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Transcriptional Regulation of *lux* Genes Transferred into *Vibrio harveyi*

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Past work has shown that transformed *Escherichia coli* is not a suitable vehicle for studying the expression and regulation of the cloned luminescence (*lux*) genes of *Vibrio harveyi*. Therefore, we have used a conjugative system to transfer *lux* genes cloned into *E. coli* back into *V. harveyi*, where they can be studied in the parental organism. To do this, *lux* DNA was inserted into a broad-spectrum vector, pKT230, cloned in *E. coli*, and then mobilized into *V. harveyi* by mating aided by the conjugative plasmid pRK2013, also contained in *E. coli*. Transfer of the wild-type *luxD* gene into the *V. harveyi* M17 mutant by this means resulted in complementation of the *luxD* mutation and full restoration of luminescence in the mutant; expression of transferase activity was induced if DNA upstream of *luxC* preceded the *luxD* gene on the plasmid, indicating the presence of a strong inducible promoter. To extend the usefulness of the transfer system, the gene for chloramphenicol acetyltransferase was inserted into the pKT230 vector as a reporter. The promoter upstream of *luxC* was verified to be cell density regulated and, in addition, glucose repressible. It is suggested that this promoter may be the primary autoregulated promoter of the *V. harveyi* luminescence system. Strong termination signals on both DNA strands were recognized and are located downstream from *luxE* at a point complementary to the longest mRNA from the *lux* operon. Structural *lux* genes transferred back into *V. harveyi* under control of the *luxC* promoter are expressed at very high levels in *V. harveyi* as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis: the gene transfer system is thus useful for expression of proteins as well as for studying the regulation of *lux* genes in their native environment.

Emission of light by luminescent bacteria results from the oxidation of a long-chain aliphatic aldehyde and a reduced flavin. This reaction is catalyzed by luciferase, an enzyme that contains two nonidentical subunits, α and β, encoded by the genes *luxA* and *luxB*, respectively. The aldehyde substrate for luciferase is provided by a fatty acid reductase complex composed of three enzymes, reductase, transferase, and synthetase, whose corresponding genes are *luxC*, *luxD*, and *luxE* (25). Detailed studies on two luminescent strains, *Vibrio fischeri* and *V. harveyi*, have shown that the five structural genes are closely linked in an operon, in the order *luxCDABE* (3, 10, 27). These five structural genes are sufficient for light production, since they can be cloned on a single DNA fragment which, when inserted into *E. coli* in a plasmid vector and preceded by a suitable promoter (for example, the T7 bacteriophage promoter), will cause the transformed bacteria to luminesce (27).

Luminescence in bacterial cultures is cell density regulated; liquid cultures are very dim until the mid-logarithmic phase of growth, and the amount of light then increases rapidly up to several thousandfold. The light system is turned on through the action of an autoinducer synthesized constitutively by the cells during early growth of the culture and accumulated in the medium (7, 9, 11, 30). The autoinducer from *V. fischeri* is freely diffusible across the cell wall (19), and when a critical concentration is reached it induces expression of the *lux* operon. Autoinducers for both *V. fischeri* and *V. harveyi* have been isolated and purified; they are similar in structure, containing a homoserine lactone linked through an amide bond to a fatty acid derivative, but differ in the fatty acid moiety (8; J. Cao and E. A. Meighen, J. Biol. Chem., in press).

The mechanism of light induction has been studied most thoroughly with *V. fischeri* (9–11). A regulatory gene, *luxI*, whose product is thought to be involved in the synthesis of the autoinducer, has been found upstream from *luxC*, closely linked to it and transcribed in the same direction. This gene and the five structural genes constitute the right operon, *luxICDABE*. A second regulatory gene, *luxR*, just upstream from *luxI* and transcribed in the opposite direction, is termed the left operon. When a sufficiently high concentration of autoinducer has been synthesized at a constitutive rate, it is hypothesized to interact with the *luxR* gene product and turn on transcription of the right operon. Further synthesis of the *luxI* gene product, producing more autoinducer, as well as synthesis of the *lux* enzymes, then proceeds in an exponential manner, leading to a rapid increase in light emission. The expression of *luxR* is turned off by high levels of the autoinducer and *luxR* gene product complex (6).

Whether or not the mechanism of light induction in *V. harveyi* is similar to that in *V. fischeri*, several pieces of evidence indicate that the arrangement of its regulatory genes is quite different. First, when cloned into wild-type *E. coli*, the *V. harveyi luxCDABE* operon, with very large flanking regions in both directions, does not produce light, either regulated or unregulated. Mutant strains of *E. coli* containing cloned *lux* DNA emitted light, but it was apparently unregulated (26). Mutation of an *E. coli* gene thus appears to affect the production of light by the cloned *lux* operon. Second, the region upstream from *luxC* has been sequenced, and for the first 637 base pairs (bp) there are numerous stop codons in each direction and no open reading frame larger than 48 bp (29). This result eliminates the possibility of a *luxI* gene being closely linked to the *luxCDABE* operon. Third, transposon mutagenesis of *V. harveyi* has recently led to the identification of a new region, unlinked to *luxCDABE*, that controls light expression (24). The locus has not been mapped, but it is thought to

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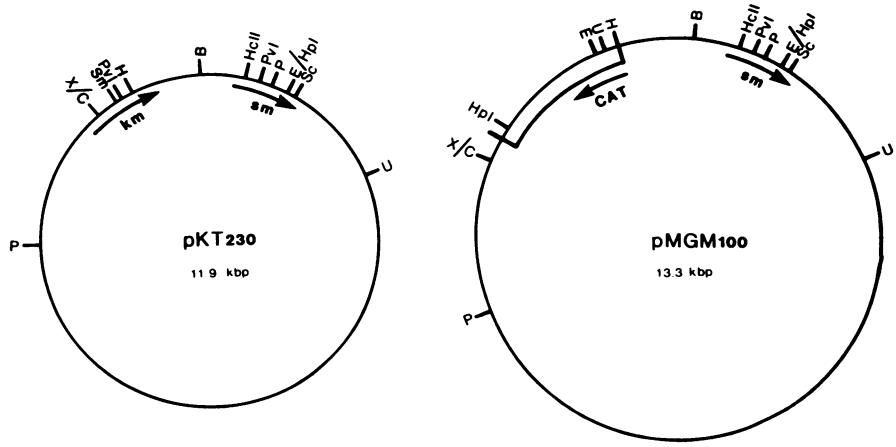

FIG. 1. Restriction map of the broad-host-range, RSF1010-derived vector pKT230 (11.9 kbp) and geneology of the vector pMGM100 (13.3 kbp), derived from pKT230. Abbreviations: sm and km, genes conferring resistance to streptomycin and kanamycin, respectively; P, *Pst*I; X, *Xho*I; C, *Cla*I; Sm, *Sma*I; PvI, *Pvu*I; H, *Hin*dIII; B, *Bam*HI; HcII, *Hin*cII; E, *Eco*RI; HpI, *Hpa*I; Sc, *Sac*I; U, *Pvu*II. *Hin*dIII and *Sma*I were used to insert the 1.6-kbp fragment (□) of pSV2cat containing the CAT gene (see Materials and Methods). The resultant vector no longer conferred Km^r. The *Cla*I site was determined to be close to *Xho*I, and the *Hpa*I site was close to *Eco*RI.

encode a regulatory protein that activates transcription of the *luxCDABE* operon in response to autoinducer, a locus similar in function to that of *luxR* in *V. fischeri*. No gene corresponding to *luxI* was identified.

Our own attempts to study the regulation of the *V. harveyi lux* operon, by complementing it in *E. coli* with various cloned DNA fragments from *V. harveyi*, have not met with success. In the present work we have therefore turned to a conjugative system to transfer clones of *V. harveyi* DNA from *E. coli* back into *V. harveyi*, where they could serve for gene complementation. *V. harveyi* DNA fragments were cloned in a broad-host-range vector (1) inserted into *E. coli* and then transferred from *E. coli* into *V. harveyi* by mating with *V. harveyi* by using a second *E. coli* culture containing a conjugative plasmid (12). This system has allowed the transfer of *V. harveyi* DNA at high efficiencies from *E. coli* into *V. harveyi*, a bacterium not easily transformed with plasmid DNA. We have used this conjugative system to complement a *luxD* (dark) mutation in the *V. harveyi* genome, with the resultant luminescence of the bacteria. In addition, the system has been used to identify a major inducible promoter upstream from the *luxC* gene of the luminescence operon.

MATERIALS AND METHODS

Bacteria and growth. *V. harveyi* B392 and the *V. harveyi luxD* mutant M17 (34) were grown in LB medium (consisting of [per liter] 10 g of tryptone [Difco Laboratories], 5 g of yeast extract [Difco], and 10 g of NaCl). Both strains were resistant to 100 μg of ampicillin per ml (Amp^r). Bacteria were subcultured to a starting cellular density of 0.05 at 660 nm and incubated at 28°C in a rotary water bath set at 250 rpm; the extent of cellular growth was determined periodically at 660 nm. In vivo luminescence was measured by using a photomultiplier calibrated with the light standard of Hastings and Weber (17); 1 light unit (LU) = 1×10^{10} quanta/s. The M17 mutant has a single point mutation in the *luxD* gene which results in an inactive transferase enzyme (27) and thus requires exogenous decanal for full expression of luminescence.

E. coli MM294 (Hfr) was obtained from the American Type Culture Collection (ATCC 33625).

Plasmids and cloning procedures. Techniques for molecular cloning have been described previously (22). Various plasmids already described (26), containing *V. harveyi lux* DNA, were the source of all the DNA cloned for this study, except for the plasmid pSV2cat (14), from which the chloramphenicol acetyltransferase (CAT) gene was obtained. Restriction endonucleases (Pharmacia, Inc.; Bethesda Research Laboratories, Inc.) were used to obtain DNA fragments. When required, DNA restriction fragments with 5′ overhangs were blunt ended through the fill-in reaction with the Klenow fragment of *E. coli* DNA polymerase (Pharmacia). When restriction resulted in 3′ overhangs, the fragments were blunt ended through the 5′-3′-exonuclease action of T4 DNA polymerase (Pharmacia). After restriction of the vector, the 5′ ends (after blunt ending, if required) were dephosphorylated with calf intestinal alkaline phosphatase (Boehringer Mannheim Biochemicals). T4 polynucleotide ligase was then used to ligate DNA fragments with the vector. DNA was purified from added enzymes, except from T4 polynucleotide ligase, by phenol-chloroform extraction and ethanol precipitation in the presence of 3 M ammonium acetate (pH 7). The pellet was washed with 90% ethanol, dried, and suspended in water. Alternatively, the DNA was separated by electrophoresis on 1% low-gelling-temperature agarose (SeaKem; FMC Corp.) gels. Gel slices containing the DNA fragments were melted at 65°C, and the DNA was purified from the gel by using cetyltrimethylammonium bromide (21) followed by P6 Bio-Gel (Bio-Rad Laboratories) spin column chromatography (33) and ethanol precipitation. All new clones were verified by extensive restriction mapping of the DNA plasmid, extracted by the gentle lysis technique, as described previously (22).

The cloning vector, pKT230, obtained from the American Type Culture Collection (ATCC 37294), a derivative of RS1010, contains the kanamycin resistance gene (Km^r) from the R6-5 miniplasmid pKT105, additional DNA from pACYC177, and the streptomycin resistance gene (Sm^r) (1). Figure 1 gives a restriction map of pKT230 (11.9 kbp), including the several unique restriction endonuclease sites available in the two antibiotic resistance genes which can be used for cloning (*Xho*I, *Cla*I, *Sma*I, and *Hin*dIII in the Km^r gene; *Hin*cII, *Hpa*I, *Eco*RI, and *Sac*I in the Sm^r gene;

TABLE 1. Plasmids used in this study

Plasmid[a]	Source of vector	Inserted DNA
pMGM100	pKT230	CAT gene
pMGM101	pKT230	B
pMGM102	pKT230	G
pMGM105	pMGM100	BU
pMGM106	pMGM100	GU
pMGM107	pMGM100	GSac
pMGM108	pMGM100	SacU
pMGM109	pMGM100	C
pMGM110	pMGM100	GH
pMGM111	pMGM110	HH
pMGM112	pMGM110	HE
pMGM113	pMGM110	EPvI
pMGM114	pMGM110	GG
pMGM200	pKT230	CAT gene
pMGM201	pKT230	B
pMGM202	pKT230	G
pMGM203	pKT230	HB
pMGM209	pMGM100	C
pMGM214	pMGM110	GG

[a] Plasmids in the series pMGM1 have the genes or promoters in the inserted DNA transcribed in the counterclockwise direction, whereas plasmids in the series pMGM2 are oriented in the same direction (clockwise) as the streptomycin gene in pKT230 (Fig. 1). The *lux* DNA described in Fig. 2, 5, and 7 was inserted in the unique *Hin*dIII site of the plasmids except for G and B DNA inserted into the *Bam*HI site and HB, BU, GU, GH, SacU, and GSac DNA inserted between the *Bam*HI and *Hin*dIII sites. Insertion of the CAT gene into pKT230 to give pMGM100 is shown in Fig. 1 and described in the text, whereas the CAT gene in pMGM200 was inserted between the *Bam*HI and *Hin*dIII sites.

*Bam*HI and *Pvu*II outside the antibiotic resistance genes). Plasmids generated in this study by insertion of cloned DNA fragments into pKT230 as well as into the newly constructed plasmids (pMGM series) are listed in Table 1. Figure 1 also depicts the newly constructed 13.3-kbp plasmid, pMGM100, which contains the CAT gene obtained from pSV2cat inserted into the Km^r gene of pKT230. The *Hin*dIII-*Bam*HI fragment (1.6 kbp) of pSV2cat includes the entire CAT gene preceded by a typical Shine-Dalgarno sequence. This fragment was obtained by restricting pSV2cat with *Bam*HI, blunt ending the resulting 5′ overhang, and then restricting with *Hin*dIII. The resultant fragment was ligated with pKT230, which had been cut at the *Hin*dIII and *Sma*I sites, and dephosphorylated. After transformation of competent *E. coli* MM294 with this plasmid (22), the bacteria were plated on LB (1.5% [wt/vol] Difco agar) containing 25 μg of streptomycin per ml. Colonies were then screened with 30 μg of kanamycin per ml, and sensitive colonies containing the desired plasmid were picked. Plasmids containing *lux* DNA (Table 1) were also selected in *E. coli* MM294 grown on LB plates containing streptomycin and screened for luminescence (plus decanal) if the *luxAB* genes were present.

Gene mobilization. Competent *E. coli* MM294 was transformed with the various recombinant plasmids, and the resultant cells were plated on LB containing 25 μg of streptomycin per ml when the inserted DNA disrupted the Km^r gene, 30 μg of kanamycin per ml when the Sm^r gene was disrupted, or both antibiotics when neither gene was disrupted. Recombinant DNA was transferred into *V. harveyi* by conjugation with transformed *E. coli* MM294; the transfer was promoted by simultaneous conjugation with pRK2013 (ATCC 37159), which is Km^r and carries the RK2 transfer system (tra^+) in a ColEl replicon (12). The method for gene mobilization into *V. harveyi* was performed exactly as described previously (16) with fresh cultures of *V. harveyi*, *E. coli* MM294 with pMGM plasmids, and *E. coli* MM294(pRK2013). Selection on LB agar containing ampicillin (100 μg/ml), kanamycin (30 μg/ml), and streptomycin (25 μg/ml) ensured that the resulting colonies were *V. harveyi* carrying pKT230 (*V. harveyi*, Amp^r; pMGM, Km^r and/or Sm^r; pRK2013, Km^r). Moreover, the plate morphology of the colonies was that of *V. harveyi* (more yellow and less convex than that of *E. coli*). Although *V. harveyi* containing recombinant DNA could usually be maintained as a pellet at −80°C after selection, it was better to store the mated cells as a pellet at −80°C before selection. When desired, a small portion of the thawed, mated cells was spread on selective LB plates. The mated cell pellet could be frozen and thawed at least three times with no effect on the results.

Assaying enzyme activities in vitro. During the growth of *V. harveyi*, portions (A_{660} × volume = 1) were withdrawn and pelleted. The cultures were allowed to grow for at least 2 h before the first portion was taken. The pellets were then either frozen at −80°C for later use or lysed immediately in 50 μl of 0.25 M Tris hydrochloride (pH 8.0) by using a Branson ultrasonicator at a speed setting of 40 for three 10-s treatments with 1.5-min intervals on ice between treatments. After centrifugation to remove cellular debris, all enzyme assays were conducted immediately. Protein concentrations of extracts measured by using a modified Lowry assay (23) were found to be relatively constant at 10 ± 4 μg/ml.

Luciferase activities were determined as described previously (15); a specific activity of 1 LU/mg is equivalent to 1×10^{10} quanta/s per mg.

Acyltransferase (the *luxD* gene product, which catalyzes the generation of fatty acids for the fatty acid reductase system of the luminescence reaction) activities were determined by cleavage of [^{3}H]myristoyl acyl carrier protein (4). The specific activity of transferase was determined as picomoles of acyl-acyl carrier protein cleaved per minute per milligram of protein assayed.

Assays for CAT activity were performed as described previously (13), with an incubation time of 15 min unless otherwise noted. Since assays were linear over only a limited range of added protein, different amounts of lysate were added to ensure proper quantitation. One unit of CAT activity in this paper corresponds to 1 nmol of chloramphenicol acetylated per min at 37°C under optimal assay conditions. When cell lysates were stored at 4°C, the CAT activity was stable for at least 2 months, unlike the luciferase and transferase activities, which fell by 40% after 24 h at 4°C. Similarly, CAT activities in lysates after freezing at −20°C and thawing were stable, whereas luciferase and transferase activities were relatively unstable.

In vivo [^{3}H]myristic acid acylation of polypeptides involved in the fatty acid reductase system was performed as described (26, 35).

RESULTS

In vivo complementation of the transferase activity of a *luxD* mutant of *V. harveyi* by using gene mobilization. A partial restriction map of *V. harveyi* DNA encompassing the known *lux* structural genes along with several of the *V. harveyi* DNA fragments used in this study are shown in Fig. 2. The DNA fragments containing the *luxCDAB* genes (G), the *luxDAB* genes (B), and the *luxAB* genes (HB) were cloned

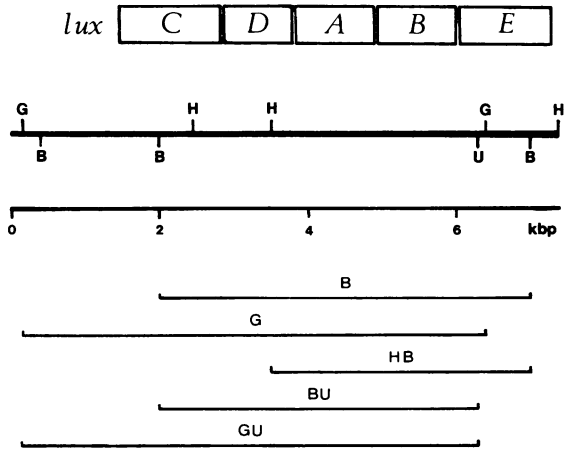

FIG. 2. Restriction map of 6.3 kbp of *V. harveyi lux* DNA including *luxCDABE* genes and locations of cloned DNA fragments. Abbreviations: G, *Bgl*II; H, *Hin*dIII; B, *Bam*HI; U, *Pvu*II. At 0 kbp, there is one of several *Cla*I sites, none of which are indicated on the map.

into pKT230 in both directions except for the HB DNA. Three plasmids contained the *lux* genes inserted clockwise into pKT230 (pMGM201, pMGM202, and pMGM203), and two clones contained the *lux* genes inserted counterclockwise into pKT230 (pMGM101 and pMGM102). Recombinant plasmids were transferred from *E. coli* MM294 into the *V. harveyi* acyltransferase mutant (M17) by using the gene mobilization system described in Materials and Methods.

Figure 3 gives profiles of the luminescence per unit cell density at increasing cell densities of M17 cells containing cloned *lux* genes and of *V. harveyi* wild-type and M17 cells. Whereas M17 alone or M17 with pMGM203, coding for only *luxAB*, gave very low luminescence in the absence of aldehyde, light emission was very high in M17 cells harboring the plasmids coding for *luxD* (pMGM101, pMGM102, pMGM201, and pMGM202), with light intensities close to that of the wild-type *V. harveyi* and 10^3 to 10^4 times higher than that of M17 cells. Isolation of the plasmid followed by restriction analysis indicated that the plasmids were unchanged. Moreover, the same isolated plasmid could again complement the defect. Clearly, the point mutation in *luxD* of M17 could be complemented in *trans* with the mobilized recombinant plasmids containing the *luxD* gene, and sufficient amounts of transferase are produced to give full expression of the luminescence system without addition of exogenous aldehyde.

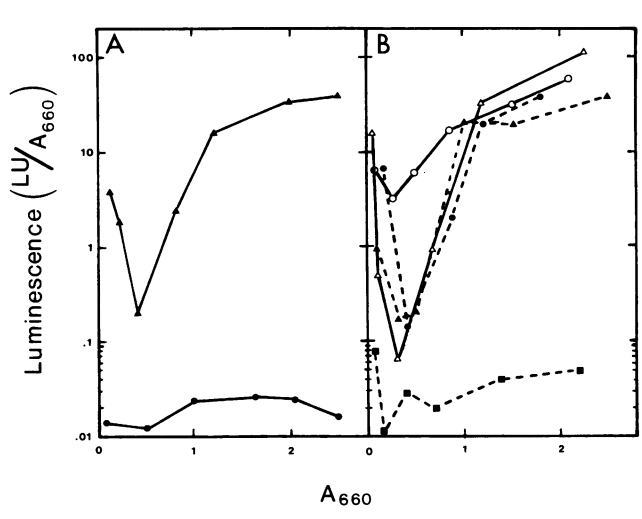

FIG. 3. In vivo luminescence (LU/A_{660}) as a logarithmic function of cell density (A_{660}). (A) Growth curves of wild-type *V. harveyi* (▲) and its transferase mutant M17 (●), which were grown in the presence of ampicillin and assayed as described in Materials and Methods. (B) Growth curves of M17 cells harboring different plasmids transferred by gene mobilization: pMGM203 (■), pMGM101 (●), pMGM201 (○), pMGM102 (▲), and pMGM202 (△).

TABLE 2. Dependence of luciferase and transferase activities on cell growth[a]

Cell	Luciferase activity (LU/mg)		Transferase activity (pmol/min per mg)	
	Before induction	After induction	Before induction	After induction
V. harveyi	1.4	16	0.1	1.5
M17	1.3	28	<0.02	<0.02
M17(pMGM201)	48	160	5.0	8.2
M17(pMGM101)	0.7	15	0.2	0.4
M17(pMGM202)	1.3	153	0.3	4.6
M17(pMGM102)	6	200	0.2	6.0[b]

[a] Identical amounts of cells (A_{660} × vol [milliliters] = 1) were lysed before (A_{660} = 0.2 to 0.3) and after (A_{660} = 2.0 to 3.0) induction of luminescence, and activities of luciferase and transferase were assayed as described in Materials and Methods.

[b] Cells were extracted at A_{660} = 1.2.

It should be noted that previous attempts to complement the *luxD* of rifampin-resistant (Rif^r) M17 cells by constructs similar to that of pMGM101 and pMGM201 containing *luxDAB* were not successful (16). The reason why complementation occurs with Amp^r and not Rif^r as the marker for *V. harveyi* cells is unknown. We also were unable to obtain light-emitting colonies when Rif^r M17 cells were used as the acceptor in the gene transfer system.

In vitro enzyme activities in the complemented *V. harveyi* system. Although induction of luminescence with cellular growth in the complemented M17 cells was observed (Fig. 3), these results may reflect simply the regulation of the *lux* genes on the *V. harveyi* genomic DNA. To test whether expression of the transferred plasmid genes was regulated, levels of transferase activity encoded by *luxD* and luciferase activity encoded by *luxAB* at increasing cell densities of the transformed M17 cells were measured in vitro (Table 2). As expected, both luciferase and transferase activities in wild-type *V. harveyi* were induced during growth, whereas only luciferase activity was induced in M17 cells and transferase activity was absent. Complementation of the M17 genome with pMGM101 or pMGM201 resulted in expression of transferase activity in M17. However, the relative increase in specific activity of transferase with cell growth (less than twofold) was much smaller than the observed induction of luciferase or transferase activity in wild-type *V. harveyi*, suggesting that expression of the *luxD* gene on pMGM101 or pMGM201 in M17 is not under the same control as expression of the genomic *lux* genes. It is interesting that the level of transferase observed in M17 with pMGM101 was significantly lower than that with pMGM201 or in wild-type *V. harveyi*. Such levels may reflect the more efficient use of plasmid promoter(s) oriented clockwise in pMGM201 (e.g., the promoter for the Km^r gene) compared with promoters running counterclockwise on pMGM101. The increase of luciferase activity with cellular growth of M17(pMGM101) to

the same level as wild-type *V. harveyi* reflects induction of expression of the *luxAB* genes primarily in the genome of *V. harveyi* rather than on the complementing plasmid. This result is consistent with the absence of strong external plasmid promoters oriented in the counterclockwise direction. In contrast, the level of luciferase activity reached in M17(pMGM201) was eightfold higher than that obtained with wild-type *V. harveyi*, and its luciferase activity increased only threefold with cellular growth compared with the 10-fold increase in wild-type *V. harveyi*; a strong external promoter on the plasmid appears to be expressed in the clockwise direction on the plasmid and is primarily responsible for the high level of expression of *luxAB*.

In contrast to the results with pMGM101 and pMGM201 in M17, insertion into M17 of plasmids pMGM102 and pMGM202 containing the *luxCDAB* genes resulted in specific activities of transferase increasing 20- to 30-fold with cell density, independently of the direction in which the DNA was inserted. Similarly, the specific activity of luciferase increased dramatically (50- to 100-fold) with cell growth. The specific activity of an enzyme constitutively expressed, alkaline phosphatase, changed less than twofold over this growth range (data not given). It appears that expression of the *lux* genes on the plasmid containing *luxCDAB* was induced in an analogous manner to the genomic *lux* DNA. These results provide strong evidence that the DNA including *luxCDAB* contains a promoter which is used very efficiently and is responsible for the growth-dependent induction of the *lux* genes on the plasmid.

Use of pMGM100, a mobilizable vector with a reporter gene. To pursue further the idea that the DNA including *luxCDAB* contains an inducible promoter, we constructed a vector containing the CAT reporter gene, which is not involved in the bioluminescence system and whose activity is stable and readily monitored. The plasmid (pMGM100) contains the CAT gene in pKT230 in the opposite orientation to the Sm^r gene (Fig. 1) so that expression would be expected to be very low without the addition upstream of a DNA fragment containing a promoter oriented in the same direction. *Bam*HI and *Hin*dIII sites upstream of the CAT gene are convenient for insertion of *lux* DNA to be tested for promoter activity (Fig. 1). *E. coli*(pMGM100) was found to be sensitive to chloramphenicol (1 μg/ml), and therefore strong promoters are not present in the plasmid upstream of the CAT gene. It is worth noting that when the CAT gene was oriented in the opposite direction relative to plasmid promoters, *E. coli* containing this plasmid (pMGM200) was resistant to chloramphenicol even at 20 μg/ml. Moreover, when the resultant vector was transferred into *V. harveyi*, the CAT activity in lysates was very high, indicating expression of the CAT gene from a pKT230 promoter on the plasmid.

Promoter studies with the mobilization vector pMGM100. DNA restriction fragments of *V. harveyi*, BU and GU (Fig. 2), encompassing *luxDAB* and *luxCDAB*, respectively, were inserted into pMGM100 upstream and in the same orientation as the CAT gene, and the resultant plasmids, pMGM105 and pMGM106, respectively, were mobilized into the *V. harveyi* mutant M17. Luciferase and transferase activities were analogous to those in M17 cells containing the same *lux* genes (pMGM101 and pMGM102 [Table 1]). Acetylation of [^{14}C]chloramphenicol increased with cell growth of M17(pMGM106) (Fig. 4, lanes 9 to 13) with the same amount of added protein, whereas there was much less acetylation (which remained constant with increasing cell density) with M17(pMGM105) (Fig. 4, lanes 5 to 8). As a control, M17(pMGM100) (Fig. 4, lanes 1 to 4) was analyzed. Almost no detectable CAT activity could be seen despite a threefold longer incubation with 2.5 times more protein. Identical results were obtained when the plasmids were mobilized into wild-type *V. harveyi*. These results indicate that there is a strong promoter in the DNA containing *luxCDAB* (pMGM106) that is expressed optimally at high cell densities, as expected for an inducible promoter of the *lux* system. In the case of the DNA fragment with *luxDAB* (pMGM105), there appears to be a weak but constitutive promoter which could account for the lower, but significant, level of CAT activity. Therefore the use of pMGM vectors with CAT as a reporter gene makes it possible to distinguish clearly between constitutive and induced expression in the luminescent bacteria.

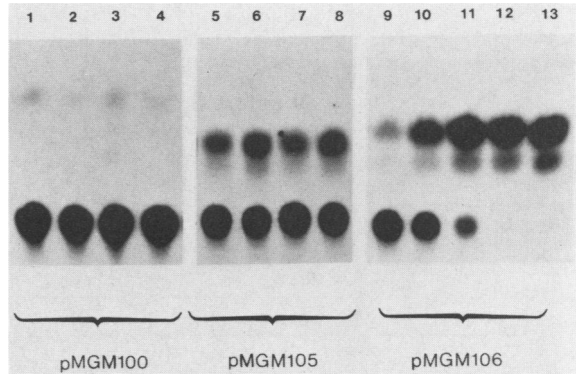

FIG. 4. Fluorography of the products of [^{14}C]chloramphenicol after assaying CAT activity in extracts from M17 containing pMGM100, pMGM105, or pMGM106. Cells (A_{660} × vol = 1) at increasing concentrations during growth were harvested, lysed, and subjected to the CAT assay as described in Materials and Methods. The nonacetylated and acetylated forms of chloroamphenicol, in order of increasing mobility on silica plates, are observed from bottom to top. Results in lanes 1 to 4 were from cells carrying pMGM100, which were assayed after withdrawing cells at A_{660} = 0.1, 0.5, 0.7, and 2.5; results in lanes 5 to 8 were from cells pMGM105 at A_{660} = 0.2, 0.9, 1.4, and 2.3; and results in lanes 9 to 13 were from cells with pMGM106 at A_{660} = 0.1, 0.2, 0.8, 1.2, and 1.8. The same amount of protein (1.6 μg) was added to 50 μl of the CAT reaction mix for 15 min for cells with pMGM105 and pMGM106, whereas 4 μg of protein was added to the 50-μl reaction mix and incubated three times longer for cells with pMGM100. When CAT assays were quantitated, cells with pMGM100 had 0.05 units/mg and cells with pMGM105 had 3 to 5 units/mg. For cells with pMGM106, activities in the assays at high cell densities were outside the linear range for the reaction. When lower levels of protein (0.2 μg) were used for pMGM106, the activity in lysates increased from 5 to 65 units/mg with increasing cell density.

Location of the *lux* promoter regulated by cell density. Since CAT expression is high and induced in *V. harveyi* containing *luxCDAB* and low and constitutive in DNA with *luxDAB*, an inducible *lux* promoter appears to be positioned between the start sites for the DNA located at 0.15 (*Bgl*II) and 2.0 (*Bam*HI) kbp on the map of *V. harveyi* (Fig. 2). These results, however, do not remove the possibility that DNA downstream of the *Bam*HI site is also required in *cis* for effective CAT expression. To test this possible requirement and to define the location of the promoter more precisely, different fragments of DNA were inserted in the pMGM100 vector upstream with *lux* DNA oriented in the same direction as the CAT gene and the resultant plasmids were transferred into *V. harveyi*. Figure 5 shows the DNA

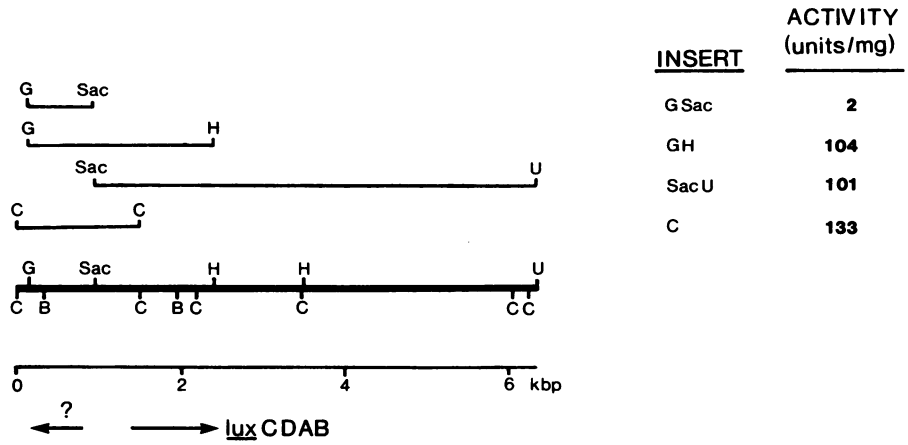

FIG. 5. Location of the strong *lux* promoter of *V. harveyi* as determined by CAT activity in *V. harveyi* transformed by various pMGM plasmids. The locations of restriction fragment inserts of *V. harveyi lux* DNA (GSac, GH, SacU, and C) cloned into the pMGM100 vector counterclockwise at the *Bam*HI and/or *Hin*dIII sites are indicated with the aid of the 6.3-kbp restriction map. Abbreviations: G, *Bgl*II; C, *Cla*I; B, *Bam*HI; Sac, *Sac*I; H, *Hin*dIII. After growth of *V. harveyi* containing these clones to A_{660} readings from 1.7 to 2.6, cells were assayed for CAT activity (units) by addition of 0.24 μg of lysate protein to the CAT reaction mix. The upstream open reading frame found in the opposite direction to the *luxCDAB* genes is indicated with a (?). This gene (615 bp) starts 637 bp above the *luxC* gene and terminates shortly before the *Bgl*II site (at 0.15 kbp). Its function and possible involvement in the *lux* system are unknown.

inserts in a more detailed restriction map and compares the CAT activities in lysates of *V. harveyi* harboring the different constructs. Identical results were obtained on gene transfer into M17. Very low CAT activity was detected for *V. harveyi*(pMGM107), in which the vector contains *lux* DNA from the *Bgl*II site downstream to the *Sac*I site (GSac).

In contrast, *V. harveyi* conjugated with plasmids containing DNA starting at the *Bgl*II site and extending 1.4 kbp past the *Sac*I site to the *Hin*dIII site (GH) in pMGM110, from the *Sac*I site to the *Pvu*II site (SacU) in pMGM108, and between two upstream *Cla*I sites (C) in pMGM109 gave very high activity. The high CAT activities in *V. harveyi* with pMGM108, pMGM109, and pMGM110, >50 times that of *V. harveyi*(pMGM107), indicate that the strong *lux* promoter is enclosed in a 500-nucleotide region between the *Sac*I and *Cla*I sites (1.0 to 1.5 kbp). Approximately the same amount of CAT activity was found in *V. harveyi* with the cloned long fragment (SacU) as with the shorter fragments (GH and C). The strength of the *lux* promoter does not appear to be diminished with increased distance from the CAT gene, and none of the downstream gene products (within 1.5 to 6.3 kbp) appear to be involved in regulation of the promoter. Since similar results were obtained on transfer of pMGM110 into the M17 mutant, it appears that active transferase is not required for efficient expression of the *lux* promoter.

Glucose repression of *lux* genes in *trans*. Luminescence in a number of marine bacteria including *V. harveyi* is under catabolite repression (31). By using the gene transfer system, it was possible to check whether glucose had any effect on the expression of *V. harveyi lux* genes mobilized into *V. harveyi*. When *V. harveyi*(pMGM108) cultures were grown to different stages of cellular growth with and without added glucose, CAT activity was induced in the absence of glucose but was constitutive in the presence of glucose (Fig. 6A). In contrast, glucose had no effect on the expression of *V. harveyi*(pMGM105) (data not shown), which is missing the inducible *lux* promoter.

In *V. fischeri*, glucose repression of luminescence is believed to act indirectly by reducing the expression of an upstream regulatory gene, *luxR*, transcribed in the direction opposite to the rest of the *lux* genes (5). It was therefore of interest to determine whether there was an analogous glucose-repressible promoter in *V. harveyi* oriented in the opposite direction to the *lux* operon. In a previous report, an open reading frame of 615 bp on the opposite strand was found (29) commencing 637 bp above the start of the *luxC* gene (Fig. 5). Its similar size and orientation to the *V. fischeri luxR* gene might suggest a *lux* regulatory role. DNA (C), which includes this open reading frame (Fig. 5), was inserted into pMGM100 with the lux DNA and CAT genes oriented in opposite directions, so that promoters in front of the open reading frame are also in front of the CAT gene (pMGM209). Only a low level of constitutive CAT activity was observed in *V. harveyi*(pMGM209) at different stages of cellular growth (specific activity, 2.8 units/mg) (Fig. 6B). Addition of glucose had no effect on its expression, clearly demonstrating that the upstream gene is not under catabolite repression.

Transcriptional termination studies. Although it has been

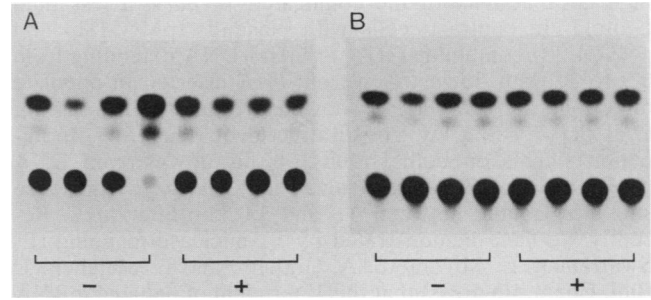

FIG. 6. Fluorography of the products of [^{14}C]chloramphenicol obtained in CAT assays of *V. harveyi* containing pMGM108 or pMGM209 plasmids grown in the presence and absence of glucose. Cells were processed as described in the legend to Fig. 4. (A) *V. harveyi*(pMGM108) in the absence of glucose (−), taken at A_{660} = 0.25, 0.7, 1.6, and 2.5, and in the presence of 0.2% (wt/vol) glucose (+), taken at A_{660} = 0.3, 0.8, 1.5, and 2.8. (B) *V. harveyi*(pMGM209) in the absence of glucose (−), taken at A_{660} = 0.2, 1.0, 1.4, and 2.2, and in the presence of glucose (+), taken at A_{660} = 0.2, 1.2, 1.9, and 2.7. CAT assays of *V. harveyi*(pMGM108) included 0.24 μg of protein per 50-μl reaction mix, whereas CAT assays of *V. harveyi*(pMGM209) included 10 times more protein.

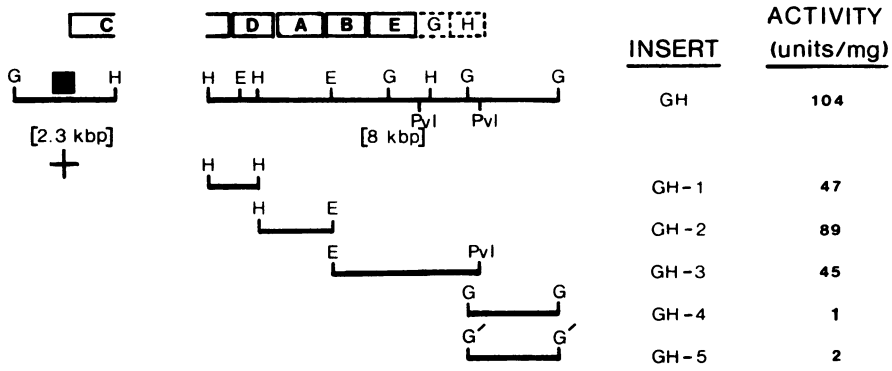

FIG. 7. Transcription termination studies of the *lux* system in *V. harveyi* as determined by assaying for CAT activity in *V. harveyi* containing pMGM110 plus various segments of downstream *lux* DNA. The pMGM110 plasmid (Fig. 5) has already been shown to contain the strong *lux* promoter (■). GH-1 through GH-5 are the restriction fragments added to pMGM110, and the extents of the DNA are indicated with the aid of the 8-kbp restriction map of *V. harveyi lux* DNA. Abbreviations: G, *Bgl*II; H, *Hin*dIII; E, *Eco*RI; PvI, *Pvu*I. The dotted boxes after *luxCDABE* indicate the recently sequenced genes *luxG* and *luxH*, which are transcribed in the same direction as *lux* mRNA (Swartzman et al., in press). The second PvI site is just before the 3′ terminal of *luxH*. CAT assays, with lysates of *V. harveyi* containing these plasmids, were performed when the A_{660} of cells reached 1.9 to 2.6. With plasmids pMGM110, pMGM111, pMGM112, and pMGM113) containing GH, GH-1, GH-2, and GH-3 inserts, respectively, the amount of added protein was 0.2 to 0.3 μg/50 μl of reaction mix, but with those containing GH-4 (pMGM114) and GH-5 (pMGM214), 10 times more protein was analyzed.

proposed that a short sequence of DNA, just past the *luxB* gene, exhibiting dyad symmetry, may be a termination signal (18, 32), we did not detect a reduction in expression when this DNA was present downstream of the inducible *lux* promoter in the gene transfer system; e.g., pMGM106, which includes DNA extending 0.3 kbp past *luxB*, still expressed the CAT gene very well (Fig. 4). To check whether various segments along the map of *V. harveyi* contained a recognizable terminator, we constructed plasmids with pMGM110 as the vector by inserting various DNA fragments at the *Hin*dIII site between the inducible *lux* promoter and the CAT gene. Figure 7 shows the various DNA restriction fragments cloned and CAT activities obtained in the gene transfer system. Addition to pMGM110 of GH-1 DNA (contiguous to GH DNA so that the total cloned DNA includes *luxC* and most of *luxD*) to give pMGM111, GH-2 DNA (encompassing *luxA*) to give pMGM112, or GH-3 DNA (containing most of *luxB*, all of *luxE*, and extending downstream 1 kbp) to give pMGM113 affected the high CAT activity of the conjugated *V. harveyi* less than twofold. In contrast, cells with plasmids (pMGM114 and pMGM214) containing GH-4 or GH-5 DNA extending from 0.9 to 3.0 kbp downstream from *luxE* inserted in opposite directions into pMGM110 showed a dramatic decrease of CAT activity to a low constitutive level. Therefore, termination signals on both strands of this downstream DNA (GH-4 and GH-5) are recognized in *V. harveyi*, and these sites are located between 0.9 and 3.0 kbp after *luxE*. Recently we have demonstrated by S1 nuclease mapping (E. Swartzman, C. Miyamoto, A. Graham, and E. Meighen, J. Biol. Chem., in press) that the 3′ terminal of induced mRNA encoding the *lux* genes of *V. harveyi* was located downstream from *luxE* just after two open reading frames (*luxG* and *luxH*; Fig. 7, dashed boxes) and a classical *rho*-independent hairpin termination site. A second hairpin on the opposite strand was also detected just beyond this location. These structures are encoded within GH-4 DNA, and the diminished CAT activities of clones containing this DNA after the inducible *lux* promoter would appear to arise as a result of these termination signals.

Expression of *lux* proteins as visualized on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Investigators describing the pKT230 mobilization system have demonstrated that there are about 15 to 20 copies of pKT230 per cell at any given time (2). Given our system, which includes an inducible *lux* promoter ahead of *lux* genes on pKT230, an increase in the gene dosage would allow a large enrichment of the *lux* proteins encoded on the plasmid. Indeed, the elevated luciferase activity found in vitro in M17 harboring different plasmids (Table 2) supports this conclusion. Therefore, we analyzed lysates of *V. harveyi* with and without pMGM106, the plasmid containing *luxCDAB* (Fig. 2) under control of the inducible *lux* promoter (Fig. 4), by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (20) to determine whether the *lux* polypeptides could be observed directly by Coomassie blue staining. The lysate of *V. harveyi* (pMGM106) (Fig. 8, lane 2) is enriched over that of *V. harveyi* without plasmid (lane 1), for reductase (R), α and β subunits of luciferase, and acyltransferase (T), the gene products of *luxC*, *luxA*, *luxB*, and *luxD*, respectively. The CAT gene product appears to run slightly faster than the transferase (T, 33 kilodaltons), but is not as readily visualized over the background of *V. harveyi* proteins. The cells with the plasmid taken at a low A_{660} reading of 0.5 gave the same profile of polypeptides (data not presented) as that seen in lane 1, indicating that the expression of these *lux* polypeptides is cell density regulated. When glucose was added to *V. harveyi*(pMGM106) cells during growth, the lysates did not contain the four *lux* polypeptides (lane 3) to the extent seen without glucose, and the profile of proteins was similar to that of *V. harveyi* without plasmid. Therefore, the gene mobilization system gives rise to a high level of expression of the *lux* genes under control of the inducible *lux* promoter; in the presence of glucose, expression of the genes is repressed, as expected from the results of CAT assays of *V. harveyi*(pMGM108) grown with and without glucose (Fig. 6A).

DISCUSSION

Use of the conjugative system to transfer cloned *V. harveyi* genes from *E. coli* back into *V. harveyi* has permitted us to identify a strong, cell-density-regulated promoter to the left of the *luxC* gene. Expression of the promoter is glucose

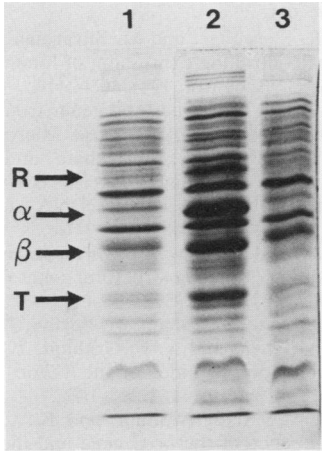

FIG. 8. Protein staining of sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of *V. harveyi* (lane 1), *V. harveyi*(pMGM106) (lane 2), and *V. harveyi*(pMGM106) in the presence of glucose (0.2%, wt/vol) (lane 3). Cells were grown, pelleted, and lysed as described in Materials and Methods. A 100-μg quantity of protein from each lysate was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a 12.5% resolving gel and a 5% stacking gel, by staining with Kodak Coomassie blue R250 (0.05%, wt/vol) in 50% methanol–10% acetic acid. The arrows indicate R (reductase), α and β subunits of luciferase, and T (acyltransferase), whose identities were based on mobility compared with protein standards including in vivo [^{3}H]myristic acid-labeled polypeptides, R, S, and T components of *V. harveyi*.

repressible. Further work is needed to fix the precise position of this promoter and the cyclic AMP receptor protein site, but together they may well be responsible for the transcription of an mRNA that spans the whole length of the luminescence operon in addition to several shorter mRNAs (28). A start site for these mRNAs 26 bases before the initiation codon of *luxC* and with a well-defined −10 consensus sequence has been recently identified (Swartzman, et al., in press). It seems that the promoter we have described may be the primary regulatory locus of the *V. harveyi* luminescence operon.

An open reading frame of 615 bp extending in the opposite direction has been found to start 637 bp upstream from *luxC*. Although it has the same direction of transcription as the *luxR* gene of *V. fischeri*, its location is somewhat different relative to the *lux* operon containing the structural genes. Moreover, when *V. harveyi* DNA from this region was inserted into the pMGM100 vector and tested for promoter activity after transfer into *V. harveyi*, there was only a low constitutive level of promotion that was unaffected by glucose addition. This is unlike the function of the *V. fischeri luxR* gene, which is under the control of catabolite repression. Recent work on transposon-generated mutants of the *V. harveyi* regulatory genes has suggested that the *luxR* function is located elsewhere on the bacterial genome and is unlinked to the structural genes (24). It appears that the reverse open reading frame upstream from *luxC* may not be associated with the luminescence system of *V. harveyi*.

The several structural genes of the *lux* operon are expressed in a coordinate manner (26). Nevertheless, there is a complex series of mRNAs present after the induction of luminescence, of which several start before *luxC*, some start before *luxD*, and others start before *luxA*; they terminate at various sites along the operon. Some of the mRNAs are present at much higher concentrations than others. These mRNAs have posed a perplexing question since they were first found (28): whether they are transcribed from different inducible promoter sites or whether they result from one or a few large mRNAs by processing. The situation is partially resolved by the observation in the present work that there do not appear to be rightward, inducible promoters in the *lux* operon downstream from the initiation codon in the *luxC* gene, a region that spans the mRNA start sites in front of *luxD* and *luxA*.

The conjugative system that we have used in this work has turned out to be a powerful tool for the analysis of promoters and terminators in the *V. harveyi lux* operon and for complementation of mutant gene function. In particular, it has allowed the analyses to be carried out in the parental organism, *V. harveyi*, thus avoiding some major problems in studying this cloned operon in *E. coli* (26). Preliminary experiments involving gene transfer of the pMGM plasmids with the inducible promoter into a *V. harveyi* regulatory mutant defective in autoinducer production have demonstrated that expression of the DNA on the plasmid is stimulated by the autoinducer. We are also continuing to use this system in attempts to find an explanation for the variety of mRNAs induced during the expression of luminescence by *V. harveyi* and to complement the *V. harveyi* mutants which are defective in regulation.

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