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Use of Regulated Cell Lysis in a Lethal Genetic Selection in *Escherichia coli*: Identification of the Autoinducer-Binding Region of the LuxR Protein from *Vibrio fischeri* ATCC 7744

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A lethal genetic selection utilizing the bacteriophage λ lysis genes (*S*, *R*, R_Z) has been developed and used in conjunction with a luminescence screen to allow the isolation and characterization of six missense mutations and two nonsense mutations in the *luxR* gene from *Vibrio fischeri* ATCC 7744. A transcriptional fusion of the lysis genes in operon$_R$ downstream of a truncated *luxI* gene allows control of cell lysis by the addition of synthetic autoinducer to the growth medium. The six missense mutations isolated resulted in changes in the LuxR protein of Asp at position 79 to Asn (hereafter designated as D79N), V82I, V109L, L118F, S123I, and H217Y. Variant LuxR proteins with amino acid changes of D79N, V82I, V82L, and H127Y were shown to require higher concentrations of autoinducer to elicit a certain amplitude response than is required by the wild-type protein. We believe that the clustering of a total of seven randomly generated missense mutations in a 49-amino-acid region of the LuxR primary sequence defines a critical portion of the LuxR protein. The observation that proteins with lesions in this region responded to elevated levels of autoinducer suggests that the autoinducer-binding site is constructed, at least in part, from several amino acid residues within the 79-to-127 region of the LuxR protein.

The regulation of bioluminescence in the marine bacterium *Vibrio fischeri* has been studied extensively through cloning and genetic manipulation of the *lux* system in *Escherichia coli* (3–5, 8–10). Expression of the *lux* genes in *V. fischeri* is controlled by a unique form of positive feedback regulation called autoinduction, and this pattern of regulation is duplicated by the cloned system in *E. coli* (8, 10). The autoinduction response is mediated by the production and accumulation of a small molecule, autoinducer, which is synthesized in the presence of the *luxI* gene product. This molecule presumably interacts with the *luxR* gene product to induce the synthesis of the enzymes required for light production. The autoinducer of *V. fischeri* has been identified as *N*-(3-oxo-hexanoyl)homoserine lactone (7) and shown to be both freely diffusible across the cytoplasmic membrane and species specific in its ability to stimulate bioluminescence (6, 17). This molecule has been synthesized in vitro and shown to function in a biological assay (7, 16).

The *lux* genes are organized into two divergently transcribed operons, termed rightward and leftward, which are separated by a common regulatory region (2, 8, 11). The *luxR* gene is the only known gene in the leftward operon (operon$_L$) and encodes a positive regulatory protein which, in the presence of autoinducer, stimulates transcription of the rightward operon (operon$_R$). This interaction has recently been shown to require the 20-base-pair *lux* operator located in the control region (3). Operon$_R$ consists of at least six genes (*luxICDABE*); the *luxI* gene encodes a protein required for autoinducer synthesis (9), the *luxC*, *luxD*, and *luxE* genes encode enzymes which provide luciferase with an aldehyde substrate (20), and the *luxA* and *luxB* genes encode the α and β subunits of the luciferase enzyme.

The current model describing the autoinduction process suggests that a low basal level of transcription of operon$_R$ leads to low-level synthesis of autoinducer by LuxI. High cell density is required for autoinducer to accumulate, since it is freely diffusible across the cytoplasmic membrane. It is by virtue of the diffusible nature of autoinducer that the expression of luminescence is cell density dependent. If the LuxR protein, whose synthesis is regulated at the transcriptional level by the cyclic AMP-catabolite gene activator protein (cAMP-CAP) system (4, 5), has also accumulated, it can form a complex with autoinducer capable of binding to the *lux* operator and stimulating transcription of operon$_R$. Positive feedback results from the placement of *luxI* in operon$_R$, since stimulation of rightward transcription by LuxR and autoinducer leads to the production of more autoinducer by increased levels of LuxI protein. This positive feedback mechanism leads to the sharp induction of the enzymes required for light production. In addition to this primary regulatory circuit, several global regulatory systems in *E. coli* have been shown to interact with the *lux* system to affect the timing of induction of bioluminescence. These systems include the heat shock (σ^{32}) system and the SOS response (24, 25). It remains unclear how these systems interact with the components of the *lux* system to influence the timing of induction.

In this paper, we describe the construction of a plasmid vector which retains an intact *luxR* gene and regulatory region but lacks all of the genes in the operon$_R$, retaining only a truncated *luxI* gene. This arrangement affords a system in which operon$_R$ transcription is controlled by the addition of synthetic autoinducer but without the positive-feedback mechanism. A potentially lethal transcriptional fusion of the lysis genes (*S*, *R*, R_Z) from bacteriophage λ was created in operon$_R$ by insertion downstream of the truncated *luxI* gene. This vector (pGS102) was used in a lethal genetic selection in conjunction with a subsequent luminescence screen to recover point mutations in the *luxR* gene.

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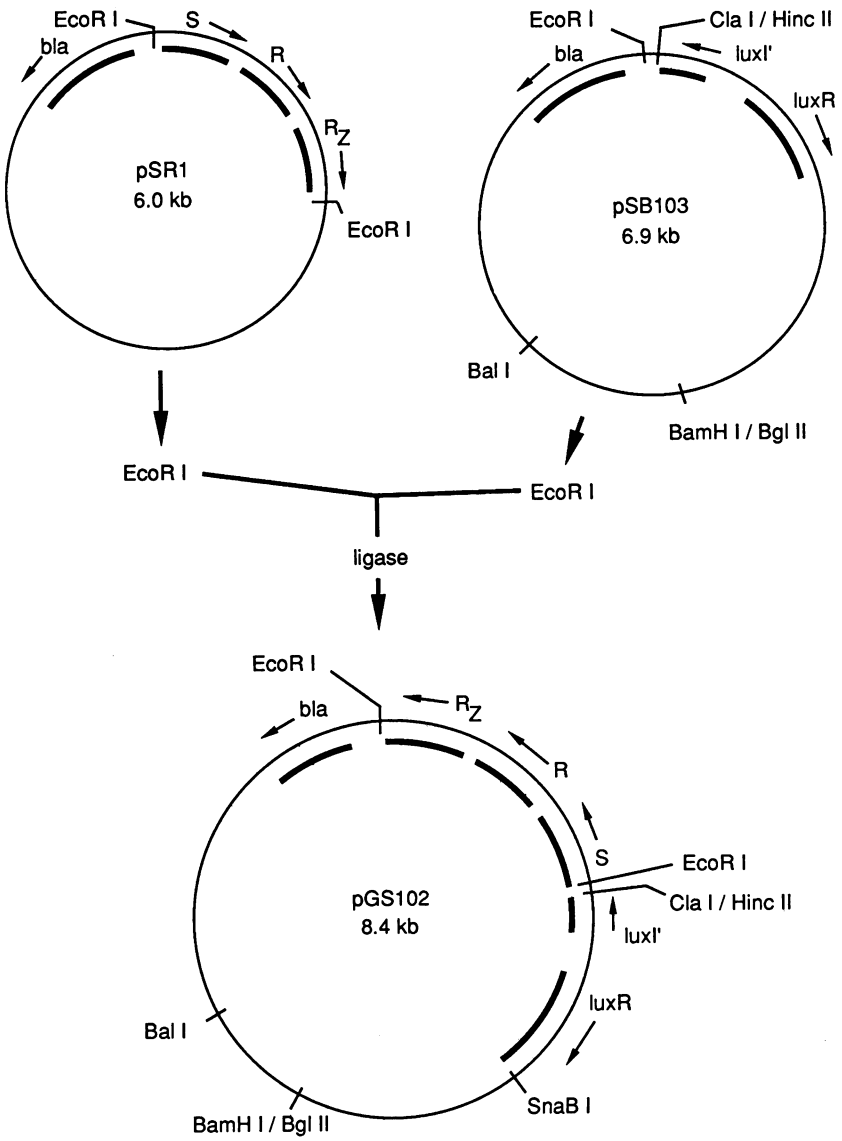

FIG. 1. Construction of the lysis vector pGS102. Small arrows indicate the 5'-to-3' direction of the gene indicated, and large arrows indicate cloning steps taken during the construction of the pGS102 plasmid. kb, Kilobases.

MATERIALS AND METHODS

Enzymes and chemicals. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs, Inc., or Boehringer Mannheim Biochemicals. Klenow fragment of *E. coli* DNA polymerase I and modified T7 DNA polymerase (Sequenase) were purchased from United States Biochemicals. ATP and deoxyribonucleotides were obtained from Pharmacia LKB. Radiolabeled dATP was obtained from Dupont, NEN Research Products. Hydroxylamine hydrochloride and *n*-decanal were obtained from Sigma Chemical Co. All other chemicals were of the highest quality commercially available.

Cell strains. All growth experiments were performed using *E. coli* TB1 [*hsdR* Δ(*lac pro*)]. *E. coli* CA8404 (*crp**; a generous gift of Pete Greenberg) was used to achieve better complementation with *luxR* in *trans*. This strain produces a mutant CAP which does not require cAMP to activate transcription from cAMP-CAP-regulated promoters (21a).

Plasmid constructions. Construction of the lysis vector pGS102 is summarized in Fig. 1. pGS102 was constructed from the plasmid pSB101, which contains a *Bgl*II restriction fragment harboring the bioluminescence genes *luxR*, *-I*, *-C*, and *-D'* cloned into the the *Bam*HI site of pBR322 with the transcription of operon$_R$ oriented opposite that of the *tet* gene. To eliminate production of the autoinducer via the *luxI* gene product, pSB101 was digested with *Cla*I, filled with Klenow fragment, partially digested with *Hinc*II, and ligated to yield plasmid pSB103 containing a truncated *luxI* gene fused at the *Cla*I site of pSB101. The lysis genes of bacteriophage λ (*S*, *R*, and R_Z) were isolated on a 1.5-kilobase *Eco*RI restriction fragment from plasmid pSR1, which is a derivative of plasmid pRG1 (21) with the unique *Hin*dIII site converted to an *Eco*RI site by using synthetic adapters. This *Eco*RI fragment was subsequently ligated into the *Eco*RI site of pSB103 to yield pGS102. Restriction analysis was used to screen for the proper orientation of the lysis genes in pGS102, which consisted of a transcriptional fusion between operon$_R$ of the *V. fischeri lux* genes and the λ lysis genes,

which were now located downstream of the truncated *luxI* gene.

A *Sna*BI-*Bal*I deletion of pGS102 was constructed to remove *V. fischeri* DNA that was downstream of the *luxR* gene in the operon$_L$. This construction was called pGS103 and exhibited the same lysis phenotype as pGS102.

The construction of plasmid pJHD500 has been described earlier (3). This plasmid is similar to pGS102, except the *luxA* and *luxB* genes, encoding the subunits for the luciferase enzyme from *Vibrio harveyi*, were cloned downstream of the truncated *luxI* gene, creating a bioluminescent transcriptional reporter for operon$_R$. DNA containing mutations in the *luxR* gene generated in pGS102 or pGS103 was subcloned into pJHD500 in order to quantitate the ability of the mutant LuxR proteins to stimulate rightward transcription.

Growth of cultures and measurement of bioluminescence in vivo. The ability of the mutant LuxR proteins to respond to autoinducer was determined by monitoring cell growth and aldehyde-stimulatable luminescence of samples withdrawn from cultures grown in 50-ml culture flasks containing (initially) 12 ml of Luria-Bertani (LB) medium with carbenicillin (100 μg/ml) at 30°C in a New Brunswick model G76 gyratory water bath shaker at 200 rpm. Inoculation was from overnight cultures grown at 30°C and diluted 1/200 (vol/vol). Samples (1 ml) were removed at various times during growth. Cell density was determined as the optical density at 600 nm (OD_{600}) by using a Milton Roy Spectronic 601 spectrophotometer. The same samples were then used for luminescence measurements in vivo by transferring the sample into a 20-ml scintillation vial, placing the vial in a photometer, and injecting 1 ml of a sonicated solution of *n*-decanal (10 μl of aldehyde per 10 ml of LB medium). Peak light emission was monitored by using a photomultiplier-photometer for which 1 light unit represents 9.8×10^9 quanta/s, based on the liquid light standard of Hastings and Weber (13).

Data depicting cell lysis were collected by monitoring culture growth in 125-ml culture flasks containing 20 ml of LB medium with carbenicillin (100 μg/ml) at 30°C in a New Brunswick Aquatherm water bath with shaking at 100 rpm. Duplicate cultures were inoculated by dilution (1/200 [vol/vol]) of an overnight culture grown at 30°C with an addition of autoinducer to one of the cultures immediately after inoculation. Cell lysis was detected as a drop in the OD_{600} of the culture.

Pure autoinducer was synthesized by the method of Eberhard et al. (7) and was stored as a 50 mM solution in water at 4°C. This stock was further diluted into the growth medium to the desired concentration. The concentration of autoinducer was calculated by using the weight of the dried, purified material and a molecular weight of 213 for autoinducer.

Colonies containing pJHD500 and its *luxR* mutant derivatives were screened for luminescence on solid medium by applying *n*-decanal to the lid of a petri dish and observing the glowing colonies in a dark room.

Mutant selection in liquid medium. A variety of growth conditions were used for lysis selection in liquid medium. Temperatures were varied between 24 and 37°C, and M9 minimal medium supplemented with glycerol (0.2%), proline (40 μg/ml), and thiamine (0.001%) was used as well as LB medium. Autoinducer concentrations were varied between 0.5 and 5 μM. In all experiments, 5-ml cultures of *E. coli* TB1 containing plasmid pGS102, prepared by inoculation from overnight cultures by dilution (1/100) into medium containing carbenicillin (100 μg/ml), were used. Duplicate cultures were incubated with and without autoinducer until cell lysis was observed visually as a loss of the turbidity of the culture. Cultures were then diluted into fresh medium, plated onto solid LB medium, and incubated at 30°C to allow growth of surviving cells. In some cases, the lysed cultures were pelleted, resuspended in fresh autoinducer-containing medium, and taken through a second lysis induction (double-induction experiment).

Hydroxylamine mutagenesis and screening on solid medium. Hydroxylamine mutagenesis was done essentially as described previously (15). Purified pGS103 (2.5 μg) was suspended in 250 μl of a 1 M hydroxylamine solution at pH 6.0. The hydroxylamine solution was prepared by mixing 125 μl each of a 2 M hydroxylamine solution (0.7 g of hydroxylamine hydrochloride dissolved in 0.56 ml of 4 N NaOH and adjusted to 5 ml with sterile water) and a 2× TE solution (200 mM Tris Cl, pH 6.0, 2 mM EDTA). This mutagenesis mixture was incubated at 65°C for 35 min, and the modified DNA was precipitated with 2 volumes of ethanol after the addition of ammonium acetate to 1 M. The pellet was suspended in TE (pH 8.0), and 0.25 μg was used to transform competent *E. coli* TB1. Transformed cells were plated in duplicate onto solid LB medium containing carbenicillin (100 μg/ml) with and without 5 μM autoinducer. Mutant colonies were screened as normal opaque colonies on autoinducer-containing plates against a background of translucent colonies (see Results).

DNA sequencing. Double-stranded plasmid DNA was prepared from overnight cultures by the alkaline lysis method (19). The DNA pellets were then treated with RNase A and precipitated with polyethylene glycol 8000. The purified DNA was then denatured with NaOH and used as template for sequencing by the dideoxy-chain termination method using modified T7 DNA polymerase (Sequenase) (23). Sequencing primers used to sequence *luxR* have been described elsewhere (3).

Site-directed mutagenesis. Site-directed mutagenesis was done by the method of Kunkel et al. (18), with slight variations. Single-stranded uracil-containing DNA isolated from phagemid-infected cells was used as a template for the mutagenesis reactions. Purification of the template was done as described earlier (3), except the starting plasmid was pVFS185, which is a derivative of pTZ18R (Pharmacia) containing a *Sac*I restriction fragment harboring most of *luxR*, all of *luxI* and *luxC*, and a portion of *luxD*.

RESULTS

Demonstration that the lysis phenotype is under *lux* control. The lysis vectors pGS102 and pGS103 both allowed expression of the lysis phenotype to be controlled by the addition of synthetic autoinducer. A *Sna*BI-*Bal*I deletion of pGS102 was done to create pGS103 (Fig. 1). The deleted sequences consisted of uncharacterized *V. fischeri* DNA and a portion of the pBR322 vector. Removal of these sequences had no effect on the induction of cell lysis by the addition of autoinducer. When autoinducer was added from the beginning of the growth experiment or at an early point in growth, a lag was observed prior to cell lysis while LuxR protein accumulated. A typical lysis curve is shown in Fig. 2. At 30°C in LB medium, with autoinducer added at the beginning of the experiment, cell lysis was observed as a decrease in the OD_{600} of the culture between 4.5 and 5 h after initial inoculation of the culture (OD_{600}, 1.2), followed by a steady decrease in OD_{600} during the next several hours. When autoinducer was added at a later point during culture growth,

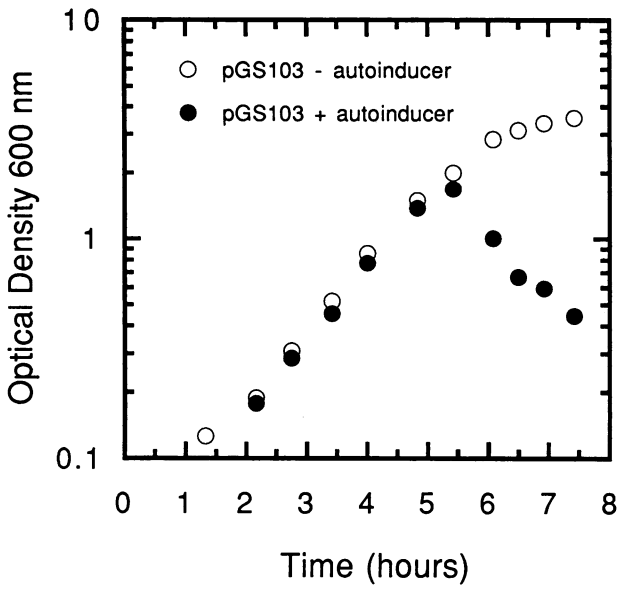

FIG. 2. Growth of cultures of *E. coli* TB1 carrying the plasmid pGS103 and demonstration of autoinducer-dependent cell lysis. Autoinducer (5 μM) was added from the beginning of the experiment where indicated.

the lag period was decreased. The lag could be almost completely eliminated if *E. coli* CRP* was used, since the stimulation of *luxR* expression by cAMP-CAP does not require the accumulation of cAMP in this strain (data not shown). These observations suggest that the timing of induction was mainly a function of the cAMP-CAP stimulation of *luxR* expression.

Selection of mutants following lysis induction in liquid medium. Selection of *lux* regulatory mutants was accomplished by the addition of autoinducer to cultures grown in liquid medium at 24°C and allowing the culture to incubate overnight (12 to 14 h) with shaking. Cells surviving the lysis induction were then grown on solid medium. Two problems were encountered with this procedure. First, a background of colonies was observed which retained the ability to lyse when screened in liquid medium for a nonlysing phenotype. Second, a considerable proportion of the colonies isolated from the selection which did retain a nonlysing phenotype carried plasmids which had suffered deletions and/or other rearrangements of the original pGS102 plasmid. The former problem was partially eliminated by pelleting the cells from the initial lysis induction, suspending them in fresh autoinducer-containing medium, and taking them through a second lysis induction under identical conditions. Dilutions plated from these cultures exhibited a lower background of surviving nonmutant colonies.

From the double-induction experiment in M9 medium at 24°C, 18 colonies, which were presumably mutant, were picked and grown overnight to isolate plasmid DNA and to screen for a nonlysing phenotype in liquid medium. All 18 mutants exhibited a nonlysing phenotype, and 13 of these 18 isolates had wild-type restriction patterns for the pGS102 plasmid. To screen for mutations which were not plasmid-borne, the plasmid DNA from the remaining 13 mutants was retransformed into wild-type *E. coli* and again screened for lysis in response to autoinducer in liquid medium. By this criterion, six of these mutants (L2S2, L2S9, L2S14, L2S18, L2S20, and L2S21) were results of non-plasmid-borne mutations and were probably results of *E. coli* chromosomal

TABLE 1. Summary of *luxR* point mutations isolated by variations of the lysis selection

Mutation designation	*luxR* nucleotide change[a]	LuxR amino acid change	Lysis selection[b]
L2S3	T-114 to A	C-38 to TGA (stop)	L
L2S5	G-325 to T	V109L	L
L2S7	G-368 to T	S123I	L
L2S8	C-352 to T	L118F	L
L2S17	T-502 to G	Y-169 to TAG (stop)	L
	C-508 to A	R170R (silent)	
XS-2	G-244 to A	V82I	S
XS-3	C-649 to T	H217Y	S
XS-4	G-235 to A	D79N	S

[a] Positions are numbered from 1 starting with the A of the AUG start codon for *luxR* indicated by Devine et al. (2).
[b] L, Lysis selection in liquid medium; S, lysis screen on solid medium after hydroxylamine mutagenesis (see Materials and Methods).

mutations which prevented cell lysis. The remaining seven mutants were judged to be plasmid borne and were next screened for *luxR* null phenotypes by checking for lysis in the presence of autoinducer with *luxR* supplied in *trans* on a compatible plasmid (pAC102). All seven of the mutants (L2S3, L2S5, L2S7, L2S8, L2S10, L2S15, and L2S17) exhibited a *luxR* null phenotype. The nucleotide sequences of the *luxR* genes from the *luxR* null mutants were determined, and the results are shown in Table 1. No mutation was identified within the *luxR* coding region for the L2S10 and L2S15 isolates.

Hydroxylamine mutagenesis and lysis screen on solid medium. Attempts to select mutants by the lysis technique on autoinducer containing solid medium resulted, as with the liquid medium selection, in a large background of colonies which retained a lysing phenotype when screened in liquid medium. The desired mutant colonies could, however, be discriminated from the background of surviving nonmutant colonies as a normal versus translucent phenotype. The translucent phenotype was presumably due to a heterogeneous population of lysed and unlysed cells. In order to increase the frequency of mutant colonies, the plasmid pGS103 was modified by reaction with hydroxylamine in vitro prior to screening for mutants on autoinducer-containing solid medium. By this method, approximately 15% of the colonies were of the mutant phenotype in the hydroxylamine experiment, whereas in the control experiment with unmodified plasmid, mutant colonies were observed only at very low frequencies. Twenty mutant colonies were isolated with this screen, and plasmid DNA was prepared for further study.

Quantitation of autoinducer response of *luxR* point mutations. To recover mutations in the *luxR* gene, restriction fragments containing portions of the *luxR* gene from the 20 mutants isolated by the hydroxylamine experiment described above were subcloned into the bioluminescent rightward reporter vector pJHD500 and screened as dim colonies on autoinducer-containing solid medium. Colonies of cells containing pJHD500 with a wild-type *luxR* were bright under these conditions. Eight dim mutants were picked from this screen, five of which possessed a wild-type restriction pattern, indicating an intact *luxR* gene. Three of these were sequenced and shown to contain point mutations in *luxR* resulting in changes in the LuxR amino acid sequence of Val at position 82 to Ile, hereafter designated as V82I, H217Y, and D79N (Table 1). In addition, the three missense mutations and the L2S3 nonsense mutation isolated by the liquid

TABLE 2. Autoinducer response of *luxR* point mutations measured in the transcriptional reporter vector pJHD500

LuxR variant of pJHD500	Light units/ml[a]		Stimulation (fold)	% Stimulation[b]
	Without autoinducer	With autoinducer (2.5 μM)		
pJHD500	8.0	265	33	100
L2S3	9.0	9.0	None	0
D79N	9.0	10.0	None	0[c]
V82I	7.5	175	23	70
V82L	8.0	20.0	2.5	8
V109L	7.0	8.0	None	0
L118F	8.0	8.0	None	0
S123I	6.5	6.0	None	0
H127Y	8.0	15.0	1.9	6
H217Y[d]	2.0	2.3	None	0

[a] Peak light emission measured from 1 ml of culture at an OD_{600} of 1.5.
[b] Values are given relative to the stimulation achieved by the wild-type LuxR protein encoded by the pJHD500 construction.
[c] At a 10-fold-higher autoinducer concentration, the response of D79N is about 1% that of the wild-type protein.
[d] The H217Y data were collected during a different growth experiment under a different set of conditions than was used for the other mutations shown in Table 1 (100 rpm, 29°C, 2.5 μM autoinducer).

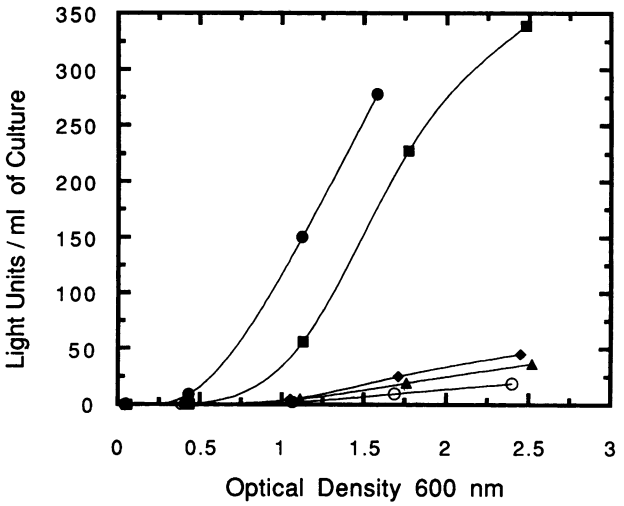

FIG. 3. Autoinducer response of pJHD500 and three LuxR⁻ pJHD500 derivatives. Symbols: ● and ○, pJHD500-wild type LuxR; ■, H127Y; ◆, V82L; ▲, V82I. Open symbols indicate no autoinducer added to the culture, and closed symbols indicate the addition of 2.5 μM autoinducer from the beginning of the experiment.

lysis induction were subcloned into pJHD500 to allow transcription from $operon_R$ to be measured. To screen for mutations which could respond to higher concentrations of autoinducer, cells containing the *luxR* mutant derivatives of pJHD500 were replica plated onto solid medium with and without 5 μM autoinducer. The LuxR protein with the amino acid change of V82I was the only mutant observed by a visual screen to respond to this (elevated) concentration of autoinducer. To further examine the autoinducer response of the change at position 82, a second mutation was introduced by site-directed mutagenesis which changed the valine at this position to leucine instead of isoleucine.

During the course of these experiments, we became aware of a similar genetic study of the LuxR protein from *V. fischeri* MJ1 by Slock et al. (22). These authors describe the isolation of several LuxR missense mutations, one of which was observed to respond to higher concentrations of autoinducer. This mutation resulted in the replacement of the histidine at position 127 with tyrosine. In order to quantitate the autoinducer response of this mutation with our reporter vector, the same mutation was constructed by site-directed mutagenesis in our laboratory and subcloned into pJHD500. The change was introduced by site-directed mutagenesis rather than by subcloning of their mutation because our two groups work on different strains of *V. fischeri* (ATCC 7744 and MJ1) which have been shown to have differences in the amino acid sequence at four positions within the LuxR protein (3). Thus, we wanted to ensure that the observed phenotype was the result of the change at position 127 and not due to strain differences in the LuxR protein.

Growth curves and luciferase assays in vivo were done for the total of nine *luxR* mutations cloned in the reporter vector pJHD500. The autoinducer response was measured as the ability of the mutations to stimulate $operon_R$ transcription in the presence of autoinducer (2.5 μM) above the basal level observed in the absence of autoinducer. The results are summarized in Table 2, and complete growth curves of those mutations which respond to autoinducer are shown in Fig. 3. The results presented in Fig. 3 and Table 2 demonstrate that the mutant V82I responded with an activity of approximately 70% that of the wild-type protein (23- versus 33-fold stimulation above the basal level at an OD_{600} of 1.5). The site-directed mutants V82L and H127Y responded to a much lower extent, giving only 8 and 6% of the stimulation of the wild-type protein, respectively. The mutant D79N was observed to reproducibly give stimulation of about 1% that of the wild-type protein at a 10-fold-higher autoinducer concentration than that used in the above experiments (data not shown). Both of the position 82 mutants, as well as the H127Y mutant, were screened for response to a range of autoinducer concentrations. With all three of these variants, it was found that elevated levels of autoinducer were required to compensate for the lesion (data not shown). None of the remaining mutants exhibited significant autoinducer-dependent stimulation (Table 2).

Location of random mutations in *luxR*. The locations of the randomly generated mutations isolated in this study are shown in Fig. 4 along with two mutations isolated by Slock et al. (22), G121R and H127Y, which reside in the same region of the LuxR primary sequence. A total of seven randomly generated missense mutations occur within a 49-amino-acid stretch of the LuxR protein spanning residues 79 to 127, labeled as the autoinducer-binding region in Fig. 4 (see Discussion). One mutation isolated during the luminescence screen, H217Y, occurred within a second critical region in LuxR spanning residues 184 to 230 and defined primarily by mutations isolated by Slock et al. (22) (Fig. 4).

DISCUSSION

Several features of the lysis gene cassette from bacteriophage λ make it generally useful as the lethal component in a lethal genetic selection. The results of an exhaustive mutational analysis of the bacteriophage λ *S* gene revealed that host mutations which confer resistance to the lethal action of the S protein are not recovered, since the S protein apparently acts alone in forming the lethal pore in the cytoplasmic membrane (21). This is not true for many other lethal proteins, which require interaction with host components in order to exert their lethal functions. The *S* gene is the only lethal gene of the three comprising the lysis cas-

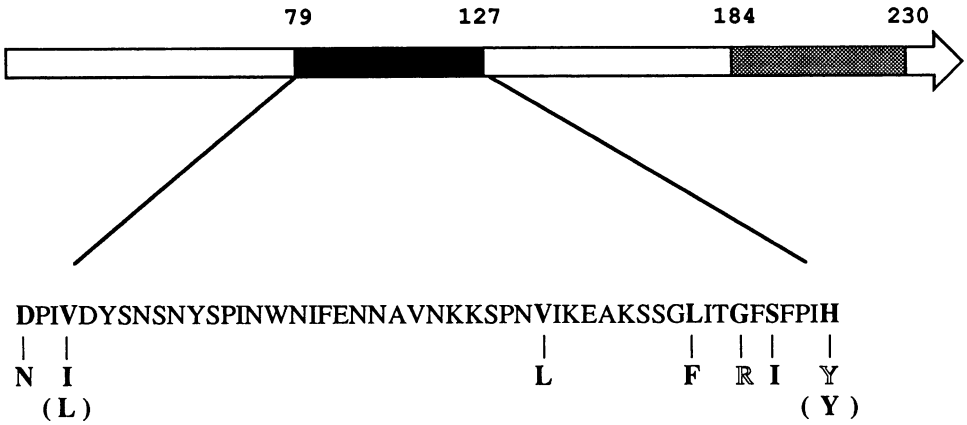

FIG. 4. Critical regions of the LuxR protein defined by random mutagenesis and the primary sequence of the autoinducer-binding region (■). Boldface type indicates random mutations isolated in this study which map between residues 79 and 127. Open type indicates random mutations isolated by Slock et al. (22) which map between residues 79 and 127. Letters in parentheses indicate amino acid changes introduced by site-directed mutagenesis. ▨, The second critical region defined by mutations isolated by Slock et al. (22) and the H217Y mutation isolated in this study.

sette, and it consists of only 107 codons. The small size of the *S* gene makes it a small target for mutation, and therefore, the frequency of recovering mutations in the lysis cassette which prevent lethality is very low. In addition, many of the codons constituting the *S* gene are not mutable by transitions to "knockout" missense mutations or nonsense mutations which result in loss of S protein function. Lastly, a simple secondary screen can distinguish between the desired transcriptional control mutants and unwanted *S* gene mutants. This screen utilizes the ability of a limited amount of chloroform to substitute for S protein pore formation by disrupting the cell membrane and allowing the diffusion of accumulated murein transglycosylase into the periplasm (12). The murein transglycosylase, the product of the λ *R* gene, is responsible for degradation of the peptidoglycan in the *E. coli* cell wall (1). Transglycosylase accumulation does not require *S* gene function; therefore, mutations which decrease transcription of the lysis cassette will not lyse even in the presence of chloroform, while mutations in the *S* gene will allow lysis in the presence of chloroform since the transglycosylase can still accumulate and is free to enter the periplasm.

Problems encountered in this study while adapting the lysis method to the *lux* system were mainly a result of different temperature requirements for the *lux* system, which is isolated from a marine bacterium, and the lysis genes, which normally function in the enteric bacterium *E. coli*. For example, when *E. coli* carrying a lysis plasmid was grown at 37°C, cell lysis was observed in the absence of autoinducer. However, cell lysis was not observed in the absence of autoinducer when the cultures were grown at 30°C or lower. This apparently resulted from either an increased basal level of transcription of operon$_R$ at 37°C or the ability of the protein products of lysis genes to function more efficiently at the higher temperatures. Temperature therefore appears to be one parameter which can be adjusted to optimize the conditions for the lysis selection and may provide a means for controlling the lethality of the lysis genes, allowing selection of a variety of mutants.

In this study, the λ lysis cassette was used to isolate mutations in the *luxR* gene from *V. fischeri* with *E. coli* as a host for the cloned *lux* genes. A transcriptional fusion was created between the bacteriophage λ lysis genes and operon$_R$ by insertion downstream of a truncated *luxI* gene. The resulting plasmid allowed cell lysis to be controlled by the addition of synthetic autoinducer to the growth medium. Mutations in the *luxR* gene generated by variations of this lysis selection were subcloned into the luminescent reporter vector pJHD500, which created both a secondary screen for defective LuxR proteins and a method for quantitating the ability of these LuxR variants to respond to autoinducer.

A total of seven randomly generated missense mutations have been characterized, and by DNA sequence analysis, the lesions have been shown to occur within a 49-amino-acid stretch of the LuxR primary sequence. An additional mutation introduced by site-directed mutagenesis changed the valine at position 82 to leucine. A change of valine to isoleucine at position 82 of LuxR resulted in a protein with 70% of the autoinducer-dependent transcriptional stimulation capacity of the wild-type protein, while changing this same valine to a leucine resulted in a protein exhibiting only 8% of the wild-type response (Table 2). The ability of such conservative changes at position 82 of LuxR, valine to isoleucine and valine to leucine, to dramatically affect the autoinducer response of the resulting proteins suggests that this residue may be involved in direct interaction with the autoinducer molecule. A total of four *luxR* mutations yielded proteins which exhibited an ability to stimulate transcription of operon$_R$, albeit to lower-than-wild-type levels, while the remaining *luxR* mutations did not allow any detectable autoinducer-dependent stimulation, even at elevated concentrations of autoinducer. The clustering of the seven randomly generated mutations within the region spanning residues 79 to 127 of the LuxR protein demonstrates that this region of the protein is critical for activity. The ability of several mutations within this region to respond to elevated concentrations of autoinducer suggests that the autoinducer-binding site is composed, at least in part, of amino acids residing within this region.

The possibility that the mutations in the proposed autoinducer-binding region may result in the production of unstable proteins which could give rise to the defective autoinducer response phenotype observed cannot be ruled out by our data. However, two additional mutations isolated by Slock et al. (22), G121R and H127Y, were shown by Western immunoblot analysis to be synthesized in vivo at levels comparable to those of the wild-type protein. These results increase our confidence that our mutant proteins are

likewise produced at wild-type levels. This is especially true of the mutations at position 82 of *luxR* which both give stimulation greater than does the H127Y protein, which is synthesized at wild-type levels. It is difficult to imagine a situation in which the proteins with position 82 mutations could give greater stimulation if they were synthesized at levels lower than wild-type levels.

The results of this mutational study of the LuxR protein, coupled with those of Slock et al. (22), demonstrate that at least two functional regions exist in the LuxR protein. One region spans amino acids 79 to 127 and is proposed to be an autoinducer-binding region, and the other region spans amino acids 184 to 230 (Fig. 4). Although no experimental evidence demonstrates that the mutations isolated by Slock et al. (22) and the mutation of H217Y isolated in this study, which resides in this second critical region, are in fact defective in DNA binding, there is some recent evidence which supports the hypothesis that a carboxy-terminal DNA-binding region exists in LuxR. Alignment of amino acid sequences similar to those of the LuxR protein by Henikoff et al. (14) revealed that a carboxy-terminal region of LuxR has a sequence similar to regions within nine other diverse bacterial proteins, including five known activator proteins (FixJ from *Rhizobium meliloti*, MalT and UhpA from *E. coli*, GerE from *Bacillus subtilis*, and RcsA from *Klebsiella aerogenes*). The apparently homologous regions revealed in this computer search have been predicted to form a helix-turn-helix DNA-binding motif at a common position (S. Henikoff, personal communication), which includes residues within the region defined by mutations residing between positions 184 and 230 in LuxR. Further biochemical evidence is required, however, before this region can be unequivocally defined as the DNA-binding region of the LuxR protein.

The primary regulatory circuit controlling the induction of bioluminescence in *V. fischeri* appears to require three interacting elements: the LuxR protein, the autoinducer molecule, and the *lux* operator. Early genetic studies in which *lux* regulatory functions were deleted by transposon insertion mutagenesis demonstrated that the *luxR* and *luxI* genes were both required for the proper induction of bioluminescence (8). Insertions in *luxR* resulted in loss of a function which could not be recovered by the addition of autoinducer, whereas insertions in the *luxI* gene were complemented by autoinducer addition. These observations led to a model which suggested that the *luxI* gene product was required for autoinducer synthesis and that the *luxR* gene product interacted with the autoinducer molecule to stimulate transcription of operon$_R$. Recent studies supported this model and demonstrated that a 20-base-pair palindrome, the *lux* operator, located within the control region is also required for autoinducer-dependent stimulation of transcription of operon$_R$ (3). Several mutations in the *luxR* gene which encoded variant LuxR proteins with altered autoinducer responses were isolated in this study. Demonstration that these mutant proteins can respond to higher concentrations of autoinducer provide the first evidence supporting the direct interaction between LuxR and autoinducer.

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LITERATURE CITED

1. **Bienkowska-Szewczyk, K., B. Lipinski, and A. Taylor.** 1981. The *R* gene product of bacteriophage lambda is the murein transglycosylase. Mol. Gen. Genet. **184**:111–114.
2. **Devine, J. H., C. Countryman, and T. O. Baldwin.** 1988. Nucleotide sequence of the *luxR* and *luxI* genes and the structure of the primary regulatory region of the *lux* regulon of *Vibrio fischeri* ATCC 7744. Biochemistry **27**:837–842.
3. **Devine, J. H., G. S. Shadel, and T. O. Baldwin.** 1989. Identification of the operator of the *lux* regulon from the *Vibrio fischeri* strain ATCC 7744. Proc. Natl. Acad. Sci. USA **86**:5688–5692.
4. **Dunlap, P. V., and E. P. Greenberg.** 1985. Control of *Vibrio fischeri* luminescence gene expression in *Escherichia coli* by cyclic AMP and cyclic AMP receptor protein. J. Bacteriol. **164**:45–50.
5. **Dunlap, P. V., and E. P. Greenberg.** 1988. Control of *Vibrio fischeri lux* gene transcription by a cyclic AMP receptor protein-LuxR protein regulatory circuit. J. Bacteriol. **170**:4040–4046.
6. **Eberhard, A.** 1972. Inhibition and activation of bacterial luciferase synthesis. J. Bacteriol. **109**:1101–1105.
7. **Eberhard, A., A. L. Burlingame, C. Eberhard, G. L. Kenyon, K. H. Nealson, and N. J. Oppenheimer.** 1981. Structural identification of autoinducer of *Photobacterium fischeri* luciferase. Biochemistry **20**:2444–2449.
8. **Engebrecht, J., K. Nealson, and M. Silverman.** 1983. Bacterial bioluminescence: isolation and genetic analysis of functions from *Vibrio fischeri*. Cell **32**:773–781.
9. **Engebrecht, J., and M. Silverman.** 1984. Identification of genes and gene products necessary for bacterial bioluminescence. Proc. Natl. Acad. Sci. USA **81**:4154–4158.
10. **Engebrecht, J., and M. Silverman.** 1986. Regulation of expression of bacterial genes for bioluminescence, p. 31–44. *In* J. K. Setlow and A. Hollaender (ed.), Genetic engineering, vol. 8. Plenum Publishing Corp., New York.
11. **Engebrecht, J., and M. Silverman.** 1987. Nucleotide sequence of the regulatory locus controlling expression of bacterial genes for bioluminescence. Nucleic Acids Res. **15**:10455–10467.
12. **Goldberg, A. R., and M. Howe.** 1969. New mutations in the S cistron of bacteriophage lambda affecting host cell lysis. Virology **38**:200–202.
13. **Hastings, J. W., and G. Weber.** 1963. Total quantum flux of isotropic sources. J. Opt. Soc. Am. **53**:1410–1415.
14. **Henikoff, S., J. C. Wallace, and J. P. Brown.** 1990. Finding protein similarities with nucleotide sequence databases. Methods Enzymol. **183**:111–132.
15. **Humphreys, G. O., G. A. Wilshaw, H. R. Smith, and E. S. Anderson.** 1976. Mutagenesis of plasmids with hydroxylamine: isolation of mutants of multi-copy plasmids. Mol. Gen. Genet. **145**:101–108.
16. **Kaplan, H. B., A. Eberhard, C. Widrig, and E. P. Greenberg.** 1985. Synthesis of N-[3-oxo-(4,5-$^3H_2$)-hexanoyl] homoserine lactone: biologically active tritium-labelled *Vibrio fischeri* autoinducer. J. Labelled Compd. Radiopharm. **22**:387–395.
17. **Kaplan, H. B., and E. P. Greenberg.** 1985. Diffusion of autoinducer is involved in regulation of the *Vibrio fischeri* luminescence system. J. Bacteriol. **163**:1210–1214.
18. **Kunkel, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154**:1367–1382.
19. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Meighen, E. A.** 1988. Enzymes and genes from the *lux* operons of bioluminescent bacteria. Annu. Rev. Microbiol. **42**:151–176.
21. **Raab, R. A., G. Neal, J. Garrett, R. Grimaila, R. Fusselman, and R. Young.** 1986. Mutational analysis of the bacteriophage lambda lysis gene *S*. J. Bacteriol. **167**:1035–1041.

21a. **Sabourin, D., and J. Beckwith.** 1975. Deletion of the *Escherichia coli crp* gene. J. Bacteriol. **122:**338–340.
22. **Slock, J., D. VanRiet, D. Kolibachuk, and E. P. Greenberg.** 1990. Critical regions of the *Vibrio fischeri* LuxR protein defined by mutational analysis. J. Bacteriol. **172:**3974–3979.
23. **Tabor, S., and C. C. Richardson.** 1987. DNA sequence analysis with modified bacteriophage T7 DNA polymerase. Proc. Natl. Acad. Sci. USA **84:**4767–4771.
24. **Ulitzur, S.** 1989. The regulatory control of the bacterial luminescence system—a new view. J. Biolumin. Chemilumin. **4:** 317–325.
25. **Ulitzur, S., and J. Kuhn.** 1988. The transcription of bacterial luminescence is regulated by sigma 32. J. Biolumin. Chemilumin. **2:**81–93.