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Identification of a Locus Controlling Expression of Luminescence Genes in *Vibrio harveyi*

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Mutagenesis with transposon mini-Mu*lac* was used to identify loci containing genes for bioluminescence (*lux*) in the marine bacterium *Vibrio harveyi*. Transposon insertions which resulted in a Lux$^-$ phenotype were mapped to two unlinked regions of the genome. Region I contained the *luxCDABE* operon which was previously shown to encode the enzymes luciferase and fatty acid reductase, which are required for light production. The other locus, region II, which was identified for the first time in this study, appeared to have a regulatory function. In Northern blot analysis of mRNA from mutants with defects in this region, no transcription from the *luxCDABE* operon could be detected. Strains with transposon-generated *lux*::*lacZ* gene fusions were used to analyze control of the transcription of these regions. Expression of luminescence in the wild type was strongly influenced by the density of the culture, and in strains with the *lacZ* indicator gene coupled to the *luxCDABE* operon, β-galactosidase synthesis was density dependent. So, transcription of this operon is responsive to a density-sensing mechanism. However, β-galactosidase synthesis in strains with *lacZ* fused to the region II transcriptional unit did not respond to cell density. The organization and regulation of the *lux* genes of *V. harveyi* are discussed, particularly with regard to the contrasts observed with the *lux* system of the fish light-organ symbiont *Vibrio fischeri*.

Luminescent bacteria are common 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 be used to attract prey, for intraspecies communication, or to escape from predators (24). Luminescence could function to provide a direct benefit to the bacteria. Light emitted by large aggregations of bacteria may attract feeders which ingest the bacteria into the nutrient-rich environment of the gut tracts. Another 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) (15). 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 (30). 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 (5, 26), so functions which 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 compared with that in dilute cultures. Density-dependent regulation of luminescence has been investigated most thoroughly with the light-organ symbiont *Vibrio fischeri* (13, 23). This bacterium synthesizes a small extracellular signal molecule, called an 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. The autoinducer from *V. fischeri* has been shown to be *N*-(β-ketocaproyl) homoserine lactone (4). The genes (*lux*) necessary for light production in recombinant hosts have been cloned from *V. fischeri* MJ-1 on one 9-kilobase (kb) fragment of DNA (5, 6). 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* were used to explore the molecular basis of luminescence control. It is clear from results of these studies that the autoinducer controls light production by inducing transcription of the *lux* operon encoding the enzymes for luminescence.

Expression of *lux* in *V. harveyi*, like that in *V. fischeri*, is dependent on the density of the cell culture, but the luminescence systems of the species differ substantially with respect to the nature of the autoinducer substances and the organization of *lux* genes. The autoinducer of *V. fischeri* is chemically defined, is produced only by *V. fischeri*, and elicits a response only in *V. fischeri*. The autoinducer activity from *V. harveyi* is labile, and the chemical formula has not been determined. In addition, other nonluminous marine bacteria such as *Vibrio parahaemolyticus* and *Vibrio anguillara* produce a substance or substances in growth supernatants which stimulate induction of luminescence in *V. harveyi* (11).

The *lux* genes encoding enzymatic functions (*luxC*, *luxD*, *luxA*, *luxB*, and *luxE*, which are transcribed in one operon in this order) are identical in arrangement in both *V. fischeri* and *V. harveyi* and share extensive DNA homology, as judged by DNA sequence determination and cross-hybridization (evidence has been reviewed in reference 17). The regulatory genes of *V. fischeri*, *luxR*, and *luxI*, are closely linked to the genes for luminescence enzymes (5, 6, 8). *luxI*, for autoinducer synthesis, is upstream from luxC in the *luxCDABE* operon; and *luxR*, for activation of transcription of *luxICDABE* in response to autoinducer, is a divergently

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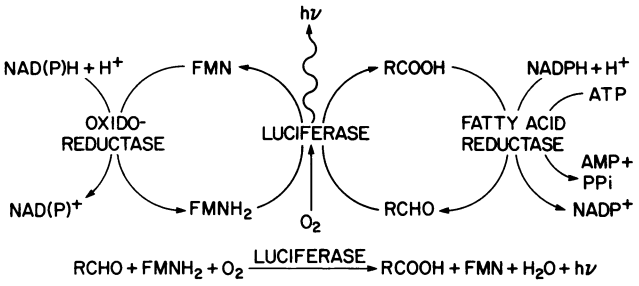

FIG. 1. Substrates, products, and enzymes of the bacterial bioluminescence reaction.

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 probably not linked to the locus containing genes for light production. Several lines of evidence support this conclusion. To date no recombinant clone, even those containing more than 20 kb of *V. harveyi* DNA, has manifested density-dependent expression in *E. coli* (2, 20). One explanation for the lack of density-dependent expression of luminescence in recombinant *E. coli* is that a *lux* regulatory gene, which is present on the cloned fragment of DNA, is not expressed or that the product of this gene does not function in the recombinant host. The 1-kbp region 5′ to *luxC* of *V. harveyi*, however, has been sequenced, and it contains a high frequency of termination codons in all three reading frames on both strands (22), suggesting the absence of an additional *lux* gene which could encode the regulatory function in this region.

In our search for the *lux* regulatory genes of *V. harveyi*, we used a direct genetic analysis of *V. harveyi*. Specifically, transposon mutagenesis was used to survey the entire genome for the genes required for luminescence. For mutagenesis we used mini-Mu*lac*, which has proven to be particularly effective for genetic analysis of undomesticated bacteria. Collections of mutants with null phenotypes can be isolated by selecting those strains expressing the transposon-encoded drug resistance; target gene::*lac* indicator gene fusions are useful for measuring transcription; transposon insertions result in large alterations in DNA structure which can be readily mapped by Southern blot analysis of restricted genomic DNA; and the transposon drug resistance determinant serves as a selective marker for cloning target gene sequences (3). We report here the isolation and characterization of Lux$^-$ mutants of *V. harveyi* generated by transposon mini-Mu*lac* insertion. The defects in these mutants defined two loci essential for luminescence. One contained the operon encoding the enzymes for light production and the other locus contained a gene or genes with regulatory function.

MATERIALS AND METHODS

Bacterial strains and media. A rifampin-resistant derivative of strain BB7 (2) of *V. harveyi* was used for this study. Cultivation was done at 30°C or less, since growth at higher temperatures resulted in the inactivation of luciferase. The medium used for general-purpose propagation and transposon mutagenesis was heart fusion medium (25 g of heart infusion broth [Difco Laboratories, Detroit, Mich.] and 20 g of NaCl per liter) or LM medium (10 g of tryptone [Difco], 5 g of yeast extract [Difco], and 20 g of NaCl per liter). NZM top agar (3) used in the mutagenesis procedure consisted of 10 g of N-Z Amine (Kraft, West Lafayette, N.J.), 20 g of NaCl, and 6.5 g of agar (Difco) per liter. Autoinducer bioassay, medium AB, described by Greenberg et al. (11) was used to grow cells and obtain supernatants for analysis of the control of *lux* gene expression. It consisted of 0.3 M NaCl, 0.05 M $MgSO_4$, and 0.2% vitamin-free Casamino Acids (Difco). After the pH was adjusted to 7.5 with KOH, the medium was sterilized by autoclaving and then allowed to cool to room temperature, after which 1 ml of sterile 1 M potassium phosphate (pH 7.0), 1 ml of 0.1 M L-arginine (free base), and 2 ml of 50% glycerol were added per 100 ml of AB medium.

Cell-free supernatants were prepared by centrifuging late-logarithmic-phase cultures grown in AB medium at 5,000 × *g* at 5°C for 5 min and then filtering the supernatant through a 0.2-μm-pore-size membrane filter unit (Nalgene Labware Div., Nalge/Sybron Corp., Rochester, N.Y.). LB medium (10 g of tryptone [Difco], 5 g of yeast extract [Difco], and 10 g of NaCl per liter) was used for cultivation of *E. coli*. When solidified medium was required, 15 g of agar (Difco) per liter was added to liquid medium. Antibiotics (Sigma Chemical Co., St. Louis, Mo.) were added to the medium at the following concentrations: 80 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. 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 an optical density at 600 nm (OD_{600}) of 1. Specific β-galactosidase activity of *lux*::*lacZ* fusion strains was determined by the method of Miller (18).

Transposon mutagenesis. For mutagenesis of *V. harveyi* with transposon mini-Mu*lac*(Tet^r), we used a modification of a method used previously to mutagenize *V. parahaemolyticus* (3). Transfer of mini-Mu*lac*(Tet^r) into *V. harveyi* was accomplished by transduction with phage P1, which infects but does not replicate this bacterium. To obtain a transducing lysate, *E. coli* MC4100 [mini-Mu*lac*(Tet^r), P1 *clr100*CM] was diluted 1:100 from an overnight culture into fresh LB medium containing 5 mM $CaCl_2$. Ca^{2+} was added to stabilize P1 phage in the presence of $CHCl_3$ (27). The lysogen was grown at 30°C to an OD_{600} of approximately 0.4, at which point P1 and transposon replication was induced by a temperature shift to 42°C for 20 min. Incubation was continued at 37°C with vigorous shaking until lysis occurred (about 90 min postinduction). Omission of the selective antibiotics (chloramphenicol and tetracycline) from the induction medium prevented carry-over into the transduction phase of the procedure and gave some improvement in the extent of lysis. After lysis, a 1/100 volume of $CHCl_3$ was added to complete lysis and to kill the remaining cells. Once the $CHCl_3$ evaporated, cell debris was removed by centrifugation, and the lysate was stored over $CHCl_3$ at 5°C. Lysates could be stored for several weeks without the loss of substantial activity.

For transduction of mini-Mu*lac*(Tet^r) into *V. harveyi*, 100 μl of lysate ($CHCl_3$ first removed by evaporation) was added to 200 μl of recipient cells, which consisted of an overnight culture of *V. harveyi* grown in LM medium to which $CaCl_2$ was added to a final concentration of 25 mM just prior to infection. Cells were absorbed to phage for 20 min at room temperature without shaking. LM medium (0.5 ml) was then added, and the culture was grown for 2 h at 30°C with

shaking to allow expression of the transposon-encoded drug resistance. Each transduction mixture was combined with 2.5 ml of molten NZM top agar (at 55°C) and immediately poured onto a heart infusion medium plate containing tetracycline. Plates were incubated at 30°C for 48 h, and Lux$^-$ mutant colonies were identified by inspection of plates in a darkroom. Recognition of nonluminous colonies also required the use of a red light, the intensity of which could be adjusted with a voltage regulator.

Cloning *lux* DNA. The *lux* regulatory region (region II) of *V. harveyi* proximal to and including the end of mini-Mu*lac*(Tet^r) containing *lacZ*, *lacY*, and *tet* (see Fig. 3C) was cloned by ligating *Pst*I-digested chromosomal DNA from transposon-generated mutant MR1101 with *Pst*I-digested vector pACYC177 and then using this mixture to transform *E. coli* DH5α [F$^-$ *endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 recA1 gyrA96 relA1* Δ(*argF-lacZYA*)U169 ϕ80d*lacZ*ΔM15 λ$^-$]. Restricted vector DNA was treated with calf intestine phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) according to the instructions of the manufacturer to prevent vector self-ligation, and transformation was done by the method of Hanahan (12). Selection for recombinants was on L-agar medium containing 10 μg of tetracycline per ml and 50 μg of kanamycin per ml. One plasmid, pRS101, which contained the nub of the regulatory locus, was used to obtain a subclone that was devoid of most of the transposon sequences. This plasmid, pMR102, contained the sequences from the right *Hin*dIII site in the transposon in strain MR1101 to the *Hin*dIII site situated at the right (downstream) of the regulatory locus (see Fig. 3B). This recombinant was obtained by analyzing the products of ligating a *Hin*dIII digest of pRS101 with a *Hin*dIII digest of vector pACYC184. Subclone pMR102 was subsequently used in Southern blot analysis to map the locations of transposon insertions.

The *lux* DNA encoding the enzymes for bioluminescence (region I operon) was also cloned, but this did not require the use of the transposon drug resistance marker because recombinants with the *luxCDABE* operon are weakly luminescent and can be recognized by inspection of recombinant libraries in a darkroom. One such recombinant, plasmid pMR2713, was isolated from a library containing *V. harveyi* DNA in cosmid pMMB33 (10). Cosmid vector arms were ligated with donor DNA by the method of Franklin (9). Donor DNA consisted of *Sau*3A partially digested *V. harveyi* DNA in the size range of 25 to 35 kb. In vitro packaging of 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 the packaged cosmid preparation and plating the bacteria onto L-agar plates containing kanamycin. Ligations, restrictions, and general recombinant DNA procedures were performed by the methods of Maniatis et al. (16). Restriction endonucleases and T4 DNA ligase were purchased from Boehringer Mannheim Biochemicals and were used according to the instructions of the supplier.

DNA and RNA hybridizations. Southern and colony hybridizations were performed as described by Maniatis et al. (16) with the following specifications. Hybridizations and washes were done at 65°C, washes were done in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate, and tRNA (type X; Sigma) was substituted for salmon sperm DNA (0.1 unit per ml of hybridization solution). DNA probes were labeled by nick translation with [α-^{32}P]dCTP and DNA polymerase I from Dupont, NEN Research Products (Boston, Mass.) according to instructions in a technical bulletin from the manufacturer (20 μCi of labeled dCTP per 0.25 μg of DNA). To perform Northern blot analysis, exponentially growing cultures of *V. harveyi* in LM medium were harvested at an OD_{600} of 0.1 (for preinduction cells) or an OD_{600} of 1.0 (for postinduction cells). Cultures were rapidly chilled by mixing them with an equal volume of a frozen solution consisting of 25% sucrose, 20 mM sodium azide, and 50 mM Tris hydrochloride (pH 8.0) with 400 μg of chloramphenicol per ml. The cells were pelleted and suspended in the remaining buffer (~0.5 ml) and placed in an RNase-free, 125-ml Erlenmeyer flask on ice. Total RNA was then isolated by a modification of the procedure of Aiba et al. (1). Five milliliters of lysis solution (20 mM sodium acetate [pH 5.5], 0.5% sodium dodecyl sulfate, 1 mM EDTA) was added to the cells, which lysed immediately. Five milliliters of hot phenol (65°C; 30 ml of 20 mM sodium acetate [pH 5.5] to 100 ml of redistilled phenol and 0.1% 8-hydroxyquinoline) was added, and the solution was swirled gently for 5 min at 65°C. The phases were separated by centrifugation, and the aqueous phase was reextracted twice more with hot phenol.

The RNA was then precipitated with three volumes of 100% ethanol at −70°C for 30 min and pelleted by centrifugation. The pellet was suspended in 500 μl of 40 mM Tris hydrochloride (pH 8.0), 10 mM NaCl, and 6 mM $MgCl_2$. One unit of RNase-free DNase (Promega Biotec, Madison, Wis.) was added, and the solution was then incubated for 20 min at 37°C. DNase was removed by three extracts with an equal volume of phenol-chloroform-isoamyl alcohol (50:24:1) at room temperature followed by one extraction with $CHCl_3$. A 0.1 volume of 3 M sodium acetate (pH 4.8) was added to the aqueous phase, and RNA was precipitated with two volumes of ethanol. The precipitate was then suspended in 500 μl of 0.2 mM EDTA. The OD_{260} was determined for the solution. Two volumes of 100% ethanol were then added, and the RNA was stored in precipitated form at −70°C. RNA (10 μg) was denatured with formaldehyde and formamide and fractionated in 0.8% agarose gels containing 0.66 M formaldehyde (25). Transfer of RNA to a membrane (pore size, 0.45 μm; Nytran; Schleicher & Schuell, Inc., Keene, N.H.) and hybridization of Northern (RNA) blots with nick-translated probes was done as described above for Southern blots.

RESULTS

Transposon mutagenesis and mapping. Transposon insertion mutants were used to define the loci-encoding functions required for luminescence in *V. harveyi*. A library of mutants was generated by mobilizing transposon mini-Mu*lac*(Tet^r) from *E. coli* into *V. harveyi* with transducing phage P1 and then selecting transductants with transposon insertions by plating P1-infected bacteria onto agar containing tetracycline (see above). Mutants with transposon insertions in the genes for bioluminescence, *lux*, were isolated by screening the library of tetracycline-resistant strains in a darkroom for those with a nonluminous, Lux$^-$ phenotype. Fifty-four Lux$^-$ mutants were found in a collection of approximately 30,000 transposon insertion mutants.

Because transposon insertion resulted in a large perturbation of target gene sequence arrangement, the location of the transposon insertions in each Lux$^-$ mutant could be mapped by Southern blot analysis. Specifically, genomic DNA from each mutant was cleaved with one or a combination of restriction enzymes, the resulting mixture of restriction fragments was fractionated on an agarose gel and the sepa-

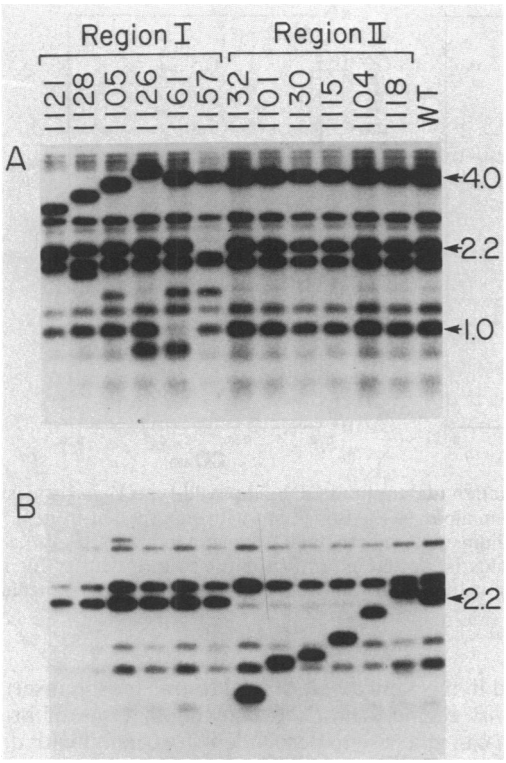

FIG. 2. Southern blot analysis of genomic DNA from Lux transposon insertion mutants. Blots containing *Hin*dIII-digested DNA from wild-type *V. harveyi* and transposon mini-Mu*lac*(Tet^r)-generated insertion mutants (MR mutant designations given above the lanes) were hybridized to a ^{32}P-labeled probe for *lux* region I (A) or *lux* region II (B). Restriction fragments containing *lux* DNA are marked by arrows on the right, with sizes given on the right in kilobases.

rated fragments were transferred to a Nytran membrane, and the blots were then hybridized to radioactive *lux* DNA probes. Recombinant clones containing part or all of the locus encoding enzymes for bioluminescence (the *luxCDABE* operon) have been isolated by several laboratories (17), so hybridization probes for mapping transposon insertion mutations in this locus are readily available. We chose to use our own collection of overlapping cosmid clones containing the *luxCDABE* region (see above). Hybridization of ^{32}P-labeled cosmid pMR2713 to genomic digests of various Lux$^-$ mutants is shown in Fig. 2A. Two classes of mutants were observed: those which contained transposon insertions in the *luxCDABE* operon, which is called *lux* region I (left lanes in Fig. 2A), and those in which the transposon insertion did not map to this region (right lanes in Fig. 2A). Perturbation of one of the three *Hin*dIII restriction fragments containing the parts of the *luxCDABE* operon is evident in each transposon mutant with an insertion in *lux* region I.

All but 9 of the 54 Lux$^-$ mutants had insertions which mapped to *lux* region I. The orientation and location of the transposon insertion in each mutant in this group was mapped by analysis of Southern blots containing genomic DNA digested with *Hin*dIII, *Eco*RI, and *Hin*dIII-*Eco*RI. The result of mapping representative insertion mutations is shown in Fig. 3A. The correspondence of the restriction map and the *luxCDABE* operon is also shown. Alignment of the restriction map with the gene map is based on data from our laboratory (2) and from the laboratory of Meighen and colleagues (19, 20, 22). They have sequenced some of the *lux* genes in region I. The closed circles above the map in Fig. 3A indicate that the transcriptional orientation of the *lacZ* gene of the transposon is from left to right, and open circles indicate an orientation from right to left. Light production could be restored in strains with transposon insertions in *lux*

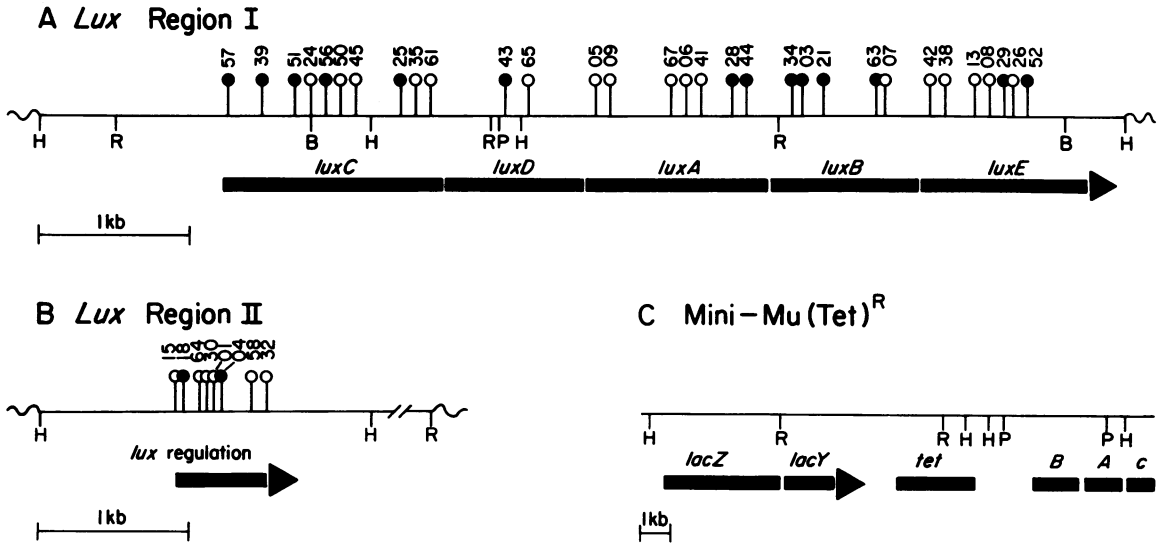

FIG. 3. Mapping transposon insertion mutations in *lux* DNA of *V. harveyi*. Southern blot analysis such as that shown in Fig. 2 was used to determine the position and orientation of insertion of transposon mini-Mu*lac*(Tet^r) mutations in *lux* region I (A) and *lux* region II (B). The transposon insertion in strain MR1146 was in *lux* region II, but the location is not shown here because the presence of a transposon-induced deletion prevented precise mapping of the defect. The symbols above the restriction maps represent the positions of transposon insertions in various Lux$^-$ mutants (only the last two numbers of the mutant designation are shown). Transposon mini-Mu*lac*(Tet^r) (C) was inserted in one of two orientations: transcription of *lacZ* oriented from left to right (●) or transcription of *lacZ* oriented from right to left (○). The location of coding regions (heavy lines) and the direction of transcription (arrows) of the various *lux* loci are shown below the restriction map. Restriction endonuclease sites are abbreviated as follows: H, *Hin*dIII; B, *Bam*HI; R, *Eco*RI; P, *Pst*I. Transposon insertions in *lux* region II were mapped relative to an *Eco*RI site 1.6 kb to the right of the locus (B).

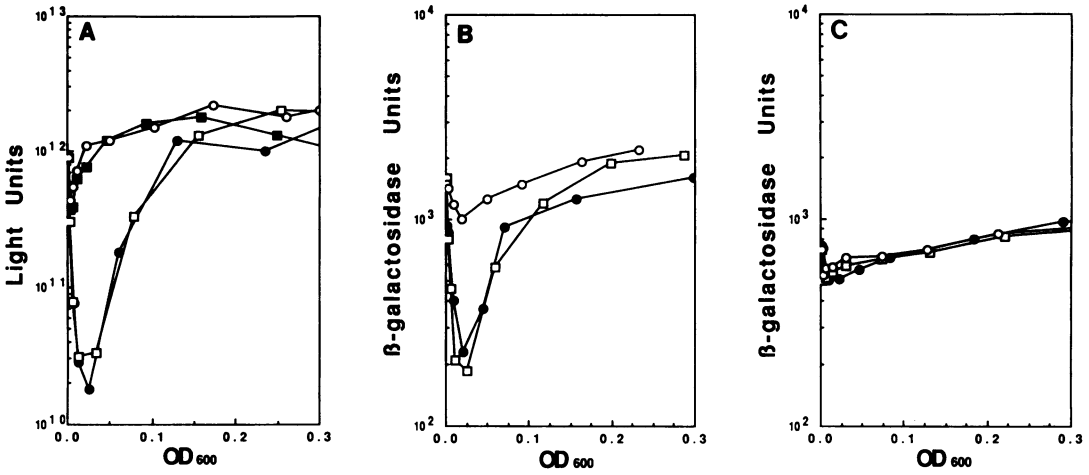

FIG. 4. Relationship of culture density to *lux* expression. Specific light production (light output/OD_{600}) by wild-type *V. harveyi* is plotted as a function of culture density (OD_{600}) (A). The culture medium was: AB medium alone (●), AB medium with supernatant from *V. fischeri* (□), AB medium with supernatant from induced *V. harveyi* (○), or AB medium with supernatant from mutant MR1104 (■). Specific β-galactosidase activity (in Miller [18] units) produced by *lux*::*lacZ* fusion strains is plotted as a function of culture density for mutants MR1144 (B) and MR1104 (C). Growth was in AB medium alone (●), AB medium with supernatant from *V. fischeri* (□), or AB medium with supernatant from *V. harveyi* (○).

region I by introducing the *luxCDABE* operon in *trans* on recombinant cosmids such as pMR2713 (data not shown). These cosmids were derived from the broad-host-range vector pMMB33 (10), which can be mobilized into *V. harveyi* by conjugation with donor *E. coli* in a triparental mating. No complementation was observed when the *luxCDABE* operon was transferred to strains with transposon-generated defects which mapped outside *lux* region I. Our collection of overlapping cosmids with the *luxCDABE* operon extended about 10 kb in either direction from this locus. Recombinant cosmids representing the extremities of this region were used as probes in Southern blots, like those shown in Fig. 2A, and no alteration in the restriction pattern of DNA from the nine mutants with unlinked insertions was observed. Therefore, we concluded that the insertions in these mutants are at least 10 kb from the *luxCDABE* operon and are therefore not closely linked to this operon.

A new probe had to be isolated to map the transposon insertions in the remaining nine mutants. The transposon-encoded drug resistance gene, *tet*, is physically linked to the target *lux* gene sequences in the mutants, so DNA from the *lux* locus in this mutants 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 with *Pst*I would yield a fragment with both *tet* and the *lux* sequences flanking one side of the site of insertion (Fig. 3C). Plasmid pMR102 (see above) contained the *lux* DNA adjacent to the site of transposon insertion in Lux^- strain MR1101. This DNA was used to probe Southern blots prepared from the Lux^- mutants, and the result obtained with some representative mutants is shown in Fig. 2B. All the mutants, including those shown in Fig. 2B, containing transposon insertions outside the *luxCDABE* locus (region I) were linked to each other because all of these contained an insertion which perturbed the arrangement of DNA sequences homologous to the probe pMR102.

These mutants defined a new locus which we called *lux* region II. The orientation and location of the transposon insertions in mutants with region II defects are shown in Fig. 3B. The extent of the target defined by these mutations was approximately 0.6 kb. The defects in all the Lux^- mutants isolated in this study were traced to transposon insertions in either *lux* region I or II. Clonal populations of bright *V. harveyi* can give rise to descendents (variants) with different luminous phenotypes ranging from extra bright to dim to dark. This genetic instability, called luminescence variation (14, 28), is particularly pronounced when bacteria are propagated from very old cultures, but we were concerned that *lux* defects would be generated by this process and could be confused with those resulting from transposon mutagenesis. Fortunately, it is apparent that variation was not the source of the mutations in our Lux^- strains. Finally, hybridization of the pMR102 probe to fragments of the *V. harveyi* genome (Fig. 2B) yielded an unexpected but possibly significant result. Plasmid pMR102 contained about 1 kb of the 2.2-kb *Hin*dIII fragment with *lux* region II (Fib. 3B), but hybridization to several other fragments was detected. This extra homology could indicate the presence of an additional *lux* locus.

A mutant of *V. harveyi* which requires exogenous addition of adenosine 3′,5′-monophosphate (cyclic AMP) for the production of light has been isolated (29). Mutants with defects in *lux* region II did not have the characteristics of catabolite repression mutants. The addition of cyclic AMP did not stimulate light production by region II mutants; and these mutants, like the wild-type parent, fermented glucose, mannitol, mannose, maltose, and cellobiose; were sensitive to phosphomycin; and synthesized functional flagella (data not shown). Catabolite repression mutants appeared to be absent in our collection of Lux^- mutants, possibly because null, transposon-generated *cya* or *crp* mutations could result in severe disturbance of the growth of such mutants.

Production of autoinducer. Light production by *V. harveyi* is strongly influenced by the density of the culture, and regulation by culture density (see above) is thought to result from the accumulation of a substance, called autoinducer, which is produced by the bacteria and which controls expression of the luminescence phenotype. The density-dependent production of light by *V. harveyi* is illustrated in Fig. 4A, which shows specific light production (total light output divided by OD_{600}) as a function of culture density (OD_{600}). The experiment was started by diluting a culture of

luminescent, postinduction bacteria in the exponential stage of growth into fresh medium. The inoculum of postinduction bacteria was obtained from a culture of *V. harveyi* grown in AB medium to an OD_{600} of 0.1. The initial luminescence was high because the cells originated from an induced culture, but specific light production decreased as the cells divided since no synthesis of luminescence enzymes was occurring. Upon reaching a particular density (and accumulation of an effective concentration of autoinducer), light production then increased rapidly because of induction of luminescence enzyme synthesis.

The initial decrease in light production did not occur if exogenous autoinducer was provided by adding sterile supernatant from a dense, induced culture of *V. harveyi* to the dilute suspension of cells. Specific light production of diluted *V. harveyi* was maintained if the growth medium was supplemented with a 30% volume of supernatant from a culture of postinduction *V. harveyi* grown in AB medium to an OD_{600} of 0.4 or from mutant strain MR1104 containing a transposon insertion in *lux* region II (Fig. 4A). Supernatants from other mutants with defects in *lux* region I or II also induced light production in a dilute suspension of *V. harveyi* (data not shown). The autoinducer from *V. fischeri* does not induce luminescence in *V. harveyi* (11), and the supernatant from a culture of this bacterium did not stimulate expression of luminescence in the experiment for which the results are shown in Fig. 4A. We conclude from such measurements that mutants with transposon insertions in either *lux* region I or II are not defective in the production of autoinducer.

Expression of gene fusions. Insertion of mini-Mu*lac*(Tet^r) can couple target gene transcription to that of the *lacZ* indicator gene of the transposon. The resulting gene fusion strains can be used to determine the direction of transcription of the target gene and to analyze control of expression of the target gene. Only one of the two possible orientations of insertion can align transcription of the *lux* target gene with transcription of *lacZ* located on one end of mini-Mu*lac* (Tet^r). *V. harveyi* is naturally Lac$^-$, so fusion strains with a Lac$^+$ phenotype (as judged by β-galactosidase cleavage of the chromogenic substrates 5-bromo-4-chloro-3-indolyl-β-D-galactosidase for an in vivo assay or *o*-nitrophenyl-β-D-galactopyranoside for an in vitro assay) have *lacZ* aligned with *lux* transcription. Transposon insertions in which the direction of transcription of *lacZ* is oriented from left to right (closed circles in Fig. 3A and B) resulted in a Lac$^+$ fusion strain, so the direction of transcription of the *lux* genes in regions I and II must also be from left to right relative to those of the maps shown in Fig. 3.

Strains with *lux*::*lacZ* fusions were used to measure the influence of culture density on expression of the *luxCDABE* operon (region I) and of the transcriptional unit located in *lux* region II. Production of β-galactosidase by strains with *lacZ* fused to each of the genes in the *luxCDABE* operon was similar to the production of light by wild-type *V. harveyi*. For example, compare Fig. 4A with Fig. 4B, which described strain MR1144 containing a *luxA*::*lacZ* fusion. At a low cell density, specific β-galactosidase activity from strain MR1144 decreased until induction of *lacZ* expression commenced at an OD_{600} of 0.025. Then, β-galactosidase activity increased rapidly until a plateau value was reached. Expression of *lacZ* was sustained at a high value in the fusion strain when growth supernatant from wild-type *V. harveyi* was added to a dilute suspension of strain MR1144 but not when supernatant from *V. fischeri* was added (Fig. 4B). The activity of β-galactosidase in strains with *lacZ* fused to the transcriptional unit in *lux* region II remained relatively constant, irrespective of the density of the culture (see strain MR1104 in Fig. 4C). The addition of supernatant from wild-type *V. harveyi* did not influence expression of the *lacZ* gene in this fusion strain, in contrast to the response to growth supernatant observed with strain MR1144 (Fig. 4B).

The influence of cell density on expression of the indicator *lacZ* gene is pronounced in strain MR1144 containing a fusion to the region I operon. Furthermore, the response to the addition of supernatant was observed only with strains such as MR1144 in which *lacZ* was coupled to the region I operon. We conclude that density-dependent control mediated by autoinducer acts as the level of transcription of the *luxCDABE* operon. The function encoded by region II could be necessary for the density-dependent response observed for the *luxCDABE* operon (see below), and it could also participate in the control of its own expression (autoregulation). The possibility that cell density could influence (in a positive or negative sense) the expression of the region II locus and that this region is itself a participant in density-dependent control was not tested by these gene fusion experiments, since mini-Mu*lac*(Tet^r) insertion inactivated the target gene and a nondefective copy of the locus was not provided in *trans*.

***lux* gene transcripts.** Recombinants with DNA homologous to the *luxCDABE* operon in region I and to the *lux* region II locus were used as probes in Northern blot analysis to measure production of *lux* gene transcripts. Plasmid pMM100 containing the *Bam*HI *lux* gene fragment shown in Fig. 3A was used to detect the region I transcript, and plasmid pMR102 (see above) was used to detect the region II transcript. RNA was isolated from wild-type *V. harveyi* grown in LM medium at a preinduction stage (OD_{600} = 0.1) and at the postinduction stage (OD_{600} = 1.0) and also from two strains, MR1101 and MR1104, with transposon insertions in region II; the last two strains were detected at the postinduction culture density. LM medium was used to cultivate *V. harveyi* for the isolation of RNA because induction of luminescence occurred at a higher cell density in LM medium, so much higher yields of preinduction-stage bacteria could be obtained. Delayed induction of luminescence in complex medium such as LM is thought to be caused by the presence of an inhibitor of autoinducer activity (11).

A transcript of about 8 kb, as well as several smaller but discrete transcripts, were detected by the *luxCDABE* probe with RNA isolated from postinduction-stage wild-type bacteria (Fig. 5A). A similar pattern of polycistronic mRNA synthesis has been reported by other investigators (21). This transcript was much less abundant in RNA from preinduction-stage cells, and it was not detected in RNA from the two strains with region II defects. Strips from the blots corresponding to the position of the largest *lux* transcript were excised, and the radioactivity was measured in a scintillation counter. Based on this comparison, the *luxCDABE* transcript was at least 75-fold more abundant in a postinduction-stage culture than in a preinduction-stage culture. When ^{32}P-labeled plasmid pMR102 DNA was hybridized to similar blots to measure RNA transcribed from *lux* region II, a transcript of approximately 1 kb was observed. It was moderately more abundant (by three- to fourfold) in postinduction-stage cells than in preinduction-stage cells and was absent in the strains with *lux* region II defects.

DISCUSSION

Transposon mini-Mu*lac*(Tet^r) insertion mutations defined two loci, *lux* regions I and II, that are necessary for biolu-

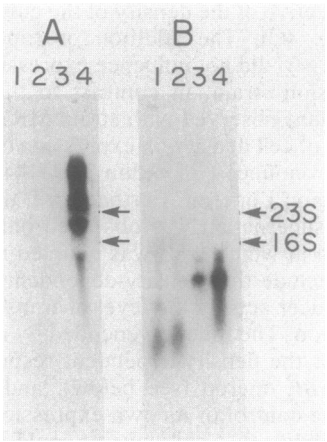

FIG. 5. Detection of *lux* transcripts. Production of *lux* mRNA encoded by *lux* region I (A) and *lux* region II (B) was measured by Northern blot analysis. Sources of RNA were postinduction-stage cells of mutant MR1104 (lanes 1); postinduction-stage cells of mutant MR1101 (lanes 2), preinduction-stage cells of wild-type *V. harveyi* (lanes 3), and postinduction-stage cells of wild-type *V. harveyi* (lanes 4). Hybridization probes are described in the text. The positions of rRNAs in the blot are shown on the right.

minescence in *V. harveyi*. DNA hybridization experiments showed that *lux* region I contains the *luxCDABE* operon, which encodes the luminescence enzymes luciferase and fatty acid reductase. The coding region delimited by transposon insertions in this locus was not of a sufficient size to accommodate additional *lux* genes; this conclusion is in agreement with other results (see above) suggesting that *lux* genes encoding putative regulatory functions are not linked to the *luxCDABE* operon. Production of β-galactosidase in strains with transposon insertions coupling the *lacZ* indicator gene to the *lux* genes in region I was affected by culture density and responded to culture supernatant from strains which synthesized autoinducer activity. This finding and the measurement of *lux* transcripts in Northern blot analysis indicated that transcription of the *luxCDABE* operon is subject to density-dependent control. We can also infer that none of the *lux* genes in region I is required for density-dependent regulation because gene fusion mutants with *lacZ* aligned with transcription of each of the region I *lux* genes were Lac^+ and exhibited density-dependent control. In addition, no mutant with a defect in region I was defective in the synthesis of autoinducer activity.

Regulatory function was encoded by *lux* region II. No mRNA transcribed from the *luxCDABE* operon (region I) was detected in mutants with defects in *lux* region II, so region II is required for expression of other *lux* genes. Mutants with transposon insertions in *lux* region II produced autoinducer activity and are, therefore, defective in a different component of the density-sensing mechanism. We hypothesize that region II encodes a regulatory protein which activates transcription of the *luxCDABE* operon in response to autoinducer. Transposon insertions in *lux* region II spanned a mutational target of 0.6 kb, but this represented a minimum size. The size of the *lux* transcript, which was approximately 1 kb and which could encode a protein with a molecular weight of 37,000, might give a more accurate estimate of the coding capacity of region II. It is important to note that definition of genes by complementation analysis, which requires the cloning of the entire region II locus and positioning of pairs of nonpolar point mutations in *trans*, has not been applied to this locus, so the number of genes here is not known.

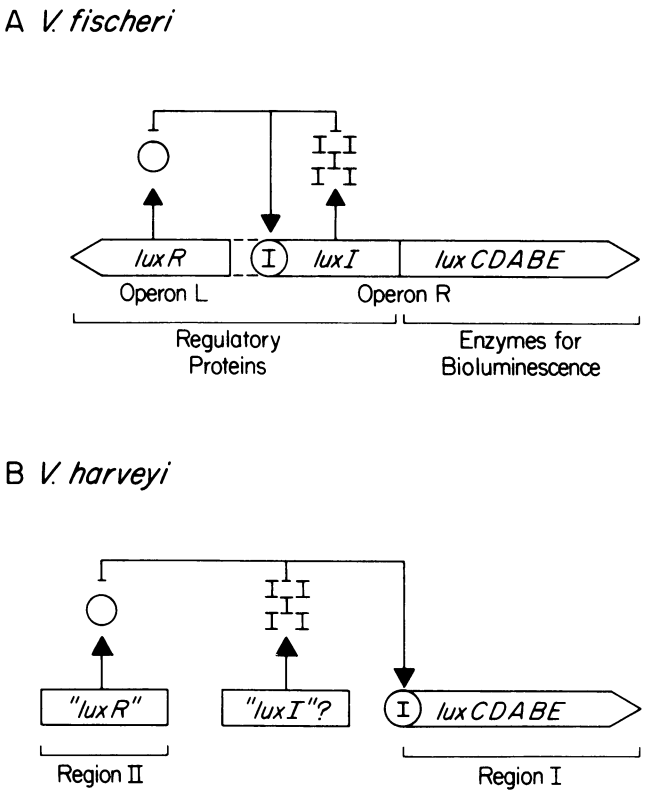

FIG. 6. Comparison of the *lux* systems of *V. fischeri* (A) and *V. harveyi* (B).

β-Galactosidase activity in mutants with *lacZ* coupled to the region II transcriptional unit was not dependent on the density of the culture and was not influenced by the addition of growth supernatant from an autoinducer donor strain. However, a three- to fourfold increase in the *lux* region II transcript was detected in postinduction-stage *V. harveyi*. This increase in the amount of region II transcript in postinduction-stage bacteria was minor compared with that observed for the *luxCDABE* transcript, but the observation suggests that density-dependent control also influences transcription of *lux* region II. The absence of a comparable effect on expression of the *lux*::*lacZ* fusion strain is consistent with involvement of the target gene itself, which was inactivated by transposon insertion, in the regulatory mechanism.

The organization of *lux* genes in *V. harveyi* is substantially different from that in *V. fischeri* (Fig. 6). The arrangement of genes encoding the luminescence enzymes was remarkably similar in both species, but this similarity did not extend to the genes encoding regulatory functions. In *V. fischeri* (Fig. 6A), regulatory control is directed by the *luxI* and the *luxR* genes (5, 6). Synthesis of the *V. fischeri* autoinducer is accomplished by the *luxI* product, and transcription of the operon encoding the luminescence enzymes is activated by the *luxR* product, probably as a result of the binding autoinducer. The *luxI* and *luxR* genes are arranged in a positive-feedback circuit such that induction of transcription results in self-amplification and an exponential increase in the synthesis of luminescence enzymes and, consequently, of light emission. Other regulatory relationships influencing *lux* expression have also been identified (7, 8, 28).

In *V. harveyi* (Fig. 6B) regulatory function is not encoded

by genes that are closely linked to the cluster of genes encoding the luminescence enzymes, the *luxCDABE* operon. The regulatory locus, *lux* region II, identified for the first time in this study is not linked to the *luxCDABE* operon, but it is necessary for transcription of this operon. We suggest that *lux* region II encodes a positive regulatory protein which activates transcription of the *luxCDABE* operon in response to the presence of autoinducer, so the function of this locus resembles that of the *luxR* gene of *V. fischeri*. Mutants of *V. fischeri* defective in *luxI* are dark and produce no autoinducer activity, but expression of luminescence in these mutants can be restored if exogenous autoinducer is provided. No mutant of *V. harveyi* with this phenotype was isolated, so we have no compelling evidence indicating the presence of a gene of *V. harveyi* analogous in function to the *luxI* gene of *V. fischeri*.

Lux^- mutants defective in autoinducer production could have been overlooked in the screening of mutants, because diffusion of autoinducer from neighboring colonies could have restored the luminescence phenotype. We picked thousands of strains with transposon insertions into the wells of microtiter dishes containing agar medium to avoid such cross-feeding and did not detect any Lux^- mutants which could be stimulated to luminescence with the growth supernatant of *V. harveyi*. Alternatively, a single mutational event might not give a mutant phenotype if autoinducer is synthesized by redundant or independent pathways or if autoinducer activity is composed of several chemical entities with different origins. On the other hand, the putative function required for autoinducer synthesis could be essential for cell viability. As mentioned above, *V. harveyi* and *V. fischeri* differ with regard to the nature of their authoinducer activities. In contrast to *V. fischeri*, luminescence of *V. harveyi* is induced by supernatants from other *Vibrio* species, including some which are not luminous (11). Considering these differences, autoinducer synthesis in *V. harveyi* might not be similar to the process in *V. fischeri*.

The entire *lux* region II locus will be cloned into an *E. coli* isolate that already contains the *luxCDABE* operon in an attempt to reconstitute density-dependent regulation in a recombinant host. If this can be achieved, genetic dissection of the *lux* system can proceed at an accelerated pace with the extensive technology applicable to *E. coli*. Involvement of additional genetic information, such as a gene or genes for autoinducer synthesis, could be required. Evidence that additional DNA homologous to *lux* region II is present in *V. harveyi* was noted (the Southern blot in Fig. 2B). We are not certain of the significance of this finding, but this homology could be an important clue in our search to identify all the regulatory relationships and elements of the *lux* system of *V. harveyi*.

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