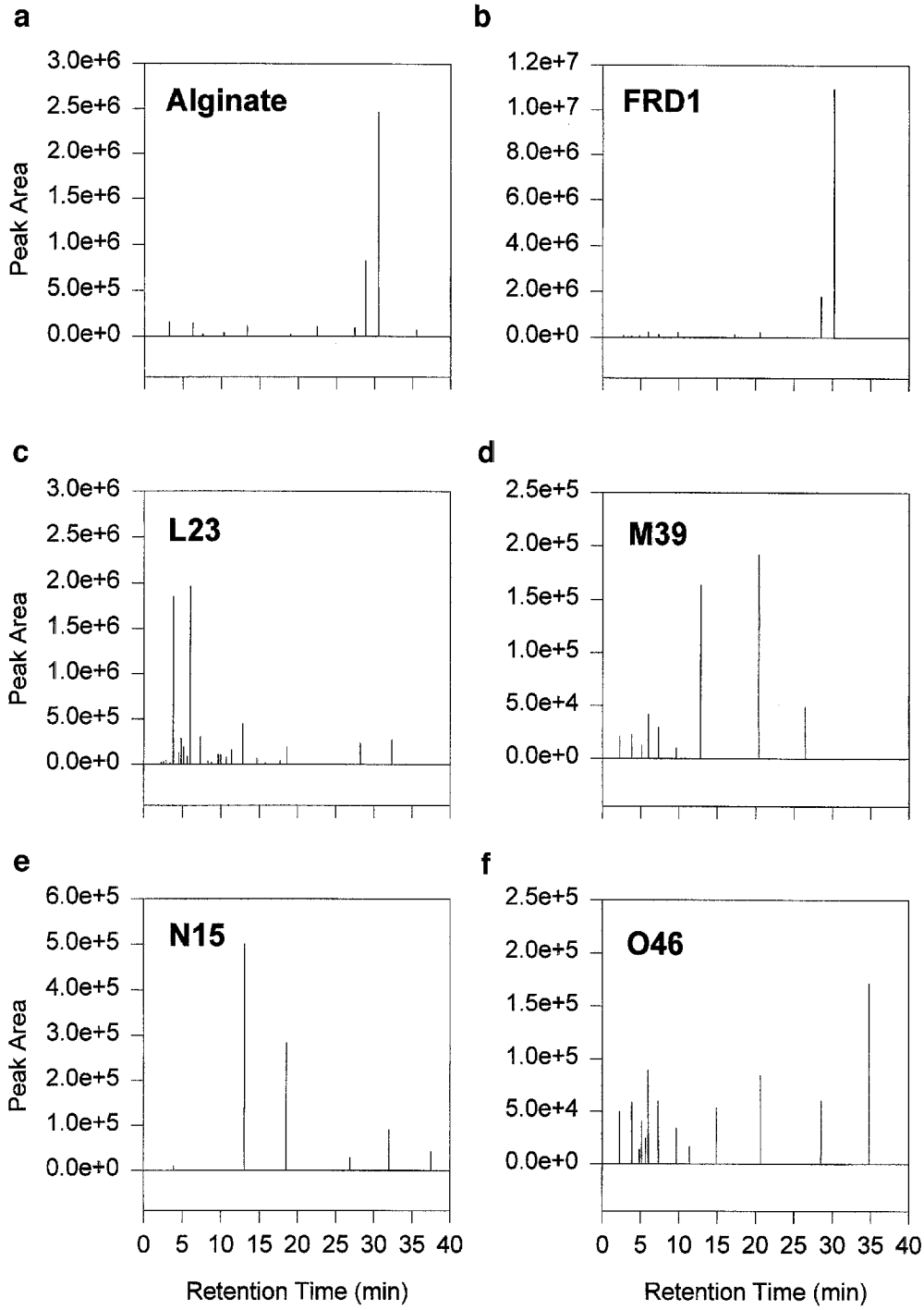

Figure 2 Ion chromatography results using the Dionex Chromatography System as described in Materials and Methods: (a) Alginate standard (1 mg ml^{-1}); (b) EPS (1 mg ml^{-1}) from FRD1 grown in APM; (c–f) EPS samples from environmental isolates (1 mg ml^{-1}), L23, M39, N15, and O46, respectively, grown in APM

FRD1, in which growth was significantly diminished in cultures containing only NO_3^- (Figure 4). In the presence of reduced levels of NH_4^+ ions, *P. fluorescens* strains M39 and N15 and *S. maltophilia* O46 each exhibited a growth lag, which was most pronounced in cultures containing 10 mM NO_3^- as the sole nitrogen source (Figure 5b–d). *P. putida* L23 exhibited only a slight growth lag in cultures in which NH_4^+ ions were limited (Figure 5a).

Shifts from 10 mM NH_4^+ to decreasing levels of available nitrogen resulted in induction of bioluminescence in *P. aeruginosa* FRD1 cultures (Tables 2 and 4). 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, which is consistent with current experimental evidence that nitrogen limitation results in induction of alginate biosynthesis [29,32]. In cultures containing

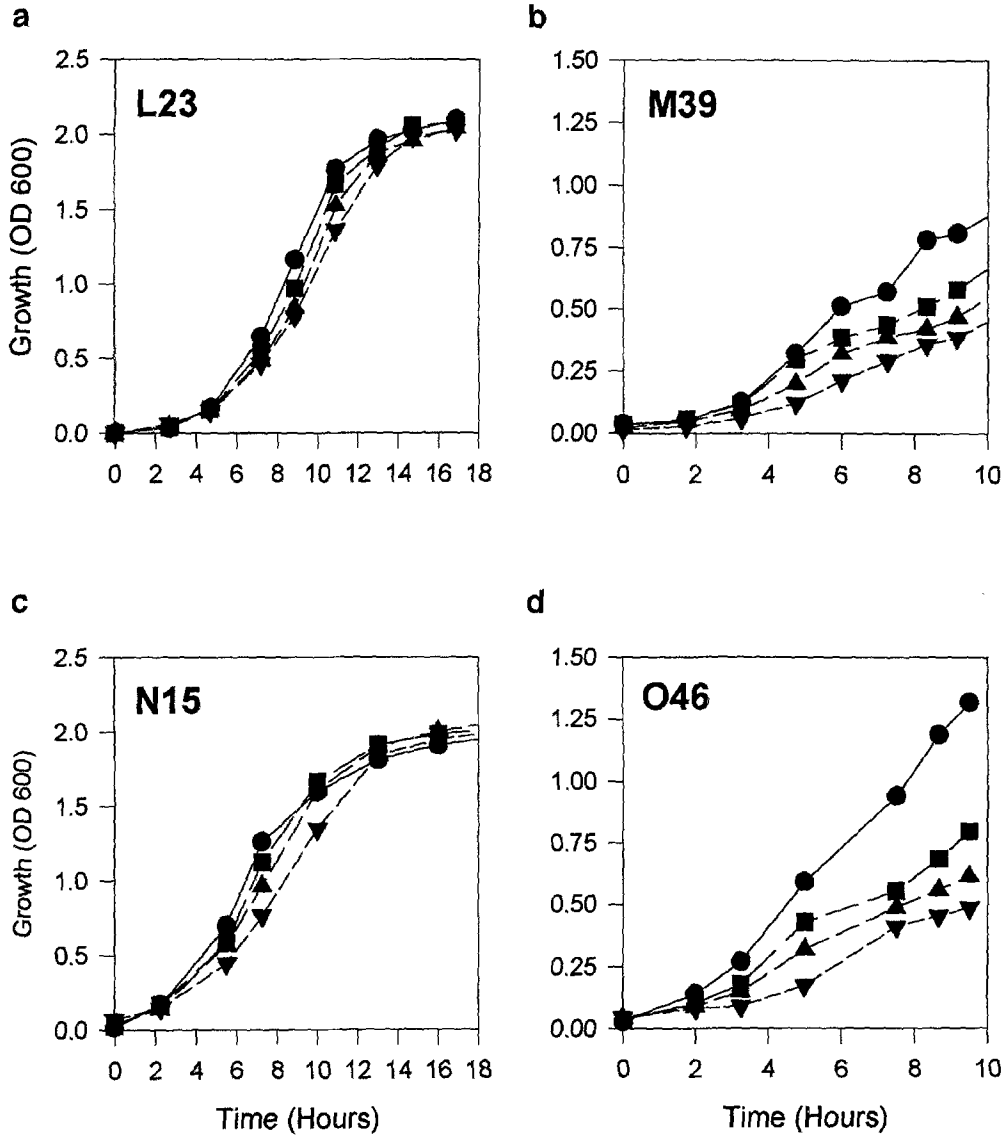

Figure 3 Growth measurements for environmental isolates grown in APM, 0.0 mM NaCl (●); APM, 100 mM NaCl (■); APM, 200 mM NaCl (▲); APM, 300 mM NaCl (▼). Bacterial growth readings were taken spectrophotometrically at 600 nm. Each point represents data from one experiment

3.25 mM NH_4^+/6.75 mM NO_3^-, a general trend of increasing bioluminescence in response to decreasing nitrogen availability was apparent (Table 4), and there was a relative induction 1.9 times the control culture (Table 2). Cultures containing only NO_3^- ions as the nitrogen source demonstrated a maximum relative induction 2.7 times the bioluminescence of the control culture (Table 2). The time of peak bioluminescence for *P. aeruginosa* FRD1 in these experiments was not variable, as was observed under conditions of increased osmolarity (Figure 4).

The environmental reporters *P. putida* L23 and *P. fluorescens* N15 demonstrated induction of bioluminescence during late log phase growth, in response to decreases in available nitrogen (Table 4, Figure 5a, c). For *P. putida* 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 2). In cultures of *P. putida* L23 containing 10 mM NO_3^-, the bioluminescence was induced 2.6-fold compared to the control cultures (Table 2). *P. fluorescens* N15 produced the greatest bioluminescence of any environmental isolate in these experiments in which nitrogen was limited (Table 4), and demonstrated the largest bioluminescence induction factors (BIF) of all the environmental isolates used in the experiments in which nitrogen was varied (Table 2). 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 no NH_4^+ ions (Table 2). The times of maximum bioluminescence for *P. putida* L23 and *P. fluorescens* N15 were not significantly altered under conditions of decreasing nitrogen availability, although *P. fluorescens* N15 demonstrated a slight shift in the times of peak bioluminescence under induced conditions (Table 4).

Induced bioluminescence, in *S. maltophilia* O46 and *P.*

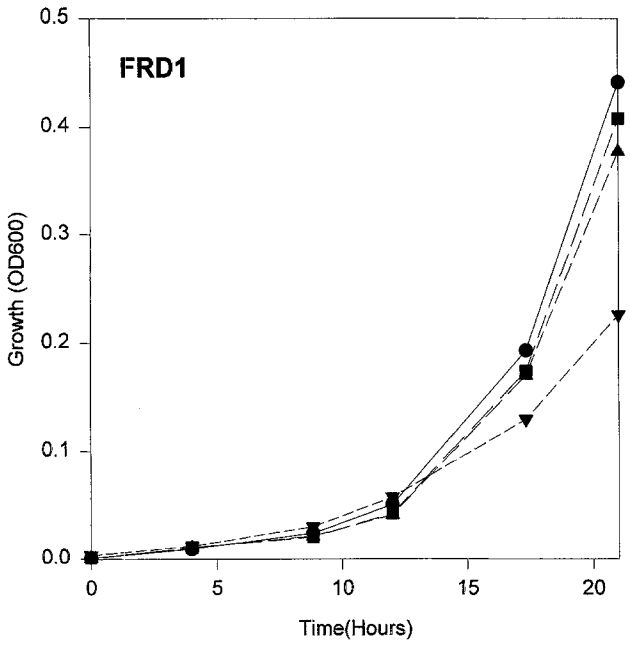

Figure 4 Growth measurements for *P. aeruginosa* FRD1 (pUTK50) growth in APM, 10 mM NH_4^+ (●); APM, 6.75 mM NH_4^+/3.25 mM NO_3^- (■); APM, 3.25 mM NH_4^+/6.75 mM NO_3^- (▲); APM, 10 mM NO_3^- (▼). Bacterial growth readings were taken spectrophotometrically at 600 nm. Each point represents the mean of two independent experiments

fluorescens M39, was observed only in the presence of 3.25 mM NH_4^+/6.75 mM NO_3^-. For these strains, no induction was observed in the presence of 6.75 mM NH_4^+/3.25 mM NO_3^- or 10 mM NO_3^- (Table 2). For *P. fluorescens* 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 *S. maltophilia* O46 (Table 4).

Preliminary characterization of the exopolymer produced by environmental isolates

Exopolymers isolated from the environmental isolates were examined using the Dionex Chromatography System to identify the major components present in the exopolysaccharides, in order to determine any similarity to the bacterial alginate produced by *P. aeruginosa* FRD1. These preliminary studies verified that for *P. aeruginosa* FRD1, alginate was present in significant amounts, and that this exopolymer was the same as was found in the alginic acid standard (Figure 2a, b). In addition, it was observed that the environmental isolates *P. putida* L23 and *P. fluorescens* N15 had a low but detectable uronic acid content (below 5%) and that for all the environmental strains other presumed polysaccharides were present in culture supernatants (Figure 2c–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 (Figure 2c–f). Major peaks for the environmental strains were considered as those containing a total peak area greater than 1E10 + 5, while all others were considered minor peaks.

Studies using biofilms

Laminar flow biofilm reactors were inoculated with the environmental isolate *S. maltophilia* O46 as described in Materials and Methods. Once the medium flow was established, bacterial biomass within the developing biofilm (estimated using the approximate amount of tryptophan present) began to accumulate (Figure 6). This biofilm growth occurred gradually over the next 4.5 days, at which time the biofilm growth began to decrease. During this period of initial growth, the bacterial activity (as measured using NADH estimates) and the bioluminescence closely paralleled the bacterial growth rate. However, once the biofilm growth rate began to diminish, a sharp increase in both the bacterial activity and the bioluminescence were observed at approximately 6 days (Figure 6). 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 decrease. This pattern continued until a maximum peak of 1.8 nA was reached at 9.6 days (Figure 6). Once the maximum bioluminescence was reached, bacterial bioluminescence decreased rapidly over the next several hours.

Discussion

Bacterial exopolymers, especially exopolysaccharides (EPS), are important in many ecological and pathogenic processes [2,6]. These extracellular macromolecules exist in a variety of forms, some consisting almost entirely of D-mannuronic and L-guluronic acids as in bacterial alginates [1,17], others may include glucose and galactose in the primary glucan backbone with varying amounts of mannose and glucuronic acid in side chains [28]. Bacterial EPS are often derivatized to varying degrees as acetate esters or pyruvate acetals under different growth conditions; they may also be associated to some degree with lipid, protein, or nucleic acid components, or derivatized with sulfate or phosphate groups [28].

The most extensive body of information addressing the molecular basis of exopolysaccharide synthesis is derived from studies on mucoid (slime-producing) strains of *P. aeruginosa* FRD1 responsible for the clinical manifestations of cystic fibrosis, and from studies on *A. vinelandii* and *X. campestris* [17,28]. On the *P. aeruginosa* chromosome, two clusters of regulatory genes (*alg*B and the *alg*R1, *alg*R2, *alg*R3 loci) are apparently responsive to environmental stimuli, and both *alg*B and *alg*R1 bear homology with bacterial superfamilies of environmentally responsive regulatory genes, including *omp*R and *ntr*C [9,10]. The protein products of the *alg*R1, *alg*R2, and *alg*R3 cluster are believed to work in concert to regulate the *alg*D promoter through topological changes in DNA structure in the far upstream sequences [16]. The *alg*D gene product GDP mannose dehydrogenase catalyzes the first step in a series of reactions that funnel sugar nucleotide precursors into a pathway committed to alginic acid synthesis [18]. Other structural genes, such as *alg*G and *alg*-76, are part of the operon controlled by *alg*D and are involved in later steps of alginic acid assimilation and modification [15]. Thus, the *alg*D promoter might serve as an indicator of the extent of alginate synthesis *in vivo*. A reporter plasmid was designed

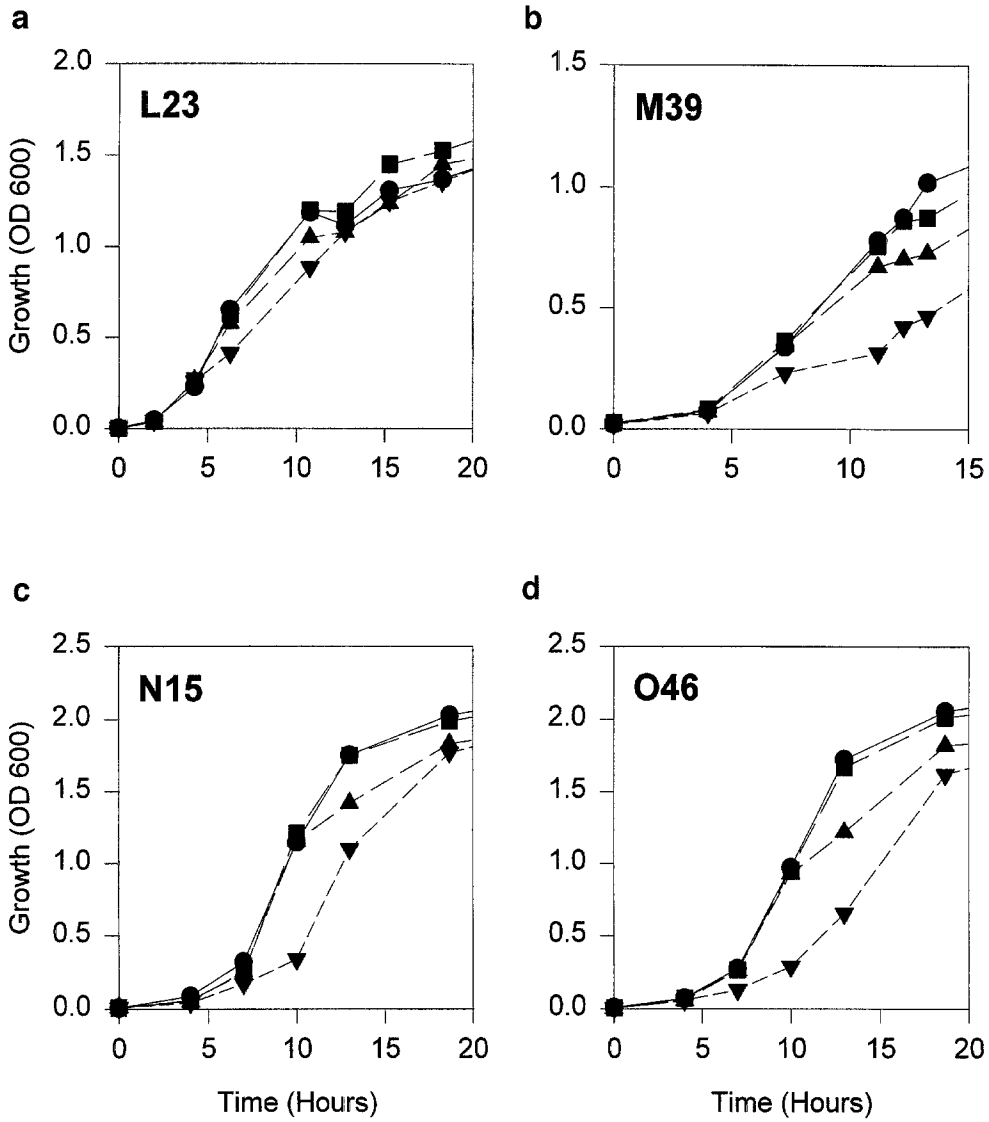

Figure 5 Growth readings for environmental isolates grown in APM, 10 mM NH_4^+ (●); APM, 6.75 mM NH_4^+/3.25 mM NO_3^- (■); APM, 3.25 mM NH_4^+/6.75 mM NO_3^- (▲); APM, 10 mM NO_3^- (▼). Bacterial growth readings were taken spectrophotometrically at 600 nm. Each point represents data from one experiment

to couple bioluminescent light output with increased activity of the *alg*D promoter, which occupies a pivotal role in the biosynthetic pathway committed to synthesis and excretion of bacterial alginate.

Initially, liquid cultures were employed to provide physiologically controlled conditions to make direct comparisons among biofilm strains and *P. aeruginosa* 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 [21,24], since bioluminescence for each culture is expressed relative to its control. This is particularly important when comparing different strains of bacteria because organisms harboring the same *lux* genes have been demonstrated to bioluminesce differently under similar environmental conditions [20].

In organisms that produce copious amounts of alginate, such as *P. aeruginosa* FRD1, the high level of alginate produced was correlated with a high capacity for bioluminescent gene expression. However, the time of onset and rate of alginic acid accumulation in the culture medium did not reflect the degree of relative induction as seen in bioluminescent light output, although alginic acid appeared to persist for a prolonged period of time in induced cultures. This suggests that the rate of EPS synthesis was not affected in this strain, but rather that it was possible for alginate to be synthesized for an extended period of time.

Factors previously associated with increased alginate

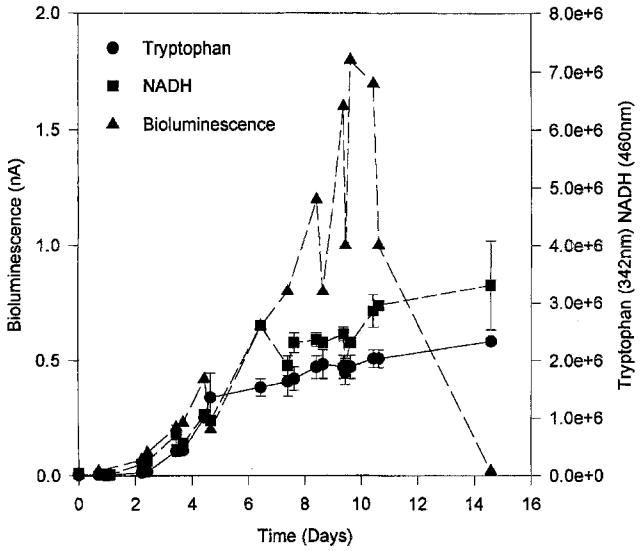

Figure 6 Bioluminescence and biomass determination in a biofilm containing *S. maltophilia* O46. Bioluminescence readings are expressed in nA (▲); fluorescence readings for tryptophan were taken at 342 nm (●) and at 460 nm for NADH estimates (■). Bioluminescence data represent the readings from the upstream port. Fluorescence readings were derived from averaging the readings from both flow cell ports. Error bars represent the standard error of this average

production, such as high osmolarity or limited nitrogen (as NO_3^-), were applied to environmental strains harboring the *alg*D::*lux* reporter plasmid. Increasing salt concentrations resulted in increased induction of bioluminescence in all environmental strains (*P. putida* L23, *P. fluorescens* N15, *P. fluorescens* M39, and *S. maltophilia* O46). Mixtures of ammonium and nitrate ions, as a limited source of nitrogen, induced bioluminescence in the environmental isolates *P. putida* L23 and *P. fluorescens* N15, and there was a definite trend of decreasing nitrogen availability vs increasing bioluminescence. However, for the other environmental strains *P. fluorescens* M39 and *S. maltophilia* O46, there was no apparent trend in induction of bioluminescence in response to decreasing nitrogen availability, and although *P. fluorescens* M39 and *S. maltophilia* 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 *P. aeruginosa* FRD1.

The evidence does not correlate bioluminescence with alginic acid synthesis in environmental isolates *P. putida* L23, *P. fluorescens* M39 and N15, and *S. maltophilia* O46. However, it does indicate that alternate polysaccharides are produced and activators of their synthesis may also induce *alg*D, and it appears that these exopolysaccharides may be regulated, transciptionally, in a manner resembling traditional global response systems, as is observed in other cellular responses such as starvation and nitrogen deprivation [11]. In fact, there is increasing evidence that alginate is regulated in response to changes in the bacteria's energy status [26] and environmental stimuli previously shown to induce global cellular changes such as the heat shock response [11]. In previous studies, the existence of alternate exopolysaccharides has been demonstrated in members of the *Pseudomonas* RNA homology group B sub-family [12], 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 these experiments, *S. maltophilia* O46 demonstrated a sustained ability for light production, under conditions necessary for biofilm growth and development. Additionally, this bacterium stably maintained pUTK50 and allowed visualization of changes in light induction throughout the course of the experiment. Consequently, these experiments demonstrate the utility of this reporter strain for studying the dynamics of *alg*D induction in response to environmental insults, during *in situ* time course experiments. Future experimentation could provide valuable information on the effects of environmental stress factors on EPS production, allowing the cellular processes involved in MIC to be studied from a molecular point of view.

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