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Genetics

Identification of genes and gene products necessary for bacterial bioluminescence

(*lux* genes/recombinant DNA/complementation/minicells)

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ABSTRACT **Expression of luminescence in *Escherichia coli* was recently achieved by cloning genes from the marine bacterium *Vibrio fischeri*. One DNA fragment on a hybrid plasmid encoded regulatory functions and enzymatic activities necessary for light production. We report the results of a genetic analysis to identify the luminescence genes (*lux*) that reside on this recombinant plasmid. *lux* gene mutations were generated by hydroxylamine treatment, and these mutations were ordered on a linear map by complementation in *trans* with a series of polar transposon insertions on other plasmids. *lux* genes were defined by complementation of *lux* gene defects on pairs of plasmids in *trans* in *E. coli*. Hybrid plasmids were also used to direct the synthesis of polypeptides in the *E. coli* minicell system. Seven *lux* genes and the corresponding gene products were identified from the complementation analysis and the minicell programing experiments. These genes, in the order of their position on a linear map, and the apparent molecular weights of the gene products are *luxR* (27,000), *luxI* (25,000), *luxC* (53,000), *luxD* (33,000), *luxA* (40,000), *luxB* (38,000), and *luxE* (42,000). From the luminescence phenotypes of *E. coli* containing mutant plasmids, functions were assigned to these genes: *luxA*, *luxB*, *luxC*, *luxD*, and *luxE* encode enzymes for light production and *luxR* and *luxI* encode regulatory functions.**

Luminescent bacteria are common in the ocean and occupy a variety of ecological niches (1, 2). *Vibrio fischeri* (strain MJ-1) colonizes the light organ of the fish *Monocentris japonicus*, and we have initiated studies to determine the genetic and functional components of the luminescence system from this bacterium. The emission of light by marine bacteria is catalyzed by the enzyme luciferase, a mixed function oxidase that has two subunits, α and β, with molecular weights of ≈40,000 each (3). In the generation of light, luciferase oxidizes a reduced flavin and a long-chain aldehyde, producing oxidized flavin and the corresponding long chain fatty acid:

$$RCHO + FMNH_2 + O_2 \xrightarrow{\text{luciferase}} RCOOH + FMN + H_2O + h\nu.$$

Other components unique to the bioluminescence system include enzymes involved in the synthesis or recycling of the aldehyde substrate. Light production occurs in dense bacterial cultures and is controlled by the synthesis of a sensory molecule termed autoinducer. This molecule is secreted into the extracellular environment where it accumulates and at a critical concentration signals expression of luminescence. This induction can result in a 10,000-fold increase in light emission per cell (4). The autoinducer from *V. fischeri* has been shown to be *N*-(β-ketocaproyl)homoserine lactone (5).

We previously isolated a 9-kilobase-pair (kbp) DNA fragment from *V. fischeri* that encoded all of the functions necessary for light production and that also contained the regulatory elements required for their expression in *Escherichia coli*. By using transposon mutagenesis, the regions on this DNA fragment that encoded aldehyde, luciferase, and regulatory functions were defined. These functions were organized into two transcriptional units, operon L and operon R. Furthermore, by using the *lacZ* gene fusions created by transposon mini-Mu insertion, transcription of operon R was found to be induced by the presence of autoinducer, whose synthesis was controlled by a gene product also encoded by this same operon. Therefore, *lux* gene expression was controlled by a positive feedback circuit, and induction resulted in a logarithmic increase in the synthesis of enzymes for the light reaction (6). Due to the polar nature of these transposon insertions, we were unable to define the individual genes that encoded these functions. We report here the identification of *lux* genes and gene products by performing complementation tests with hydroxylamine-generated point mutations and by programing protein synthesis in the *E. coli* minicell system.

MATERIALS AND METHODS

Bacterial Strains, Cloning Vehicles, and Media. *E. coli* strain ED8654 (*supE supF met hsdR*$^-$ *hsdM*$^+$) was used for propagation of recombinant plasmids. Complementation tests were performed in HB101 (*hsdS recA ara proA lac galK rpsL*). *E. coli* strain P678-54 (*thr ara leu azi tonA lacY tsx minA minB gal malA thi xyl rpsL*) was used for the production of minicells for the protein programing. DNA fragments were subcloned into various sites in vehicle pBR322 (7) and pACYC184 (8). Plasmids were recovered by transformation (9) and selection for transformants containing recombinants in pBR322 was on Luria agar plates containing 80 μg of ampicillin per ml and recombinants in pACYC184 was on Luria agar containing 50 μg of chloramphenicol per ml. Hybrid plasmids in complementation studies were propagated on Luria agar plates containing 80 μg of ampicillin per ml and 50 μg of chloramphenicol per ml. Restriction endonucleases were purchased from New England Biolabs and T4 DNA ligase was purchased from Bethesda Research Laboratories. Antibiotics were purchased from Calbiochem.

Hydroxylamine Mutagenesis. Mutagenesis was as described for phage by Berman *et al.* (10). Five micrograms of pJE202 (6) or pJE737 DNA was incubated at 37°C in 0.5 M NH_2OH, pH 6/5 mM Tris/0.5 mM EDTA in a final volume of 50 μl. Five-microliter samples were removed at 60-min intervals, and the reaction was stopped by the addition of 95 μl of 100 mM $CaCl_2$. This DNA was then used to transform the strain HB101. Since mutagenesis was *in vitro* and no plasmid replication could occur, transformants with a mutant luminescence phenotype were the result of independent

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Abbreviation: kbp, kilobase pair(s).

mutations. To insure that transformants arose from a single mutant plasmid, plasmid DNA was prepared (11) from each mutant strain and used to retransform strain HB101. These transformants were subsequently reexamined for the mutant phenotype. With mutagenized pJE737 DNA, transformation was into HB101 containing plasmid pJE502 (*luxR*). Minimal amounts of DNA were used to avoid the isolation of multiple transformed bacteria. Seventy-four independent dark (no detectable light production) or dim (*ca.* 1% of wild-type light production) mutant plasmids were isolated from the pJE202 mutagenesis. Twenty mutant plasmids—i.e., no complementation of the *luxR* defect on the plasmid pJE502—were isolated from the pJE737 mutagenesis. To insure that the recombinant plasmids contained single mutations, hydroxylamine treatment was adjusted to yield a frequency of Lux^- mutants of about 5%. Approximately 240 min of exposure to hydroxylamine was necessary to achieve this level of mutagenesis.

Complementation Tests. Complementation of Tn*5* mutations on hybrid plasmids (pJE300s) (6) with hydroxylamine-generated mutations on hybrid plasmids (pJE500s) was performed by cotransforming pairs of plasmids into a Rec^- *E. coli* strain, HB101, as described (6). To perform additional complementation tests, *lux* mutations had to be transferred by reciprocal recombination to another hybrid plasmid with a compatible replicon. *lux* mutations on a pACYC184 replicon (pJE600s) were made by cotransforming pJE500 plasmids (pBR322 replicon) and pJE212 (*Sal* I *lux* fragment in pACYC184) into a Rec^+ *E. coli* strain, ED8654. Plasmid DNA was isolated from these strains and retransformed into strain HB101 with selection for the pACYC184 replicon. Approximately 1% of the transformants had the mutant phenotype of the donor plasmid (pJE500s). Complementation in *trans* of *lux* gene mutations was then measured by cotransforming plasmids of the pJE500 and pJE600 series into HB101. Light production was measured in these strains visually, by autoradiography or with a LKB 1211 Minibeta scintillation counter in the chemiluminescence mode, and complementation was scored as positive if light production was >10% of wild-type light production (HB101 containing plasmid pJE202 or pJE212) and negative if <10%. Hybrid plasmids containing amber mutations in specific genes were generated by hydroxylamine mutagenesis. Strains with mutant plasmids were selected on the basis of complementation (to identify the gene defect) and suppression with strain ED8654 (to recognize amber mutations).

Minicell Preparation and Electrophoresis of Gene Products. Minicells were made from strain P678-54 as described by Matsumura *et al.* (12). The hybrid plasmids were introduced by transformation. Two milliliters of a stationary culture was used to inoculate 1 liter of Luria broth containing 80 μg of ampicillin per ml or 50 μg of chloramphenicol per ml and incubated on a 37°C shaker overnight. The culture was centrifuged for 5 min at 5000 × *g* to remove whole bacteria, and the supernatant was centrifuged for 10 min at 10,000 × *g* to concentrate minicells. The resulting pellet was suspended in 1.5 ml of buffered saline gelatin (0.85% NaCl/0.03% KH_2PO_4/0.06% Na_2HPO_4/100 μg of gelatin per ml). The cells were then sedimented on a 5–20% sucrose gradient for 20 min at 5000 × *g*. The top band was collected, pelleted, and resuspended in 1.5 ml of buffered saline gelatin. This suspension was rerun on a second 5–20% sucrose gradient, the upper band was collected, and the minicells were pelleted. The pellet was suspended to $\approx 2 \times 10^9$ minicells per ml in minimal salts medium with 0.5% glycerol and 2 μg of amino acids (minus methionine) per ml. Minicells (0.5 ml) were incubated for 30 min at 30°C, and then 10 μCi (1 Ci = 37 GBq) of [^{35}S]methionine with a specific activity of 970 Ci/mmol (New England Nuclear) or 10 μCi of ^{3}H-labeled L-amino acid mixture with a specific activity of ≈50 Ci/mmol (New England Nuclear) was added. After 20 min, the minicells were pelleted and then frozen at −20°C. The details of Na$DodSO_4$/polyacrylamide gel electrophoresis have been described (13). The gel was incubated in Autofluor (National Diagnostics, Somerville, NJ) before drying and autoradiography.

RESULTS

Seventy-four dark or dim mutants of strains carrying pJE202 mutagenized with hydroxylamine were isolated (see *Materials and Methods*). As observed previously with transposon mutants (6), there were four classes of mutant phenotypes: (*i*) mutants that could not produce or respond to autoinducer; (*ii*) those that could respond to autoinducer but could no longer produce autoinducer; (*iii*) those that were dark but produced light with the addition of exogenous aldehyde; and (*iv*) those that were dark but could not respond to the addition of aldehyde. Mutants in categories *i* and *ii* were defective in functions required for regulation of *lux* gene expression. Mutants in categories *iii* and *iv* produced and responded to autoinducer but had defects in enzymes for light production. Mutants in categories *i–iii* were dim (1% or less of wild-type light production), whereas mutants in category *iv* were generally dark (no detectable light production). To generate a linear map of the hydroxylamine mutations, complementation tests were performed with *lux* plasmids containing Tn*5* insertions that were previously mapped by restriction analysis (6) (see Fig. 2*A*). Due to the polar nature of these transposon insertions, genes within an operon downstream from the transposon insertion would not be transcribed, whereas any genes upstream would be expressed. Therefore, in complementation tests with hybrid plasmids containing *lux* point mutations in *trans* with hybrid plasmids containing transposon insertions, complementation would not occur if the point mutation were in a gene identical to one (in *trans*) inactivated by transposon insertion (target gene or distal gene), but complementation would occur if the point mutation were in a gene identical to a gene (in *trans*) proximal to transposon insertion. All 74 mutant plasmids were characterized by this test, and Table 1 shows the result of this type of analysis. Thus, *lux* mutations on hybrid plasmids were mapped to six regions of the cloned DNA fragment.

Nonsense mutations resulting from hydroxylamine mutagenesis would be expected to show a degree of polarity on transcription of downstream genes. However, none of the point mutations tested showed polarity of the extent exhibit-

Table 1. Complementation of polar mutations

Hydroxylamine mutation (pBR322)	Tn*5* mutation (pACYC184 replicon)* 331	337	307	309	301	320
501	−	+	+	+	+	+
502	−	+	+	+	+	+
560	+	−	+	+	+	+
561	+	−	+	+	+	+
525	+	−	−	+	+	+
528	+	−	−	+	+	+
546	+	−	−	−	+	+
547	+	−	−	−	+	+
508	+	−	−	−	−	+
510	+	−	−	−	−	+
505	+	−	−	−	−	+
515	+	−	−	−	−	+
537	+	−	−	−	−	−
545	+	−	−	−	−	−

Complementation of *lux* defects was measured in $RecA^-$ strains harboring pairs of mutant plasmids (+, light producing; −, little or no light produced).

*Location of transposon Tn*5* insertions in *lux* operons is shown in Fig. 2*A*.

ed by the Tn*5* transposon mutations (see Table 1). Thus, hybrid plasmids containing these point mutations could be used in complementation tests to define individual genes. To perform these tests, it was first necessary to transfer point mutations to a second compatible replicon (pJE212, derivative of pACYC184) via recombination with the original plasmid mutations (pJE500s). These hybrid plasmids were designated pJE600s and would replicate in the same cell as the pJE500s (see *Materials and Methods*). Fifty *lux* mutations from the pJE500 series were transferred to the pACYC184 replicon (pJE600 series). Complementation studies were then undertaken by introducing pJE500 and pJE600 plasmids into the same *E. coli* strain. To reduce the number of complementation tests, the entire matrix of *lux* mutation combinations (50 pJE500 × 50 pJE600 series plasmids or 2500 strains) was not constructed. Instead, subgroups of plasmids with linked *lux* mutations (generally 6 pJE500 × 6 pJE600 series plasmids or 36 strains) were used in complementation tests to define individual genes. Pairs of mutant plasmids representing each complementation group were then assembled into the matrix shown in Fig. 1. Light production occurred when the mutations on the plasmids complemented. Those cells that produced little or no light harbored plasmids with noncomplementing mutations. Since mutations in the same gene did not complement and seven groups of noncomplementing mutations were observed, we concluded that there were seven *lux* genes encoded by the fragment cloned from *V. fischeri*. These genes are designated *luxR*, *luxI*, *luxC*, *luxD*, *luxA*, *luxB*, and *luxE*. The locations of these genes in operon L and operon R, as determined by data such as those in Table 1, are shown in Fig. 2*B*. The function of each gene was inferred from the phenotypes of mutants isolated in this study and from the properties of transposon mutants analyzed in a previous report (6). The products of *luxR* and *luxI* regulate expression of luminescence; *luxI* encodes a function required for the synthesis of autoinducer and *luxR* encodes a function necessary for response to autoinducer. The *luxC*, *luxD*, and *luxE* gene products function to provide the aldehyde substrate, and *luxA* and *luxB* encode the α and β subunits of luciferase.

The *E. coli* minicell system has been used extensively to identify plasmid-encoded gene products. Since only small replicons such as the hybrid *lux* plasmids segregate with the minicells during their formation, protein synthesis in purified minicell preparations is directed exclusively by hybrid plasmid genes. To use this system to program the synthesis of *lux* gene products, we constructed hybrid plasmids that contained subclones of the *lux* gene region and also generated chain-terminating (amber) mutations in specific genes in hybrid plasmids (see Fig. 2*C*). These hybrid plasmids were then transferred into the minicell strain by transformation, and minicells were prepared for protein labeling experiments. Fig. 3 shows an autoradiogram of a polyacrylamide gel containing ^{35}S-labeled polypeptides synthesized in these hybrid plasmid-containing minicells. Plasmid pJE202 contains the entire *lux* region, and this plasmid programed the synthesis of polypeptides of approximately 53,000, 42,000, 40,000, 38,000, 33,000, and 25,000 M_r. When hybrid plasmid pJE544, containing an amber mutation in *luxB*, was used to direct protein synthesis in minicells, the 38,000 M_r protein was not synthesized, and we concluded that this polypeptide was the product of *luxB*. *luxB* has been shown to encode the β subunit of luciferase (6, 14, 15) and the molecular weight obtained from the programing experiment agrees well with the published size for this subunit (16). In our preparation of protein this *luxB* polypeptide often appeared as a doublet band on gels. However, since in pJE544 both bands disappeared, we assumed that both were encoded by *luxB*. When a hybrid plasmid containing a *luxD* amber mutation, pJE547, was used to program protein synthesis in minicells, the 33,000 M_r protein was not present. It was, therefore, concluded that this polypeptide was the product of the *luxD* gene. Plasmid pJE209 contains *luxB* and *luxE* and in minicells directed the synthesis of 42,000 and 38,000 M_r polypeptides. By elimination, the 42,000 M_r protein was assigned as the product of the *luxE* gene. Plasmid pJE208, which contains *luxA* alone, directed the synthesis of a 40,000 M_r protein. *luxA* encodes the α subunit of luciferase (6, 14, 15), which has a reported size of ≈40,000 M_r (16). Plasmid pJE709 contains *luxR*, *luxI*, and *luxC* and programed polypeptides of 53,000 and 25,000 M_r. When a plasmid encoding *luxR* and *luxI*, pJE743, was used to direct protein synthesis in minicells, the 53,000 M_r protein was not detected, and it was, therefore, assigned as the *luxC* gene product. Since the *luxR* gene product was not

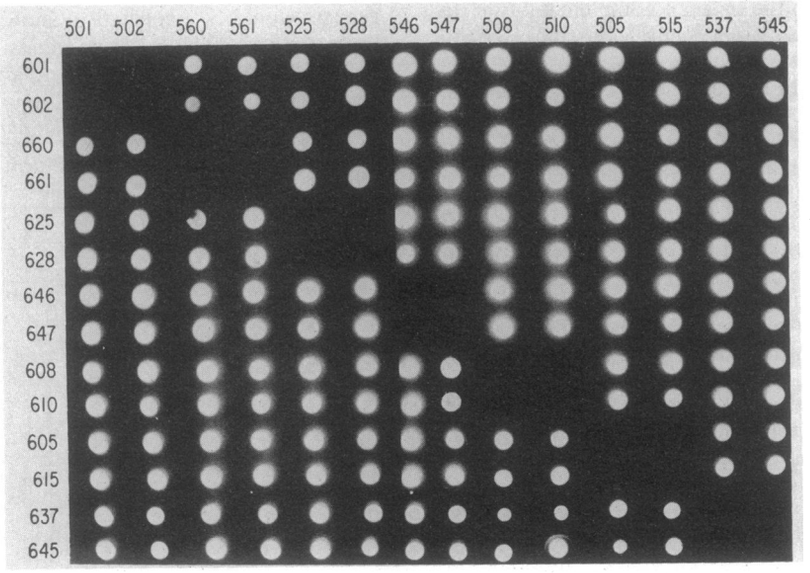

FIG. 1. Complementation analysis. *E. coli* strains containing pairs of hybrid plasmids with *lux* mutations (one of each of the pJE500 and pJE600 series) were grown on a nutrient agar surface and photographed by their own light. The mutant plasmids assembled for this test contained *lux* mutations representing all of the complementation groups defined in an extensive analysis of the complementation of 50 independent *lux* mutations. The second and third numbers of the plasmid designation refer to the *lux* allele—i.e., pJE501 and pJE601 contain the same *lux* mutation. Strains with noncomplementing mutations were dark and do not appear in the photograph of the matrix.

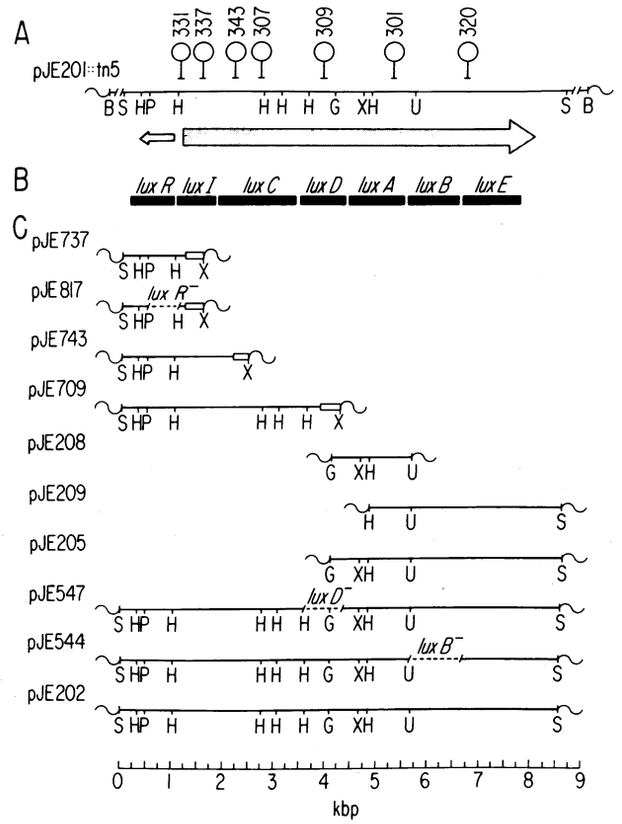

FIG. 2. Restriction maps of *lux* region with transposon insertions, *lux* genes, and hybrid plasmids. (*A*) Plasmid pJE201 (*Bam*HI fragment from *V. fischeri*) with locations of Tn*5* insertions used for mapping *lux* point mutations. Restriction sites are B, *Bam*HI; S, *Sal* I; H, *Hin*dIII; P, *Pst* I; G, *Bgl* II; X, *Xho* I; U, *Pvu* II. Horizontal arrows denote direction of transcription and location of operon L (left) and operon R (right). (*B*) The location of *lux* genes. The length of rectangles represents the relative size of *lux* genes as calculated from the apparent molecular weights of the *lux* gene products (Fig. 3). (*C*) The composition of hybrid plasmids used for programing protein synthesis in minicells. Hybrid plasmids pJE737, pJE817, pJE743, and pJE709 contain DNA fragments subcloned from hybrid plasmids with Tn*5* insertions. Restriction sites in the transposons were required for subcloning, and open rectangles represent DNA derived from the transposons.

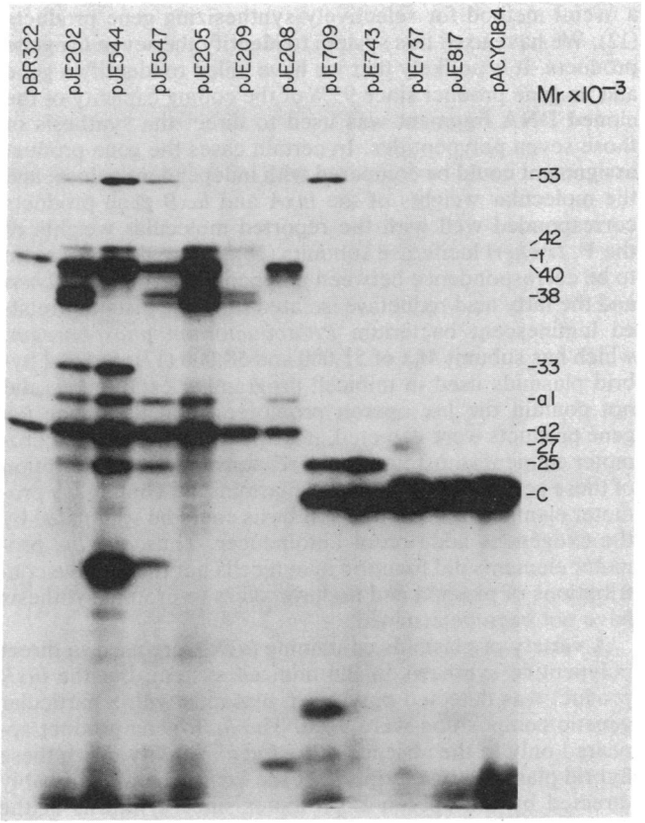

FIG. 3. Protein synthesis in minicells. An autoradiogram of ^{35}S-labeled proteins (24 hr of exposure) synthesized in hybrid plasmid containing minicells is shown. The composition of the hybrid plasmids (top) is described in Fig. 2*C*. Molecular weight assignments are shown at right. Polypeptides encoded by plasmid vehicles are t, *tet* gene product; a1 and a2, *bla* gene products; and c, *cam* gene product.

usually expressed (see below), the 25,000 M_r protein was assigned as the *luxI* product.

The *luxR* gene product (27,000 M_r) was observed when *luxR* alone was present on hybrid plasmid pJE737. This polypeptide was not detected when other plasmids were used (pJE202, pJE544, pJE547, pJE709, pJE743). Plasmids that contained *luxR* but that did not direct synthesis of the 27,000 M_r polypeptide invariably contained the *luxI* gene. To verify that *luxR* encoded the 27,000 M_r polypeptide, *luxR* point mutations in plasmid pJE737 were isolated. Specifically, hydroxylamine-generated *lux* mutations on plasmid pJE737 that did not complement in *trans* with a *luxR* mutation on plasmid pJE502 were collected. When a hybrid plasmid with a *luxR* mutation, pJE817, was used to direct protein synthesis in minicells, the 27,000 M_r polypeptide was no longer synthesized (Fig. 3). Since negation of *luxR* genetic activity resulted in loss of the capacity to direct synthesis of the 27,000 M_r protein, *luxR* apparently encoded the 27,000 M_r protein. ^{3}H-labeled polypeptides were also synthesized in hybrid plasmid containing minicells to insure that any *lux* gene products that did not contain methionine would be observed. No additional polypeptides were synthesized under these conditions (data not shown).

DISCUSSION

Seven *lux* genes required for light production in recombinant *E. coli* were identified in this study. These genes, which were cloned from *V. fischeri* on a single DNA fragment, are located in two operons (see Fig. 2) and encode regulatory functions and activities necessary for the light reaction. These include the *luxA* and *luxB* genes for the α and β subunits of luciferase, the *luxC*, *luxD*, and *luxE* genes for the synthesis or recycling of the aldehyde substrate, and two genes, *luxI* and *luxR*, that regulate light production by synthesizing and responding to autoinducer. Functions were assigned to genes by examining the phenotypes of mutants. For example, a mutant with a *luxA* defect was dark even in the presence of exogenous aldehyde, whereas a mutant with a *luxC* defect was dark but produced light when exogenous aldehyde was added. Regulatory mutants produce little or no autoinducer, but mutants defective in *luxI* could respond to autoinducer added to the culture, whereas mutants with *luxR* defects could not respond. (See ref. 6 for more complete details of the positive feedback circuit controlling light production.) The bioluminescence system probably interfaces with other cellular systems that furnish common organic intermediates. Since the *de novo* synthesis of autoinducer, *N*-(β-ketocaproyl)homoserine lactone, could require a series of biochemical reactions, the *E. coli* host probably provides a precursor that can then be converted to autoinducer by the *luxI* gene product. Aldehyde precursors and reducing power generated via an oxidoreductase must also be furnished by the *E. coli* host.

The hybrid plasmid minicell system has been shown to be

a useful method for selectively synthesizing gene products (12). We have used this system to identify the seven *lux* gene products. It is unlikely that we have failed to identify a gene and its gene product since 95% of the coding capacity of the cloned DNA fragment was used to direct the synthesis of those seven polypeptides. In certain cases the gene product assignment could be compared with independent values, and the molecular weights of the *luxA* and *luxB* gene products corresponded well with the reported molecular weights of the *V. fischeri* luciferase subunits (16). There does not seem to be correspondence between polypeptides from *V. fischeri* and the fatty acid reductase isolated from the distantly related luminescent bacterium *Photobacterium phosphoreum*, which has subunit M_rs of 51,000 and 58,000 (17). Several hybrid plasmids used in minicell programing experiments did not contain the *lux* operon promoter elements. Since *lux* gene products were detected, it is apparent that plasmid promoter elements must have been responsible for transcription of these genes. However, other plasmids did contain *lux* promoter elements and protein synthesis could be stimulated by the exogenous addition of autoinducer. Thus, the *lux* promoter elements did function in minicells but the relative contributions of plasmid and *lux* promoters to protein synthesis have not been determined.

A variety of plasmids containing *luxR* were used to direct polypeptide synthesis in the minicell system, but the *luxR* product was detected only when plasmids with a particular genetic composition were used. The *luxR* gene product appeared only in the absence of *luxI* gene activity. With these hybrid plasmids transcription of the *luxR* gene was probably directed by the operon L promoter since synthesis of the *luxR* product was independent of alignment with defined plasmid promoters. It is possible that expression of operon L (containing *luxR*) is repressed by the *luxI* gene product. Operon R, containing *luxI* and genes for light reaction enzymes, is positively regulated by the combined actions of the *luxR* and *luxI* gene products (6). The possibility that bioluminescence is controlled by both negative and positive regulatory circuits must be explored.

The definition of gene functions resulting from this study is in agreement with the general categories of function inferred from the earlier use of transposon-generated mutations (6). With the identification of *lux* genes and gene products, we can now begin to dissect further this system. The subcellular location of these *lux* gene products can now be determined by using labeled polypeptides synthesized in the *E. coli* minicell system. Knowing the identity of *lux* gene products should assist in the purification and biochemical analysis of poorly understood components of the bioluminescence system such as those for aldehyde cycling and for the synthesis, excretion, and sensing of autoinducer.

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