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Cloning and Nucleotide Sequence of *luxR*, a Regulatory Gene Controlling Bioluminescence in *Vibrio harveyi*

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Mutagenesis with transposon mini-Mu*lac* was used previously to identify a regulatory locus necessary for expression of bioluminescence genes, *lux*, in *Vibrio harveyi* (M. Martin, R. Showalter, and M. Silverman, J. Bacteriol. 171:2406–2414, 1989). Mutants with transposon insertions in this regulatory locus were used to construct a hybridization probe which was used in this study to detect recombinants in a cosmid library containing the homologous DNA. Recombinant cosmids with this DNA stimulated expression of the genes encoding enzymes for luminescence, i.e., the *luxCDABE* operon, which were positioned in *trans* on a compatible replicon in *Escherichia coli*. Transposon mutagenesis and analysis of the DNA sequence of the cloned DNA indicated that regulatory function resided in a single gene of about 0.6-kilobases named *luxR*. Expression of bioluminescence in *V. harveyi* and in the fish light-organ symbiont *Vibrio fischeri* is controlled by density-sensing mechanisms involving the accumulation of small signal molecules called autoinducers, but similarity of the two luminescence systems at the molecular level was not apparent in this study. The amino acid sequence of the LuxR product of *V. harveyi*, which indicates a structural relationship to some DNA-binding proteins, is not similar to the sequence of the protein that regulates expression of luminescence in *V. fischeri*. In addition, reconstitution of autoinducer-controlled luminescence in recombinant *E. coli*, already achieved with *lux* genes cloned from *V. fischeri*, was not accomplished with the isolation of *luxR* from *V. harveyi*, suggesting a requirement for an additional regulatory component.

Luminescent bacteria are widespread in the marine environment, where they exist planktonically and as parasites and light organ symbionts. Light production by symbiotic bacteria living in association with higher organisms may serve to attract prey, for intraspecies communication, or to escape from predators (34). Luminescence could also function to provide a direct benefit to the bacteria. One possibility is that the luminescence system is used as a terminal oxidase when the cytochrome electron transport system cannot be synthesized (low iron availability) or cannot function (low oxygen tension) (26). Luciferase, a mixed function oxidase consisting of α and β subunits, catalyzes the emission of light (Fig. 1). In the generation of light, luciferase oxidizes a reduced flavin, $FMNH_2$, and a long-chain fatty aldehyde producing oxidized flavin and the corresponding fatty acid (22). A fatty acid reductase unique to the bioluminescence system functions to synthesize or recycle the aldehyde substrate. Expression of cloned genes for luciferase and fatty acid reductase is sufficient for the production of light in a variety of nonluminous bacterial hosts (12, 37), so functions that supply reduced flavin and precursors of the fatty aldehyde substrate are apparently not unique to the bioluminescence system.

Light production by most species of luminous bacteria is strongly influenced by the density of the cell culture. Light emission per cell can be as much as 1,000-fold higher in dense cultures than in dilute cultures. Density-dependent regulation of luminescence has been investigated most thoroughly with the light organ symbiont *Vibrio fischeri* (9, 25, 33). This bacterium synthesizes a small extracellular signal molecule, called autoinducer, which accumulates in the growth medium and induces expression of the luminescence phenotype. It is the concentration of autoinducer and not cell density per se which directly affects expression of luminescence. Autoinducer from *V. fischeri* has been shown to be *N*-(β-ketocaproyl)homoserine lactone (10). The genes (*lux*) necessary for light production in recombinant hosts have been cloned from *V. fischeri* (strain MJ-1) on one 9-kilobase (kb) fragment of DNA (12, 13). This fragment contains genes encoding regulatory functions and the luciferase and fatty acid reductase enzymes. Regulation of light production in recombinant *Escherichia coli* containing *lux* genes mirrored that observed in *V. fischeri*, so the refined genetic techniques developed for *E. coli* have been used to explore the molecular basis of luminescence control. It is clear from these studies that autoinducer controls light production by inducing transcription of the *lux* operon encoding the enzymes for luminescence.

Expression of *lux* in *Vibrio harveyi*, as in *V. fischeri*, is dependent on the density of the cell culture, but the luminescence systems of these species differ substantially with respect to the nature of the autoinducer substances and the organization of *lux* genes. The autoinducer of *V. fischeri*, *N*-(β-ketocaproyl)homoserine lactone, is produced only by *V. fischeri* and elicits a response only in *V. fischeri*. The autoinducer from *V. harveyi* is different and has recently been determined to be *N*-(β-hydroxybutryl)homoserine lactone (3). Also, other nonluminous marine bacteria such as *Vibrio parahaemolyticus* and *Vibrio anguillarum* produce a substance in growth supernatants which stimulates induction of luminescence in *V. harveyi* (18). The *lux* genes encoding enzymatic functions (*luxC*, *luxD*, *luxA*, *luxB*, and *luxE*, transcribed in one operon in this order) are identical in arrangement in both *V. fischeri* and *V. harveyi* and also share extensive DNA homology, as judged by DNA sequence determination and cross-hybridization (evidence reviewed in reference 30). The regulatory genes of *V. fischeri*, *luxR*, and *luxI*, are closely linked to the genes for luminescence enzymes (12, 13, 15). *luxI*, for autoinducer synthesis, is upstream from *luxC* in the *luxCDABE* operon, and *luxR*, for

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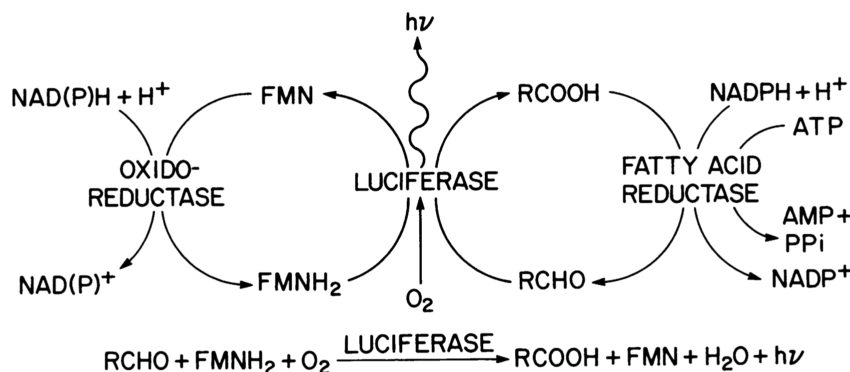

FIG. 1. Substrates, products, and enzymes of the bacterial bioluminescence reaction.

activation of transcription of *luxICDABE* in response to autoinducer, is in a divergently transcribed operon adjacent to *luxI*. However, genes encoding the putative regulatory functions in *V. harveyi* are not highly homologous to those of *V. fischeri* (no cross-hybridization with *luxR-luxI* as a probe of genomic DNA), and they are not linked to the *luxCDABE* cluster as in the case in *V. fischeri* (32).

Mutagenesis of *V. harveyi* with transposon mini-Mu*lac* was used to survey the genome for genes required for luminescence. Transposon insertions which resulted in the Lux^- phenotype were mapped to two unlinked regions of the genome (28). One region contained the *luxCDABE* operon encoding the luciferase and fatty acid reductase enzymes for light production, but genes encoding additional *lux* functions were not located in this region. These results supported previous sequence analysis of the 1-kb region immediately 5′ to *luxC* which indicated the absence of an open reading frame that could encode a *lux* regulatory gene (32). The second *lux* region defined by transposon insertions appeared to have a regulatory function because mutants with defects in this locus did not support transcription of the unlinked *luxCDABE* operon. We report here the cloning and characterization of this locus, including DNA sequence determination and analysis, localization of regulatory function by transposon Tn*5* mutagenesis, and examination of the effect of the cloned regulatory gene (i.e., *luxR*) on expression of the *luxCDABE* operon in *trans* in recombinant *E. coli*.

MATERIALS AND METHODS

Bacterial strains and media. A rifampin-resistant derivative of *V. harveyi* BB7 (1) was used for the isolation of chromosomal DNA. Cultivation of *V. harveyi* was done at 30°C or less in heart infusion medium (25 g of heart infusion broth [Difco Laboratories, Detroit, Mich.], 20 g of NaCl per liter). *E. coli* DH5α [F^- *endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 recA1 gyrA96 relA1* Δ(*argF-lacZYA*)U169 φ80d *lacZ*ΔM15 λ^-] was used as a general host strain. Strain DH5αF′ (DH5α with an F′ plasmid [Bethesda Research Laboratories, Inc., Gaithersburg, Md.]) was used for propagation of M13, and strain CC118 [*araD139* Δ(*ara leu*)*7697* Δ*lacX74* Δ*phoA20 galE galK thi rpsE rpoB argE*(Am) *recA*1] was used as a host strain for Tn*5* mutagenesis using λ467 (λ *b221rex*::Tn*5 c*I857 *O*am29 *P*am80). LB medium (10 g of tryptone [Difco], 5 g of yeast extract [Difco], 10 g of NaCl per liter) was used for the general cultivation of *E. coli*. H medium (10 g of tryptone [Difco], 8 g of NaCl) was used for the propagation of strains containing M13. NZC medium (10 g of NZ amine [Scheffield Products, Memphis, Tenn.], 5 g of NaCl, 2 g of $MgCl_2 \cdot 6H_2O$, 1 g of Casamino Acids [Difco] per liter) was used for growth of strain CC118 for transposon mutagenesis. When solidified medium was required, 15 g of agar (BBL Microbiology Systems, Cockeysville, Md.) per liter was added to liquid medium. For top agar, 8 g/liter was added (agarose [Bethesda Research Laboratories] top agar was used in the case of plaque lifts). Antibiotics (Sigma Chemical Co., St. Louis, Mo.) were added to the medium at the following concentrations: 100 μg of ampicillin per ml, 50 μg of kanamycin sulfate per ml, 10 μg of tetracycline per ml, and 20 μg of chloramphenicol per ml.

Recombinant genetic methods. DNA manipulations were done according to Maniatis et al. (27) unless otherwise stated. DNA transformations were done according to Hanahan (20). The DNA hybridization and nick translation procedures used have been described elsewhere (28). Restriction endonucleases, T4 DNA ligase, and calf intestine alkaline phosphatase were purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.) and were used according to the instructions of the supplier. A cosmid library was constructed by ligating *Hin*dIII partially digested *V. harveyi* chromosomal DNA in the size range of 15 to 25 kb to *Hin*dIII-digested pLAFRII DNA (16) that had been doubly phosphatase treated with calf intestine alkaline phosphatase. In vitro packaging of T4-ligated DNA was accomplished with Gigapack Gold (Stratagene, La Jolla, Calif.) λ DNA packaging extracts according to the instructions of the manufacturer. Recombinant clones were obtained by infecting strain DH5α with a packaged cosmid preparation and plating the bacteria onto L-agar plates containing tetracycline. Individual colonies were picked onto 50 L-agar tetracycline plates in a 7-by-7 grid to form a bank of ~2,500 colonies. The library was then replica plated to make several copies. One copy was immediately frozen in LB medium with 10% dimethyl sulfoxide (Schwarz Mann Biotech, Cleveland, Ohio) in microdilution dish wells to preserve the spatial separation of the colonies. Several other sets of the cosmid library were transferred to filter paper (Whatman 541; Whatman Ltd., Maidstone, England) and processed according to the colony blot method (27).

Plasmid pMR102 (28) containing part of the *lux* regulatory locus was used to prepare a hybridization probe to detect clones with homologous DNA in the cosmid library. DNA of pMR102 was restricted with *Hin*dIII and fractionated on a 0.6% agarose gel. The band corresponding to the insert DNA was excised from the gel under UV light and isolated from the gel fragment on an analytical electroeluter (International Biotechnologies Inc., New Haven Conn.) according to the

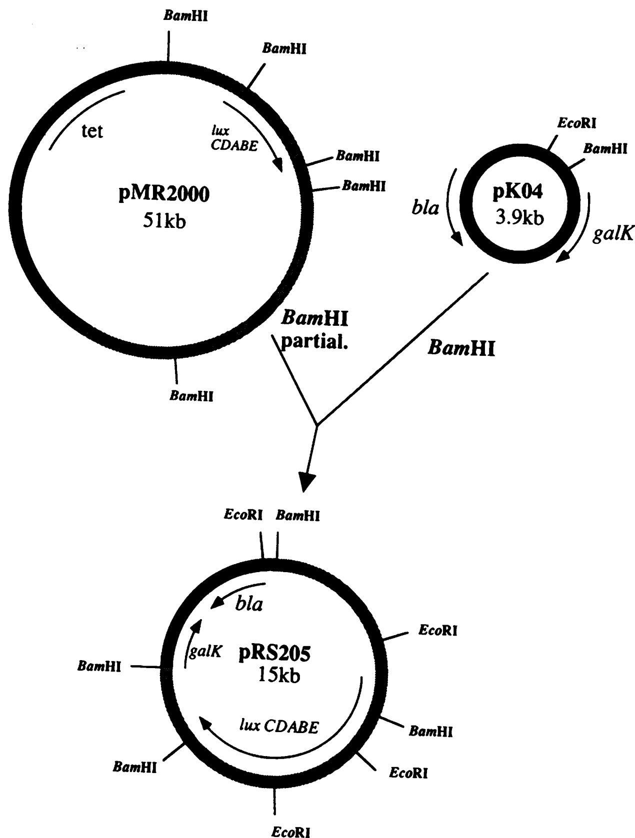

FIG. 2. Construction of plasmid pRS205. The influence of exogenous promoters on the transcription of *lux* genes was eliminated by subcloning the *luxCDABE* operon from cosmid pMR2000 into promoter-probe vector pK04. Symbols: ■, vector DNA; ▒, genomic DNA from *V. harveyi*.

instructions of the manufacturer. This DNA was then ^{32}P labeled by nick translation and used to probe the 50 colony filter blots of the cosmid bank. Plasmid pMR105 was constructed at the same time as pMR102 and contains the 2.1-kb *Hin*dIII fragment adjacent in the genome to the *Hin*dIII fragment cloned into pMR102. This *Hin*dIII fragment was also isolated and used to probe another set of colony filter blots. Positive clones were isolated from the frozen set of the cosmid library for the preparation of plasmid DNA. Clone DNA was restricted with *Hin*dIII, size fractionated on a 0.7% agarose gel, blotted to a Nytran membrane (pore size, 0.45 mm; Schleicher & Schuell, Inc., Keene, N.H.), and hybridized to the same probes used to detect the original clones in the cosmid library. The library was also screened in the dark room in the presence of aldehyde (decanal) vapor to detect weakly luminescent cosmid clones that contained the *luxCDABE* operon. Plasmid pRS156, a subclone containing *luxR* on a 2.2-kb *Hin*dIII fragment, was constructed by ligating *Hin*dIII-digested pMR1403 to *Hin*dIII-digested and phosphatased pACYC184.

Plasmid pRS205 containing the *luxCDABE* operon in promoterless vector pK04 (29) was constructed (Fig. 2) as follows. DNA from cosmid pMR2000 (*luxCDABE*) was restricted for different lengths of time with a constant amount of *Bam*HI restriction enzyme. The digestions were terminated by phenol extraction, and the DNA was then precipitated with ethanol. A sample of each reaction was run on a 0.6% agarose gel, and samples with suitable partial restriction fragments were pooled and used for ligation. Plasmid pKO4 was cut to completion with *Bam*HI, phosphatased, and ligated to the partially digested pMR2000. The ligated DNA was then transformed into strain DH5α with selection for recombinants on L-agar plates containing ampicillin. The recombinant colonies were then exposed to aldehyde vapor, and weak Lux$^+$ colonies were picked for plasmid DNA preparation. Restriction analysis with *Eco*RI and *Bam*HI revealed that plasmid pRS205 had the minimum number of *Bam*HI fragments containing the entire *luxCDABE* operon in the proper orientation in relation to the *galK* gene in plasmid pK04.

Transposon mutagenesis. Cosmid pMR1403 in strain CC118 was used for transposon mutagenesis of the *lux* regulatory locus. Tn*5* mutagenesis was done according to De Bruijn and Lupski (6) with the following changes. The λ467 lysates were prepared according to Davis et al. (5). Cosmid-containing cells were grown in 10 ml of NZC medium overnight at 37°C, diluted 1:5 into 20 ml fresh medium, and grown to an optical density at 600 nm (OD_{600}) of >0.8. Bacteriophage stocks were added to 1-ml samples of bacteria for a multiplicity of infection of ~1, and phage were allowed to absorb at room temperature for 2 h. After adsorption of the phage, the cells were allowed to express kanamycin resistance by growing in 2 ml of LB medium with 10 μg of tetracycline per ml at 30°C for 2 h. Then 3 ml of LB medium containing 10 μg of tetracycline per ml and 100 μg of kanamycin per ml (for a final concentration of 60 μg of kanamycin per ml) was added, and growth was continued with shaking at 37°C overnight. A 3-ml sample of each culture was used in an alkaline mini-plasmid preparation procedure, and each preparation represented a different mutagenesis. The DNA from each minipreparation was used to transform strain DH5α, which already contained pRS205 (*luxCDABE* operon). Selection for recombinants was done at 37°C on LB plates containing tetracycline (for pMR1403 resistance), kanamycin (for Tn*5* resistance), and ampicillin (for pRS205 resistance). The plates were cooled to room temperature to allow the luminescence enzymes to function and were then examined in the dark under a red light to categorize clones with respect to the production of light. To map the Tn*5* insertions in the cosmid DNA, the mutated cosmids had to be separated from plasmid pRS205 by retransformation of strain DH5α. Mini-plasmid preparations were made from the original transformants, and the cosmid and plasmid DNA were then retransformed into DH5α at a low concentration of DNA. Selection was on LB agar containing tetracycline and kanamycin but not ampicillin in order to eliminate the presence of plasmid pRS205. DNA was again prepared for each of the Tn*5*-mutagenized cosmids and then restricted with several combinations of *Hin*dIII, *Bam*HI, *Cla*I, *Pst*I, and *Eco*RI enzymes and fractionated on a 0.7% agarose gel; the position of the Tn*5* insertions in each cosmid was mapped according to these digests.

Sequencing of the *luxR* gene. Several Tn*5* mutants in pMR1403, all with transposon insertions within the 2.2-kb *Hin*dIII fragment, were used for cloning appropriate subfragments into M13mp9. DNA from each mutant was digested with *Pst*I and *Hin*dIII and ligated to double-stranded M13mp9 digested in the same way. The ligation mixtures were used to transfect strain DH5αF′, which was then plated with 200 μl of exponentially growing DH5αF′ lawn cells in H top agarose on H-agar plates. M13 plaques were transferred to nitrocellulose (Schleicher & Schuell) filter circles, baked at 80°C under vacuum for 1 h, and probed with the 2.2-kb *Hin*dIII fragment isolated from pRS156, using standard

hybridization procedures. Only those clones containing the DNA between the *Pst*I site from Tn*5* and the *Hin*dIII site from the 2.2-kb *luxR* DNA fragment should hybridize to the probe. Six clones that hybridized to the probe from each of the ligations described above were grown up for preparation of double-stranded M13 DNA. Because the insertion site of each Tn*5* insertion was known and the Tn*5* arm added ~600 base pairs (bp) of DNA from the *Pst*I site to the insertion point, restriction analysis with *Hin*dIII and *Pst*I allowed identification of the right- and left-hand fragments for each Tn*5* insertion (see Fig. 5). Using an oligonucleotide homologous to the end of Tn*5* as a primer, 3'-GTTCATCGCAG GACTTG-5' (11), single-stranded M13 DNA was sequenced by the dideoxy-chain termination procedure of Sanger et al. (36), using Sequenase version 2.0 (United States Biochemical Corp., Cleveland, Ohio) with [^{35}S]dATP (Dupont, NEN Research Products, Boston, Mass.) according to the instructions of the manufacturer. This strategy allowed sequencing of both strands of the *luxR* gene without need of additional clones in another M13 vector. Sequence analysis was performed by using the University of Wisconsin Genetics Computer Group software package version 6.1, August 1989 (7).

Measuring expression of bioluminescence. The luminescence of appropriately diluted cultures of bacteria was measured in a minibeta scintillation counter (1211; LKB Instruments, Inc., Rockville, Md.) by using the single-photon-event, or chemiluminescence, mode and is reported as specific light units, which are the scintillation counts per minute normalized to 1 ml of culture at OD_{600}. Strain RS201, containing pRS205 (*luxCDABE* operon) and pMR1403 (*luxR*) in DH5α, was used to measure the expression of bioluminescence in *E. coli*. Autoinducer bioassays and cell-free culture supernatant preparation have been described elsewhere (18, 28). The medium used for the autoinducer bioassay was LB broth. Cell-free supernatants were made from *E. coli* DH5α and RS201 and from wild-type *V. harveyi* BB7 grown in LB medium to an OD_{600} of 1.0. Mid-exponential-phase cells of *V. harveyi* and strain RS201 were diluted to an OD_{600} of ~0.01 into 100% cell-free supernatant from cultures of various strains and also into fresh LB medium. Samples were removed each hour to determine the OD_{600} and the light production of the culture. All cultures of *V. harveyi* and *E. coli* used for these experiments were grown at 30°C.

RESULTS

Cloning of the *lux* regulatory locus. Transposon mutagenesis was used previously to define a locus in *V. harveyi* necessary for transcription of the operon (*luxCDABE*) containing the genes encoding the luminescence enzymes, and one mutant with a transposon insertion in this region was used to construct recombinants containing parts of this locus which could then be used as hybridization probes to obtain other recombinants with the entire *lux* regulatory region. The transposon mini-Mu*lac* (Tet^r)-encoded drug resistance gene, *tet*, is physically linked to the target *lux* sequences in the mutants, so DNA from the *lux* regulatory locus could be isolated by cloning a fragment containing the selectable, drug resistance marker. Cleavage of genomic DNA from a mini-Mu*lac* (Tet^r) insertion mutant (i.e., MR1101) with *Pst*I yielded a fragment with both *tet* and the *lux* sequences flanking one side of the transposon insertion. Details of the construction of plasmid pMR102, which contains part of the 2.2-kb *Hin*dIII fragment spanning the regulatory locus, and of pMR105, which contains the adjacent downstream *Hin*dIII fragment (both fragments are shown in Fig. 4), are given in Materials and Methods and in reference 28.

Recombinant clones with the entire regulatory locus were obtained from a cosmid library constructed by ligating a partial *Hin*dIII restriction digest of the genome of *V. harveyi* with cosmid vector pLAFRII, followed by in vitro packaging and recovery of recombinants by transduction. Plasmid pMR102, which contains part of the 2.2-kb genomic *Hin*dIII fragment spanning the *lux* regulatory locus, also hybridized to other restriction fragments in the genome of *V. harveyi* (28). This cross-hybridization interfered with unambiguous detection of clones with regulatory DNA, so plasmid pMR105 containing the adjacent *Hin*dIII genomic fragment was also used as a probe. Insert DNAs from plasmids pMR102 and pMR105 were isolated, labeled by nick translation, and hybridized to separate filter blots of the cosmid library. Of about 2,500 recombinants in the library, 43 cosmid clones hybridized to DNA from pMR102, and 17 of these hybridized to DNA from pMR105. Restriction analysis and Southern blot analysis of *Hin*dIII-digested cosmid DNA showed that all 17 cosmids contained two *Hin*dIII fragments in common: a 2.2 kb-fragment that hybridized to pMR102 DNA and a 2.1-kb *Hin*dIII fragment that hybridized to pMR105 DNA.

The 2.2-kb *Hin*dIII fragment, present on the 17 recombinant cosmids, encodes a function necessary for expression of luminescence in *V. harveyi*, but does this function influence expression of the *luxCDABE* operon in recombinant *E. coli*? To test this, each of the cosmids with the regulatory locus was positioned in *trans* in *E. coli* with a compatible vector containing the *luxCDABE* operon, i.e., pMR2713 based on vector pMMB33 (28). Colonies of *E. coli* containing the *luxCDABE* operon in *trans* with a control cosmid produced no visible light, but those with *luxCDABE* in *trans* with any of the 17 cosmids with regulatory function encoded by the cloned DNA produced visible bioluminescence. To obtain a more refined measurement of the influence of regulatory function on *luxCDABE* expression, the *luxCDABE* operon was recloned into promoter-probe vector pK04 (29), where it was shielded from the effect of extraneous vector promoters. Construction of the resultant plasmid, pRS205, is shown in Fig. 2. Control and regulatory locus cosmids were positioned in *trans* with plasmid pRS205. In this arrangement, the presence of regulatory function resulted in visibly luminescent recombinant colonies and stimulated light production by at least 10,000-fold.

Expression of luminescence. The presence of the cloned regulatory locus in *trans* with the *luxCDABE* operon resulted in a substantial increase in luminscence by the recombinant *E. coli*, but has a functionally complete regulatory system been reassembled in the recombinant? Specifically, has the level of light production and the autoinducer-mediated, density-dependent control characteristic of *V. harveyi* been reproduced with cloned DNA in *E. coli*? The production of light by *V. harveyi* and recombinant RS201, containing the cloned regulatory locus and the *luxCDABE* operon in *trans*, is shown in Fig. 3. Specific light production in light units is plotted as a function of culture density for bacteria that were inoculated from a mid-logarithmic-phase culture into a variety of media, including fresh LB medium, and cell-free culture supernatants prepared from suspensions of *V. harveyi* and *E. coli* DH5α or recombinant RS201. Specific luminescence of *V. harveyi* cells grown in fresh LB medium declined rapidly during growth at low density (below OD_{600} of 0.1) but climbed as the culture density increased (Fig. 3A), eventually reaching a level characteristic of dense cultures.

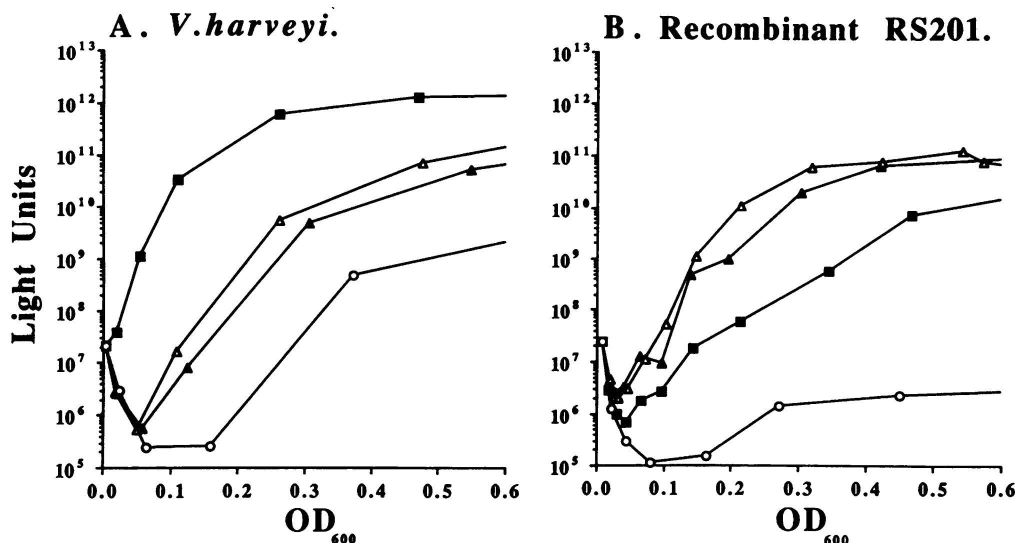

FIG. 3. Induction of bioluminescence in *V. harveyi* (A) and in recombinant *E. coli* RS201 containing the *lux* regulatory locus on cosmid pMR1403 and the *luxCDABE* operon on plasmid pRS205 (B). Bacteria from cultures with an intermediate level of bioluminescence were diluted into various media for measurement of growth and light production. Specific light production (light output per OD_{600}) is plotted as a function of culture density (OD_{600}). Symbols: ○, cells grown in fresh LB; ▲, cell-free supernatant from *E. coli* DH5α grown in LB to an OD_{600} of 1.0; △, supernatant from strain RS201 grown in an OD_{600} of 1.0; ■, supernatant from *V. harveyi* grown in LB to an OD_{600} of 1.0.

Luminescence increased rapidly to a maximal level without an initial decline when *V. harveyi* was grown in cell-free supernatant from *V. harveyi*. These observations, which are consistent with results of other studies (9, 18, 25), indicate the presence of an extracellular inducer of luminescence, autoinducer, which accumulates in the growth medium and at a critical concentration causes expression of luminescence genes. Induction of light production in *V. harveyi* grown in cell-free supernatant from *E. coli* DH5α occurred earlier, at a lower OD_{600}, than in fresh medium (Fig. 3A). However, *E. coli* does not produce autoinducer (18), and the modest stimulatory effect observed with this supernatant could have resulted from the removal of an inhibitor of luminscence that has been reported to be present in complex media (9). Supernatant from strain RS201 with the cloned *lux* genes was similarly ineffective at inducing luminescence of *V. harveyi*, so we conclude that the recombinant was producing little or no autoinducer activity.

Light production by recombinant *E. coli* RS201 appeared to be influenced by the density of the culture, and the maximal level of luminescence approached (i.e., was about 10- to 100-fold less, depending on the medium used) the level obtained for *V. harveyi* (Fig. 3B). However, the supernatant from *V. harveyi* was not a potent inducer of luminescence of the recombinant and was less effective at stimulating expression of luminescence than cell-free supernatant from either *E. coli* DH5α or recombinant strain RS201. Production of light by the recombinant was therefore not specifically responsive to the presence of autoinducer. Rather, the effect of culture density on the luminescence of strain RS201 could have been solely due to the removal of the inhibitor of luminescence, which also appears to be a factor influencing the luminescence of *V. harveyi*. It is clear that the *lux* regulatory locus is necessary for expression of luminescence in both *V. harveyi* (28) and recombinant *E. coli*, the latter of which produced 10,000-fold more light when the regulatory locus was present in *trans* with the *luxCDABE* operon. However, the presence of the cloned regulatory locus in recombinant *E. coli* was not sufficient to reproduce the regulatory control characteristic of the native *V. harveyi*.

Tn5 mutagenesis. Mutagenesis with transposon Tn5 was used to precisely locate the region of cloned DNA responsible for regulatory function. Recombinant cosmid pMR1403 was chosen for mutagenesis, which involved infecting cosmid-bearing *E. coli* with a λ::Tn5 incapable of replicating or integrating in that host, selecting for Kan^r transductants, recovering cosmid DNA from pools of transductant bacteria, and retransforming *E. coli* containing pRS205 with cosmid DNA to score for the capacity to stimulate expression of the *luxCDABE* operon. Several hundred mutated cosmids were screened. Nine pMR1403::Tn5 cosmids that did not stimulate light production in *E. coli* with plasmid pRS205 (i.e., *luxCDABE* in *trans*) and seventeen control cosmids that retained the capacity to stimulate light production were reisolated, and each was subjected to restriction analysis to determine the location of Tn5 insertion. The regulatory region delimited by Tn5 mutations comprised about 0.8 kb of DNA entirely within the 2.2-kb *Hin*dIII fragment present in all of the cosmids with regulatory activity. The location of this region (Fig. 4A) corresponds to the region defined earlier by using mini-Mu*lac* (Tet^r) insertions in the genome of *V. harveyi* (28) (Fig. 4B). As expected from these results, a recombinant plasmid containing only the 2.2-kb *Hin*dIII fragment (i.e., pRS156) was equally effective at increasing expression of the *luxCDABE* operon. Also, recombinant cosmids containing the regulatory region, when transferred by conjugation, could restore the Lux^+ phenotype to all mutants of *V. harveyi* containing mini-Mu*lac* insertion in the *lux* regulatory region (data not shown).

DNA sequence analysis. The strategy used to sequence about 1,200 bp of the regulatory region was to use Tn5 insertions throughout the region to provide well-spaced, defined restriction fragments terminated with a unique sequence (part of the Tn5 arm), to which one primer oligonucleotide could be hybridized for the M13-dideoxynucleotide sequencing method. The Tn5 insertions used and the DNA strands sequenced are shown in Fig. 5. Analysis of the DNA sequence revealed only one open reading frame in the three reading frames in either orientation of transcription which was larger than 222 bp. This candidate for the *lux* regulatory

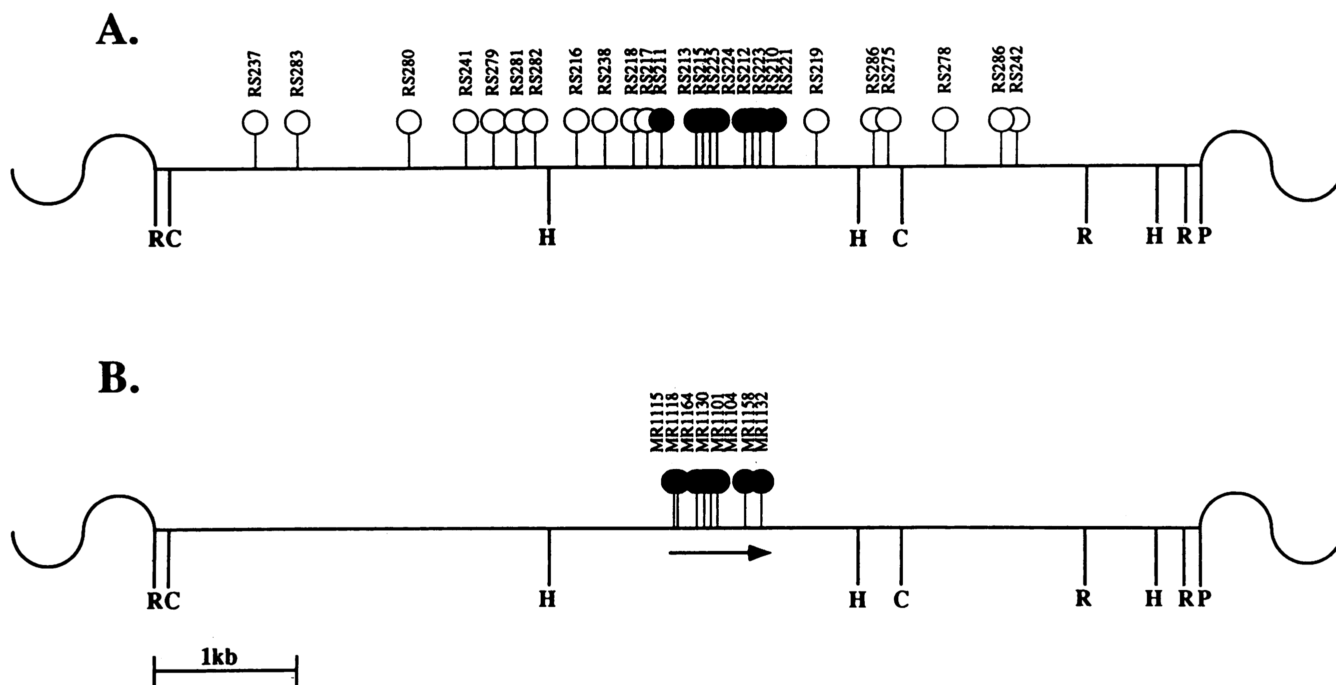

FIG. 4. Location of DNA encoding regulatory function by transposon mutagenesis. (A) Locations of transposon Tn*5* insertions in the portion of cosmid pMR1403 that contains the cloned *lux* regulatory locus from *V. harveyi*. (B) Locations of transposon mini-Mu*lac* (Tet^r) insertions in the region of the genome of *V. harveyi* containing the *lux* regulatory locus (see reference 28). Symbols: ●, insertion mutations that eliminate regulatory function, i.e., give a Lux$^-$ phenotype; ○, mutations outside the regulatory region that do not; →, direction of transcription determined by analysis of *lux*::*lac* fusions (28). Restriction sites: R, *Eco*RI; C, *Cla*I; H, *Hin*dIII; P, *Pst*I.

gene is 615 bp long and encodes a protein of 205 amino acids (Fig. 6). Its size and location are in close alignment with the regulatory region defined by Tn*5* mutagenesis of the cloned locus and mini-Mu*lac* mutagenesis of the genome of *V. harveyi* (28), and the orientation of transcription of this open reading frame is the same as that deduced from analysis of *lacZ* transcriptional fusions generated previously by mini-Mu*lac* mutagenesis. We conclude that regulatory activity is encoded by this open reading frame, a single gene which we name *luxR*.

Features upstream of this open reading frame resemble *cis*-acting control sites. The ATG codon at position 318 is preceded with a 6-bp spacing by the sequence AAG GAAAA, which is similar (five of eight bases, which are underlined) to the consensus Shine-Dalgarno sequence of *E. coli*, AAGGAGGT (38). Candidates for promoter sequences are present upstream of the open reading frame in the vicinity of Tn*5* insertion RS211 which eliminates regulatory function. The putative −35 and −10 sequences of three promoters were compared, using the statistical method of Harley and Reynolds (21), to the canonical *E. coli* promoter sequence (23, 35); i.e., the −35 hexamer is TTGACA separated by 17 bp from the −10 hexamer which is TATAAT. The promoter sequence starting at position 205, i.e.,

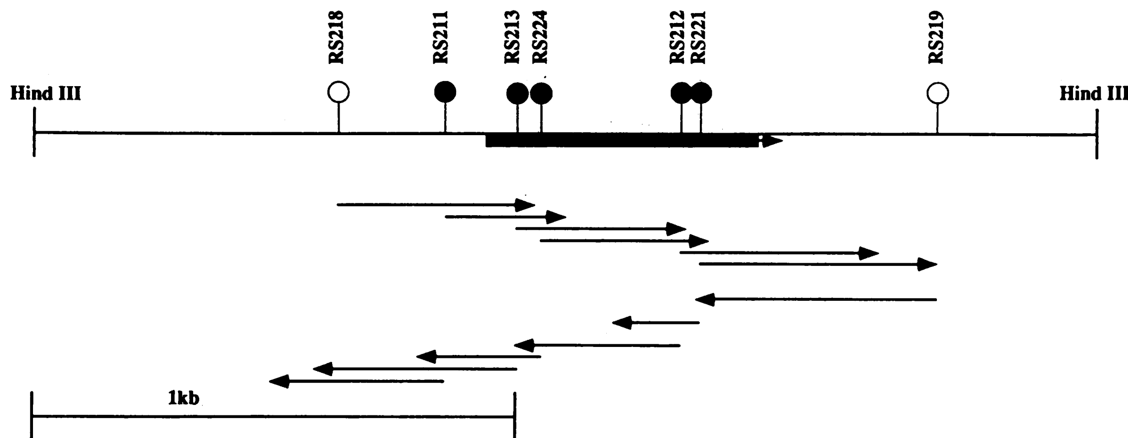

FIG. 5. Sequencing of the *lux* regulatory region. The sequencing strategy described in the text used cosmids each with a transposon Tn*5* insertion in this region of DNA. Locations of the Tn*5* insertions are shown above the restriction map. Arrows below the map represent the overlapping segments of DNA from both strands that were sequenced.

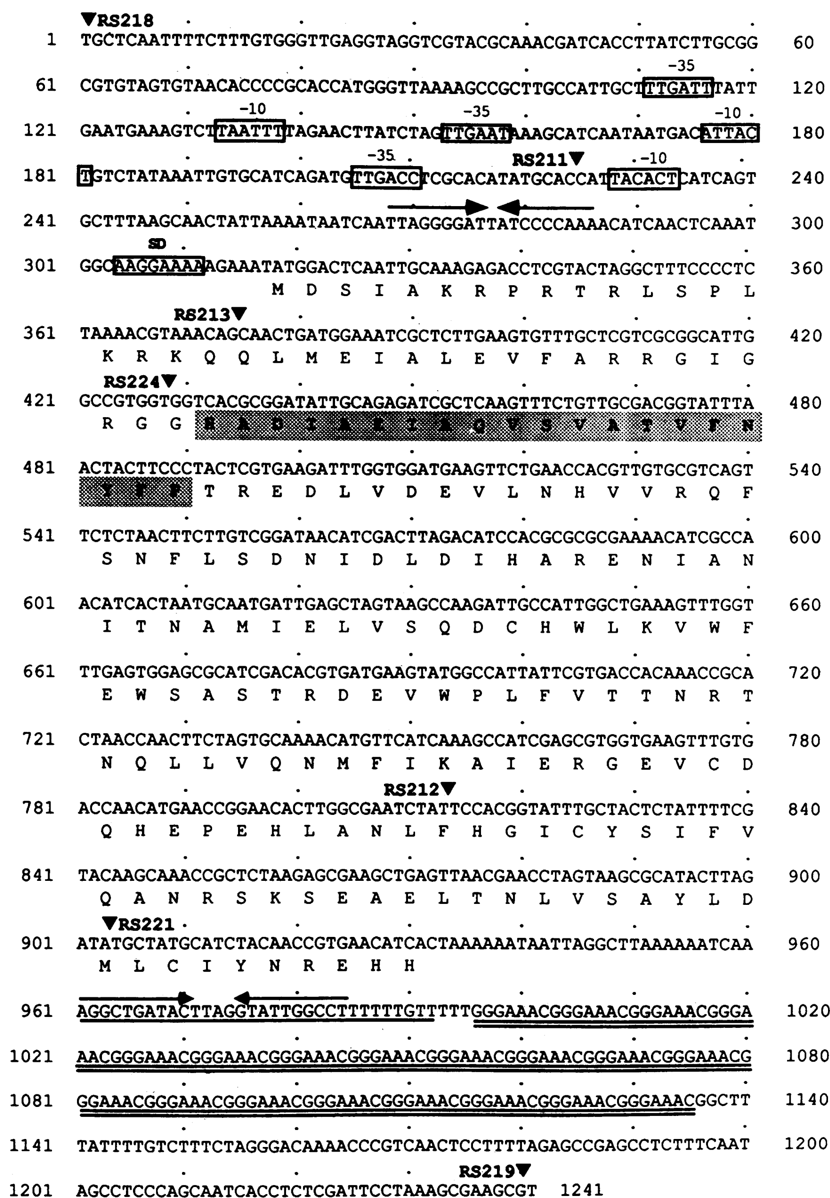

FIG. 6. DNA sequence of the *lux* regulatory region. The amino acid sequence of the *luxR* product is shown below the DNA sequence. The −35 and −10 sequences of three putative *lux* promoters and a candidate for a Shine-Dalgarno ribosome-binding site are boxed. Vertical arrows show the location of transposon Tn*5* insertion mutations; horizontal arrows show the locations of inverted repeat sequences. The possible DNA-binding domain in the LuxR sequence is shaded. A single underline marks the location of a sequence similar to a *rho*-independent termination site; a double underline marks the 20 copies of a heptamer sequence.

TTGACC-17 bp-TACACT, is most similar to the canonical sequence of *E. coli* (promoter homology index of −2.1). Other noteworthy features include two inverted repeat regions, one centered at position 277 which could serve as a DNA-binding site and a second inverted repeat after *luxR* which resembles a *rho*-independent transcription termination site (2, 35). Also, there is an unusual region following *luxR* which contains 20 copies in direct repeat configuration of the sequence GGGAAAC (Fig. 6). The significance of the 7-mer direct repeats is not apparent, but an extensive repeat of another hexamer sequence has also been found after the P1 porin protein gene of *Neisseria gonorrhoeae* (17).

The sequence comparison programs of the University of Wisconsin Genetics Computer Group (7) were used to search the GenBank and EMBL data bases for similarity between *luxR* and its product and other DNA and protein sequences. This search revealed no significant homology of *luxR* or the *luxR* product of *V. harveyi* to other DNA or amino acid sequences in the data base, including those of the *luxR* gene and gene product of *V. fischeri*. The weight matrix method of Dodd and Egan (8) was used to compare LuxR with Cro-like DNA-binding proteins, and a 20-amino-acid segment (Fig. 6) resembling a Cro-like DNA-binding domain was found (i.e., score of 1672 for this segment). Few genes of *V. harveyi* have been sequenced, so there is no compilation of codon usage data for genes expressed at high, moderate, or weak levels to use as a reference. If the codon usage in *luxR* (Table 1) is compared with the codon usage preference of highly expressed genes of *E. coli*, some resemblance is evident. The codon preference statistic calculated by the

TABLE 1. Codon usage for the *luxR* gene of *V. harveyi*

	U			C			A			G		
	Codon	Amino acid	No. of times used	Codon	Amino acid	No. of times used	Codon	Amino acid	No. of times used	Codon	Amino acid	No. of times used
U	UUU	Phe	3	UCU	Ser	4	UAU	Tyr	0	UGU	Cys	1
	UUC	Phe	7	UCC	Ser	1	UAC	Tyr	4	UGC	Cys	3
	UUA	Leu	4	UCA	Ser	1	UAA	End	1	UGA	End	0
	UUG	Leu	3	UCG	Ser	2	UAG	End	0	UGG	Trp	4
C	CUU	Leu	3	CCU	Pro	3	CAU	His	3	CGU	Arg	9
	CUC	Leu	0	CCC	Pro	0	CAC	His	6	CGC	Arg	4
	CUA	Leu	6	CCA	Pro	1	CAA	Gln	7	CGA	Arg	0
	CUG	Leu	3	CCG	Pro	1	CAG	Gln	2	CGG	Arg	0
A	AUU	Ile	6	ACU	Thr	4	AAU	Asn	2	AGU	Ser	0
	AUC	Ile	9	ACC	Thr	1	AAC	Asn	12	AGC	Ser	4
	AUA	Ile	0	ACA	Thr	2	AAA	Lys	4	AGA	Arg	1
	AUG	Met	5	ACG	Thr	2	AAG	Lys	2	AGG	Arg	1
G	GUU	Val	6	GCU	Ala	4	GAU	Asp	7	GGU	Gly	4
	GUC	Val	0	GCC	Ala	2	GAC	Asp	4	GGC	Gly	2
	GUA	Val	5	GCA	Ala	6	GAA	Glu	11	GGA	Gly	0
	GUG	Val	5	GCG	Ala	4	GAG	Glu	5	GGG	Gly	0

method of Gribskov et al. (19) was 0.870 for *luxR*, compared with an average codon preference for a random DNA sequence of the same composition of 0.459, which ranks the *luxR* gene with moderately expressed genes of *E. coli*. However, the *luxC*, *-D*, *-A* and *-B* genes of *V. harveyi*, most of which can be induced to an extremely high level of expression, have been sequenced (4, 24, 31, 32), and comparison of their codon preference with that of *E. coli* by this method suggests only a weak to moderate level of expression. The strong preference for particular codons in *luxR* indicates that this DNA is a coding sequence, but it may not be accurate to estimate the level of *luxR* expression by extrapolation from the pattern of codon usage in *E. coli*.

DISCUSSION

Transposon mini-Mu*lac* (Tet^r) mutagenesis defined two loci, *lux* regions I and II, that are necessary for bioluminescence in *V. harveyi* (28). Region I contains the *luxCDABE* operon, which encodes the luminescence enzymes luciferase and fatty acid reductase; region II encodes a regulatory function necessary for transcription of the *luxCDABE* operon. We have now cloned and analyzed this regulatory locus. The cloned locus stimulated expression of the *lux CDABE* operon positioned in *trans* on a compatible replicon in recombinant *E. coli*. The capacity to stimulate expression of this operon was localized to one open reading frame, which we called *luxR* (*R* to denote regulatory function). The *luxR* coding sequence corresponds very closely in size, location, and orientation of transcription with the regulatory region defined previously by transposon mini-Mu*lac* mutagenesis of the genome of *V. harveyi* and in this study by transposon Tn*5* mutagenesis of the cloned DNA. Also, the *luxR* gene is flanked by *cis*-acting sites that could function in regulation of transcription and translation.

We speculated earlier that the regulatory locus of *V. harveyi* encodes an activator of *lux* transcription analogous to that encoded by *luxR* of *V. fischeri*. The product of *luxR* of *V. harveyi* does have an amino acid sequence similar to that of the DNA-binding domain of Cro-like proteins, but there is no indication of amino acid or DNA sequence similarity between *luxR* of *V. harveyi* and *luxR* of *V. fischeri*. The regulatory function encoded by the cloned *luxR* gene of *V. harveyi* in *E. coli* was not responsive to the presence of autoinducer, supplied exogenously in a culture supernatant, which property contrasts with that of the regulatory activity encoded by the cloned *luxR* gene of *V. fischeri* (12, 14). Furthermore, the *luxI* gene of *V. fischeri*, which is required for synthesis of *V. fischeri* autoinducer, is linked to the *luxR* gene (and to the *luxCDABE* operon as well in *V. fischeri*), but there was no evidence for an analogous gene linked to *luxR* in the regulatory locus of *V. harveyi*. Specifically, no region of the cloned DNA other than *luxR* was found by transposon Tn*5* mutagenesis to be necessary for expression of luminescence in the recombinant, and the regulatory region did not direct the synthesis of an autoinducer activity.

Control of luminscence in the two marine bacteria, *V. harveyi* and *V. fischeri*, appears to be similar because expression of luminescence in both is stimulated by an extracellular signal substance. But it is clear that the luminescence systems from these bacteria are considerably different with regard to the chemical structure and spectrum of activity of the respective autoinducers, to *lux* gene organization, and to the regulatory functions encoded by these genes, so comparisons between the two systems could be of limited usefulness in guiding the course of experimentation. We will continue to search for a gene in *V. harveyi* analogous in function to *luxI* of *V. fischeri*, but a more general approach for identifying genes encoding new regulatory functions could be more fruitful. For example, if the *lux* regulatory system of *V. harveyi* were not reconstructed in *E. coli* because another gene or genes was required, we could identify the missing function by cloning additional *V. harveyi* DNA into *E. coli* that already contains *luxR* and the *luxCDABE* operon together on another replicon.

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