Annu. Rev. Genet. 1994. 28:117–139

GENETICS OF BACTERIAL BIOLUMINESCENCE

E. A. Meighen
Department of Biochemistry, McGill University, Montreal, Quebec, Canada, H3G 1Y6

KEY WORDS: luminescence, lux genes, homoserine lactones, autoinduction, luciferase

CONTENTS

INTRODUCTION

The emission of light by living organisms is of special interest in terms of the development and maintenance of a genetic system that requires high amounts of chemical energy. The genetic systems required for luminescence are complex. Genes are required that code for the enzyme catalyzing the light-emitting reaction (luciferase) and for the enzymes that convert the standard physiological metabolites into the high-energy substrates (luciferins) for the luminescent reaction.

In luminescent bacteria, twenty one different genes from at least three genera (*Photobacterium, Vibrio*, and *Photorhabdus*) have been implicated in the lu-

0066-4197/94/1215-0117$05.00

minescent (lux) system and have been designated as *lux* genes, although only five of these *lux* genes have been found in all luminescent bacteria (49, 50). Light emission is expected to be under strict regulation and to involve multiple controls for integrating light emission with energy metabolism and the growth and survival of the organism, as well as allowing response to environmental and nutritional signals. Indeed, luminescence in most bacteria is under cell density-dependent regulation. The light intensity is induced at the late stages of cellular growth similar to the control of metabolic processes involved in secondary metabolism and/or developmental changes triggered in many other bacteria as cells enter stationary phase (36). Identification of the *lux* autoinducers in *Vibrio* species as N-acyl homoserine lactones (12, 24) and the discovery that identical or related homoserine lactones are produced by plant and animal pathogens—including *Erwinia* (4, 5, 65), *Pseudomonas* (38, 62, 81), and *Agrobacterium* (64, 86) that affect virulence and other properties—has generated considerable interest in the *lux* genetic system and the regulation of luminescence in bacteria.

Luminescent bacteria are the most abundant and widespread of the luminescent organisms found in marine, freshwater, and terrestrial habitats. Their primary habitat is in the ocean in symbiotic, saprophytic and parasitic relationships as well as in a free-living mode. The luminescent species that have been most extensively investigated are the marine bacteria; *Vibrio harveyi, Photobacterium (Vibrio)*[1] *fischeri, Photobacterium phosphoreum* and *Photobacterium leiognathi*, and the terrestrial bacterium, *Photorhabdus (Xenorhabdus) luminescens*. Other luminescent bacteria that have been recognized but studied less include some strains of the freshwater pathogen, *V. cholera*, the aerobic species, *Shewanella hanedai*, and the obligate symbionts of the flashlight fishes, the deep-sea angler fishes, and the pyrosomes (35).

ORGANIZATION AND IDENTIFICATION OF THE *LUX* GENES

$$FMNH_2 + O_2 + RCHO \rightarrow FMN + H_2O + RCOOH + Light \qquad 1.$$

As depicted below, the bacterial luminescence reaction involves relatively simple compounds as substrates ($FMNH_2$, O_2, and a fatty aldehyde, RCHO) that are closely related to the basic metabolites of the cell. In this regard, the substrates for bacterial luciferase are different from the luciferins of other organisms with O_2 in that they are the only common element involved in the

[1]*Vibrio fischeri* and *Xenorhabdus luminescens* have recently been reclassified as *Photobacterium fischeri* (39) and *Photorhabdus luminescens* (10), respectively. Consequently most references refer to these species as *V. fischeri* and *X. luminescens*.

light-emitting reaction in different luminescent organisms. Consequently, the bacterial luciferase genes (*lux*A and *lux*B) and their gene products are not related in sequence to those from other organisms. Bacterial luciferase is a heterodimeric enzyme($\alpha\beta$) of 78 kDa containing two nonidentical subunits, α and β, with ~30% sequence identity coded by the *lux*A and *lux*B genes, respectively. Both subunits are required for luminescence; reports of low activity for individual subunits (73) have not been verified by other laboratories (46). A blue-green light is emitted with a maximum intensity at 480 to 490 nm.

Common lux Genes

Aside from the luciferase genes (*lux*AB), only three other genes (*lux*C, D, and E) have been associated with all lux systems in luminescent bacteria. The *lux*C, D, and E genes code for three proteins, which divert tetradecanoic acid away from the fatty acid biosynthesis pathway into the long chain fatty aldehyde substrate for the luminescent reaction. LuxD, the first thioesterase whose crystal structure has been elucidated (41), cleaves tetradecanoyl-ACP. The fatty acid product is activated by LuxE with ATP to form fatty acyl-AMP. In the presence of LuxC, the acyl group is transferred from acyl-AMP to LuxE and then to LuxC before being reduced with NADPH (49).

$$\underset{}{\overset{\text{LuxD}}{\text{RCO-X}\rightarrow\text{RCOOH}}}\overset{\text{LuxE}}{\rightarrow}\underset{\text{ATP}}{\overset{\text{LuxC}}{\text{RCO-AMP}}}\rightarrow\text{RCO-}\textit{Lux}\text{E}\rightarrow\underset{\text{NADPH}}{\text{RCO-}\textit{Lux}\text{C}}\rightarrow\text{R-CHO}$$

The three proteins form a weakly associated fatty acid reductase complex in *P. phosphoreum* that generates tetradecanal (85). Shorter chain aldehydes, in particular decanal and dodecanal, are routinely used in the in vitro assays for luciferase activity, although tetradecanal is thought to be the natural substrate for the luminescent reaction, consistent with the specificity of the fatty acid reductase complex.

Species-specific lux Genes

Although the luciferase and fatty acid reductase genes are the only common *lux* genes in luminescent bacteria, new *lux* genes are continually being discovered in specific luminescent bacteria, adding to the complexity and diversity. Their discovery and the identification of their roles help in understanding how the luminescent system is connected with other metabolic pathways and related to the overall physiology of the different bacteria.

Figure 1 depicts the *lux* operons containing the common *lux* structural genes (*lux*ABCDE) of different species and shows their organization and their relationship to other genes that are part of, or closely linked to, the primary *lux*

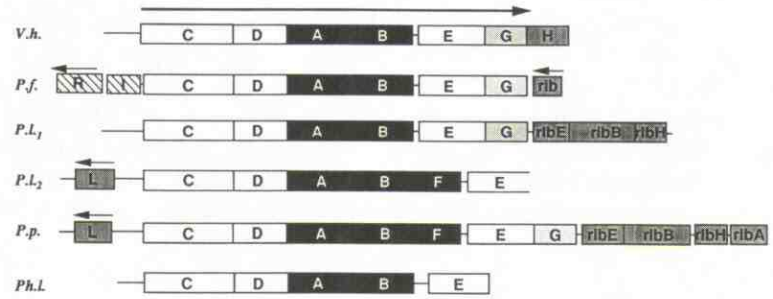

Figure 1 Organization of the *lux* operons of bioluminescent bacteria. *Lux* operons in order from the top are: *V. harveyi* (V.h.), *P. fischeri*(P.f.), two strains or subspecies of *P. leoignathi* (P.l.$_1$ and P.l.$_2$), *P. phosphoreum*(P.p.), *Ph. luminescens*(Ph.l.). Transcription (indicated by arrows) is from left to right unless indicated otherwise. Single letters refer to *lux* genes and linked genes connected to riboflavin synthesis are indicated by *rib*.

operon. In all lux systems, the luciferase genes (*lux*AB) are flanked by the *lux*CDE genes in the order of *lux*CDAB...E.

In the marine *Photobacterium* and *Vibrio* genera, the *lux*E gene is immediately followed by the *lux*G gene (77, 79), which codes for a protein related in sequence to enzymes involved in electron transport and flavin reduction (3). However, *lux*G has not been directly demonstrated to be a flavin reductase and recent evidence has implicated another enzyme in the production of the $FMNH_2$ substrate for the luminescent reaction (82). These results together with the absence of *lux*G in the *lux* operon of the terrestrial *Photorhabdus* genus (52) leave the role of *lux*G in luminescence unresolved.

In some *Photobacterium* species, a gene (*lux*F) related in sequence to the luciferase genes is present between *lux*B and *lux*E (6, 47). LuxF is a nonfluorescent flavoprotein containing a flavin adduct covalently linked with tetradecanoic acid, as demonstrated by X-ray crystallography (59); the function of LuxF is unknown. Interestingly, only *Photobacterium* strains with *lux*F have another gene, *lux*L, located about 600 bp upstream of the start of the *lux* operon transcribed in the opposite direction (Figure 1), distinguishing *P.l.*$_1$ strains from *P.l.*$_2$ and *P.p.* strains (43). *lux*L codes for the lumazine protein that binds lumazine and/or riboflavin and modulates the spectrum and efficiency of light emission in these species (45). Another gene, *lux*Y, related in sequence to *lux*L but not linked to the *lux* operon, has also been discovered in certain strains of *P. fischeri*. LuxY results in a shift of light emission to longer wavelengths rather than to the shorter wavelengths found for LuxL (27).

Aside from regulatory genes, only the *lux*H gene in *V. harveyi*, located immediately after *lux*G (79), is part of the *lux* operon. This gene has a strong

sequence similarity to *rib*B in *Escherichia coli*, which codes for dihydroxy-4-butanone phosphate (DHBP) synthetase, an enzyme involved in the riboflavin biosynthetic pathway. Recent experiments have clearly shown that the *rib*-EBHA genes are closely linked downstream of *lux*G in *P. phosphoreum* and *P. leiognathi* (Figure 1) and that the protein products have riboflavin synthetase, DHBP synthetase, lumazine synthetase, and GTP cyclohydrolase II activities, respectively (42). In *P. fischeri,* a convergent gene is present (44), whose protein product is related in sequence to RibG in *Bacillus subtilis*, while in *Ph. luminescens, rib* genes are not linked to the *lux* genes.

That so many of the genes linked to or part of the lux system are related in sequence indicates that gene duplication played a strong role in evolution of the luminescence system (61). Among the sets of proteins related in sequence are: (*a*) the luciferase subunits (LuxA and LuxB) and the nonfluorescent flavoprotein (LuxF); (*b*) the lumazine protein (LuxL), the yellow fluorescent protein (LuxY), and riboflavin synthetase (RibE), and (*c*) DHBP synthetase (RibB) with the amino and carboxyl terminal domains related to LuxH and RibA, respectively (42, 61).

Regulatory lux Genes

The only regulatory genes linked to the *lux* structural genes are found in the lux system in *P. fischeri*. The *lux*I gene is cotranscribed with the *lux* structural genes as part of the right operon (Figure 1) and is required for generation of the autoinducer, N-(3-oxohexanoyl) homoserine lactone, involved in regulating the expression of luminescence in this bacterium (24, 28, 29). The *lux*R gene in *P. fischeri*, transcribed in the opposite direction on the left operon, is located 219 bp upstream of *lux*I and codes for a receptor protein for the autoinducer (2, 29, 70, 74). In other species, a noncoding region extends upstream for over 400 bp (43, 52, 56). However, unlinked regulatory genes have been discovered in *V. harveyi* (7, 8, 9, 48). These include genes that code for proteins proposed to be involved in autoinducer synthesis (*lux*L*M), and reception (*lux*N and *lux*Q), and as repressors (*lux*O), or activators (*lux*R*). The LuxL* and LuxR* proteins in *V. harveyi* are not related in sequence or function to the LuxL and LuxR proteins in the *Photobacterium* species.

Are All lux Genes Involved in Luminescence?

The relationship of a gene (and its protein product) to luminescence can be defined by various criteria including genetic linkage, specific functional properties associated with, or necessary for, light emission and/or a common regulatory mechanism. The identification of the *lux* genes and their organization was initially based on cloning of a 9-kbp DNA fragment in *P. fischeri* that on transfer into *E. coli* resulted in a luminescence phenotype regulated in the same manner as in *P. fischeri* (28, 29). Complementation in *E. coli* of compatible

plasmids containing *lux* DNA from *P. fischeri* with polar transposon insertions (Tn*5* and mini-Mu) and/or nonpolar single point mutations helped to define the genes in the left (*lux*R) and right operons (*lux*ICDABE). Sequence and functional analysis has confirmed this assignment of *lux* genes in the two operons (29) and later resulted in the recognition of another gene, *lux*G, after *lux*E (77). 3′-S1 nuclease experiments identified a bidirectional terminator centered 24 bp downstream of *lux*G for overlapping mRNAs from *lux*G and a convergent gene related in sequence to *rib*G (44, 77).

In contrast to *P. fischeri,* transfer of the *lux* structural genes in *V. harveyi* (*lux*CDABEGH) along with flanking regions extending up to 5 kbp into *E. coli* did not generate a luminescence phenotype regulated in the same manner as in *V. harveyi* (54). Indeed, the level of light emission was extremely low and therefore suggested that unlinked regulatory elements were missing. The organization of the *lux* genes in *V. harveyi* was thus initially defined by sequence and functional analyses. Northern blots showed that *lux* mRNA extended well past the end of *lux*E (53), leading to the discovery of two additional genes, *lux*G and *lux*H (79), after *lux*E with a termination site found 52 bp after *lux*H by 3′ S1-nuclease mapping. Polycistronic mRNAs starting in front of *lux*C and *lux*D/*lux*A and terminating after *lux*B and *lux*H may partially account for the higher expression of the luciferase genes (*lux*AB) than of the flanking fatty acid reductase genes (*lux*CDE) (55, 79).

Transposon mutagenesis using mini-Mu*lac* transduced into *V. harveyi* with phage P1 demonstrated that insertions in two different loci resulted in dark mutants (48). One locus was defined by the five common structural genes (*lux*CDABE) and the second locus coded only for the unlinked *lux*R* gene. As dark mutants with insertions in *lux*G and *lux*H were not detected, these results also raise the question of whether the LuxG and LuxH proteins are actually involved in the luminescent system or are only required under certain physiological conditions.

Only sequence and functional analyses have been used to identify the *lux* genes in *P. phosphoreum, P. leoignathi,* and *Ph. luminescens.* In *Photobacterium species,* a transcription termination site in the *lux* operon before the downstream *rib*E gene could not be identified (42). Initial studies have indicated that synthesis of luciferase and RibE (riboflavin synthetase) are induced at the same time during growth of *P. phosphoreum.* This finding raises the possibility that the downstream *rib*EBAH genes are under control of the lux promoter and part of the same operon (11).

The lux system in *Ph. luminescens* has been cloned and sequenced for three different strains. The nucleotide sequences of the *lux*CDABE genes in two of the strains were 85–90% identical but diverged completely 350 bp upstream of *lux*C and immediately after *lux*E (52). This divergence suggests that the lux system in terrestrial bacteria may have evolved via horizontal gene transfer of

the five common *lux* genes into different locations on the genome of *Ph. luminescens*.

Intergenic Elements in the lux Operons

The *lux* genes are closely linked in the *lux* operon, with less than 50 bp separating most genes. The *lux*E and *lux*G genes are so closely linked in some species that the *lux*G ribosome binding site is located in the coding region of *lux*E. Only between *lux*E and the gene upstream (*lux*B or *lux*F) is there a relatively large intergenic region of 80 bp extending up to 300 bp in some strains of *Ph. luminescens*. This region contains in *Photobacterium* and *Vibrio* species a relatively conserved palindromic sequence (6, 43, 51) that could allow the transcribed mRNA to form strong hairpin loops and stabilize upstream mRNA from 3′-exonuclease digestion.

In the *lux* operon in *Ph. luminescens*, a 126-bp repetitive unit is found between *lux*B and *lux*E (52). This 126-bp unit, which has been referred to as an enteric repetitive intergenic consensus (ERIC) sequence or intergenic repetitive unit (IRU), is present in single copies in the intergenic regions of specific operons and in multiple copies in the genomes of other bacteria including *E. coli* and *Serratia marcescens* (37, 71). In strains of *Ph. luminescens* isolated from soil or insects, a single IRU or ERIC unit was detected between *lux*B and *lux*E, whereas in strains isolated from humans, multiple copies were detected in one operon, including two copies between *lux*B and *lux*E and one copy between *lux*D and *lux*A (52). The function of this repetitive unit is unknown, and expression in *E. coli* of the *lux* operons of *Ph. luminescens* isolated from humans and insects was identical.

CELL DENSITY-DEPENDENT INDUCTION OF THE LUMINESCENT SYSTEM

The dependence of expression of the lux system on cell density, reflected in high levels of luminescence only at the late stages of cellular growth, provides perhaps the most ideal system for investigating developmental changes in bacteria that occur in dense cultures. As light intensity can be instantaneously and continuously measured across an extremely wide range without disruption of the cellular structure, the lux system provides by far the greatest opportunity to monitor the contributions of the structural and regulatory genetic elements to the biological function.

The induction of luminescence in *V. harveyi* with cell growth is illustrated in Figure 2A, which shows the lag in luminescence at low cell densities followed by a large change in light intensity that can be readily measured over a millionfold range. The key component is a small autoregulator, referred to as the *lux* autoinducer and excreted into the media, that allows

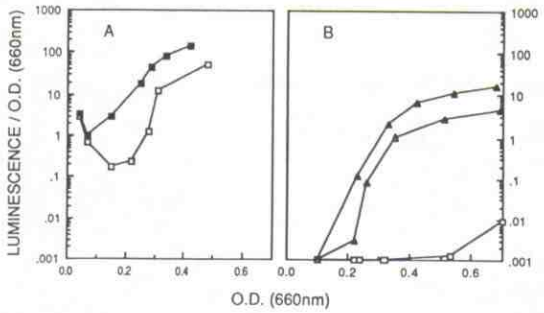

Figure 2 Dependence of luminescence of *V. harveyi* on growth and the *lux* autoinducer. A. *V. harveyi* grown in complex media plus (■ = filled square) and minus (❑ = open square) conditioned media (20% v/v) containing autoinducer. B. An autoinducer-deficient mutant of *V. harveyi* grown in complex media (❑ = open square) or in the presence of conditioned media (▲ = filled triangle) or synthetic N-(3-hydroxybutanoyl) homoserine lactone (Δ = open triangle).

the sensing of the culture density in the immediate microenvironment and thus allows communication between cells. Perhaps the pivotal experiment was the recognition that addition of media conditioned by the prior growth of cells of *V. harveyi* decreased the lag in induction of luminescence (23; Figure 2A). Extraction of the media followed by extensive purification has led to the identification of N-(3-hydroxybutanoyl) homoserine lactone (12) and N-(3-oxohexanoyl) homoserine lactone (24) as autoinducers of *V. harveyi* and *P. fischeri*, respectively. Figure 2B shows a similar but more sensitive assay for autoinducer production involving the use of an autoinducer-deficient mutant of *V. harveyi* (13). Both conditioned media and synthetic autoinducer N-(3-hydroxybutanoyl) homoserine lactone considerably increase light intensity.

The primary genetic elements involved in the *lux* regulatory systems of *P. fischeri* and *V. harveyi* and their proposed roles in the autoinduction of luminescence in these two species are outlined in Figure 3. In spite of the similar structures of the *lux* autoinducers in *P. fischeri* and *V. harveyi,* completely different regulatory systems have been proposed to explain the cell density-dependent induction of luminescence in these species. In *P. fischeri*, the left (*lux*R) and right (*lux*ICDABEG) operons are controlled by the autoinducer and the LuxR regulatory protein, with the primary effect being activation of the right operon (28, 69). In *V. harveyi,* a complex system of unlinked *lux* genes is implicated involving more than one autoinducer acting in conjunction with

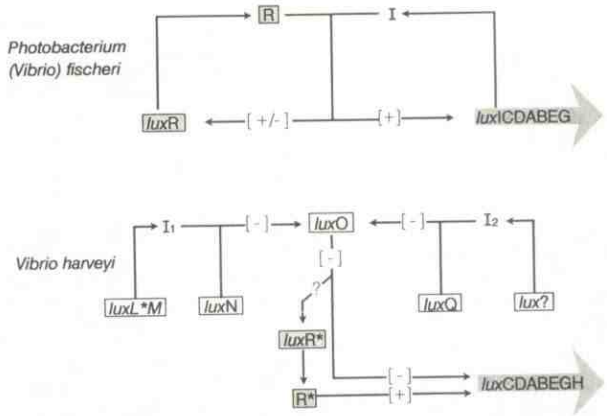

Figure 3 Models depicting the role of the lux regulatory proteins and autoinducer (I) in controlling expression of the *lux* operons in *P. fischeri* and *V. harveyi*. Activation and repression indicated by [+] and [−], respectively. Adapted from models by Silverman and coworkers (8, 9, 29).

a set of regulatory proteins as repressors coupled with a positive activator, LuxR* (7–9, 48).

Regulation of the lux System in Photobacterium (Vibrio) fischeri

In *P. fischeri,* the first gene on the right operon, *lux*I, is required for production of the autoinducer, N-(3-oxohexanoyl) homoserine lactone (I). At low cell density, the autoinducer slowly accumulates in the medium until it reaches a threshold concentration. It then interacts with the LuxR protein (R) coded by the left operon generating a positive feedback loop (Figure 3) that stimulates transcription of the *lux*ICDABEG operon (28, 69). Synthesis of the *lux* autoinducer is positively autoregulated (25). In addition, evidence exists that the autoinducer-LuxR pair inhibit as well as activate expression of the left (*lux*R) operon (22, 66, 67).

The transcription start sites for the right and left operons were located by S1 nuclease mapping 21 and 43 bp before the initiation codons of *lux*I and *lux*R, respectively (31; Figure 4). Primer extension has revealed two additional transcription start sites for *lux*R located 8 to 10 bp on each side of the initial site, with the two proximal sites to *lux*R being dependent on the presence of cAMP and CRP (67). Sequences similar to the −10 and −35 promoter recognition sites in *E. coli* could be identified for the left operon, but only the −10 Pribnow box could be readily recognized for the right operon (31).

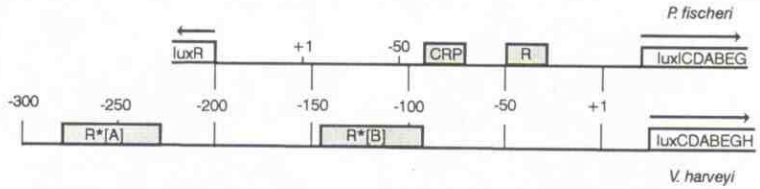

Figure 4 The *lux* operator/promoter regions. The number of nucleotides from transcription start sites (+1) for the *lux* operon of *V. harveyi* (*lux*CDABEGH) and the right *lux* operon of *P. fischeri* (*lux*ICDABEG) are indicated in the center and from the transcription start site for the left *lux* operon of *P. fischeri* (*lux*R) at the top. Binding sites for the regulatory proteins, R, R*, and CRP are represented by boxes.

CONTROL OF THE RIGHT *LUX* OPERON In *E. coli*, transformed with the *lux* regulatory region and *lux*R (in *cis* or in *trans*), addition of autoinducer stimulates expression of the promoter of the right (*lux*ICDABEG) operon about 100-fold providing that the DNA extends upstream through *lux*R (17). The specific site for binding of the LuxR-autoinducer complex has been identified as a 20-bp palindrome centered about 40 bp upstream of the transcription start site (Figure 4). Deletion of 12 bp or point mutations in the palindrome significantly dropped the basal level of expression and abolished the effects of autoinducer in most instances. Removal of DNA immediately upstream of the palindrome including *lux*R (now supplied in *trans*) increased basal expression tenfold in *E. coli* with autoinducer now stimulating expression only about twofold. These results have demonstrated a critical role for the 20-bp palindrome but also raise the question of the involvement of the DNA immediately upstream of the palindrome with respect to interactions with the LuxR-autoinducer complex and expression of the right operon. In addition, LexA has also been implicated in the inhibition of the luminescence system (83), possibly by acting as a competitor of LuxR for the 20-bp palindrome in the *lux* operator (69).

CONTROL OF THE LEFT *LUX* OPERON Evidence has been obtained for both positive and negative regulation of *lux*R by the autoinducer-LuxR complex, which can be decreased by mutation or deletion of the 20-bp palindrome required for LuxR binding. Stimulation of *lux*R expression by autoinducer and LuxR was observed if most of the right operon was removed or if low concentrations of autoinducer and LuxR were present (66). Negative regulation by LuxR and autoinducer could only be observed when the concentration of LuxR was high and DNA from the right promoter extending downstream from *lux*I and *lux*C into *lux*D was present (17). It has been proposed that there is a weak binding site for LuxR in *lux*D that interacts through DNA looping

with LuxR bound to the *lux* operator (68). Deletion of the negative element results in a shift from negative to positive regulation by the autoinducer-LuxR complex, although the basal level of expression is also significantly decreased.

INTERACTION OF LUXR AND AUTOINDUCER Interaction of LuxR and the autoinducer was initially indicated by the ability of high concentrations of autoinducer to compensate for the defects of some amino terminal domain mutants of LuxR in activation of the *lux*ICDABEG promoter (70, 74). Complete but not partial deletion of the amino terminal domain of LuxR produced a truncated protein that could activate the *lux*ICDABEG promoter in the absence of autoinducer. Binding of autoinducer to the amino-terminal domain was thus proposed to block interaction with the carboxyl-terminal domain and allow the latter region to bind to the *lux* operator (15). Direct support for this proposal has come from experiments demonstrating that the purified carboxyl terminal domain of LuxR is soluble and can specifically bind to the *lux* operator region in the presence of RNA polymerase (75). As LuxR is located in the membrane, it is likely that the amino-terminal autoinducer-binding domain is bound to the cytoplasmic membrane with the carboxyl-terminal DNA-binding domain extending into the cytoplasm (40).

Interestingly, negative autoregulation of the *lux*R promoter was abolished by all amino terminal deletions of LuxR except for removal of the first few amino acids (15). Moreover, partial deletion of the carboxyl terminal DNA-binding domain of LuxR, which eliminated activation of the right operon, did not block negative autoregulation of the left operon (16). These results raise further questions about the role of the autoinducer-LuxR complex in the inhibition of transcription of the promoter of the left operon.

More direct evidence has been obtained for interaction of LuxR and autoinducer by demonstrating the accumulation of autoinducer in *E. coli* cells cotransformed with both *lux*R and the *gro*ESL genes (2). These results indicate that the GroESL chaperones are necessary for folding of LuxR into a suitable conformation to bind autoinducer, particularly when present at high concentrations. The low level of luminescence in *htp*R⁻*E. coli* cells missing the σ_{32} transcription factor required for expression of the *gro*ESL genes is consistent with this result (84). Indeed, *htp*R⁻ cells with the *gro*ESL genes expressed by a σ_{32}-independent promoter, are highly luminescent (1, 18).

CRP-cAMP REGULATION A CRP-binding site has been located between the transcription start sites of the left and right *lux* operons (Figure 4) by sequence analysis and DNAse footprinting (69). Transformation of CRP and cAMP mutants of *E. coli* with plasmids containing the *lux* regulon from *P. fischeri* with the transposon mini-Mu(*lac*Z⁺) inserted into *lux*R resulted in low β-galactosidase activity with cAMP stimulating expression in the cAMP⁻ mutant

(19). Using luciferase as a reporter gene for the right operon, luminescence was two- to threefold higher in the absence of CRP or cAMP, although expression was very low due to the absence of a functional LuxR. If *lux*R was added in *trans* under control of the cAMP-independent *tac* promoter then expression of the right operon was high and independent of the presence of cAMP and CRP (20). Mutation of the CRP-binding site decreased expression of the left operon but did not prevent stimulation by the LuxR-autoinducer pair.

These results are consistent with cAMP-CRP activating expression of the left operon containing *lux*R; the production of LuxR, along with the autoinducer in turn, then activates expression of the right operon and induces luminescence. As cAMP-CRP may also inhibit expression of the right operon to some degree and because expression of the left operon appears to be inhibited by autoinducer-LuxR, regulation of expression of the promoters of the right and left operons in *P. fischeri* may reflect a form of competitive balance between these sets of transcriptional regulators (20).

Regulation may be even more complex as expression of the *lux* regulon in *E. coli* is dependent on the FNR transcriptional activator under anaerobic conditions (60). A potential FNR-binding site sequence has been recognized between the CRP-binding site and the *lux*R transcription start site (Figure 4), which suggests that *lux*R may be under positive transcriptional control by a FNR-like protein (60).

SOME CAUTIONARY NOTES The interpretation of these results is complex as the dependence of expression of the *lux*R promoter on the autoinducer-LuxR pair is usually small (two- to threefold), dependent on the extent of growth, and is measured in *E. coli* often with high levels of LuxR and with genetic constructs that vary in expression depending on their length. Moreover, the left operon may also be under negative regulation at the posttranscriptional level (30). The experiments are complicated further by the fact that deletion of DNA in the coding region of *lux*R near the *lux* operator greatly enhances the basal expression of the promoters of both the left and right operons (6, 17). Some caution is also warranted in interpreting the results with the right *lux* promoter inasmuch as cell density-dependent modulation of expression of the *lux*ICDABEG promoter in *E. coli* has been observed in the absence of LuxR and autoinducer (21).

Regulation of the lux System in Vibrio harveyi

The regulatory system for control of luminescence in *V. harveyi* differs substantially from that for *P. fischeri* despite similar structures for the autoinducers. Based on genetic studies, a model has been recently proposed (7, 8, 9) that involves two signaling systems with different autoinducers (I_1,I_2)

and sensors. Figure 3 presents a model that encompasses all the implicated *lux* regulatory genes, including the LuxR* activator.

*LUXR** CODES FOR AN ACTIVATOR Transposon mutagenesis of the genome in *V. harveyi* demonstrated that mutation of a locus other than the *lux*CDABE locus resulted in loss of light (48). The second locus contained a single gene, *lux*R*, which coded for a 24-kDa regulatory protein (72). Complementation of *E. coli* transformed with the *lux* operon from *V. harveyi* by *lux*R* resulted in a 10,000-fold stimulation in luminescence. However, the characteristic cell density-dependent induction of light and the dependence on autoinducer observed in *V. harveyi* could not be reconstituted and light levels in *E. coli* were still 10- to 100-fold lower than in the native strain.

BINDING OF LUXR* TO THE *LUX* OPERATOR IN *V. HARVEYI* Binding of LuxR* occurs at two sites (A and B) located about 100 and 250 bp upstream of the transcription start site of the *lux*CDABEGH operon (78; Figure 4). In *E. coli* transformed with the *lux* operon, a transcription start site was located in the B region; complementation with *lux*R then generated the same transcription start site as found in *V. harveyi* and greatly increased *lux* mRNA concentrations (80). Transconjugation of the *lux*CDABEGH promoter with the chloramphenicol acetyl transferase (*cat*) gene as a reporter group back into *V. harveyi* has shown that removal of the A site decreases expression by about twofold. However, deletion of only part of the B site decreases expression to background levels (58). As the LuxR* binding sites are quite large as defined by DNAse protection experiments using extracts of *V. harveyi* (78), other proteins may bind to this site in a cooperative interaction with LuxR*.

Expression of luminescence in *V. harveyi* is repressed by glucose; the same result is also observed for expression of the *cat* gene under control of the *lux* promoter in *V. harveyi* (57). Interestingly, the only putative CRP-binding sites in the *lux*CDABEGH operator region based on weak sequence homology are located in the A and B binding sites. These results may suggest that a CRP site is located upstream of the *lux* promoter or that other components required for expression of the *lux* operon, such as *lux*R*, are under CRP-cAMP control. Mobility shift analyses of the luxR* operator/promoter DNA have indicated that two different proteins can bind upstream of the *lux*R promoter (14).

NEW REGULATORY GENES MODULATING EXPRESSION OF THE *LUX* OPERON IN *V. HARVEYI* To screen for additional genes, Bassler et al (7) used cosmid vectors containing large (20-kbp) regions of the *V. harveyi* genome that restored light on transconjugation into spontaneous dim mutants. One mutant was complemented by a DNA fragment containing the closely linked genes *lux*L*, M, and N (7). The location of the genes was established by transposon mutagenesis

of the inserted DNA in the cosmid vector, followed by recombination back into the *V. harveyi* genome. Surprisingly, all transposon mutants in *lux*L*MN could restore luminescence to colonies grown on solid media. However, analyses in liquid cultures demonstrated that the cell density-dependent induction had been altered with insertions in *lux*L*M that rendered the cells defective in autoinducer synthesis and with insertions in *lux*N that prevented response to the N-(3-hydroxybutanoyl) homoserine lactone autoinducer (I_1). Further analyses also revealed that the *lux*L*M mutants still excreted a compound(s) that could cause partial induction of the luminescence system; this finding suggests that a second autoinducer (I_2) may be produced (7; Figure 3).

These same researchers (9) recently identified another locus, designated *lux*Q, that appears to reflect part of a second signaling system. By nitrosoguanidine mutagenesis of a strain with a defect in the first signaling system(I_1^-), dim mutants were found that could not respond to conditioned media containing I_2 although all mutants could produce I_2, indicating that only the sensor function had been mutated. Complementation of one of these mutants with genomic DNA from *V. harveyi* resulted in identification of the *lux*Q locus, which contained two adjacent genes, *lux*P and *lux*Q. The ability of these two genes to restore luminescence on complementation was blocked on transposon insertion; the insertions in *lux*P may reflect polar effects on the *lux*Q gene located immediately downstream. Recombination of a defective *lux*Q locus back into wild-type *V. harveyi* gave bright cells that produced but did not respond to I_2.

A third locus, which restored light on conjugation into certain dim mutants of *V. harveyi* (8), contained the *lux*O gene, whose location was defined by transposon insertions. Recombination of the *lux*O transposon mutants into the genome of wild-type *V. harveyi* gave bright cells with constitutive expression of the lux system, which did not respond to either autoinducer (I_1 or I_2). From these latter results it was proposed that LuxO is a negative regulator of the *lux*CDABEGH operon in *V. harveyi* and that effects of both inducer systems were mediated through LuxO, presumably by also acting as negative regulators (see Figure 3). The potential interaction of these components with LuxR*, and, in particular, LuxO, should be considered. Recent experiments have suggested that the expression of *lux*R is also controlled by N-(3-hydroxybutanoyl) homoserine lactone (C Miyamoto & E Meighen, unpublished data). LuxO could therefore decrease the expression of the *lux*CDABEGH operon in *V. harveyi* by inhibiting the expression of *lux*R*.

The sequence of LuxO is related to the response regulator in the two component signal-response phosphorylation systems possessing a C-terminal domain for binding DNA and a N-terminal domain for interaction with the sensor (8). LuxN and LuxQ, on the other hand, appear to have kinase and regulatory domains but no DNA-binding domain (7, 9). A potential membrane-

spanning domain in the amino terminus of LuxN has been proposed as an autoinducer binding site for I_1. LuxP may serve this role for I_2 because such a domain is absent in LuxQ and LuxP is related in sequence to a periplasmic ribose binding protein (9).

HOMOSERINE LACTONES AS GENERAL SIGNAL TRANSDUCTANTS

The discovery that N-substituted homoserine lactones (HL) are produced by nonluminescent bacteria and control such properties as virulence of plant and animal pathogens has greatly stimulated interest in the role of the homoserine *lux* autoinducers in cell density-dependent regulation of luminescence (32). Figure 5 compares the structures of the homoserine lactones discovered in luminescent and nonluminescent bacteria and shows the structures of some of the closely related 2,3-disubstituted butyrolactones that function as autoregulators in *Streptomyces* species (36).

In the plant pathogen, *Erwinia carotovora*, OHHL controls synthesis of the antibiotic carbapanem and the exoenzymes involved in virulence, which are

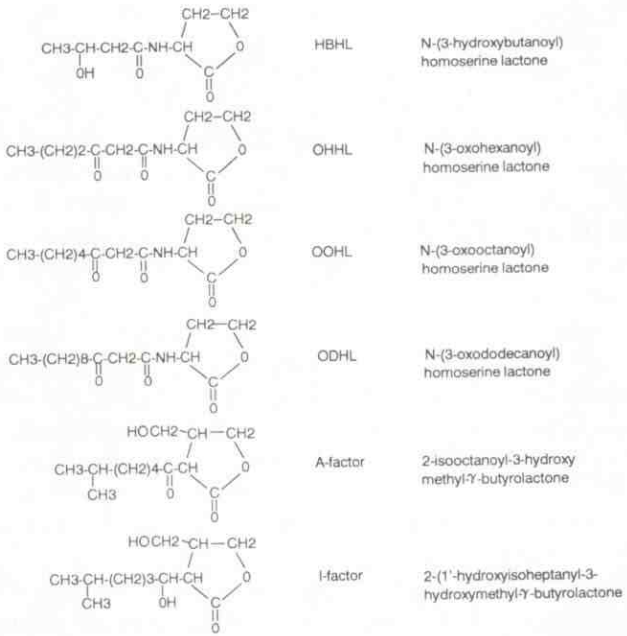

Figure 5 Butyrolactone autoregulators. HBHL and OHHL are autoinducers for the lux systems in *V. harveyi* and *P. fischeri*, respectively, while OOHL and ODHL as well as OHHL have been identified in nonluminescent bacteria. A-factor and I-factor are from *Streptomyces* species.

needed to degrade the cell walls of plants to allow propagation of the bacteria (4, 38, 65). Carbapanem production and a set of degradative enzymes are under cell density-dependent regulation; their synthesis is induced as the cells enter stationary phase in a manner similar to the induction of luminescence in the light-emitting bacteria. OHHL-negative mutants of *E. carotovara* (*exp*I) are avirulent and cannot damage or propagate on tobacco leaves (65) or potato tubers (38). Virulence and carbapanem production is restored to *exp*I mutants either by the addition of OHHL or by complementation with *exp*I from *E. carotovora* or *lux*I from *P. fischeri.* Synthesis of the degradative enzymes is restored and the concentration of the respective mRNAs increases correspondingly. A plasmid containing the *lux* regulon from *P. fischeri* with a frameshift in *lux*I resulted in luminescence in wild-type but not *exp*I mutant cells of *E. carotovora.* Thus *exp*I can also replace *lux*I (65).

Pseudomonas aeruginosa is an opportunistic human pathogen that infects damaged tissues and causes infection, particularly in immunocompromised individuals. Elastase, an extracellular protease, is a primary virulence factor produced during the late log and stationary phases. In separate reports, OHHL and ODHL have been isolated from culture media of *P. aeruginosa* and shown to control expression of elastase (38, 63). In the latter report (63), OHHL and OOHL were also detected, but ODHL was much more effective in activation of the promoter of *las*B that controls synthesis of elastase. This activation indicates that a set of N-3-oxoacyl homoserine lactones are produced and that ODHL is the natural autoinducer. A gene responsible for synthesis of the autoinducer in *P. aeruginosa, las*I, whose gene product is homologous with LuxI, has been identified on a transcript separate from, but adjacent to *las*R, whose gene product is homologous with LuxR (62). Both the *las*R-ODHL and *lux*R-OHHL pairs could be interchanged and stimulated expression in *E. coli* of the promoters for the elastase gene from *P. aeruginosa* and the right *lux* operon from *P. fischeri* (33).

OOHL has been identified as the conjugation factor of *Agrobacterium tumefaciens*, which behaves as a secondary messenger by transmitting information to the *tra* genes responsible for conjugal transfer (86). Transfer is also dependent on TraR, which is related in sequence to LuxR (64). A gene responsible for OOHL synthesis, *tra*I, has also been identified with the proposed TraR-autoinducer complex that also activates expression of *tra*R and *tra*I in a manner analogous to the positive feedback loops controlling induction of luminescence in *P. fischeri* (32).

The production of OHHL by other bacteria has been investigated by transformation with a plasmid containing *lux*R from *P. fischeri,* the intergenic operator region and the promoter for the right operon linked to the *lux*AB genes from *V. harveyi*, which function as a light-emitting sensor (81). The production of OHHL (or its equivalent) by the bacteria then causes activation

of the *lux* promoter and the expression of luciferase, which can readily be detected on addition of aldehyde. *lux*AB from *V. harveyi* rather than *lux*AB genes from *P. fischeri* are used because of the greater thermal stability of the luciferase from *V. harveyi*. This assay system has shown that cell density-dependent luminescence can be generated in *E. caratovora, Enterobacter agglomerans, Hafnia alvei, Rahnella aquatilis* and *S. marcescens*. It also demonstrates that genes comparable to *lux*I are present and that they appear to be under growth-dependent regulation (81). Previous studies showing the production of OHHL by *Erwinia, Enterobacter*, and *Serratia* support this conclusion (5). Complementation of the sensor plasmid transformed into *E. coli* with genomic libraries from these strains all resulted in selection of colonies that were highly luminescent on addition of aldehyde. A LuxI homolog must therefore be present. Sequence analyses of the clones from *E. caratovora* and *Enterobacter agglomerans* identified genes, *car*I and *eag*I, whose gene products have 25% sequence identity with LuxI.

Five closely related homologues (CarI, EagI, TraI, LasI, ExpI) of LuxI have been cloned from four different genera (*Erwinia, Enterobacter, Pseudomonas, Agrobacterium*), with 28 to 35% sequence identity (32, 81). Proteins corresponding to the receptor LuxR in *P. fischeri* have been sequenced for *P. aeruginosa* and *A. tumefacians* (LasR and TraR) (31a). In addition, homologs of LuxR have been recognized in *E. coli* (SdiA, previously UvRC-28) and *Rhizosphere leguminosarum* (RhiR), which are involved in regulation of nitrogen fixation and cell division, respectively. However, the inability to stimulate a *lux* sensor for OHHL indicates that *E. coli* has very low levels, if any, of this particular homoserine lactone. It will be of interest to determine if similar but not cross-reactive homoserine autoregulators are present.

Control of Polyhydroxybutyrate Biosynthesis in V. harveyi *by HBHL*

During the investigation of potential morphological changes in *V. harveyi* on induction of luminescence, lipid-like granules have been identified in the cells by electron microscopy and chemical analyses as polyhydroxybutyrate (PHB) granules (11, 76). This result is unexpected as *V. harveyi* is reported to be PHB-negative. The accumulation of PHB in the cells was dependent on cell density, with the levels increasing over 20-fold to above 2% of the dry weight at the late stages of cellular growth. Moreover, the level of PHB was very low in autoinducer-deficient mutants even at high cell density and could be stimulated up to 20-fold by the addition of HBHL. These results have indicated that the *lux* autoinducer, HBHL, in *V. harveyi* is actually a general signal transducer that apparently controls expression of more than one metabolic pathway (luminescence and PHB synthesis) as the cells enter the late phases of growth. Earlier work indicated that other bacterial species may also produce

HBHL or closely related analogs (34). *V. harveyi* may thus be part of a more extended family with cross-reactive autoregulators.

Relationship of Homoserine Lactones to Other γ-butyrolactone Autoregulators

The autoregulators of *Streptomyces* species and the homoserine lactones display remarkable structural similarity, with both sets of compounds being γ-butyrolactones (Figure 5). The A-factor from *Streptomyces* species has a keto group substituent whereas the homoserine lactones have an imino substituent at the 3-position of the butyrolactone ring. Factors from other *Streptomyces* species including Factor I from *S. viridochromogenes* have a hydroxymethylene substituent at this position with side chains of CH_3-$(CH_2)_{3\text{-}5}$-CH(OH)–, CH_3-CH(CH_3)-$(CH_2)_{2\text{-}5}$-CH(OH)– or CH3-CH_2-CH(CH_3)-CH_2-CH(OH)–. Specificity for the keto group or hydroxymethylene is high whereas less specificity is shown for the side chain (36). A similar selectivity has been observed for the *lux* autoinducers; the 3-hydroxybutanoyl (homoserine) lactone is used only by *V. harveyi* whereas the 3-ketohexanoyl (homoserine) lactone is specific for *P. fischeri*, with some stimulation by shorter and longer chain analogs (26).

A-factor in *S. griseus* controls a number of metabolic steps expressed at the later stages of development. These steps include sporulation and antibiotic synthesis. A-factor relieves repression by binding to a repressor, thereby activating these processes. Although the mechanism of activation by A-factor appears different from that for the *lux* autoinducer (OHHL) in *P. fischeri* and other N-(3-ketoacyl) homoserine lactones, a closer relationship may exist between the regulatory mechanisms for the *lux* autoinducer (HBHL) in *V. harveyi* and the autoregulators from *Streptomyces*. Genetic experiments have suggested that HBHL binds to LuxN and blocks expression of a repressor, LuxO, which, in turn, inhibits expression of the *lux*CDABEGH operon either directly or indirectly by inhibiting expression of *lux*R*. Because LuxO as well as LuxN and LuxQ have been related in sequence to the two-component signal response phosphorylation systems (7, 8, 9) and because the phosphorylation pattern of proteins in *S. griseus* is dependent on A-factor (36), both systems may involve signal relays that occur via protein phosphorylation.

PERSPECTIVES: Past and Future

The first luciferase genes were cloned in the early 1980s, an event that started the development of the *lux* genetic system. In a little over a decade, the functions of new *lux* genes from a number of species of marine and terrestrial bacteria were identified and luminescence was connected to genetic elements involved in fatty acid and flavin metabolism. The discovery that *lux* autoinducers are N-acylhomoserine lactones controlling the growth-dependent induction of the

luminescent system coupled with their identification in nonluminescent bacteria indicated that the *lux* autoinducers are part of a new and broadly based class of bacterial pheromones acting as general signal transductants to control processes expressed at the later stages of cellular growth. The identification of the *lux* regulatory genes in *P. fischeri* and *V. harveyi* demonstrated the amazing diversity between genetic elements regulating identical functions under apparently similar control. Further genetic and biochemical studies on the reception and biosynthesis of the *lux* autoinducers and the differences and similarities in how this signal is transmitted in *V. harveyi* and *P. fischeri* to induce luminescence are clearly important and of great interest. The remarkable sensitivity, range, and ease for instantaneous and continuous measurement of light as a sensor of perturbances of the structural and regulatory genetic elements of the *lux* system without disruption of the cellular structure, provide these studies with insight into regulation that cannot be approached in other systems. Such studies, in turn, have the potential to allow us to determine how the regulatory and structural elements of one metabolic system interface with the genetic elements controlling the basic physiology of the cell.

ACKNOWLEDGMENTS

The author gratefully acknowledges the support of his colleagues and thanks Bonnie Bassler, Peter Greenberg, Ken Nealson, Michael Silverman, and Ulrich Winkler for providing information and manuscripts prior to publication. Special thanks go to Joyce Keating for her patience and skill in preparation of this review. Support by the Medical Research Council of Canada is gratefully acknowledged.

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