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The *Lux* Genes and Riboflavin Genes in Bioluminescent System of *Photobacterium leiognathi* Are under Common Regulation

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The key riboflavin synthesis genes are located immediately downstream of *lux*G in the *lux* operon from *Photobacterium leiognathi*. It is of interest that a site capable of forming a *rho*-independent terminator does not appear to be present between *lux*G and *rib*E in our previous data. These results raise the question of whether the transcription of *lux* and *rib* genes is integrated or not. In order to answer the question, *in vivo* transcriptional assay and Southern blot were examined. These studies demonstrate that neither transcriptional terminator nor promoter site is present in the intergenic region between of *lux* and *rib* genes as well as that the riboflavin genes are single copy in a chromosome of *Photobacterium leiognathi*.

key words: bioluminescence, *lux*, *Photobacterium*, riboflavin,transcription

INTRODUCTION

The open reading frames have been found in the region downstream of the *lux*G in the *Photobacterium lux* operon [1-3]. These genes (*rib*E, B, H, and A in Figure 1) are not only closely linked to the *lux* operon and transcribed in the same direction, but also show the same organization and code for proteins homologous in sequence to the gene products of second, third, and fourth gene of the *rib* operon of *Bacillus subtilis*, respectively [2, 3].

The luminescent reaction ($FMNH_2 + O_2$ + long chain aldehyde FMN + H_2O + long chain fatty acid + light) in luminous marine bacteria is catalyzed by luciferase whose two subunits are coded by two adjacent genes (*lux*A and B in Figures 1 and 2) in the same operon [4, 5].

The detection of the genes just downstream of *lux*G corresponding in sequence to the key riboflavin synthesis genes may be particularly relevant to luminescence in *Photobacterium* species as this genus produces the highest level of light intensities of any bioluminescent bacteria with luciferase levels reaching up to 20 % of the soluble proteins [6]. As $FMNH_2$ is the substrate for the light-emitting reaction in bioluminescent bacteria (Figure 2), the study on the riboflavin synthesis genes linked to the *lux* operon may provide important clues concerning the regulation of flavin supply for the light-emitting reaction.

In this regards, it is of interest that a site capable of forming a *rho*-independent terminator does not appear to be present between *lux*G and *rib*E [1]. These results raise the question of whether or not the *rib* genes are part of the same transcriptional unit as the *lux* genes. In addition, Southern blot to see if the riboflavin genes are single copy, may help to put the riboflavin biosynthesis story in a clear text in regard to regulation of the *lux* operon.

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MATERIALS AND METHODS

Materials

Restriction enzymes, T4 DNA ligase were purchased from Pharmacia. [^{14}C]-chloramphenicol was obtained from Du Pont-New England Nuclear. Riboflavin, ampicillin, and kanamycin were obtained from Sigma Chemical Company. The bacterial strains used in these studies, *Photobacterium leiognathi* ATCC25521, *Vibrio harveyi* B392, *Escherichia coli* K38-1, and *E. coli* HB101. Plasmids pMGM, constructed from pKT230 [7], were used as cloning vectors for transcriptional termination assay.

Cell growth and lysis

The growth was initiated with an inoculum of luminescent cells to give an initial absorbance at 660 nm (A_{660}) of 0.05. Bacterial growth and luminescence *in vivo* were followed by the increase in A_{660} and in light units (LU) per ml of culture medium, respectively, where 1 LU is equivalent 9×10^9 quanta/sec, based on the standard of Hastings and Weber [8].

Southern blotting

Southern blotting was performed essentially as described by Southern [9]. *P.leiognathi* chromosomal DNA was incubated with desired restriction endonuclease or combination of endonuclease under conditions recommended by the supplier. The immobilized DNA was then hybridized with a restriction fragment isolated from a clone of *P. leiognathi lux* operon which had been labeled ^{32}P by nick translation and denatured. The samples were applied

into 1.0% agarose gel, and run at 25 V for 4 hours in running buffer (50 mM NaOH - 1 mM EDTA). Hybridization were carried out with *P. leiognathi Eco*RV-*Xba*I DNA fragment labelled with [γ-^{32}P]CTP (0.1 pmol, 1.2×10^6 cpm/pmol) at 65°C for overnight.

Transcriptional termination assay

Transcription termination assay was measured by using an "*in vivo*" gene expression assay as previously described [7]. The *Hind*III DNA fragment was inserted into the *Hind*III site of pMGM in front of the CAT gene in the presence/absence of *lux* promoter and transferred by conjugation into *V. harveyi* as well as *P. leiognathi*. Expression of CAT activity was measured as previously described [7].

RESULTS AND DISCUSSION

Four genes immediately downstream of *lux*G in the *P. leiognathi lux* operon (*rib*EBHA in Figure 1) have been sequenced and identified their functions of gene products as riboflavin synthase (RibE), lumazine synthase (RibH), and DHBP synthase (RibH), and GTP cyclohydrolase II (RibA), respectively [1, 3]. It is very important to show that the *lux* genes and the key *rib* genes are integrated in the same operon since riboflavin is the direct precursor of riboflavin 5'-monophosphate (FMN), which reduced form is the substrate of bacterial bioluminescence reaction (Figure 2).

In order to find out the potent transcriptional terminator for *lux* operon, the nucleotide sequence containing the *lux* and *rib*

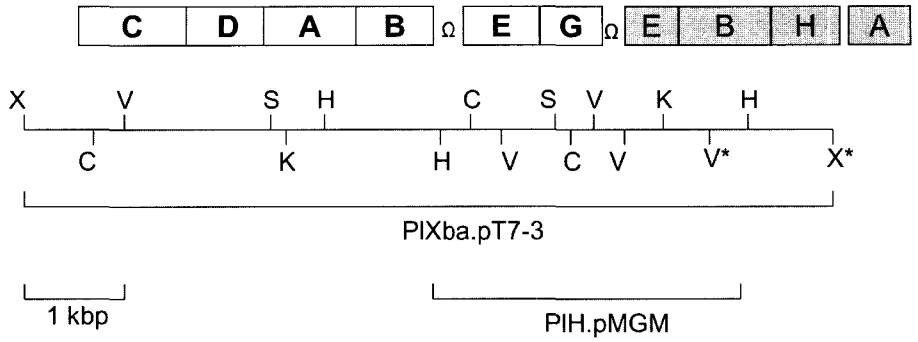

Figure 1. Physical map of the *lux* operon of *P. leiognathi*. The genes involved in the bioluminescence reaction are shown in the capital letter inside in the open box (*lux*CDABFEG) whereas the riboflavin genes are marked in the closed box (*rib*EBHA). The *rib*A gene, confirmed by the recent study [3], is located in outside of PlXba recombinant plasmid in pT7-3 originally obtained by the previous work [10]. The DNA fragment using transconjugation termination assay is shown to PlH.pMGM. Ω indicates the stem-loop structure located in the intergenic region shown in Figure 3. Restriction sites are labeled as follows: B, *Bam*HI; C, ClaI; H, *Hind*III; K, *Kpn*I; S, *Sac*I; V, *Eco*RV; X, *Xba*I. V*-X* is the radioactive DNA fragment for the Southern blot in Figure 5.

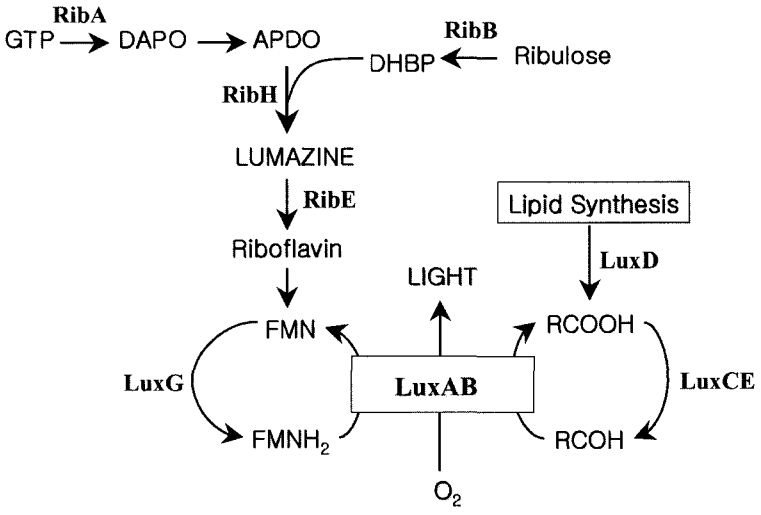

Figure 2. The genes involved in the bioluminescence reaction and the riboflavin synthesis were found in the *lux* operon from *P. leiognathi*. APDO, 5-amino-6-ribitylamino-2,4-pyrimidinedione; DAPO, 2,5-diamino-6-(5'-phosphoribosylamino)-4-pyrimidineone; DHBP, 3,4-dihydroxy-2-butanone 4-phosphate; Lumazine, 6,7-dimethyl-8-ribityllumazine; Ribulose, ribulose 5'-phosphate; RCOOH, fatty acid; RCOH, fatty aldehyde. LuxC, LuxD, and LuxE, represent for acyl-CoA reductase, acyl-transferase, and acyl protein synthetase, respectively. *lux*AB and *lux*G code for hetero-dimer of luciferase and flavin reductase, respectively.

genes were carefully checked from our previous sequence paper [1, 10]. 15 nucleotides upstream of *lux*E, a potent secondary structure element (-23.5 kcal/mol) (Figure 3A) was identified in an intergenic non-coding DNA segment of 83 base pairs separated from *lux*B and the initiation codon of *lux*E [10]. The stem-loop is separated from the initiation codon of *lux*E by 15 nucleotides segment that is very A/T rich. There is a relatively large intergenic region of 80 bp between *lux*E and the gene upstream (*lux*F or *lux*B) in *Photobacterium* and *Vibrio* species. This region contains a relatively conserved palindromic species [11] that could allow the transcribed mRNA to form strong stem-loop structure and stabilize upstream mRNA from 3'-exonuclease digestion [12, 13].

The first riboflavin gene *rib*E starts from 108 bp after *lux*G in the *lux* operon of *P. leiognathi*. In the intergenic regions, it was initially reported a sequence capable of forming a strong stem-loop structure was not present [1]. Careful examination revealed that three relatively weak secondary structures (less than -15 kcal/mol) capable of stem-loop structure, shown in the Figure 3B, were present in the intergenic region between *lux*G and *rib*E. In order to determine whether these segments work as a transcriptional terminator or not, *in vivo* termination assay with CAT reporter gene, was performed using conjugational methods.

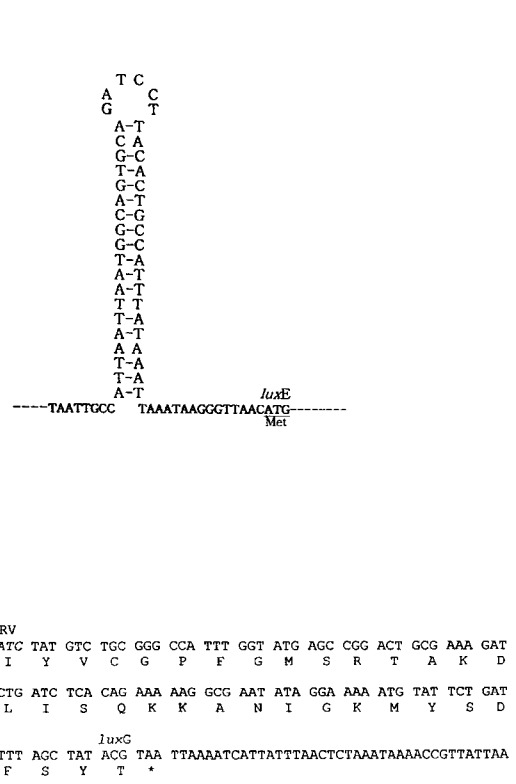

Figure 3. Stem and loop structure located between *lux*B and *lux*E (A), and the intergenic DNA sequence between *lux*G and *rib*E (B) from *P. leiognathi*. The original DNA sequences were obtained from the previous papers [1,10]. Sequencing Analysis of data was performed using the DNASIS and PROSIS programs of Hitachi Software Engineering Co., Ltd.

The *P. leiognathi* DNAs (PlH) containing the possible stem-loop structures shown in Figure 3A and 3B were inserted between a strong *lux* promoter and a reporter gene (chloramphenicol acetyltransferase, CAT) in pMGM plasmid and transferred by conjugation into *V. harveyi* and *P. leiognathi*. Both cases of *V. harveyi* (Vh) and *P. leiognathi* (Pl), the expression of the reporter gene were not significantly reduced by the insertion of the *P. leiognathi* DNA (PlH) extending from *lux*B to *rib* between a strong *lux* promoter and a reporter gene (lane 1 and 2 in Figure 4). Combined with the previous data [7, 14] that the CAT activities in this system were drastically decreased to less than 1 % on the insertion of the DNA containing the transcriptional terminator for the *lux* operon from *V. harveyi* and *V. fischeri*, it suggests that this region does not possess the transcriptional terminator.

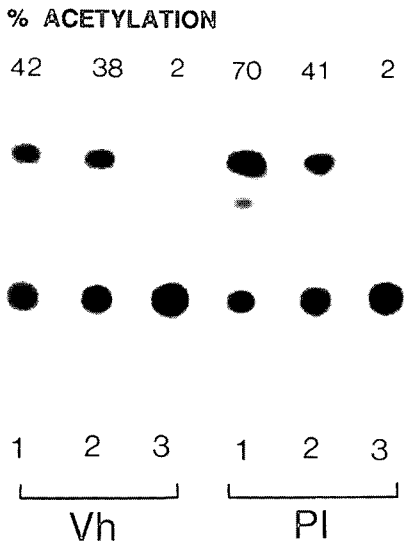

Figure 4. Fluorography of the ^{14}C-labelled substrates and products in the CAT assay resolved by thin layer chromatography. CAT activity was assayed from extracts of *V. harveyi* (Vh) *and P. leiognathi* (Pl), containing pMGM with strong *lux* promoter (GH, lane 1), and pMGM110 with insertion of the *P. leiognathi Hind*III DNA (GH-PlH, lane 2) fragments between the *lux* promoter and CAT reporter gene in pMGM 110, and the plasmid just containing *P. leiognathi Hind*III DNA fragment constructed by eliminating the strong *lux* promoter (PlH, lane 3). All sample were collected from the same amount cells around at OD_{660}= 3.5. The CAT activity was shown as % acetylation from extracts of *V. harveyi* and *P. leiognathi* containing pMGM. CAT assay was performed according to the previously described procedure [14].

However, the CAT genes were not expressed at all (lane 3 in Figure 4) in an analogous construct missing the *lux* promoter region in *V. harveyi* (Vh) as well as *P. leiognathi* (Pl). In other words, the CAT gene in recombinant pMGM plasmid could not be expressed without the appropriate DNA containing promoter activity. This result indicates that a promoter for expression of the *rib* genes was not present in this region.

Recently, functional analysis illustrated that the specific segments lay behind the *rib*H and *rib*A gene might form potential loops Ω_{oT} and Ω_{T1}-Ω_{T2}. The stem-loop structure Ω_{oT}, located in the intergenic region of *rib*H and *rib*A, is functioned as mRNA stability or/and for subregulation by alternative modulation, whereas the stem-loop structure Ω_{T1}-Ω_{T2}, located downstream of *rib*A, could be the transcriptional terminator of the *lux* operon [3].

In order to answer the question of whether the transcription of *lux* genes and *rib* genes is intergrated or not, it would be necessary to demonstrate directly that a polycistronic mRNA extends across the *lux*G-*rib*E region. As a RNase minus mutant of *P. leiognathi* does not exist, it was failed to get the Northern blot that do not completely across even all the *lux* genes. However, it is shown that a single strand DNA extending across the *lux*G to *rib*B is apparently fully protected by *P. phosphoreum* mRNA from S1 nuclease digestion (C. Y. Lee *et al.*, unpublished data). Taken together, it can be considered that these results are also correlated with the transcriptional termination analysis in the present study showing that the intergenic region does not contain any termination sites as well as promoter sites.

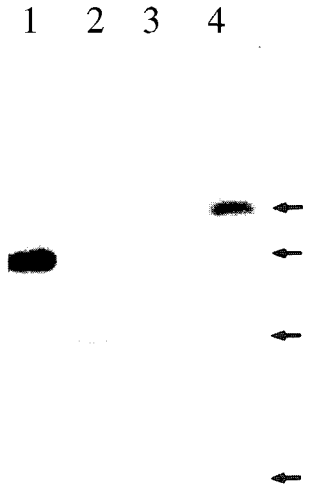

Figure 5. Fluorograph of Southern blot of *P. leiognathi* chromosomal DNA hybridizes to a labeled *lux* DNA. A radioactive *Eco*RV-*Xba*I *P. leiognathi* DNA (marked in V*-X* in Figure 1) encoding part of *lux*G to *rib*H was used to probe for *P. leiognathi* chromosomal DNA (10-20 μg) digested with various restriction endonucleases. The chromosomal DNA were digested with *Eco*RV-*Xba*I (lane 1), with *Eco*RV-*Xba*I-*Hind*III (lane 2), with *Kpn*I-*Xba*I-*Hind*III (lane 3), and with *Kpn*I-*Xba*I (lane 4). Asterisks indicate the size of hybridization to radioactive DNA, corresponding to 2.1, 1.7, 1.2, and 0.5 kbp, respectively.

Introduction of the polar mutation into the *lux* operon is restricted due to lack of the genetic studies on *P. leiognathi*. Therefore, Southern blot to test if riboflavin genes are single copy was performed. The chromosomal DNA of *P. leiognathi* was digested with with various different restriction enzymes and then probed with ^{32}P-labelled 1.7 kbp *Eco*RV-*Xba*I fragment contained *rib*B and *rib*H genes. As shown in Figure 5, a strong hybridization to a single DNA fragment 1.7 kbp *Eco*RV-*Xba*I (lane 1), or 2.1 kb *Kpn*I-*Xba*I fragment (lane 4), fragment was observed in each lane. Similarly, additional digestion with different enzyme gave small fragment DNA bound to the radioactive DNA (lanes 2 and 3 in Figure 5). No weakly large size of extra band was visible. This result shows that the riboflavin gene is present as a single copy in the chromosome of *P. leiognathi*.

In conclusion, the *lux* regulon in the *Photobacterium* species may therefore be quite complex consisting of the luciferase gene (*lux*AB), of the genes involved in supplying for long chain fatty aldehyde substrate (*lux*CDE) and in supplying riboflavin substrate (*lux*G and *rib*EBHA). And also, it can be suggested that the *rib* genes in *P. leiognathi* are part of the *lux* operon and are under the common regulation.

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