Microb Ecol (1994) 27:225–239

MICROBIAL ECOLOGY

An *algD*-Bioluminescent Reporter Plasmid to Monitor Alginate Production in Biofilms

W.H. Wallace, J.T. Fleming, D.C. White, G.S. Sayler

Department of Microbiology and The Graduate Program in Ecology, Center for Environmental Biotechnology, The University of Tennessee, 10515 Research Drive, Knoxville, TN 37932, USA

Received: 3 May, 1993; Revised: 21 February 1994

Abstract. A broad-host range *algD-lux* bioluminescent reporter plasmid was developed to examine the role of exopolymer production in biofilm function. The *algD-lux* reporter plasmid will allow rapid on-line in situ detection of environmental factors that induce alginate biosynthesis. The *algD* promoter was stimulated by factors previously reported to induce alginate production, including ethanol and NaCl, and differences were observed with different nitrogen sources. With growth on minimal media with either glucose or succinate as a carbon source, succinate had a greater inductive effect on the *algD* promoter. An increase in light output of 1.3-fold and 1.7-fold was seen with cultures amended with 50 and 150 m*M* NaCl, respectively, compared to cultures with succinate alone. NaCl induction of the *algD* promoter was confirmed by *algD* RNA slot blots. Light output increased 2.0-fold and 1.7-fold with 0.25% and 0.5% ethanol, respectively, compared with controls grown with succinate only. While the rate of *algD* promoter response was initially similar when either NH_4 or NO_3 was used as a nitrogen source, NH_4-grown cultures maintained a higher light output during late log phase compared to NO_3-grown cultures.

Introduction

Exopolymers are known to be responsible for attachment of bacterial cells to surfaces [2]. The initial association to the surface by the bacteria is through bacterial exopolysaccharide. The cell then divides and the daughter cells are trapped within the exopolysaccharide layer, forming microcolonies. Eventually the surface is covered by a microbial biofilm that entraps other cells and macromolecules [2]. The exopolymer alginate, a copolymer of D-mannuronic acid and L-guluronic acid joined in β-1,4 linkages [15], has been shown to be important in bacterial adhesion [4,27]. Alginate was recently shown to be more corrosive to copper than

Correspondence to: G.S. Sayler.

gum arabic, culture supernatant from a marine bacteria, and *Pseudomonas atlantica* exopolymer [17].

Research pertaining to alginate biosynthesis has been dominated by studies with mucoid strains of *P. aeruginosa* isolated from the respiratory tract of cystic fibrosis patients [1,3,5,6,23,25], which has led to characterization of the alginate biosynthetic pathway and the genes encoding those enzymes in this organism [7,8,10,14,16,18]. There are numerous reports of documented environmental conditions such as osmolarity, nitrogen source, oxygen tension, and nutrient limitation (stress factors) which regulate transcription of the *algD* gene, the step at which precursors are committed to alginate synthesis [6,7,8,9,22,27]. The *algR* and *algQ* gene products (alternatively known as *algR1* and *algR2*), located at 8 minutes on the *P. aeruginosa* chromosome, are homologous to the bacterial two-component response regulators and histidine kinases, respectively, which positively regulate transcription of the *algD* gene [22,28]. A third gene, *algB*, located at 13 min is also homologous to response regulator proteins and regulates *algD* transcription [33]. The regulatory genes *algS, algT*, and *muc* are located at 68 minutes and are involved in the switching between mucoid and nonmucoid phenotypes [14]. Although many details remain unclear, it appears that these diverse sensory and regulatory components may interact in a complex fashion that may explain the range of responses to varied environmental conditions reported with different strains [7,28].

While alginate production has been characterized in a small number of bacterial species, recent studies have shown that alginate genes are present throughout the genus *Pseudomonas* even though they are not normally expressed. Of 23 bacterial species probed for the presence of *P. aeruginosa* alginate genes, i.e., *algA* (phosphomannose isomerase and GDP-mannose pyrophosphorylase), *pmm* (phosphomannomutase), *algD* (GDP-mannose dehydrogenase), and *algR* (alginate regulatory gene), all group I *Pseudomonas* species, except *P. stutzeri*, contain homologous sequences to all the *alg* probes [12]. Other bacterial species such as *Xanthomonas campestris, Azomonas macrocytogenes*, and *Azotobacter vinelandii* demonstrated DNA homology to at least one of the *alg* genes [12]. In another DNA hybridization study with 120 bacterial isolates obtained from corroded metal surfaces, 10 isolates showed homology to *P. aeruginosa algD, algB* (regulatory gene), *algG* (epimerase), and *alg76* (polymerase) genes [32].

The primary objectives of this research were to create a bioluminescent (*lux*) transcriptional fusion of the alginate biosynthetic genes to facilitate analysis of environmental factors controlling exopolymer gene expression and to permit online bioluminescent sensing of alginate production during the establishment of biofilms associated with microbially influenced corrosion. Recent results have demonstrated the potential for correlating reporter gene light emission with microbial biomass and activity in biofilm colonization by a naphthalene degrading *Pseudomonas fluorescens* containing a *nahG-lux* transcriptional fusion [21].

DNA sequence data of the *algD* gene and deletion studies determining upstream regions necessary for regulation of the *algD* gene allowed us to mobilize the 1.2 kb promoter region containing the far upstream sequences (FUS) region of the *algD* gene in front of the bioluminescent gene cassette [22]. Fusion of this environmentally responsive promoter to the bioluminescent genes of *Vibrio fischeri* will allow nondisruptive in situ quantitative analysis [30] of environmental factors that induce

Table 1. Bacterial strains and plasmids

Strain	Genotype and phenotype	Source
Bacteria *E. coli*		
DH5	F-, *end*A1 *hsd*R17 *sup*E44 *thi*-1 λ-*rec*A1 *gyr*A96 *rel*A1 (*arg*F-*lac*ZYA) U169 80d*lac* AM15	Gibco BRL
HB101	*pro*A2 *leu*B6 *thi*-1 *hsd*R *hsd*M *rec*A13 *sup*E44 *rps*L20	P. Phibbs
P. aeruginosa		
FRD1	Prototrophic, Alg+ cystic fibrosis isolate	D. Ohman
Plasmids		
pUCD615	IncW, Promoterless *lux* probe vector	C. Kado
pRK2013	ColE1-Tra (RK2)+ Km^r	
pCC27	IncP1 cos oriT Tc^r with 23 kb *P. aeruginosa* DNA containing *algD*	D. Ohman
pUTK50	*algD* promoter linked to *lux* genes in plasmid pUCD615	This study
pDJW126	*algD* structural gene is pBluescript	D. Ohman

algD transcription. The *algD-lux* bioluminescent reporter plasmid can be transferred to environmental strains containing the requisite alginate structural and regulatory genes to determine what environmental factors induce alginate biosynthesis in these bacteria.

Materials and Methods

Bacterial Strains and Plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. All *Escherichia coli* and *Pseudomonas* strains were grown on Luria broth [10 g Bacto-Tryptone (Difco Laboratories, Detroit, Mich.), 5 g Difco Yeast Extract, 5 g NaCl per liter]. Antibiotics were prepared as sterile stocks and used at the following concentrations for *E. coli:* tetracycline, 10 μg/ml; kanamycin, 25 μg/ml; ampicillin, 50 μg/ml; concentrations for *P. aeruginosa* were: kanamycin, 300 μg/ml; carbenicillin, 200 μg/ml.

Plasmid DNA Isolation and Genetic Manipulations

Plasmid DNA was isolated as previously described [26]. DNA modifications were performed by conventional procedures [29] and as recommended by the suppliers of the enzymes (Bethesda Research Laboratories, Rockville, Md.; New England Biolabs, Beverly, Mass.).

Cloning Strategy

A 1.2 kb *Hind*III-*Eco*R1 *algD* promoter fragment was obtained by restriction from pCC27 which contains the *algD* promoter and structural gene cloned from *P. aeruginosa* [3]. This *Hind*III-*Eco*R1 fragment was inserted in the multicloning site

of pBluescript (Stratagene, La Jolla, Calif.) and the resultant plasmid was used to transform competent *E. coli* cells. The insertion of cloned fragments interrupt the *lacZ* gene in pBluescript, permitting transformants with inserts to be chosen as white colonies on X-gal petri plates with selection by ampicillin. This promoter fragment was in turn excised from pBluescript by restriction using *EcoR*1 and *Hinc*II, and gel purified by agarose electrophoresis. The addition of a *Hinc*II restriction site permitted directional insertion of the *algD* promoter fragment into the *EcoR*1 and *Sma*I sites of the promoterless *lux* reporter plasmid pUCD615. The resultant construction of pUTK50 was used to transform *E. coli* cells under selection by ampicillin. The presence of the *algD* insert and orientation was determined by restriction analysis of mini-prep DNA.

Bacterial Mating

Mobilization of plasmid constructs into *Pseudomonas* hosts was by triparental matings using helper plasmid pRK2013 by methods previously described [13].

Bioluminescent Measurements in Batch Cultures

Starter cultures containing 25 ml Vogel-Bonner (VB) liquid media containing 0.8 m*M* $MgSO_4 \cdot 7H2O$, 1 m*M* citric acid H_2O, 57 m*M* K_2HPO_4, 16.8 m*M* $NaNH_4HPO_4 \cdot 4H_2O$ [31], with 25 m*M* glucose and 50 μg/ml kanamycin were grown overnight at 28°C. A 5% inoculum was added to 30 ml experimental liquid media with 50 μg/ml kanamycin in 250-ml Nephlo flasks (Baxter, Atlanta, GA) and grown at 28°C. Experimental cultures were monitored for light and growth for 36 h or until bioluminescence ceased. Bioluminescence was measured with an Oriel digital display (model 7070) with a photomultiplier tube (model 77340) (Milton, Roy, Rochester, NY) in conjunction with a fiber optic cable. Bioluminescence was standardized for cell density by dividing the photomultiplier amperage by the absorbance of the culture (namps/OD units). Absorbance was determined using a Bausch and Lomb spectrophotometer model Spectronic 70. Absorbance readings were taken at 420 nm for all experimental trials except for the nitrogen source experiments. Absorbance was determined at 550 nm for the nitrogen source experiments because excessive background absorbance at 420 nm was encountered with the nitrogen source media.

Effect of Environmental Stimuli on algD-lux *Reporter Activity*

To test the effect of osmolarity on the *algD-lux* reporter, cultures that had been grown in 30 ml of VB liquid medium with 30 m*M* succinate were amended with 50 m*M* or 150 m*M* NaCl. To test the effect of ethanol, cultures grown in 30 ml of VB liquid medium with 30 m*M* succinate were amended with 0.25% or 0.5% ethanol. To test the effect of the nitrogen source on *algD* promoter activity, cultures were grown in minimal medium containing 100 m*M* potassium phosphate buffer, pH 7.4, 10 m*M* potassium sulfate, 2 μ*M* $FeSO_4$, 1.5 m*M* $MgCl_2$, 1.5 m*M* $CaCl_2$, 8 μ*M* Na_2MoO_4, and 5 μ*M* $MnCl_2$, 55m*M* glucose, and either 20 m*M* KNO_3 or 15 m*M* $(NH_4)_2SO_4$ as the nitrogen source [7].

Messenger RNA Isolation

Starter cultures containing 25 m*M* glucose were prepared as described above and used to inoculate three 250-ml flasks containing 30 m*M* succinate in Vogel and Bonner minimal media supplemented with kanamycin (50 μg/ml). NaCl was added to two of the flasks to bring the concentrations to 50 and 150 m*M*, respectively. At periodic intervals 2-ml aliquots were taken for light readings, and cell densities were determined by checking the absorbance at 600 nm. At each time point 0.6 ml of each culture was chilled on ice, the cells were pelleted and immediately frozen at −80°C. Total bacterial RNA was isolated by the hot phenol method. Bacterial cell pellets kept on ice were vortexed in 1.5-ml eppendorf tubes with 400 μl 50 m*M* sodium acetate, 50 m*M* sodium chloride, 5m*M* EDTA, pH 5.2, 200 μl phenol equilibrated with the same buffer, and 200 μl chloroform/isoamyl alcohol (24:1) preheated to 60°C for 1 min. The solution was then warmed at 60°C for 5 min, vortexed for 1 min, and immediately put on ice. The solutions were then centrifuged, the supernatants reextracted with phenol/chloroform, and precipitated after the addition of 0.3 *M* sodium acetate with 2.5 volumes of ethanol. The pellets were washed with 70% ethanol, air dried, resuspended in 50 μl 10 *M* Tris-HCl, 1 m*M* EDTA, pH 8.0 (TE), DNase treated, extracted again with phenol/chloroform, reprecipitated, resuspended in diethylpyrocarbonate (DEPC)-treated water [29], and the optical density determined at 260 nm.

Messenger RNA Slot Blots

Plasmid pDJW126 contains a 1.4kb *Hind*III-*Eco*RI fragment of the *algD* structural gene and promoter cloned into the multicloning site of the pBluescript (KS), which permits both sense and anti-sense strands to be transcribed in vitro from the T7 and T3 promoters, respectively, that border the multicloning site. Sense strand *algD* RNA was synthesized in vitro from the T7 promoter using T7 RNA polymerase and transcription kit components according to the manufacturer's recommendations (Stratagene, La Jolla, Calif.). The absolute amount of sense RNA synthesized was calculated by the percent incorporation of ^{32}P-labeled UTP (ICN, Costa Mesa, Calif.) during the transcription reaction, as determined by trichloroacetic acid precipitation on glass fiber filters as described in the Ribonuclease Protection Assay (RPA)II manual (Ambion, Austin, Texas). This permitted an *algD* RNA sense strand standard curve to be constructed using known amounts of *algD* RNA. Aliquots (5 μg) of each RNA sample were denatured in a solution with a final concentration of 50% formamide, 7% formaldehyde, 0.15 *M* NaCl, 15 m*M* sodium citrate, pH 7.0 (1 × SSC) at 68°C for 15 min, after which they were cooled on ice and two volumes of 3.0 *M* NaCl, 0.3 *M* sodium citrate, pH 7.0 (20 × SSC) were added. The denatured RNA samples were applied to Biotrans (0.2μ) nylon membranes (ICN, Costa Mesa, Calif.) using a (Bio-Rad, Melville, NY) slot blot apparatus with a 30 cm H_2O vacuum followed by washing of the slot wells with 500 μl 1.5 *M* NaCl, 0.15 *M* sodium citrate (10 × SSC). The blot was air dried, fixed by UV light from a transilluminator, and baked at 80°C for 30 min. The blot was prehybridized for 2 h with 30 ml of solution containing 50% deionized formamide, 0.0375 *M* NaCl, 2.5 m*M* $NaHPO_4$, 0.25 m*M* EDTA, pH 7.4 (5 × SSPE), 0.1%

Ficol, 0.1% polyvinylpyrrolidone, 0.1% BSA (5 × Denhardt's) (100 μg/ml). A ^{32}P-labeled *algD* anti-sense RNA probe with a specific activity of 10^9 cpm/μg was generated from the T3 promoter of pDJW126 by the incorporation of [^{32}P]UTP using the transcription kit described above. The labeled *algD* anti-sense probe so constructed was added to the prehybridization mixture and hybridized for 18 h at 65°C. The blot was then washed with 0.3 *M* NaCl, 0.03 *M* sodium citrate, pH 7.0 (2 × SSC), and 0.1% sodium dodecyl sulfate (SDS) vigorously at room temp for 2 × 15 min followed by washing with 0.1 × SSC, 0.1% SDS at 65°C for 2 × 15 min, and placed on X-ray film (Eastman Kodak, New Haven, CT, X-Omat RP) with intensifying screens at −80°C. After development the autoradiogram was scanned with a Visage 110 computer assisted imager (Millipore, Bedford, Mass.) and the amount of *algD* RNA determined for each sample on the basis of the *algD* sense strand standard curve.

Results

Effect of Carbon Source

Construction of the *algD* promoter region with the bioluminescence (*lux*) genes of *Vibrio's fischeri* is depicted in Fig. 1. The far upstream sequences (FUS) from the mRNA start site were included to ensure the proper regulation of the *algD* promoter by the host cell's response to environmental signals. This genetic construction produced very little light response in transformed *E. coli* cells even when challenged with 300 m*M* NaCl. However, when pUTK50 was transferred to *P. aeruginosa* FRD1 and grown on *Pseudomonas* isolation agar plates, a medium known to induce alginate production [7], bioluminescence was visible to the dark adapted eye. When grown in shake flasks on VB minimal media, light production from the *algD* promoter differed when host cells were grown on either succinate or glucose as the sole source of carbon and energy (Fig. 2a). While growth on both succinate and glucose was exponential (Fig. 2b), the succinate-grown culture produced approximately 25% more light at most points during the 25 h time period. Light did not resume after the 25-h period as the cells reached stationary phase of growth (data not shown).

Effect of Environmental Stimuli

Sodium Chloride Effect. The bioluminescent response of bacteria containing the *algD-lux* construction was examined in the presence of 50 m*M* and 150 m*M* NaCl (Fig. 3a). Cultures containing the 50 m*M* NaCl concentration demonstrated a rapid induction and produced approximately a 1.3-fold increase in the amount of light over succinate-grown cultures throughout the entire growth period. Cultures containing 150 m*M* NaCl were rapidly induced to high levels and demonstrated a similar pattern of bioluminescence as cultures containing no NaCl (succinate-grown control) or cultures with 50 m*M* NaCl, but yielded a 1.7-fold increase in light production throughout the experiment. Bacterial cultures containing 50 m*M* and 150 m*M* NaCl produced light for a longer time period compared to control cultures with only succinate (Fig. 3a). Compared to the succinate-grown cultures,

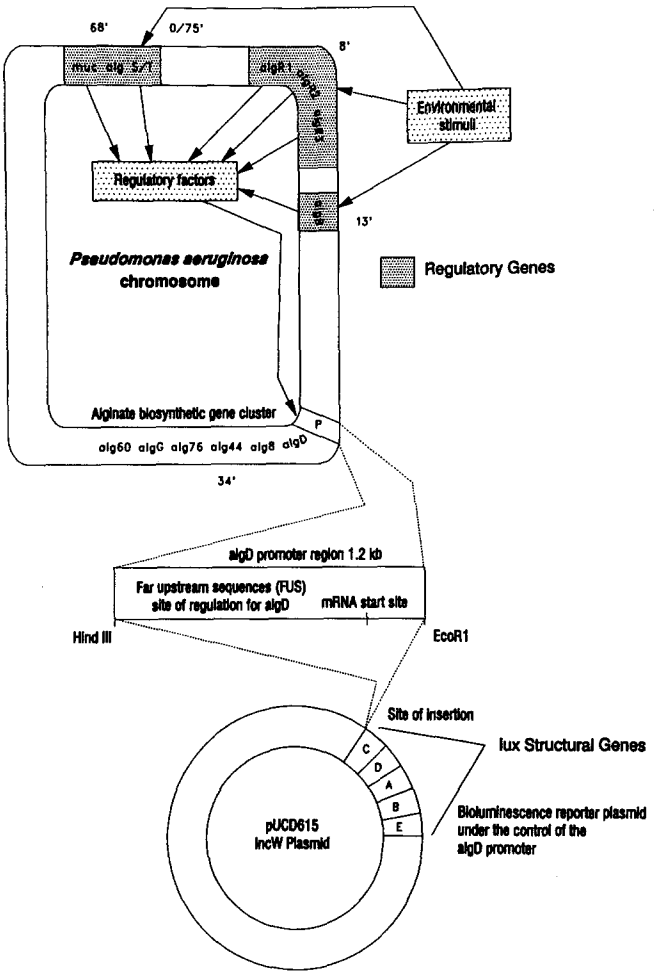

Fig. 1. Genetic arrangement of regulatory and structural genes for alginate biosynthesis on *P. aeruginosa* chromosome and construction of *algD* promoter with the bioluminescence genes in plasmid pUCD615.

the cultures containing 50 and 150 m*M* NaCl resulted in slower growth rates (Fig. 3b). Induction of *algD* mRNA mirrored the results observed with the *lux* reporter; an increase in *AlgD* expression was observed with 50 and 150 m*M* NaCl compared to the succinate control (Fig. 3c). A significant difference was observed in the time course of *algD* mRNA expression by 150 m*M* NaCl compared to the bioluminescence output of the *lux* reporter. The *algD* mRNA displayed a peak at 5 h, corresponding to a *lux* reporter luminescence peak at approximately the same time point. A second, higher luminescent peak at 24 h had no corresponding *algD* mRNA peak; at 24 h the mRNA levels for the 50 m*M* and 150 m*M* NaCl-induced cultures were only 25% and 33% of their earlier peak values.

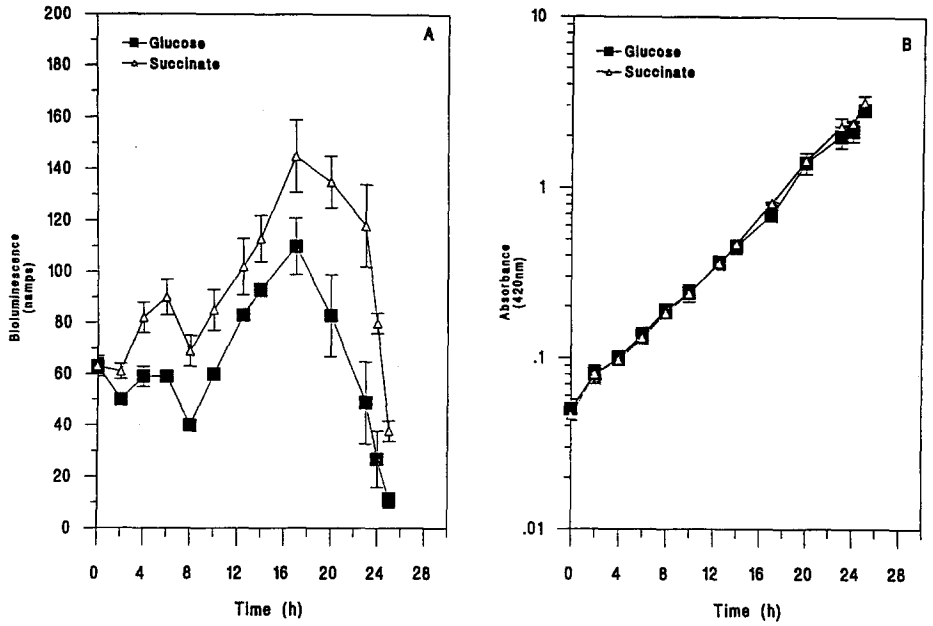

Fig. 2. **a** Bioluminescent response of *P. aeruginosa* strain FRD1 grown in 30-ml batch cultures on Vogel and Bonner (VB) minimal media with 30 m*M* glucose (■) or 30 m*M* succinate (▲) as the sole source of carbon and energy. Bioluminescence was standardized for cell density by dividing the photomultiplier amperage by the absorbance of the culture (namps/OD). Each point represents the mean from 3 independent experiments. **b** Absorbance at 420 nm of *P. aeruginosa* strain FRD1 over the period of bioluminescent measurements.

Ethanol Effect. To examine the effects of ethanol on the stimulation of *algD* promoter activity, cultures were grown in the presence of 0.25% and 0.5% ethanol. Cultures containing 0.25% and 0.5% ethanol showed a stimulatory effect of the *algD* promoter over control cultures grown on succinate alone, though light production was greater in the 0.25% ethanol (2-fold) cultures compared to the 0.5% (1.7-fold) (Fig. 4a). Bacterial growth rate was only slightly inhibited with the addition of 0.25% and 0.5% ethanol (Fig. 4b).

Nitrogen Source Effect. The effect of altering nitrogen source on the *algD*-reporter was investigated by providing cultures with either KNO_3 or $(NH_4)_2\ SO_4$ as their sole source of nitrogen (Fig. 5a). For the first 9 h, the *algD* reporter responded similarly with either nitrogen source. However, after 9 h a significant and immediate decrease in light production was observed for cultures containing KNO_3 as the sole nitrogen source, which correlates to an inflection in the growth curve at the same time point (Fig. 5b). Light production was approximately four-fold less for the cultures grown with KNO_3 compared to those grown with $(NH_4)_2\ SO_4$ at the 15 h time point. Following the rapid decrease of bioluminescence at 15 h, the KNO_3-grown culture produced light at a moderate level for an additional 12 h before a sharp decline and complete loss in light production. The bacterial culture containing $(NH_4)_2\ SO_4$ had a bioluminescence peak at 15 h and then rapidly declined, while at the 27 h time point the KNO_3 culture was producing approximately 10

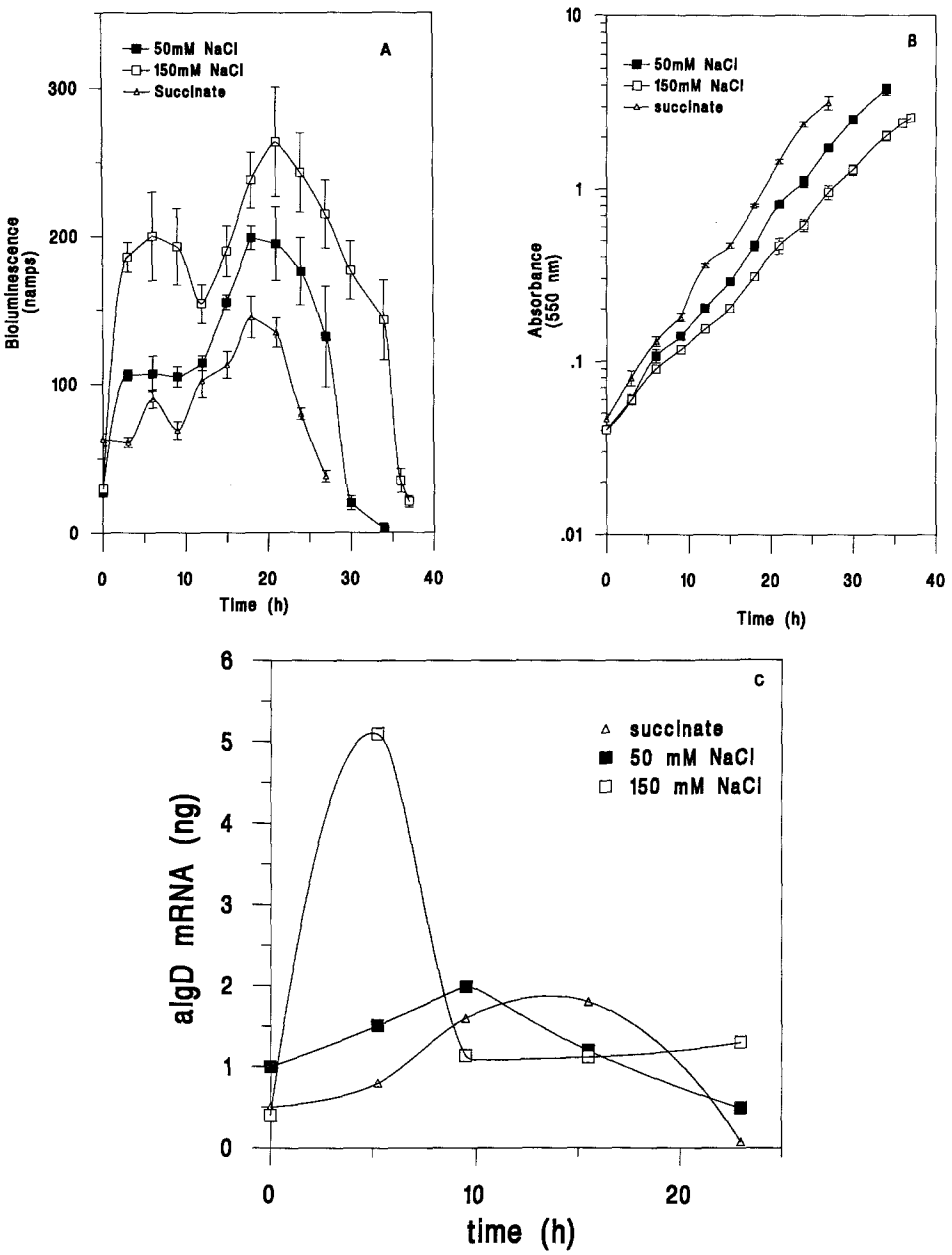

Fig. 3. **a** Bioluminescent response of *P. aeruginosa* strain FRD1 grown in 30-ml cultures on VB minimal media, 30 m*M* succinate (▲); VB, 30 m*M* succinate, 50 m*M* NaCl (■); VB, 30m*M* succinate, 150 m*M* NaCl (□). Bioluminescence was standardized for cell density by dividing the photomultiplier amperage by the absorbance of the culture (namps/OD). Each point represents the mean from 3 independent experiments. **b** Absorbance at 420 nm of *P. aeruginosa* strain FRD1 over the period of bioluminescent measurements. **c** Induction of *algD* mRNA in *P. aeruginosa* strain FRD1 grown in 30-ml batch cultures on VB minimal media, 30 m*M* succinate (△); VB, 30 m*M* succinate, 50 m*M* NaCl (■); VB, 30 m*M* succinate, 150 m*M* NaCl (□). Plotted mRNA values were obtained by quantifying *algD* mRNA slot blots against a standard curve of in vitro transcribed *algD* sense strand RNA. Each point represents data obtained from one experiment.

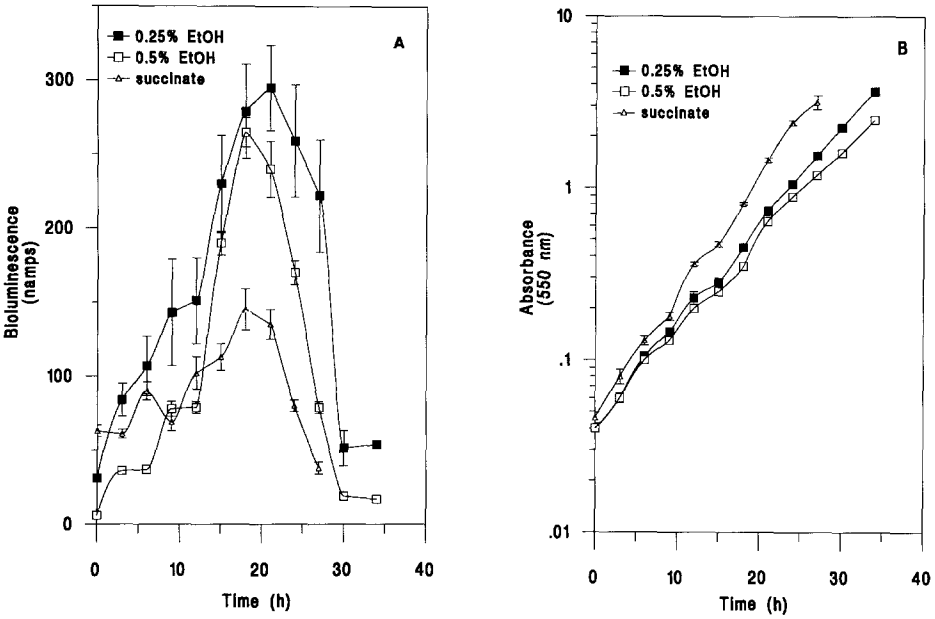

Fig. 4. **a** Bioluminescent response of *P. aeruginosa* strain FRD1 grown in 30-ml batch cultures on VB minimal media, 30 m*M* succinate (△); VB, 30 m*M* succinate, 0.25% ethanol (■); VB, 30 m*M* succinate, 0.5% ethanol (□). Bioluminescence was standardized for cell density by dividing the photomultiplier amperage by the absorbance of the culture (namps/OD). Each point represents the mean from 3 independent experiments. **b** Absorbance at 420 nm of *P. aeruginosa* strain FRD1 over the period of bioluminescent measurements.

times more light than the NH_4 culture. While early growth rates were similar for cultures grown with either nitrogen source, the NO_3 cultures demonstrated a 20% lower cell density compared to the NH_4-grown cultures at the 28 h time point (Fig. 5b). The fact that absorbance readings for the nitrogen source experiments were taken at 420 nm compared to 550 nm for the other experiments did not result in significantly different cell density readings for the nitrogen experiments compared to other experiments over similar time periods (Figs. 4b and 5b).

Discussion

In order to investigate the role of the exopolysaccharide alginate in bacterial adherence and biofilm formation we have constructed a bioluminescent reporter gene fusion with the *algD* gene (GDP-mannose dehydrogenase), which is a key step in the production of alginate by *P. aeruginosa*. Regulation by environmental factors such as osmolarity, nitrogen source, ethanol dehydration of membranes, carbohydrate metabolism, and other general stress factors appear to induce alginate biosynthesis through regulation of *algD* gene transcription.

Other researchers using either *algD-cat* or *algD-xylE* fusions have studied the induction of *algD* by parameters such as increase in NaCl concentrations, nitrogen source, and carbohydrate source [1,7,10]. However the *algD-cat* or *algD-xylE*

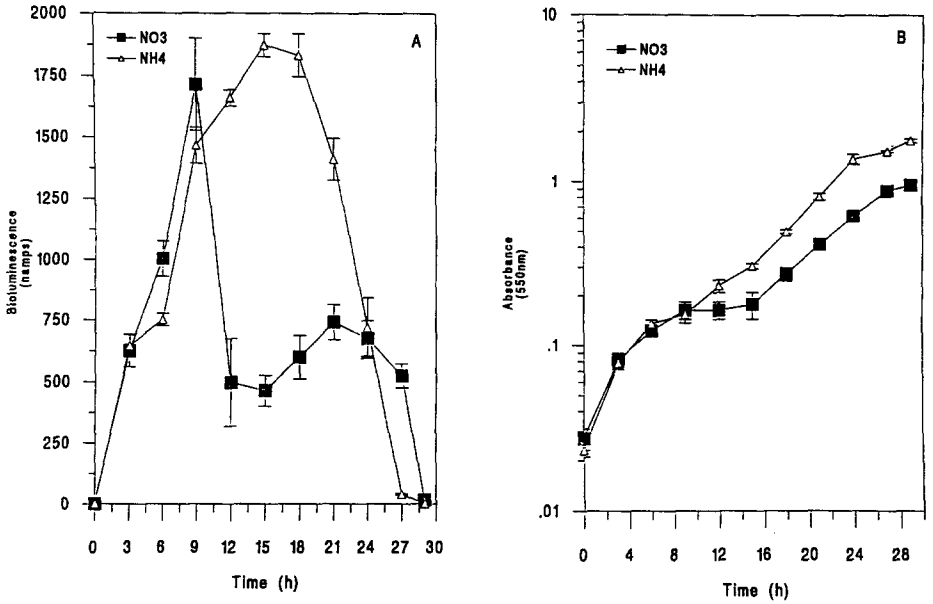

Fig. 5. **a** Bioluminescent response of *P. aeruginosa* strain FRD1 grown in 30-ml batch cultures on minimal media [7], 55 m*M* glucose, 20 m*M* KNO_3 (■); minimal media, 55 m*M* glucose, 15 m*M* $(NH_4)_2SO_4$ (□). Bioluminescence was standardized for cell density by dividing the photomultiplier amperage by the absorbance of the culture (namps/OD). Each point represents the mean from 3 independent experiments. **b** Absorbance at 420 nm of *P. aeruginosa* strain FRD1 over the period of bioluminescent measurements.

reporter system requires that cells grown under conditions for alginate induction be harvested and ruptured in order to assay for enzymatic activity of the fusion protein, thus precluding in situ analysis. In contrast the *algD-lux* reporter allows in situ, nondestructive, real time analysis of environmental conditions that affect *algD* gene expression.

To determine if the *algD-lux* fusion was a valid gene reporter system, we analyzed *P. aeruginosa* strain FRD1 containing pUTK50 under conditions previously shown to induce the *algD* gene. A high solute concentration is known to stimulate the biosynthesis of alginate through induction of the *algD* promoter [1]. In this regard the response of the *algD-lux* reporter to changes in osmolarity was similar to that of previous studies [1,7]. The *algD-lux* fusion showed a 30% increase in gene expression in the presence of 50 m*M* NaCl compared to control cells grown without added NaCl. NaCl concentrations of 150 m*M* gave an additional stimulatory effect of approximately 40% over the 50 m*M* NaCl cultures. *AlgD* promoter induction as indicated by increased bioluminescence was verified by *algD* mRNA slot blots. The *algD* mRNA peak appears to correlate with the light output peak at 6 h. If this observation is reproducible in other transformed strains under similar inducing conditions the early light peak may be used to accurately estimate transcript levels. The second light peak at 20 h most probably correlates with maximum *lux* activity, and the decrease as the cells enter stationary phase to either stability of the enzyme or an inhibitory effect on the *lux* pathway. Attempts to

correlate alginate production with light output have been inconclusive. However, preliminary results suggest that bioluminescence does not necessarily coincide with alginate production (data not shown). This is not particularly surprising in that nonmucoid as well as mucoid strains of *P. aeruginosa* can display *algD* inducibility [1].

Previous studies have demonstrated a stimulation of alginate biosynthesis in the presence of ethanol [10]. While ethanol also stimulated *algD* gene transcription, a 0.5% ethanol concentration produced less light compared with cultures grown at 0.25% ethanol. It may be possible that ethanol has a detrimental effect on the *lux* enzymes, therefore reducing light output, but there have been no studies that specifically examine this issue.

Compared to induction by EtOH or NaCl, induced light output from strain FRD1 pUTK50 was six-fold greater when grown on a minimal medium described by Deretic et al. [7] (Fig. 5a). This increase in induction was most likely not due to the stress of nitrogen limitation; compared to VB medium which contains 16.8 m*M* NH_4, the medium used in this experiment contained 30 m*M* NH_4 and 20 m*M* NO_3. The increase in light output is most probably due to the presence of additional glucose or trace metals in the second medium, though experiments to study the different effects of the individual medium components on *algD* promoter activity were not performed. However, attributing an inductive effect to glucose conflicts with initial experiments that suggested that glucose actually represses the expression of the *algD* gene approximately 25% compared to the number of cells grown on succinate (Fig. 2a), confirming previous reports of a glucose inhibitory effect on alginate production (A.M. Chakrabarty, personal communication). The differences between glucose- and succinate-grown cells cannot be attributed to differences in growth rate on the two substrates, because the growth curves are almost identical (Fig. 2b). The increase in light output from succinate-grown cells may, therefore, be due to the buffering effect of succinate in the growth medium rather than an inhibitory effect of glucose.

While both NO_3 and NH_4 resulted in induction of the *algD*, NH_4 had a longer lasting stimulatory effect. Previous studies have reported an opposite effect; the *algD* gene was expressed at significantly reduced levels during growth on NH_4 compared with NO_3, presumably because NO_3 is a poorer nitrogen source [7]. Several explanations may account for this discrepancy. If one were to consider only the 9-h time point, one would interpret the data differently suggesting the NO_3 cultures induced *algD* and the NH_4 cultures did not. In addition, several major differences exist between previous nitrogen source studies and those reported here. In the experiments of Deretic et al. [7] cultures were sampled once from agar plates as opposed to continuous readings from batch cultures as reported in this manuscript. If the early part of the luminescence curve at 6 h is taken to correlate with the actual *algD* mRNA levels, as suggested by the NaCl induction experiment, then a 25% increase in light output occurred with NO_3 as the nitrogen source as compared to NH_4. Finally, considering the great regulatory complexity of alginate production, differences in the genetic background of the host strain used in these experments, FRD1, from that of the strain used by Deretic and coworkers may account for differences in response to different nitrogen sources. KNO_3 and $(NH_4)_2$ SO_4 were used as the source of NO_3 and NH_4, respectively, in both this study and Deretic's, the only other published study on the effect of nitrogen source on *algD*

induction. The correlation of a decrease in light output from the NO_3-treated cells at 9 h corresponding to an inflection in the growth rate at the same time might be explained on this basis. Conceivably, the K^+ and SO_4^{-2} ions from the salts used here may affect the bioluminescence response, but no experiments were performed to investigate their specific role.

Under all conditions tested, pUTK50 in the host strain FRD1 exhibited a decrease in light output in late log phase. The drop in light output was mirrored by a decrease in *algD* mRNA, which actually preceded the drop in bioluminescence output. Compared to other alginate producing strains, a decrease in *algD* promoter activity may be idiosyncratic to FRD1, because many strains are reported to produce copious amounts of alginate in late log or early stationary phase. We have transferred pUTK50 to several environmental bacterial isolates, and preliminary results indicate significant *algD* promoter activity in early stationary phase. In addition, bacterial physiology is an important factor to consider when measuring bioluminescence because of the effect factors such as energy state, aldehyde substrate availability, reducing equivalents, and oxygen have on the *lux* reaction [20,24]. For *Vibrio* species, stationary phase cultures emit less light per cell than those in log phase [11,24]. In addition, various strains of bacteria with identical *lux* genes bioluminesce differently, presumably because of the influence of cell physiology [19]. Considering these factors, reports of strain specific variability in *lux* reporter bioluminescence may not be unexpected.

Having characterized the response of pUTK50 in *P. aeruginosa* FRD1 both in liquid media and biofilms, we intend, in future work, to move this *algD-lux* construction into several environmental bacterial isolates, thus allowing the study of *algD* gene expression in environmentally relevant bacteria, particularly those associated with microbial biofilms and corrosion. The *algD-lux* reporter, as a nondestructive technique, will facilitate study of the genetic mechanisms activated when bacteria attach to surfaces.

Acknowledgments. The authors gratefully acknowledge Dr. Dennis Ohman for providing the 23 kb DNA fragment containing the structural genes for alginate biosynthesis in *P. aeruginosa* and pDJW126. We also thank Dr. Clarence Kado for the *lux* vectors. This research was supported by Electric Power Research Institute Grant RP8011-02 and in part by the U.S. Departments of Energy Office of Health and Ecological Research grant DOE DE-FG05-91ER61193.

References

1. Berry A, Devault JD, Chakrabarty AM (1989) High osmolarity is a signal for enhanced *algD* transcription in mucoid and non-mucoid *Pseudomonas aeruginosa* strains. J Bacteriol 171:2312–2317
2. Characklis WG, Marshall KC (eds) (1990) Biofilms. (Wiley series in ecological and applied microbiology) John Wiley and Sons, New York, pp 341–394
3. Chitnis CE, Ohman D (1990) Cloning of *Pseudomonas aeruginosa algG*, which controls alginate structure. J Bacteriol 172:2894–2900
4. Costerton WJ, Cheng KJ, Gessey GG, Ladd TI, Nickel JC, Dasgupta M, Marrie TJ (1987) Bacterial biofilms in nature and disease. Annu Rev Microbiol 41:435–464
5. Deretic V, Dikshit R, Konyecsni WM, Chakrabarty AM, Misra TK (1989) The *algR* gene, which regulates mucoidy in *Pseudomonas aeruginosa*, belongs to a class of environmentally responsive genes. J Bacteriol 171:1278–1283

6. Deretic V, Gill JF, Chakrabarty AM (1987) Gene *algD* coding for GDP-mannose dehydrogenase is transcriptionally activated in mucoid *Pseudomonas aeruginosa*. J Bacteriol 169:351–358
7. Deretic V, Govan JRW, Konyecsni WM, Martin DW (1990) Mucoid *Pseudomonas aeruginosa* in cystic fibrosis: mutationsin the *muc* loci affect transcription of the *algR* and *algD* genes in response to environmental stimuli. Mol Microbiol 4:189–196
8. Deretic V, Konyecsni WM (1989) Control of mucoidy in *Pseudomonas aeruginosa:* transcriptional regulation of *algR* and identification of the second regulatory gene, *algQ*. J Bacteriol 171:3680–3688
9. Deretic V, Konyecsni WM, Mohr CD, Martin DW, Hibler NS (1989) Common denominators of promoter control in *Pseudomonas* and other bacteria. Bio/Technology 7:1249–1254
10. DeVault JD, Kimbara K, Chakrabarty AM (1990) Pulmonary dehydration and infection in cystic fibrosis: evidence that ethanol activates gene expression and induction of mucoidy in *Pseudomonas aeruginosa*. Mol Microbiol 4:737–745
11. Engebrecht J, Simon M, Silverman M (1985) Measuring gene expression with light. Science 227:1345–1347
12. Fialho AM, Zielinski NA, Fett WF, Chakrabarty AM, Berry A (1990) Distribution of alginate gene sequences in the *Pseudomonas* rRNA homology group 1-*Azomonas-Azotobacter* lineage of superfamily B procaryotes. Appl Environ Microbiol 56:436–443
13. Figurski D, Helinski DR (1979) Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc Natl Acad Sci USA 76:1648–1652
14. Flynn JL, Ohman DE (1988) Use of a gene replacement cosmid vector for cloning alginate conversion genes from mucoid and nonmucoid *Pseudomonas aeruginosa* strains: *algS* controls expression of *algT*. J Bacteriol 170:3228–3236
15. Gacesa P (1988) Alginates. Carbohydrate Polymers 8:161–182
16. Goldberg JB, Ohman DE (1987) Construction and characterization of *Pseudomonas aeruginosa algB* mutants: role of *algB* in high-level production of alginate. J Bacteriol 169:1593–1602
17. Jolley JG, Gessey GG, Hankins MR, Wright RB, Wachlacz PL (1989) In situ, real-time FT-IR/CIR/ATR study of the biocorrosion of copper by gum arabic, alginic acid, bacterial culture supernatant, and *Pseudomonas atlantica* exopolymer. Appl. Spectrosc. 43:1062–1067
18. Konyecsni WM, Deretic V (1990) DNA sequence and expression analysis of *algP* and *algQ*, components of the multigene system transcriptionally regulating mucoidy in *Pseudomonas aeruginosa: algP* contains multiple direct repeats. J Bacteriol 172:2511–2520
19. Meighen EA (1988) Enzymes and genes from the *lux* operons of bioluminescent bacteria. Annu Rev Microbiol 42:151–176
20. Meighen EA (1991) Molecular biology of bacterial bioluminescence. Microbiol Rev 55:123–142
21. Mittleman MW, King JMH, Sayler GS, White DC (1992) On-line detection of bacterial adhesion in a shear gradient with bioluminescence by a *Pseudomonas fluorescens (lux)* strain. J Microbiol Meth 15:53–60
22. Mohr CD, Martin DW, Konyecsni WM, Govan JRW, Lory S, Deretic V (1990) Role of the far-upstream sites of the *algD* promoter and the *algR* and *rpoN* genes in environmental modulation of mucoidy in *Pseudomonas aeruginosa*. J Bacteriol 172:6576–6580
23. Mohr CD, Hibler NS, Deretic V (1991) *AlgR*, a response regulator controlling mucoidy in *Pseudomonas aeruginosa*, binds to the FUS sites of the *algD* promoter located unusually far upstream from the mRNA start site. J Bacteriol 173:5136–5143
24. Nealson K, Hastings J (1979) Biochemistry and physiology of bioluminescent bacteria. Microb Rev 43:496–518
25. Ohman DE, Chakrabarty AM (1981) Genetic mapping of chromosomal determinants for the production of the exopolysaccharide alginate in a *Pseudomonas aeruginosa* cystic fibrosis isolate. Infect Immun 33:142–148
26. Ohman DE, West MA, Flynn JL, Goldberg JB (1985) Method for gene replacement in *Pseudomonas aeruginosa* used in construction of *recA* mutants: *recA*-independent instability of alginate production. J Bacteriol 162:1068–1074
27. Ramphal R, Pier GB (1985) Role of *Pseudomonas aeruginosa* mucois exopolysaccharide in adherence to tracheal cells. Infect Immun 47:1–4
28. Roychoudhury S, Sakai K, Chakrabarty AM (1992) *AlgR2* is an ATP/GTP-dependent protein

kinase involved in alginate synthesis by *Pseudomonas aeruginosa*. Proc Natl Acad Sci USA 89:2659–2663
29. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, second ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. Shaw JJ, Kado CI (1986) Development of a *Vibrio* bioluminescence gene-set to monitor phytopathogenic bacteria during the ongoing disease process in a non-disruptive manner. Bio/Technology 4:60–64
31. Vogel HJ, Bonner DM (1956) Acetylornithinase in *Escherichia coli:* partial purification and some properties. J Biol Chem 218:97–106
32. Wallace W, Rice J, Sayler GS (1992) Distribution of alginate genes in bacterial isolates from corroded metal surfaces. Microb Ecol 27:213–223
33. Wozniak DJ, Ohman DE (1991) *Pseudomonas aeruginosa algB*, a two-component response regulator of the *NtrC* family, is required for *algD* transcription. J Bacteriol 173:1406–1413