Bacterial Bioluminescence: Isolation and Expression of the Luciferase Genes from *Vibrio harveyi*

Abstract. *Genes for the luciferase enzyme of* Vibrio harveyi *were isolated in* Escherichia coli *by a general method in which nonluminous, transposon insertion mutants were used. Conditions necessary for light production in* E. coli *were examined. Stimulation of transcription of the genes for luciferase (*lux A *and* lux B*) was required for efficient synthesis of luciferase. To enhance transcription bacteriophage promoter elements were coupled to the cloned* lux *gene fragments.*

Luminous bacteria are ubiquitous in the marine environment and are found as planktonic forms and in symbioses with fish, squid, and other organisms. The planktonic forms are taxonomically related to those isolated as gut or light organ symbionts, and bacteria in the marine ecosystem probably move between planktonic and symbiotic niches (*1*). Bioluminescence can consume a significant portion of the cellular energy (*2*), and the expression of this phenotype is tightly regulated by physiological factors (*3*). To better understand how the luminescence system functions to provide an adaptive advantage to the bacterium and to know what genetic mechanisms regulate the expression of this property, we are isolating the genetic determinants for the luminescence system of the marine bacterium *Vibrio harveyi*. By reconstructing this system in *Escherichia coli*, the genetic components of the system can be identified and the regulatory mechanisms that govern the expression of these genes can be examined.

All luminous bacteria encode biochemically similar luminescence systems. The in vitro reaction is:

$$FMNH_2 + RCHO + O_2 \xrightarrow{\text{luciferase}} FMN + RCOOH + H_2O + \text{blue-green light}$$

All bacterial luciferases that have been purified consist of heterodimers containing an α (~ 42,000 molecular weight) and a β (~ 37,000 molecular weight) subunit. The aldehyde (RCHO) substrate is furnished by the action of a fatty acid reductase on the corresponding fatty acid (RCOOH), and the reduced flavin may be generated by an NAD or NADP-dependent oxidoreductase (*4*). Expression of the genes for luciferase (*lux*) occurs in late log phase and has been shown to be induced by a substance (autoinducer) that accumulates in growth medium (*5*). The production of light in many species is genetically unstable; nonluminous variants arise at high frequencies (*6*).

We have initiated genetic analysis with a seawater isolate of *Vibrio harveyi* (strain BB7). *Vibrio* mutants defective in light production were isolated by transposon mutagenesis with the use of a derivative of transposon Tn5 (Tn5-132), which encodes tetracycline resistance (*7*). Two of these mutants, Lux 21 and Lux 97, were defective in the synthesis of the bacterial luciferase and were used to clone DNA fragments containing the luciferase genes, *lux A* and *lux B*, which encode the α and β subunits. The *lux* genes are closely linked on the *Vibrio* genome, and a DNA fragment containing all of the *lux A* gene and part of the *lux B* gene has been isolated by an independent method (*8*). This DNA clone (pAG101) was used as a probe to hybridize with restricted *V. harveyi* DNA on nitrocellulose paper (*9*) to locate the transposon insertions in the Lux mutants (Fig. 1A). The transposon insertion mutations were located in the region NH_2-terminal to the *lux A* gene (*lux 21*) and near the NH_2-terminal portion of the *lux B* gene (*lux 97*). Since the DNA fragments containing the *lux* genes in the mutant strains also contained the transposon encoded tetracycline resistance, these fragments could be isolated by selecting for recombinant clones in *E. coli* which expressed tetracycline resistance. This selectable property of transposon mutations was used to clone the Bam HI restriction fragments containing the transposon insertions into the Bam HI site in plasmid pMC874 (*10*). Plasmid pBB101 contained the *lux 21* Bam HI fragment and pBB102 contained the *lux 97* Bam HI fragment (Fig. 1B). In the presence of exogenously added aldehyde, both clones manifest little or no light production (see Fig. 2).

This cloning strategy did not require the expression of the *lux* genes, but to examine *lux* gene expression the transposon mutation had to be removed to restore the integrity of the *lux* gene region. To accomplish this, two plasmids, each of which contained one of the *lux*::Tn5 fragments, were introduced into the same strain, thus allowing recombinational exchange to regenerate a functional complement of *lux* genes. Specifically, the Bam HI fragment was recloned from plasmid pBB101 into a compatible plasmid, pK04. The resultant plasmid, pBB105, was used to transform a strain containing pBB102. A tetracycline-sensitive derivative of pBB102 was found among the plasmid molecules isolated from this strain. Restriction enzyme analysis of this plasmid, pBB110, showed that the transposon had been

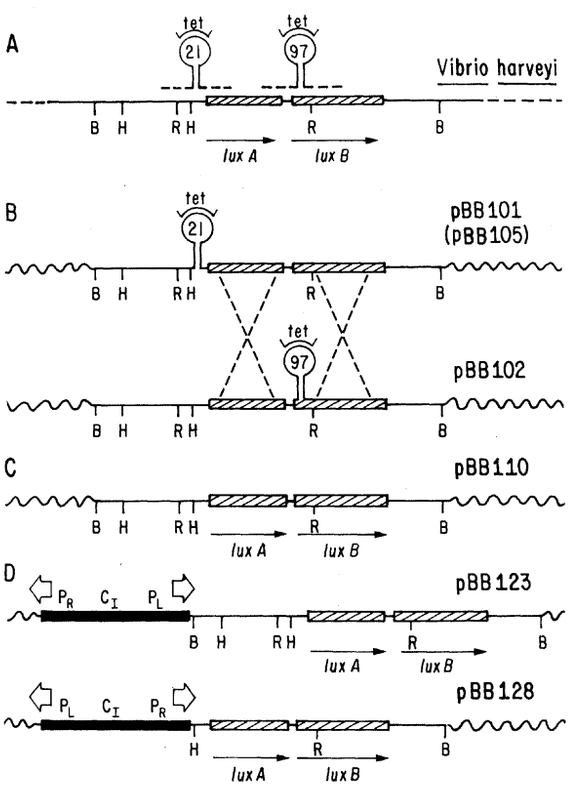

Fig. 1. Restriction map and strategy for cloning *lux* genes. (A) Restriction enzyme sites in the region of the *Vibrio harveyi* genome containing the genes for the α (*lux A*) and β (*lux B*) subunits of the luciferase protein. Restriction sites are: B, Bam HI; R, Eco RI; and H, Hind III. The Bam HI fragment containing the *lux* genes was 5 kilobase pairs (kbp) in length. Arrows under genes denote the direction of transcription. The location of transposon insertions is shown above the *Vibrio* map. (B) Cloned DNA fragment containing transposon inserts. The dashed lines demark the recombinational crossover events required to restore the continuity of the *lux* gene region as shown in (C). Restriction sites in the 5.6-kbp transposon and in the vehicle DNA (wavy lines) are not shown. Recombinant plasmids with the *lux* gene fragments aligned with bacteriophage λ promoter elements are described in (D). The Eco RI DNA fragment containing these promoters (P_L and P_R) was cloned into vector pBR322, and *lux* gene fragments were then introduced into this plasmid (pGMC12). Heavy arrows show direction of transcription from these promoter elements.

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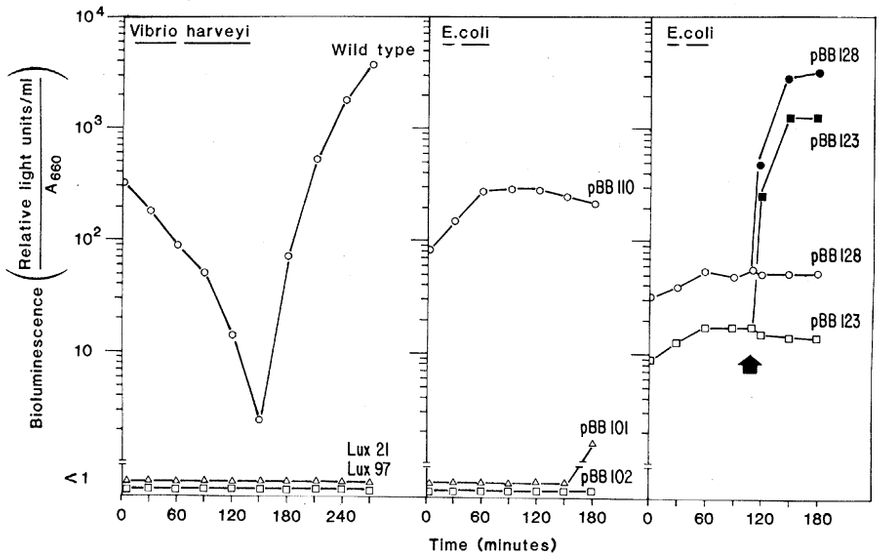

Fig. 2. Time course of expression of luminescence in *Vibrio* strains and in *E. coli* strains containing the recombinant plasmids shown in Fig. 1. Bacteria were grown in LB broth (*15*) for *E. coli* and in LB broth containing 2 percent NaCl for *Vibrio harveyi*. The *E. coli* host strain was ED8654 (*16*). Luminescence from 0.1-ml samples was measured with a photomultiplier apparatus (*5*), and 1 μl of C_{14}-aldehyde (tetradecanal) was added to all *E. coli* samples before measurement. To convert bioluminescence measurements to quanta per second the experimental value (Fig. 2) is multiplied by 5.5×10^8. The light-measuring apparatus was calibrated with the light standard of Hastings and Weber (*17*). Cultures were inoculated to an A_{660} of 0.03, and grown at 30°C except where noted. At 120 minutes *Vibrio* strains had grown to an A_{660} of 0.15. Heat induction commenced at 110 minutes (heavy arrow) at an A_{660} of 0.12 and continued until 120 minutes, at which time incubation was continued at 39°C. The spectrum of light emission from *E. coli* containing recombinant plasmids was identical to that from *V. harveyi*.

precisely removed (Fig. 1C). Furthermore, on addition of exogenous aldehyde the *E. coli* strain harboring this plasmid produced light, albeit at levels considerably less intense than that produced by *V. harveyi* (Fig. 2). The *lux* gene dosage was considerably higher in *E. coli* because of the multicopy (>20 per cell) nature of the plasmid vector.

The relatively weak light production measured from *E. coli* containing plasmid pBB110 might reflect limiting transcription from the cloned *lux* genes. Luminescence in *V. harveyi* cultures does not occur until late in logarithmic growth, at which time the accumulation of an "autoinducer" substance triggers expression of luciferase and the requisite substrates (Fig. 2A). Luminescence in *E. coli* appeared to be constitutively expressed. The regulatory elements responsible for induction may not be present or may not function in the recombinant clone pBB110. If *lux* gene transcription limited light production in *E. coli*, insertion of these genes into a vector designed to enhance transcription should result in increased bioluminescence. Two recombinant plasmids, pBB123 and pBB128, with the *lux* gene fragments aligned with the bacteriophage λ promoters P_L or P_R are shown in Fig. 1D. The vector used was a derivative of one constructed by Hedgpeth *et al.* (*11*), and transcription initiated at P_L and P_R is regulated by a thermosensitive repressor, also encoded by the vector. Thus, at 30°C light production with *E. coli* containing pBB123 and pBB128 (in the presence of exogenous aldehyde) was similar to that measured in pBB110. Upon heat induction, however, very considerable increases (~ 100 times) in light production were measured (Fig. 2).

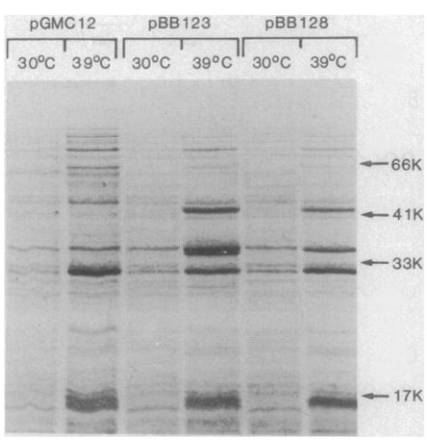

Fig. 3. Synthesis of ^{35}S-methionine–labeled polypeptides directed by *E. coli* containing recombinant plasmids. Bacteria were grown as indicated in Fig. 2 except that a minimal salt medium (*18*) was used. Labeling began 5 minutes after heat induction of cultures. Samples for electrophoresis were harvested after 30 minutes of labeling. Details of polypeptide labeling, electrophoretic separation, and autoradiography were as described (*18*). The position of molecular weight standards (in thousands) is shown to the right of the figure.

Since light production could be stimulated to an intensity similar to that measured for *V. harveyi* by coupling the *lux* genes to strong promoter elements, it appeared that expression of luminescence in *E. coli* was limited by inefficient transcription of the *lux* genes. The availability of substrates for the luciferase reaction might also limit light production in *E. coli*. In vitro measurements with extracts from cultures (Fig. 2) were performed as described (*12*). Since reduced flavin and aldehyde were provided in excess in vitro, the quantity of light production reflected the amount of luciferase present. Extracts prepared from cultures grown to the last time point (Fig. 2) produced quantities of light closely proportional to those values found in vivo. Two exceptions were light measurements from heat-induced cultures of pBB123 and pBB128 for which in vitro measurements were proportionally greater than in vivo measurements by a factor of approximately 3. This indicates that bioluminescence from in vivo induced cultures of *E. coli* with plasmids pBB123 or pBB128 which contained relatively large quantities of luciferase was limited by the availability of substrate, possibly reduced flavin.

Since light production was increased approximately 100-fold under the influence of the vector promoters, it was possible to identify the actual polypeptide products synthesized during heat induction. *Escherichia coli* cells containing pBB123 and pBB128 were labeled with ^{35}S-methionine during the heat-induction period. The labeled polypeptides were separated by electrophoresis on SDS-polyacrylamide gels and visualized by autoradiography (Fig. 3). The appearance of two prominent ^{35}S-labeled polypeptide bands was concomitant with heat induction and the presence of the *lux* genes. The molecular weight of these two polypeptides agreed well with that reported for *V. harveyi* luciferase subunits. Since both α and β contain nine methionine residues each (*13*), the intensity of labeling indicated the relative amounts of the two polypeptides synthesized; in this experiment approximately equimolar amounts of the putative gene products were synthesized. An additional polypeptide band with a molecular weight of ~ 35,000 was formed in heat-induced pBB123 cells but not in pBB128 cells; at present, no evidence connects this polypeptide with the luminescence system.

Our method for isolating the *lux* genes

was entirely general and could be applied to any gene for which transposon mutations were available. Since no requirement for *lux* gene expression was necessary in the course of the cloning procedure, we have not selected those recombinant plasmids which, because of mutation or manipulation, produced light in *E. coli*. *Lux* gene expression in *E. coli* as measured by light production was inefficient compared to that in *Vibrio* even though the *lux* genes in *E. coli* resided on a multicopy plasmid. If *lux* gene dosage were taken into account, the luminescence at 30°C in *E. coli* containing pBB123 was at least three orders of magnitude lower than *Vibrio*. It is difficult to assess the contribution of *lux* gene promoter (or promoters) to this low level of expression in *E. coli* since plasmid promoters can initiate transcription of cloned genes (*14*). Since luciferase activity could be greatly enhanced by the provision of bacteriophage λ promoters as in plasmid pBB123 and pBB128, *lux* gene expression in *E. coli* was probably limited by transcription from the *lux* gene promoter (or promoters). Accessory regulatory factors probably were not cloned with the *lux* genes or, alternatively, do not operate in *E. coli*. Since *V. harveyi* luciferase could be synthesized in *E. coli*, it should be possible to eliminate the need to supply exogenous aldehyde by cloning genes whose products fulfill the aldehyde requirement. Other genes required for expression of luminescence, such as those involved in autoinduction, could be isolated as well.

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Complex Venation Patterns in the Leaves of *Selaginella*: Megaphyll-Like Leaves in Lycophytes

Abstract. *Venation patterns of the leaves of two lycophytes,* Selaginella adunca *and* Selaginella schaffneri, *do not fit the definition of microphylls as having a single, unbranched vein. Although* S. adunca *has a simple pattern,* S. schaffneri *has a complexity matching that of many megaphylls, with numerous branching veins. The veins of* S. schaffneri *undergo an average of 13 branchings (range, 8 to 21), and reticulation between veins is frequent. The discovery of this radical departure from the familiar microphylls of lycophytes indicates that complex venation patterns in leaves do not necessarily arise from fusion of whole branches. The microphyll may not be as structurally stable as formerly believed.*

The two leaf categories traditionally recognized in vascular plants, microphylls and megaphylls, are separated on the basis of venation patterns. These categories have been of major importance in plant morphology and phylogeny for distinguishing major evolutionary clades. A microphyll has a single unbranched vein running more or less medially through the blade; a megaphyll has a complex, branching venation pattern. Only by reduction can the megaphyllous pattern be simplified to resemble that of a microphyll. Such convergence can be readily recognized by comparisons with near-relatives or other leaves on the same plant. Most plant morphologists regard the single vein as a primitive condition that has remained static since the Devonian (*1*). Microphylls have been valuable for identifying living and fossil members of the large group of plants generally known as Lycophyta or Microphyllophyta.

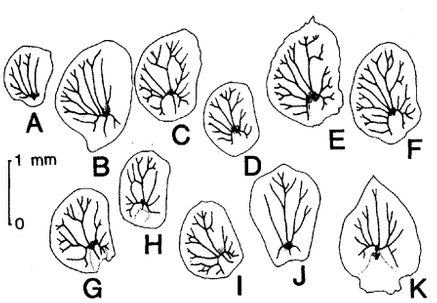

Fig. 1. Leaves of *Selaginella schaffneri*, showing complex venation patterns. (A to I) Lateral leaves. (J) Leaf at stem branching. (K) Sporophyll [specimens collected by J. G. Schaffner in 1879 in San Luis Potosí, Mexico (University of Michigan Herbarium)].

The microphyll is believed to have originated separately from the megaphyll. According to the enation theory, which is accepted by most morphologists, the microphyll arose as a nonvascularized superficial outgrowth of the stem (*1, 2*). The single vein of the microphyll arose de novo, as an extension of the vascular cylinder of the stem. The megaphyll arose by amalgamation of numerous stems, each with a vascular strand. According to the telome theory, branches, or telomes, aggregated and became modified to form leaves (*2*). Thus the veins of a megaphyll are traces of ancient stems once separate but now assembled into a united photosynthetic lamina, flattened for maximum efficiency (*3*).

Lycophyta have a long and complex history, for which there is extensive fossil documentation, including the remains of several orders now extinct (*1*). The group is represented today by three orders: Lycopodiales, the club mosses (about 400 species); Selaginellales, the spikemosses (about 800 species); and Isoetales, the quillworts (about 50 species). We describe here two species of *Selaginella* which show branching venation. One species possesses a ternate venation pattern with a midrib and two laterals. The other has a much more complex pattern with many branches, some of which form networks—a venation as complex as that of many unquestioned megaphylls. We studied fragments of dried herbarium specimens cleared for 24 hours in 5 percent sodium hydroxide solutions and bleached in dilute solutions of sodium hypochlorite.

The simpler pattern is shown by *Se-*

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