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Identification of a Regulatory Region within the *luxR* Structural Gene in a Marine Symbiotic Bacterium, *Vibrio fischeri*

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The light-organ symbiont of pine cone fish, *Vibrio fischeri*, senses its presence in the host and responds to environmental changes by differentially expressing its symbiosis-related luminescence genes. The *V. fischeri* luminescence genes are activated by LuxR protein in the presence of an autoinducer. In an effort to elucidate the mechanism of regulation of *luxR*, a plasmid containing *luxR* was mutagenized *in vitro* with hydroxylamine and a *luxR* mutant plasmid was isolated by its ability to activate luminescence genes cloned in *E. coli* in the absence of the autoinducer. The specific base change identified by DNA sequencing was only single base transition at +78 from the transcriptional start of *luxR*. Based on a Western immunoblot analysis, the nucleotide change directed the synthesis of much higher level of LuxR protein without any amino acid substitutions. The results suggest that the region including the +78th base is presumably internal operator required for autorepression of *luxR*, and the increased cellular level of LuxR results in activation of luminescence genes by autoinducer independent fashion.

The luminescent marine bacterium, *Vibrio fischeri*, can be isolated from seawater and it also occurs as the bacterial symbiont in the light organs of certain marine fishes (2, 12, 41, 42, 43). Rapid advances in molecular and genetic analyses of the *V. fischeri* luminescence system were made possible by the isolation of a 9-kilobase (kb) fragment of *V. fischeri* DNA that encodes luminescence enzymes and contains regulatory elements necessary for their expression in *Escherichia coli* (15, 16, 18). The functions were organized in two contiguous but divergent transcriptional units, *luxR* and the luminescence genes (*luxICDABE* operon) (Fig. 1). In the *lux* operon, *luxI* encodes autoinducer synthase; *luxC*, *luxD*, and *luxE* specify components of a fatty acid reductase system for synthesis of aldehyde substrate of luciferase; and *luxA* and *luxB* encode the α and β subunits of luciferase. The *luxR* gene encodes a polypeptide (LuxR) which, along with autoinducer, is necessary for the activation of the luminescence genes transcription (15, 16).

The luminescence of *V. fischeri* is inducible. The inducer, N-(3-oxohexanoyl)homoserine lactone, termed autoinducer, is a diffusible metabolite produced by *V. fischeri* that accumulates at equal concentrations in the culture medium and in cells during growth (14, 28, 38). When a sufficient amount of autoinducer is present, autoinducer is thought to bind to LuxR, a receptor, and the autoinducer-LuxR complex is thought to activate transcription of the luminescence genes and then induction of luminescence commences (20, 29, 30). The induction of the luminescence genes by the autoinducer has been referred to as autoinduction. At high population densities, such as the high cell density environment (10^{10}~11^{10} cells per ml) of a light organ , autoinducer can reach the critical concentration necessary for induction and luminescence will occurs. In sea water where *V. fischeri* exists at less than 10^2 cells per ml, autoinduction of luminescence would not be expected (20, 35, 36, 41). Thus autoinduction system can be considered as an environmental sensing system highly responsive to the cell density of *V. fischeri* (12, 20).

Previous studies revealed that cyclic AMP receptor protein (CAP) and cAMP activate transcription from *luxR* promoter (9, 10, 11). It is presumed that by this activation, sufficient LuxR protein is present prior to induction to interact with autoinducer and potentiate the induction of luminescence (11). Furthermore, control of the luminescence system by LuxR is complex. Expression of *luxR* is negatively and positively autoregulated by LuxR at the level of transcription (4, 46, 47, 48); a postt-

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ranscriptional autoregulation has also been reported (17).

The *luxR* gene has been sequenced and LuxR protein predicted from the DNA sequence is 250 amino acid residues in length (7, 18). The carboxy-terminal one-third of LuxR shows sequence similarity to a group of known or suspected transcriptional regulator proteins, the so-called LuxR family of transcriptional regulators (24). This group includes members of the so-called two component environmental-sensing systems such as the transcriptional activators, UhpA, FixJ, and NarL (3, 19, 27, 50). Although LuxR activity *in vitro* has not yet been demonstrated, a general view of some of the important feature of this protein has been developed by studying the activity of mutant LuxR proteins *in vivo* (4, 5, 6, 20). Recently Choi and Greenberg verified that LuxR is modular. There is an N-terminal regulator module required to block binding of a C-terminal activator module to the appropriate *lux* DNA. Within the regulator module, there is an autoinducer-binding region that is proximal to the C-terminal module. Autoinducer binding to the regulatory region is required to allow the C-terminal module to bind DNA and to activate *luxICDABE* transcription (4, 5). The distal end of this N-terminal module is required for autorepression of *luxR* by DNA-bound LuxR (4).

There are 155 bp of DNA between the *luxR* and *luxI* transcriptional start sites (7). Within this DNA segment, a sequence matching the consensus for a cyclic AMP receptor protein binding site is located at −59 from the *luxR* transcriptional start site. Based on deletion and point mutational analyses, it appears that a 20-bp inverted repeat centered 40 bp upstream of the *luxI* transcriptional start site is the LuxR binding site (8). It was hypothesized that this site is required for *luxICDABE* activation and *luxR* repression (8). For *luxR* autoregulation, however, the distance between this presumptive LuxR-binding site and *luxR* transcription start point is 115 bp and seems so far that direct interaction of LuxR with RNA polymerase initiation complex can not be expected. Meanwhile Shadel *et al* demonstrated another negative acting *cis* element within the *luxD* gene using deletion analysis. They also proposed a model describing autorepression of *luxR* promoter by forming a DNA-looping between LuxR bound to the 20-base repeat sequence and this suspected operator (48). However, none of the analyses were performed to *luxR* structural gene and the function of this region in *luxR* autoregulation is not yet obvious. The research described herein was initiated to gain any evidence for the presence of functional *cis* element in *luxR* for the regulation of *luxR* expression. In fact, the results of the random mutational analyses of *luxR* suggest that like other transcriptional regulation, expression of *luxR* is also governed by an internal operator sequence located within the *luxR* structural gene.

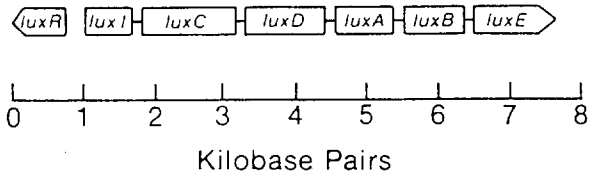

Fig. 1. Organization of *V. fischeri lux* genes.
The *luxR* gene and the *luxICDABE* luminescence genes are transcribed divergently.

MATERIALS AND METHODS

Bacterial Strains, Plasmids and Culture Conditions

The *E. coli* strain used was DH5α (22). The plasmids used were pHK555, which contains a p15A replicon, a chloramphenicol resistance marker, and a complete *lux* gene cluster (Fig. 1) with a Mu dI1681 insertion roughly in the middle of *luxR* (29); pJR551, a pHK555 derivative with a nonpolar mutation in *luxI* that inactivates this gene (13); pPD749 contains a colEI replicon (Fig. 2), an ampicillin resistance marker, and *luxR* under control of the *tac* promoter (11); and the pSC749 and pSC500 plasmids, which were derived from pPD749 as described below.

Cultures were grown in LB (Luria-Bertani) broth or on LB agar containing the appropriate antibiotic for plasmid screening or maintenance (100 μg of ampicillin or 30 μg of chloramphenicol per ml) as described previously (10). Where indicated, isopropyl-β-D-thiogalactoside

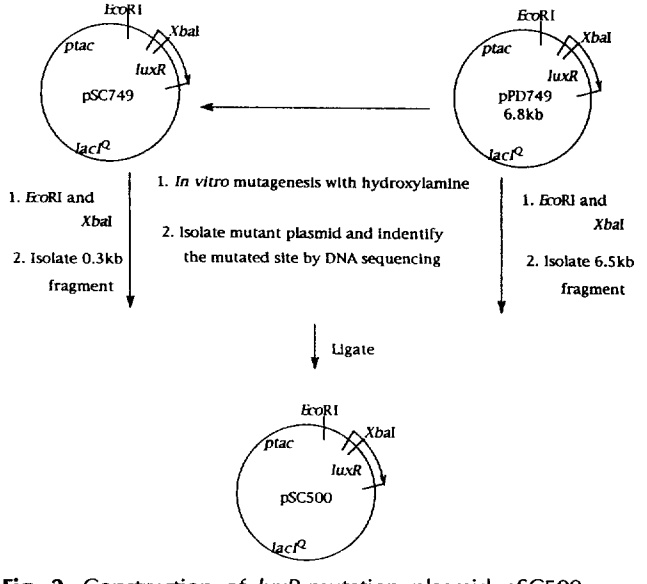

Fig. 2. Construction of *luxR*-mutation plasmid pSC500.
The 0.3 kb DNA fragment from pSC749, and the 6.5 kb DNA fragment from pPD749 were isolated after separation by electrophoresis through low-melting temperature agarose.

(IPTG; final concentration, 1 mM) was added to cultures (11). All broth cultures and plates were incubated at 30°C.

Plasmid Purification and Transformation Procedure

Plasmids were purified by the procedure described by Kraft *et al.* (31). Manipulations of plasmids were performed according to the methods of Sambrook *et al.* (44). The transformation procedure used was described by Hanahan (22).

Isolation of pPD749 Derivatives with Mutations

The construction of pSC749 and pSC500, the *luxR* mutant plasmid used in this study, is depicted in Fig. 2. Mutagenesis of plasmid DNA was according to the procedure described by Engebrecht and Silverman (16). To generate mutations on *luxR*, 3 μg of pPD749 was incubated in 50 μl of a mixture containing 0.5 M hydroxylamine, 0.5 mM EDTA, and 5 mM Tris (pH 6.0) for 8hr at 37°C. The reaction was stopped by the addition of 950 μl of 100 mM $CaCl_2$, and the hydroxylamine-mutagenized DNA was then used to transform *E. coli* DH5α containing pJR551. The transformants were plated on LB agar containing ampicilin, chloramphenicol and IPTG. Colonies that appeared luminescent after 2 days at 30°C were picked. Plasmid DNA from the luminescent strains was isolated and used to transform *E. coli* DH5α, and ampicillin-resistant, chloramphenicol-sensitive transformants, presumed to contain only the *luxR* mutant plasmid were selected. Plasmids from these transformants showed *Eco*RI and *Xba*I restriction patterns that were identical to those for pPD749. One of the resultant plasmids, pSC749 was sequenced and saved for further studies.

Construction of pSC500 Containing a Mutation within *luxR* Structural Gene

The *luxR* mutant plasmid containing a mutation only in *luxR* structural gene not in plasmid DNA was constructed (Fig. 2). This was accomplished by subcloning the *luxR* gene fragment excised from pSC749, which has the mutated-sequence identified by sequencing, into pPD749 digested with *Eco*RI and *Xba*I. The resulting plasmid pSC500, was confirmed by DNA sequencing again.

DNA Sequencing

The *luxR* gene in pPD749 and the mutant *luxR* genes in pSC749 and pSC500 were sequenced by the chain termination method described by Sanger *et al.* (45). The sequence of the *luxR* open reading frame, through the upstream promoter and downstream *rrn*B transcription terminator, was confirmed by DNA sequencing as described elsewhere (4, 5).

Gel Electrophoresis and Western Immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out by the procedure established by Laemmli (32), as described elsewhere (30). The resolving gel contained 10% acrylamide. Western immunoblotting involved the procedure described by Brahamsa and Greenberg (3) with a 1 : 200 dilution of preabsorbed LuxR antiserum to probe the nitrocellulose blot.

Determination of Cellular Luminescence

Broth cultures for luminescence and cell growth measurements were grown essentially as described previously (10). Cultures were grown in 50 m*l* of medium contained in 250 m*l* flasks with shaking (about 200 cycles per min). Cultures were inoculated to an initial cell density of 0.002 to 0.008 (OD660). Inocula were from cultures that had been grown in LB broth to a density of 0.5 (OD660) that were themselves inoculated from overnight cultures. The light-measuring equipment and standard to calibrate the equipment have been described elsewhere (23), as have the procedures for measuring luminescence of broth cultures.

RESULTS AND DISCUSSION

Sequence of the *luxR* Gene from Selected pPD749 Mutants

Because the pJR551 has no functional *luxI* to produce autoinducer, LuxR protein coded from wild type pPD 749 is not able to activate *luxICDABE* genes, and *E. coli* containing both pPD749 and pJR551 are not luminous even in the presence of IPTG without external addition of autoinducer (13). One of the pPD749 derivatives, pSC749, activated luminescence genes on pJR 551 to produce light without addition of autoinducer in this *luxI*$^-$ background. It seemed possible that the pSC749 has a mutant *luxR* and its product activate the promoter of luminescence genes by autoinducer independent fashion.

The specific base change in the pSC749 was determined by DNA sequencing through the upstream promoter and downstream terminator of the *luxR* open reading frame. Only single base transition at +78 from the transcriptional start of *luxR* was identified. The nucleotide change and the resulting amino acid substitution in the encoded LuxR are shown in Fig. 3. As expected from

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          +60                                  +80
pPD749; ATG AAA AAC ATA AAT GCC GAC GAC.....
          M   K   N   I   N   A   D   D

pSC500; ATG AAA AAC ATA AAT GCT GAC GAC.....
          M   K   N   I   N   A   D   D
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Fig. 3. Nucleotide sequences and corresponding translated amino residues of wild type and mutant *luxR* genes.
The nucleotide substitution located at +78 from the transcriptional start point of *luxR* is boxed.

wobble hypothesis, C-to-T transition of the triplet coding alanine (5'-GCC-3') does not result in amino acid substitution. Thus the LuxR coded by pSC749 in *E. coli* is presumably the same wild type protein with alanine at 6th amino acid.

The mutant plasmid was derived by *in vitro* mutagenesis (49) of pPD749 whole plasmid with hydroxylamine. Thus mutant could be assumed to have resulted from any changes in vector DNA itself such as promoter, terminator and replication origin sequence. For instance, although amino acid sequence of the pSC749-encoded LuxR protein was not different from that of wild type, the increased copy number of the plasmid could affect on the expression of luminescence genes on pJR551 by some reasons. To eliminate these possibilities, pSC 500 where the vector DNA sequence from pPD749 remained intact was constructed as mentioned in Materials and Methods. DNA sequencing of pSC500 ensured that the promoter sequence was followed by a *luxR* mutant gene with the 78th base substitution. Consequently, pSC500 was used throughout my subsequent experiments.

Cellular Luminescence in *E. coli* Containing pSC500 and pJR551

Levels of luminescence in *E. coli* containing pSC500 plasmid and pJR551 grown with IPTG were compared to levels in *E. coli* containing pPD749 and either pHK555 or pJR551 (Fig. 4). The autoinduction response in cultures of *V. fischeri* (35) and recombinant *E. coli* containing the *V. fischeri* luminescence gene cluster (10) is characterized by an initial decrease in luminescence during the early logarithmic growth phase that is followed by a rapid increase in luminescence, which occurs once autoinducer has reached the required concentration of about 10 nM as a result of the activity of basal levels

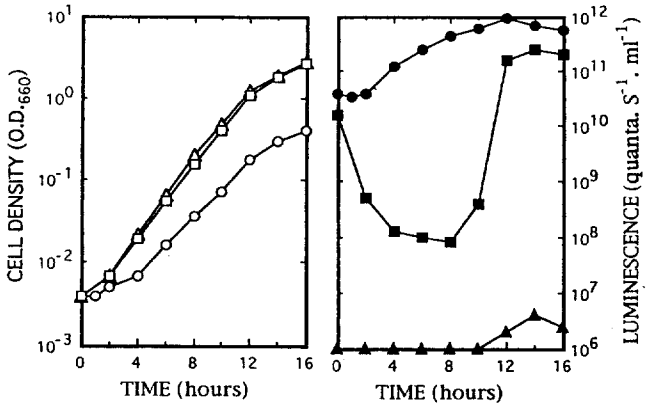

Fig. 4. Luminescence and cell growth of *E. coli* containing plasmids as indicated.
E. coli (pSC500, pJR551); ○, ●, *E. coli* (pPD749, pJR551); △, ▲, *E. coli* (pPD749, pHK555); □, ■. Cell densities are indicated by open symbols and luminescences by solid symbols and conditions for cultures are described in Materials and Methods.

of the *luxI* gene product. Thus it appears that autoinduction of luminescence resulted from the dependency of LuxR activity on the cellular level of autoinducer.

The product of pPD749, which has a wild-type *luxR* gene, appeared to be autoinducer-dependent based on the relationship between culture growth and luminescence. Measurements of cellular luminescence of *E. coli* containing pPD749 and pHK555 exhibited an initial drop before induction and then stimulation of cellular luminescence in the late logarithmic growth phase. This characteristic autoinduction response indicated autoinducer-dependent activation of *luxICDABE* genes by pPD 749 *luxR* gene product. In the meanwhile, in *E. coli* containing pPD749 and pJR551, luminescence in whole growth phase was not detected. This was expected because pJR551 is a *luxI*$^-$ *luxCDABE*$^+$ derivative of pHK 555, it does not direct synthesis of autoinducer.

For culture containing pJR551 and pSC500, which has *luxR* mutant gene without amino acid changes, the initial decrease in luminescence was not evident (Fig. 4). The results of luminescence measurements and cell growth were similar to those obtained with cultures of *E. coli* containing pJR551 and pSC162 (4). pSC162 has mutant *luxR* coding autoinducer-independent truncated LuxR protein in which autoinducer binding region is deleted (4). Luminescence is the phenotypic consequence of *luxICDABE* genes transcriptional activation. Thus, although there are alternative explanations, the results indicate that pSC500 *luxR* gene product activate luminescence genes by autoinducer-independent fashion. However, it is remarkable that a nucleotide transition in *luxR* exhibited autoinducer-independent activation of luminescence genes and appeared to direct the synthesis of wild type LuxR protein.

Intracellular Levels of LuxR Proteins from pPD749 and pSC500

From the experiments described above it is apparent that pSC500 *luxR* gene product has wild type amino acid sequence but shows autoinducer-independent activity in *E. coli*. It was interesting how the mutation can change the apparent autoinducer dependency of LuxR. To demonstrate that *E. coli* containing a pSC500 and pJR551 encoded wild type LuxR protein and to estimate the level of LuxR protein in the cells, a Western immunoblot analysis was performed (Fig. 5). The molecular mass of the product encoded by pSC500 was in agreement with that of wild type LuxR; that is about 28 kilodaltons (4, 5, 29). However, as judged from band intensities, the pSC500 mutant *luxR* plasmid produced much higher cellular level of LuxR protein than pPD749. Although the cellular level of LuxR encoded by pPD749 was sufficient for full induction of luminescence (Fig. 4), its band intensities on Western immunoblot were

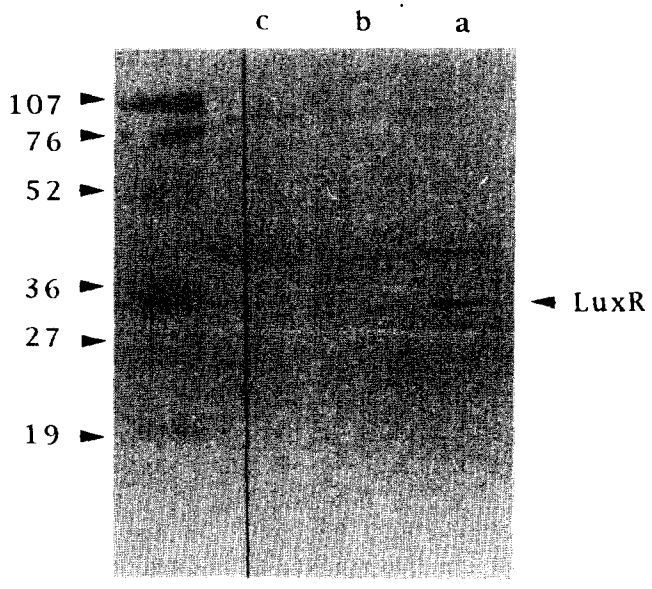

Fig 5. Western immunoblot analysis of whole cell extracts of *E. coli* containing pSC500 & pJR551 (lane a); pPD749 & pJR551 (lane b); pPD749 & pHK555 (lane c).
The migrations of protein molecular size standards are indicated to the left in kilodaltons.

even too faint to be observed clearly.

As mentioned previously, it is not yet known whether any *trans* factor including LuxR itself might bind to any sequence within the *luxR* structural gene. Nevertheless, *luxR* has been known as the only *V. fischeri* genetic element necessary for the regulation of *luxR* gene by *E. coli* (4, 17, 46, 47, 48). Thus, it is reasonable to suppose that LuxR encoded from pPD749 occurred even in induced cells at such low levels because it was autoregulated negatively. It seems likely that the *luxR* in pSC500 with a base transition is released from this negative regulation to encode a much higher level of LuxR protein. This indicates that the specific region, including the 78th base from the start of *luxR* transcription, is required for the negative autoregulation of *luxR*. Obviously, the involvement of this region in authoregulation of *luxR* whether at a transcriptional or at a post transcriptional level can not be precisely defined because of the lack of a Northern analysis for *luxR* expression. However, assuming LuxR specifically interact with *luxR* DNA, it is possible that LuxR binds to the region including the +78th base to repress *luxR* transcription and the base change prevent this interaction. In support of this hypothesis, repression of transcription from DNA sites located at substantial distances from the promoter-transcription initiation region is not uncommon in bacterial systems (1, 21, 33, 40).

In is also possible that the change in sequence results in physical alterations in DNA such as the sequence around the promoter being bent, and this alteration then may facilitate open complex formation at transcription initiation. It has been reported that the transcriptional regulations of several genes in *E. coli* are accomplished through allosteric effects caused by CAP or IHF (integration host factor)-mediated DNA bending (25, 26).

As appears to be true of other transcriptional activators (34, 37, 39), for LuxR, it has been thought to have two conformation, active form and inactive form (4). These two forms are in equilibrium and the autoinducer can change theis equilibrium. In the absence of autoinducer, a large portion of the protein in the cell exist as an inactive form and the portion of active form is too small to activate transcription. With accumulation of autoinducer in the cell, the equilibrium assume to move toward active conformation. Presumably, even the equilibrium is still favored to inactive form in the absence of autoinducer, the absolute level of LuxR with active conformation can reach to critical point simply by increasing the whole level of LuxR. Thus it seemed likely that the apparent autoinducer-independency of LuxR coded by pSC500 was possible by changing the intracellular concentration resulted from the derepression of *luxR*, rather than by generating a new conformation of the protein.

Although specific DNA binding of LuxR *in vitro* has not yet been demonstrated, the approach of using *in vitro* random point mutagenesis coupled with *in vivo* LuxR activity analysis has allowed me to develop a preliminary view of internal regulatory region for *luxR* autoregulation. Clearly, developing an *in vitro* assay for LuxR binding on *luxR* DNA would be interesting. It is also likely that *in vivo* footprinting could provide valuable information on the interaction between the LuxR protein and *luxR* DNA and could confirm the role of the region around +78th base of *luxR* in this interaction. Furthermore, these studies will provide much additional insight into the autoinduction system and further our understanding of the molecular basis of the expression of genes involved in specific interactions between bacteria and animals.

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