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Monitoring of Naphthalene Catabolism by Bioluminescence with *nah-lux* Transcriptional Fusions

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We have demonstrated the efficacy of a light-generating genetic construction in describing the induction of a *nah* operon for the catabolism of naphthalene. A fragment from plasmid NAH7, which contains the promoter for the upper pathway of degradation, was transcriptionally fused to the *lux* genes of *Vibrio fischeri*. A *Pseudomonas* strain containing this construction is inducible to high levels of light production in the presence of a suitable substrate and the *nahR* regulatory gene product. This system was used to examine catabolic activity in a unique manner under a variety of growth conditions. Induction of bioluminescence was demonstrated to coincide with naphthalene degradation in all cases through the use of mineralization assays. A significant delay in bioluminescence and biodegradation was observed when naphthalene was added to batch cultures that were growing exponentially. These results suggest that the metabolism of naphthalene by this *Pseudomonas* strain is optimal when the growth rate of the culture is slow and is greatly reduced during exponential growth.

Gene expression has been quantitatively measured by using a variety of gene fusions and biochemical assays for the gene products. Chief among these is the assay for β-galactosidase from fusions to the *lacZ* gene. This versatile reporter gene technique has also been used for the postcultivation detection of *Pseudomonas fluorescens* in soils and on root surfaces (5). Extension of this reporter gene concept to bioluminescence using *lux* gene fusions can greatly expand investigations on in situ physiological processes and activities of environmental importance. Assays for bioluminescence have important advantages of being noninvasive and nondestructive and also offer high sensitivity and real-time analysis. This report describes the fusion of the *lux* genes of *Vibrio fischeri* to a promoter from plasmid NAH7, demonstrating the efficacy of this light-producing system, and the use of this reporter to facilitate both biochemical and environmental study.

The use of bioluminescence as a genetic marker in biological systems has increased with the cloning of the *lux* genes from naturally bioluminescent organisms and genetic analysis of their components (14). In *V. fischeri*, the *lux* genes are organized into two operons, *luxICDABE* and *luxR*. Regulatory functions have been described for *luxR* and *luxI*, whereas the structural genes comprise a heterodimeric luciferase, *luxAB*, and a fatty acid reductase, *luxCDE* (14). In the light-producing reaction, the luciferase converts an aldehyde to a carboxyl group by using molecular oxygen. The reductase regenerates the aldehyde, which provides the substrate necessary for continuous light production. At least two groups have reported construction of a fusion between the *lac* promoter and the *lux* genes (7, 12). These plasmid-bearing *Escherichia coli* cells produce light at high levels when induced with an appropriate substrate. Fusions of *lux* to promoters from *Bacillus* and *Streptomyces* species have demonstrated specific induction of light production during developmentally regulated growth (3, 16). A *laf-lux* fusion in *Vibrio parahaemolyticus* produced light only when the genes for motility were activated (2). In general, these studies demonstrate the utility of *lux* fusions as reporter genes.

This study exploits the regulation of the genes for the degradation of naphthalene from the environmentally relevant plasmid NAH7. These genes are organized into two operons, comprising an upper and a lower pathway of degradation (21). The upper pathway, *nahABCDEF*, encodes the genes for the degradation of naphthalene to salicylate, whereas the lower pathway, *nahGHILNJK*, converts salicylate to acetaldehyde and pyruvate. Both the upper pathway promoter, P_{nah}, and the lower pathway promoter, P_{sal}, have a site at approximately −70 base pairs that is recognized by the *nahR* gene product, the only known regulatory gene for these operons (17, 20, 22). The NahR protein binds to this site and activates transcription only when salicylate is present (19, 24). It is assumed that when naphthalene is present, low constitutive levels of the upper-pathway enzymes generate salicylate, at which time full induction can occur. The actual mechanism of induction is still not completely understood. An excellent review of plasmid NAH7 has recently become available (23).

This report describes the subcloning of the promoter from the upper pathway of NAH7 to form a fusion with the *luxCDABE* genes of *V. fischeri*. We demonstrate the utility of this system for describing genetic expression in a unique manner, i.e., real-time analysis of a growing culture. Our results demonstrate that the upper-pathway promoter is fully induced only in the presence of an appropriate substrate and that light production is correlated with metabolism of naphthalene. Furthermore, observations from this system suggest that naphthalene metabolism is greatest when the growth rate is slow, such as during stationary phase, or in nutrient-poor medium.

MATERIALS AND METHODS

Bacterial strains. All strains and plasmids used are described in Table 1.

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TABLE 1. Bacterial strains and plasmids

Strain (plasmid)	Description	Source
E. coli		
DH5α	*rec lac* Nal^r	Bethesda Research Laboratories, Inc.
DH5(pUTK9)	P_{nah}-*lux*	This work
DH5(pUTK10)	P_x-*lux*	This work
HB101(pUCD615)	Promoter probe vector	C. L. Kado
P. putida		
PB2440	Plasmidless	M. Bagdasarian
HK53	PB2440(NAH7)	This laboratory
RB1356	PB2440(pUCD615)	This work
RB1359	HK53(pUCD615)	This work
RB1358	PB2440(pUTK10)	This work
RB1364	HK53(pUTK10)	This work
RB1357	PB2440(pUTK9)	This work
RB1351	HK53(pUTK9)	This work

Media and reagents. Luria-Bertani (LB) medium has been described previously (13). Yeast extract-peptone-salicylate-succinate (YEPSS) medium contains, in grams per liter: yeast extract, 0.2; polypeptone, 2.0; NH_4NO_3, 0.2; sodium salicylate, 0.5; and sodium succinate, 2.7. Plates were made by adding 1.5% Bacto-Agar (Difco Laboratories, Detroit, Mich.). Basal salts medium (pH 7.2) contains, in grams per liter: KH_2PO_4, 1.5; Na_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; $NaNO_3$, 4.0; $FeCl_2$, 0.005; and $CaCl_2$, 0.01. Chemicals and antibiotics were purchased from Sigma Chemical Co., St. Louis, Mo., except for naphthalene (scintillation grade; >99% pure), which was from Aldrich Chemical Co., Milwaukee, Wis. Restriction endonucleases, T4 DNA ligase, and DNA molecular size markers were obtained from Bethesda Research Laboratories, Inc., Gaithersburg, Md., except for calf intestinal alkaline phosphorylase (Sigma) and *Xho*II (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). [1-^{14}C]naphthalene with a specific activity of 8.0 mCi mmol^{-1} and a purity in excess of 98% was purchased from Sigma.

Growth conditions. *E. coli* strains were grown in LB broth at 37°C in a water bath with constant shaking at 150 rpm with antibiotic selection. *Pseudomonas* strains were grown in LB broth at 25°C in a growth chamber (Environmental Growth Chambers, Winnipeg, Manitoba, Canada) with constant shaking. Kanamycin (50 mg liter^{-1}) and ampicillin (50 mg liter^{-1}) were added for selection where appropriate. Cell growth was monitored either by plate counts after serial dilution or by light scattering, using a Klett meter (Klett Manufacturing Co., New York, N.Y.).

Recombinant DNA methods. The techniques of Maniatis et al. (13) were followed for restriction enzyme digestion, mapping of fragments, dephosphorylation of DNA, and related procedures. Ligation conditions recommended by Dardel (4) were used throughout. For some cloning procedures, DNA fragments were separated by agarose gel electrophoresis and bands were cut out of the gel. The DNA was extracted by using the Gene-Clean (Bio 101, La Jolla, Calif.) procedure. Large DNA preparations were extracted by the procedure of Anderson and McKay (1) for *Pseudomonas* species and an alkaline lysis (13) procedure for *E. coli*; cesium chloride-ethidium bromide density gradient centrifugation was used to purify plasmids. The technique of Holmes and Quigley (11) was used for screening plasmid minipreps.

The procedure of Hanahan (10) was used for transformation of *E. coli*. Electroporation was used to introduce plasmids into *Pseudomonas* species. A BTX Transfector 100 with a 400-μF capacitor was coupled with an Optimizer (BTX, San Diego, Calif.) and set for 14 kV cm^{-1} with a pulse length of 5 ms. Approximately 10^8 mid-log-phase cells were suspended in 10% sterile glycerol and layered between the electrodes. A typical pulse demonstrated a medium-specific resistivity of 25 kohm cm with an exponential decay curve. After pulsing, the cells were diluted in LB broth supplemented with 20 mM glucose and incubated for 1 h at 25°C. Successful transformants were selected on LB medium with antibiotic selection. Colony hybridization (9) was used to demonstrate the presence of plasmid DNA, in conjunction with the Genius (Boehringer Mannheim) system of nonradioactive detection.

Plasmid construction. The nucleotide sequences of the upper-pathway promoter and the *nahA* gene have been determined (18; B. Ensley, personal communication). The *nahA* gene encodes a naphthalene dioxygenase and is composed of four cistrons in this order: a flavoprotein, a ferredoxin, and the α and β subunits of an iron-sulfur protein. This information was used to obtain the appropriate promoter fragment. Figure 1 shows the subcloning procedure for construction of plasmids used in this study. A 2.7-kilobase-pair *Pst*I fragment of pE317 (8) that contains the upper-pathway promoter (18) was cloned into pUC18, with selection for colonies growing on LB plates with ampicillin and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal). White colonies were screened for the correct fragment by restriction digest. This construction, pUTK8, was used to subclone a 2.3-kilobase-pair *Xho*II fragment into the dephosphorylated *lux* plasmid vector, pUCD615 (15). This step also required cloning of the pUC18 fragment between the *Pst*I and *Bam*HI (*Xho*II) sites, but this adds only a few base pairs of vector DNA to the fragment and should not affect the fusion. Both orientations were obtained in this manner: the *nah* promoter and the first three cistrons of *nahA* were transcriptionally fused to the *lux* genes, forming pUTK9; pUTK10 has the opposite orientation.

The natural host for the NAH7 plasmid is *Pseudomonas putida* PpG7. Constitutive light-generating plasmid constructions have been introduced into PpG7, revealing that this strain is a poor host for light generation (unpublished data). This characteristic is probably due to a lack of appropriate aldehyde substrate in this strain, since addition of *n*-decyl aldehyde (Sigma) allows the expected light production to occur. Since the difficulties inherent in the use of this aldehyde are great (including toxic effects on the cells), it was decided to utilize *P. putida* PB2440, which has demonstrated light-producing potential in the absence of added aldehyde (unpublished data). Plasmid NAH7 was mated into PB2440, forming strain HK53. Since this strain grows on naphthalene or salicylate as a sole carbon and energy source, the catabolic pathways appear to function normally in this host. This strain and the parent strain PB2440 were used as hosts for the pUCD615 plasmid and for the constructed plasmids pUTK9 and pUTK10, which were introduced by electroporation. The resulting eight strains are listed in Table 1. Strains were maintained on LB or YEPSS agar medium.

Light measurement. An Oriel (Stratford, Conn.) digital display model 7070 with a photomultiplier tube model 77340 connected to a flexible liquid-light pipe and collimating beam probe was used for all light measurements. Light detection is measured as a current from a photoelectric effect and is sensitive to 10^{-12} A of induced current. The amperage is convertible to power units (watts) by multiplying the amperes by 3.08×10^3 A/W^{-1}, the conversion factor at the peak

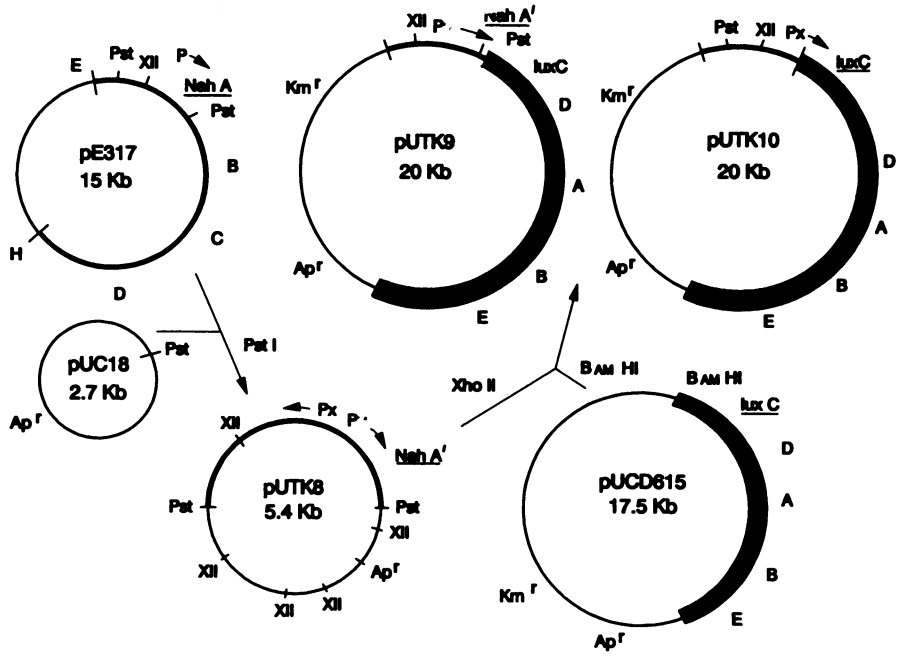

FIG. 1. Subcloning of the *nah* promoter and construction of pUTK9 and pUTK10. A *Pst*I fragment of pE317 (8) was cloned into the *Pst*I site of pUC18, creating pUTK8. A *Xho*II fragment of pUTK8 was cloned into the *lux* vector pUCD615 at the *Bam*HI site to create both pUTK9 and pUTK10. P, *nah* upper-pathway promoter, P_x, putative divergent promoter; XII, *Xho*II; E, *Eco*RI; Ap^r, ampicillin resistance, Km^r, kanamycin resistance. Arrows show direction of transcription. kb, Kilobase pairs.

wavelength for the expressed light (about 500 nm). For convenience, the light measurements in this work are reported as amperes. The photomultiplier was connected to an IBM PS/2 computer through a RS232 port, allowing continuous sampling of photomultiplier output over long periods of time. The software was written by Rod Bunn, University of Tennessee. The photomultiplier was set inside the growth chamber, which was maintained in the dark at constant temperature throughout the experiment.

Mineralization. Naphthalene metabolism was monitored by using a mineralization procedure to measure conversion of [^{14}C]naphthalene to $^{14}CO_2$. Overnight cultures of RB1351 were washed once in basal salts medium and then suspended at a concentration of 10^7 ml^{-1} in either LB or basal salts medium in 25-ml EPA vials with Teflon-lined silicone septa and caps (Pierce Chemical Co., Rockford, Ill.). Unlabeled naphthalene was added to the vials at a concentration of 100 mg liter^{-1}, and 100,000 dpm of [^{14}C]naphthalene was dissolved in 2 μl of acetone and added before the vials were sealed. $^{14}CO_2$ was collected in a 7.5-ml glass vial containing 0.5 ml of 0.4 M NaOH maintained inside the larger cultivation vial. The vials were shaken at 100 rpm in the dark at 25°C. Duplicate vials were taken at each time point, after determining the light output as described above. The assay was terminated by addition of 0.5 ml of 0.2 N H_2SO_4 injected into the large vial. After 30 min, the NaOH solution was removed to 10 ml of scintillation fluid (Beckman Instruments, Inc., Fullerton, Calif.) and counted in a Beckman LS3801 scintillation counter. The H# method as described by Beckman Instruments was used for automatic quench compensation and disintegrations-per-minute conversion of counts-per-minute data on the basis of standard quench curves. The incubation medium was then extracted with 5 ml of hexane. A 0.5-ml sample from the hexane phase was used to determine the remaining naphthalene by counting the sample in 10 ml of Econofluor (Dupont, NEN Research Products, Boston, Mass.). The ^{14}C-labeled polar metabolites were determined by taking a 0.5-ml sample of the aqueous phase and counting in Beckman Ready-Protein scintillation cocktail.

Continuous culture. The reactor system consisted of a New Brunswick Scientific BiOFLO model C30 chemostat modified with a Teflon top and tubing. The reactor liquid volume was 350 ml, with a 10-liter feed tank adding the medium at a rate of 72 ml h^{-1}. Temperature was maintained at 25°C, agitation was held at 100 rpm, and sterile aeration was held at 0.1 liter min^{-1}.

RESULTS

pUTK9 produces light in response to induction. The promoter from the upper pathway of NAH7 was subcloned into pUCD615 according to the scheme outlined in Fig. 1. The identities of these plasmid constructions were confirmed by restriction digest and subsequent Southern blotting of the fragments (Fig. 2). DNA hybridization with NAH7 showed the expected pattern in each construction. Each of the *Pseudomonas* strains listed in Table 1 was grown overnight as a single colony on LB agar medium. Table 2 reports the light output from isolated colonies on plate assays in both the presence and absence of naphthalene, which was supplied as vapor from naphthalene crystals. As expected, most strains did not produce any measurable amount of light, either because they do not contain the *lux* genes or because they lack the *nahR* regulatory gene from the NAH7 plasmid. It has been demonstrated that the *nahR* gene is necessary for induction of the upper-pathway promoter (17).

Strain RB1351, which harbors both pUTK9 and NAH7, contrasted sharply with these other strains. Expression of the upper pathway was constitutive at low levels, and expression increased substantially (about 24-fold) when naphthalene vapor was present (Table 2). Figure 3 demon-

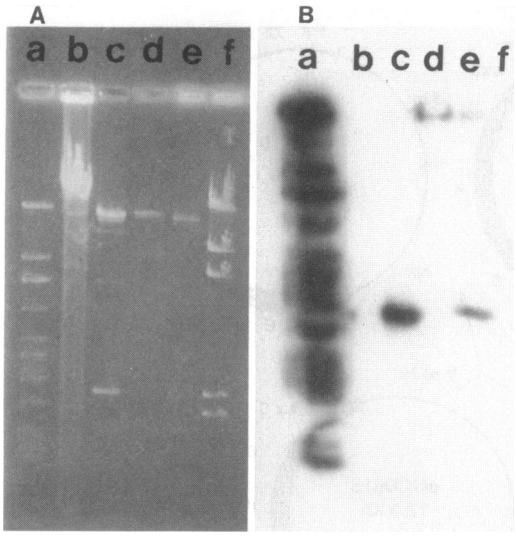

FIG. 2. Restriction analysis of pUTK9. (A) Restriction digests of the plasmids used in this study; (B) autoradiograph of the gel when NAH7 plasmid DNA was used as the probe. Lanes: a, NAH7 digested with *Eco*RI; b, pUCD615 digested with *Eco*RI and *Bam*HI; c, pUTK10 digested with *Eco*RI and *Bam*HI; d, pUTK9 digested with *Eco*RI and *Bam*HI; e, pUTK9 digested with *Eco*RI and *Xba*I; f, lambda phage DNA digested with *Hin*dIII.

strates the rapid bioluminescent response of the strain after naphthalene induction. An increase in light production was apparent within 3 min and reached its peak within 10 min. Light production was stable for at least 24 h on these plate assays, varying by approximately 10% during this time. Plasmid pUTK10 was created to determine whether a divergent promoter overlaps P_{nah}, much as the promoters for the lower pathway and *nahR* overlap. Strain RB1364, which contains both NAH7 and pUTK10, produced a very low level of light (less than 10% of the uninduced P_{nah}) that was apparently unaffected by the presence of naphthalene (Table 2). There did not appear to be sufficient evidence for a promoter in this orientation.

Light production of RB1351 in liquid media. It was anticipated that liquid batch cultures of RB1351 could be induced with naphthalene in the same way that colonies were induced. RB1351 was grown in LB broth with kanamycin selection to a mid-log growth phase, at which time naphthalene was added. Figure 4 demonstrates the growth curve and light output of this strain. Two observations are significant from this experiment. (i) Light was not produced until several hours after naphthalene addition, which corresponds to a late logarithmic phase of the growth curve. This was true whether naphthalene was added during early or mid-log phase. Addition of naphthalene at a stationary phase resulted in a gradual increase in light production, much the same as was seen for the colonies (data not shown). (ii) The light response curve showed a complex pattern. A control culture to which no naphthalene was added demonstrated a light pulse during the late log phase; this pulse was apparently due to the induction of the upper-pathway promoter, even though an inducing substrate had not been added. When naphthalene was added, a large, relatively short-lived peak of light production occurred first, followed by a smaller, more stable plateau. The response of this short-lived peak was approximately the additive value of the control peak and the naphthalene-specific plateau. Light production during the plateau was very stable, remaining almost unchanged as long as naphthalene was present.

TABLE 2. Light production by *Pseudomonas* strains[a]

Strain	Light production (10^{-9} A of induced current)	
	− Naphthalene	+ Naphthalene
PB2440	0	0
HK53	0	0
RB1356	0	0
RB1359	0	0
RB1358	0	0
RB1364	0.060	0.080
RB1357	0	0
RB1351	0.860	21.0

[a] Light production was determined as described in Materials and Methods. Naphthalene was supplied as vapor from crystals, and measurements were taken after approximately 30 min. Each strain was present as a single colony grown overnight at 25°C on LB agar. A value of 0 indicates that less than 10^{-12} A was detectable on the photomultiplier.

It was expected that the bioluminescent response would occur soon after addition of the substrate. A trivial explanation of these results would be that the aldehyde substrate required for the light-generating reaction was not available at the time of naphthalene addition. However, addition of *n*-decyl aldehyde to growing cultures as described previously (6) at a final concentration of 0.001% produced no significant changes in light output (data not shown). This finding raised the possibility that bioluminescence was not correlated with naphthalene metabolism in this system, which would be surprising but possible. The other major possibility is that bioluminescence is correlated with naphthalene metabolism but that there is a catabolite repression of the upper pathway in rich medium that is exhausted by late log phase or that perhaps naphthalene catabolism is growth rate regulated.

Naphthalene degradation is correlated with light production. To determine when naphthalene was being utilized during culture growth on rich medium, and by extension whether bioluminescence was correlated with naphthalene metabolism, a series of mineralization experiments was performed by using radiolabeled naphthalene. This arrangement allowed determination of the fraction of naphthalene converted to CO_2 (mineralized), the fraction of unused naphthalene, and the fraction that was still cell associated (biomass, or naphthalene uptake). Excess headspace in the vials provided ample oxygen during the experiment. Figure 5A shows the fate of naphthalene when basal salts medium was used. Naphthalene utilization was readily apparent, both from the removal of initial naphthalene and from the increase in radiolabeled CO_2. Light output increased gradually throughout the experiment, as expected. However, the viable cell count was unchanged throughout the course of the experiment. Catabolism of the naphthalene substrate appeared to be used for cell maintenance. A separate experiment in which the growth medium was supplemented with 6 mM glucose showed similar results (data not shown), indicating that there was no catabolite repression with this substrate. When this experiment was repeated with use of LB broth, a very different pattern appeared (Fig. 5B). Cell growth was evident throughout this experiment, as was observed with the batch experiments described above. The production of light was delayed until the late log phase, also as expected from previous results. It is evident from the data on the utilization of naphthalene, however, that there was

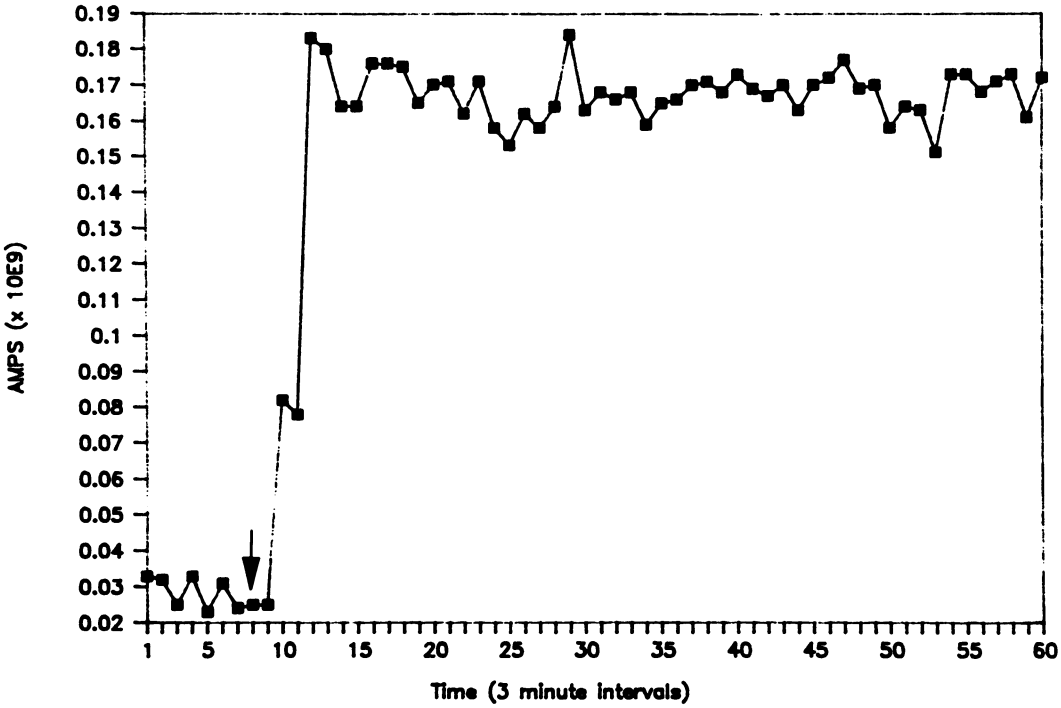

FIG. 3. Response of RB1351 colonies to naphthalene. *Pseudomonas* sp. strain RB1351 was grown to mature colonies on LB agar medium. Naphthalene was supplied as vapor from crystals placed on the lid of an inverted plate at time point 8 (arrow). E, Exponent.

very little catabolism during the log phase. This finding is supported by the data from the unused naphthalene and from the generated CO_2 fractions. These data demonstrate that naphthalene metabolism is closely correlated with bioluminescence, whether in rich or in minimal medium.

Bioluminescence is greatest at a slow growth rate. The correlation of bioluminescence with naphthalene utilization leaves open either of two major possibilities: that there is a catabolite repression of the upper pathway, or that there is a preferred growth rate for the utilization of naphthalene. It has been suggested that there is no catabolite repression of the NAH7 pathway (20), although data demonstrating this effect in LB medium are lacking. To determine whether catabolite repression was present, a culture of RB1351 was grown to a mid-log phase in LB broth, washed twice with basal salts medium, and resuspended in basal salts medium with naphthalene. No light was observed from this culture even after several hours (data not shown). Since it was suggested that this procedure could induce a stringent response that might mask true catabolite repression, a second

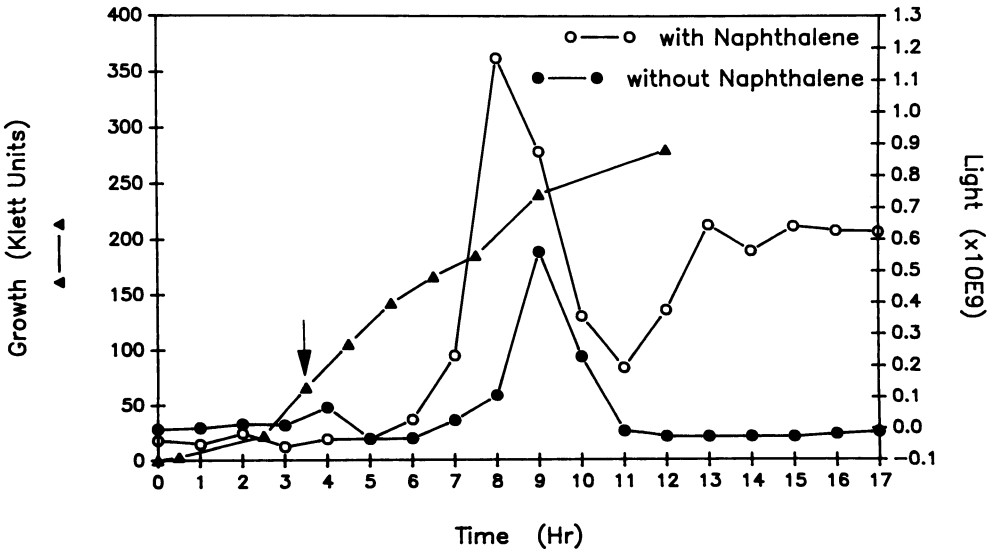

FIG. 4. Response of RB1351 batch cultures to naphthalene. *Pseudomonas* sp. strain RB1351 was grown in LB broth at 25°C with constant stirring. Naphthalene crystals were added at 1% (wt/vol) final concentration (arrow). Culture growth was determined by using a Klett meter. E, Exponent.

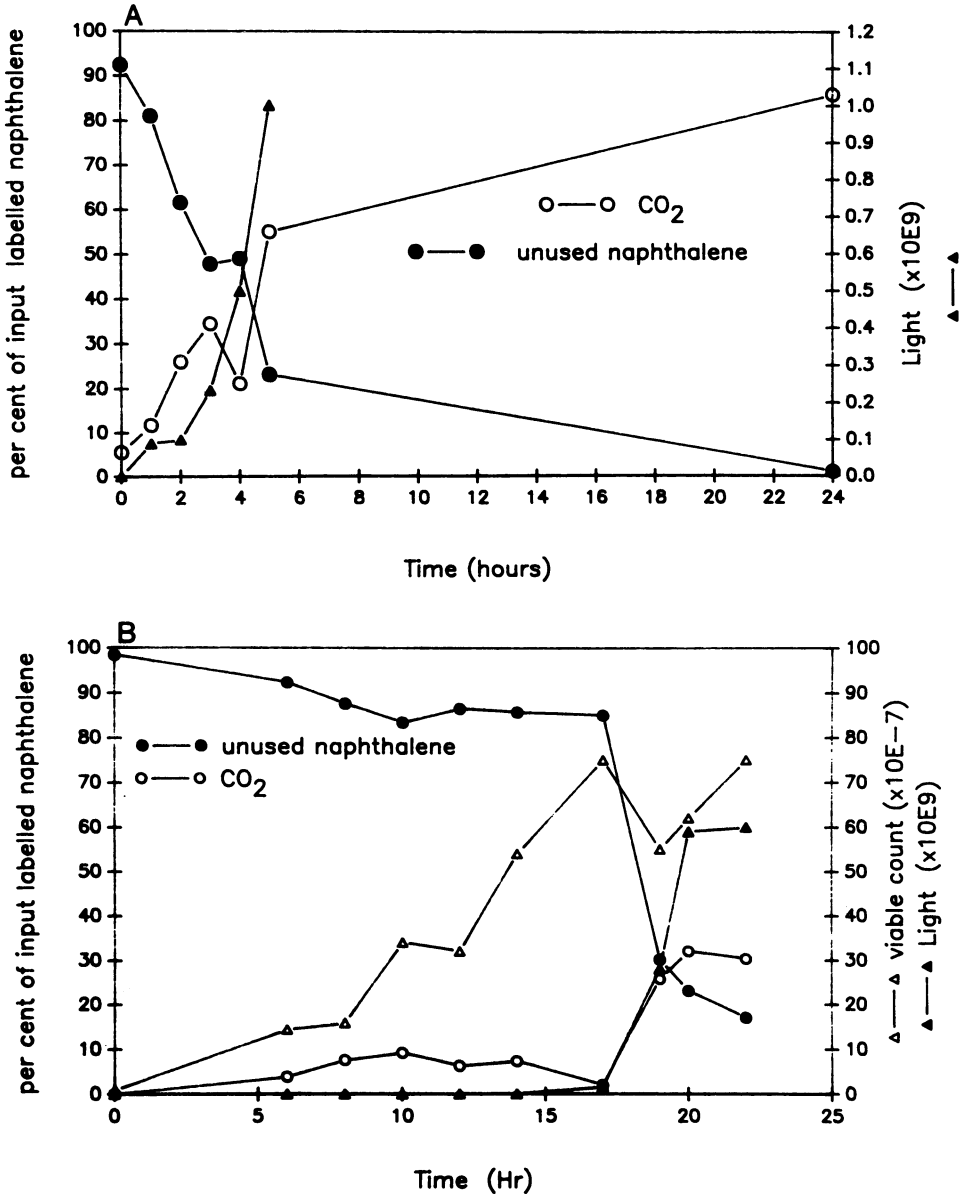

FIG. 5. Mineralization assay with RB1351. Duplicate vials were incubated with [^{14}C]naphthalene in either basal salts medium (A) or LB medium (B). See Materials and Methods for analysis of fractions. Light output was measured immediately before fraction analysis. Cell concentration in panel A remained at 3×10^7 throughout the experiment. E, Exponent.

method was used. A batch culture of RB1351 was grown overnight in LB medium saturated with naphthalene in order to allow the cells to reach a stationary phase and to deplete the medium. Identical 0.5-ml samples were collected and centrifuged to form a pellet. One sample was resuspended in 9 ml of spent medium, and the other was resuspended in 9 ml of fresh LB-naphthalene medium. Both flasks were shaken at 25°C and periodically examined for light output and cell growth (using a Klett meter). The spent medium was unable to support much additional growth, but there was sufficient naphthalene present to permit high levels of light output (Fig. 6). The fresh medium, in contrast, allowed the culture to reenter log phase growth, but the light output decreased dramatically. Interestingly, while both cultures were in the lag phase the light output per cell was almost identical, but the light output from the fresh medium culture fell off sharply with the first time point clearly in log phase. If catabolite repression was functioning in this system, it would be expected to have a noticeable effect during the first hour after addition of fresh medium. These results strongly suggest that there is no catabolite repression, but that a slow rate of growth is preferred for activation of the *nah* genes.

Chemostat cultures can generate stable light production. If the hypothesis stated above is correct, a chemostat culture growing at a sufficiently slow rate should allow the maximum level of light production from RB1351. Figure 7 depicts a chemostat culture of RB1351 grown in a basal salts-succinate (20 mM) medium and an LB medium. The dilution rate was very low, approximately 0.21 hr^{-1}, with a doubling time of approximately 3.3 hr instead of 0.7 hr during exponential growth. Addition of either naphthalene or salicylate to the reaction vessel gave the same bioluminescent response, both

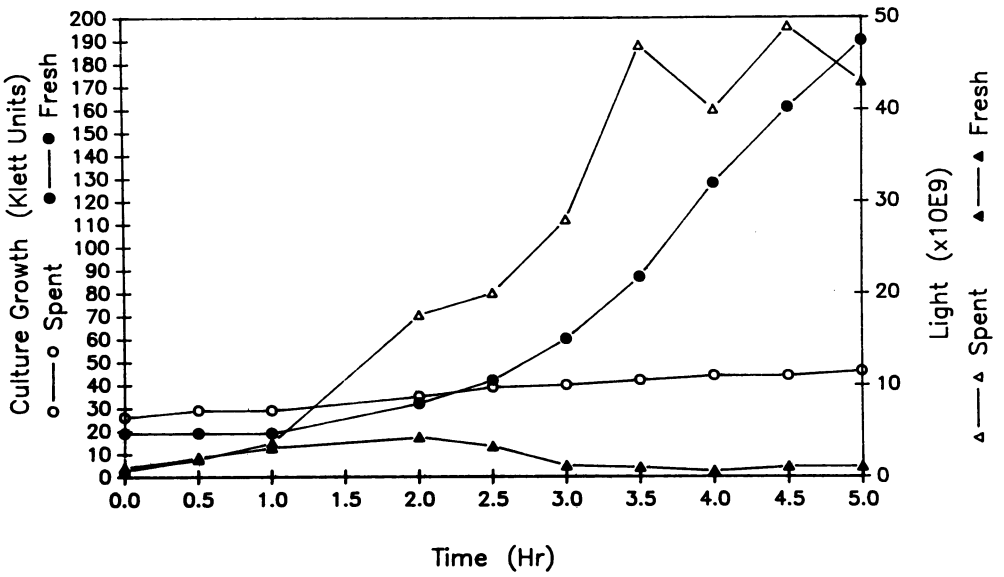

FIG. 6. Light output after resuspension in rich medium. A stationary phase culture of RB1351 in LB-naphthalene medium was resuspended in fresh medium. A separate fraction was reincubated in its own spent medium. Light output and culture growth (using a Klett meter) were examined at each time point. E, Exponent.

in magnitude and in duration. Since substrate can be lost by cell metabolism, by washout from the vessel, or through air stripping, this response was transient. However, when the reservoir was amended with naphthalene the response was continuous and stable. This response was very similar to that seen in the naphthalene-induced colonies, and the physiological state of cells in colonies and in this continuous culture may be similar as well. The difference in magnitude between the LB and basal salts media is attributable to the difference in titer; after a correction for this factor was made, the responses were identical.

DISCUSSION

The results of this investigation demonstrate the construction of a bioluminescent reporter plasmid, pUTK9, for naphthalene catabolism. The production of light was achieved by transcriptional fusion of the promoter for the upper pathway and part of the *nahA* gene with the promoterless *luxCDABE* cassette in pUCD615. Regulated light production from pUTK9 was accomplished by concerted action of the *nahR* gene product from the plasmid NAH7 that was comaintained with pUTK9 in *P. putida* RB1351. This strain has the advantage that it can grow on naphthalene as a sole carbon and energy source while active for bioluminescence. Stimulation of light production by RB1351 in the presence of naphthalene or salicylate was consistent with known mechanisms of salicylate induction of the two NAH7 operons for naphthalene catabolism. Constitutive light production was observed for isolated colonies and was increased more than 20-fold upon exposure to naphthalene vapor. For liquid cultures, either batch or continuous, no light production was observed in the absence of induction. For resting cell cultures in naphthalene minimal liquid medium, light production was directly correlated with naphthalene catabolism.

An anomalous result was found for light production in exponentially growing cells in rich medium in that transient light was produced in late logarithmic phase in the absence of naphthalene. This spurious light production remains unexplained and may be associated with a gratuitous induction. In rich medium in the presence of naphthalene, stable light production was achieved in late logarithmic or stationary phase. This result was not due simply to the increased cell titer at this late phase, since it was demonstrated that RB1351 cells in minimal medium could completely degrade naphthalene quickly, with no appreciable increase in cell numbers. This delay did not appear to be a catabolite repression event or diauxic growth and was not reproduced by addition of glucose or succinate to naphthalene minimal medium; it may indicate ease of substrate conversion.

To overcome growth state variability associated with non-steady-state colony or batch culture growth conditions, RB1351 was subjected to chemostat cultivation in a continuous-flow bioreactor on either LB or minimal medium. Single-pulse additions of naphthalene at 100 mg liter^{-1} (essentially saturation conditions) to the reactor vessels resulted in stable light production decreasing proportionally with substrate washout, approximately one to two hydraulic retention times (5 to 10 h). Light emission on a per-cell basis was essentially identical for LB- or minimal medium-grown cells under these conditions. In the absence of naphthalene, no light production was detected under continuous cultivation. Inclusion of naphthalene on a continuous basis in the reactor feed was accompanied by a nearly constant light production. Steady-state light production at a relatively slow growth rate of 0.21 hr^{-1} appears to correlate with onset of light production in batch cultures at late logarithmic or stationary phase.

Plasmid pUTK10 was unable to generate any bioluminescence under all of the conditions examined. Inspection of the nucleotide sequence (18) from this region reveals a putative promoter (P_x in Fig. 1) with good homology to *E. coli* promoters as well as to the promoter for *nahR*. Like the *nahR* promoter, P_x overlaps with the promoter for the divergently transcribed pathway. On the basis of this superficial similarity, it might be expected that P_x would be as

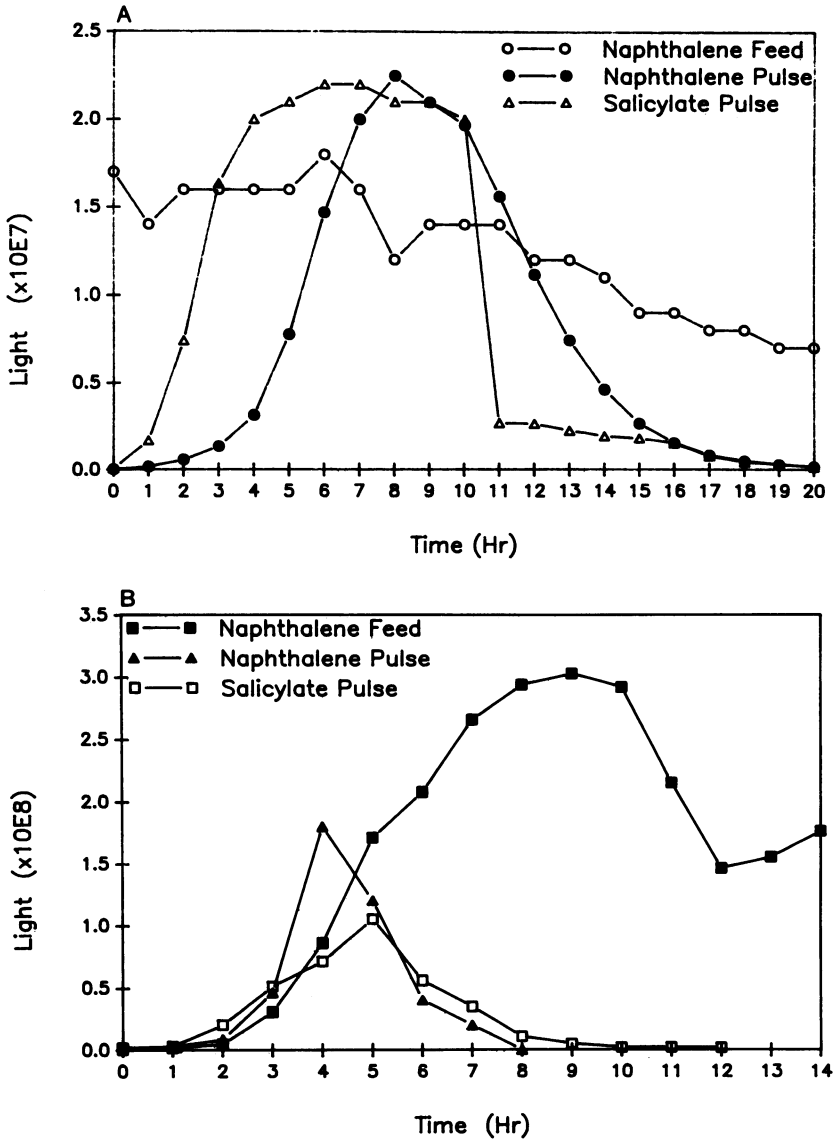

FIG. 7. Continuous culture of RB1351. *Pseudomonas* sp. strain RB1351 was grown in a chemostat apparatus in LB medium (A) or basal salts medium (B). Naphthalene or salicylate was added at saturating amounts either to the reactor vessel (pulse) or to the 10-liter feed tank (feed). Titer of the LB chemostat was approximately 10 times greater than for the basal salts chemostat. E, Exponent.

active as the *nahR* promoter. This does not seem to be an accurate comparison, however, and it seems more likely that there is no biological activity associated with this sequence. A comparison with the *nahR* promoter might help to explain the regulation of this promoter.

It is possible that the naphthalene degradative pathway evolved to eliminate toxic compounds, such as antibiotics, and not primarily as a growth pathway. This would explain why there is no catabolite repression observed. Bacterial cells in the soil are unlikely to grow at an exponential rate since there are few unused resources available in large quantity. Competing microorganisms often produce antibiotics as secondary metabolites when their growth rate is slow; a stationary-phase system of detoxifying compounds would not be unexpected. If the appropriate nutrients were found in abundance, allowing exponential growth, it is unlikely that toxic compounds would be present as well; thus, genes may be repressed under these conditions. What is clear from this work is that the presence of the inducing substrate is not always sufficient for catabolic activity; whether there is regulation at the transcriptional or posttranscriptional level remains as the subject of continuing research in this laboratory.

The immediate objective of development of a bioluminescent reporter plasmid for an important physiological activity, naphthalene catabolism, has been met by this work. An appropriate strain capable of stable plasmid maintenance and light production without antibiotic selection has been shown to respond to naphthalene exposure under a diversity of culture conditions, producing sufficient light for remote sensing by fiber optic or liquid-light pipe technology. Numerous practical applications exist in the area of environmental sensing of catabolic activities and ecological interactions among competing species by using bioluminescent reports of physiological activity. It is also anticipated that pUTK9 will be a useful tool in studying relationships with

other catabolic pathways and evolutionarily linked regulatory networks.

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