Overproduction and purification of the luxR gene product: Transcriptional activator of the Vibrio fischeri luminescence system

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ABSTRACT Expression of Vibrio fischeri luminescence genes requires an inducer, termed autoinducer, and a positive regulatory element, the luxR gene product. A plasmid containing luxR under control of a tac promoter was engineered to overproduce this gene product. The overproduced luxR gene product was active in vivo, and its apparent monomeric molecular weight was indistinguishable from that of the protein encoded by luxR under control of its own promoter (Mr, 27,000). The new tac-luxR construct directed the synthesis of large quantities of the luxR gene product in induced Escherichia coli cells lacking other lux genes. In the presence of the other lux genes, overexpression of the tac-luxR construct was not detected. The overproduced luxR gene product, which formed cytoplasmic inclusion bodies, was purified and used in subsequent studies. Nonequilibrium pH gradient electrophoresis indicated that the protein was basic, and the amino-terminal 15 amino acids were sequenced. DNA-binding activity was detected by membrane filter binding assays; under the conditions used, the binding was not lux DNA-specific. Binding of tritium-labeled autoinducer to the luxR gene product was not detected, and autoinducer enhancement of the binding of the luxR gene product to DNA could not be detected reproducibly.

Vibrio fischeri, a luminescent marine bacterium, can be isolated from seawater and has been identified as the bacterial symbiont in the light organs of certain marine fishes (1-3). The luminescence of V. fischeri is inducible; the inducer, N-(3-oxohexanoyl)homoserine lactone, termed "autoinducer," is a diffusible metabolite produced by V. fischeri that accumulates at equal concentrations in the culture medium and in cells during growth. When autoinducer reaches a critical concentration of a few molecules per cell, induction of the light-emitting enzyme luciferase and other enzymes involved in luminescence commences (4-7). At high cell densities, autoinducer can accumulate, and induction of the V. fischeri luminescence system will occur. Presumably this is the case in the light-organ symbiosis, where V. fischeri occurs at densities of \(\approx 10^{10}\) cells per ml of light organ fluid. In low-cell-density habitats such as seawater, where V. fischeri exists at \(< 10^2\) cells per ml, autoinduction of luminescence would not be expected (1-3, 8). Thus, autoinducer serves as the chemical signal or pheromone in this cell density-dependent control system that provides a rapid and sensitive cellular response to changes in the external environment (4, 6).

The cloning of a fragment of V. fischeri DNA that encodes regulatory functions and enzymatic activities necessary for light production in Escherichia coli enabled a genetic characterization of the regulation of luminescence (9, 10). A positive regulatory element was identified, the product of luxR, that is required along with autoinducer to activate transcription of a contiguous but divergently transcribed operon encoding five polypeptides required for luminescence (the luxC,D,A,B,E gene products) and one polypeptide for autoinducer synthesis (the luxI gene product). The luxR gene product can be considered the autoinducer receptor, since it is the only V. fischeri gene product required in E. coli for response to autoinducer (9, 10). Induction of luminescence also has been found to require cAMP receptor protein (CRP) and cAMP (11). There is an additional control mechanism not yet understood; the presence of either lux (which is adjacent to luxR) or its product suppresses synthesis of the luxR gene product (10). This was an important consideration in our attempts to overproduce the luxR gene product.

We report here the construction of a plasmid that directs overproduction of the luxR gene product in E. coli and a procedure for purifying this overproduced protein. Using the material purified from E. coli, we established that the LuxR protein has DNA-binding activity, and, using the luxR expression vector, we demonstrated that there is a regulatory region within the luxR structural gene.

MATERIALS AND METHODS

Bacterial Strain, Plasmids, and Culture Conditions. The E. coli strain used was JM109 (12). The plasmids used are listed in Table 1. Unless otherwise specified, E. coli cultures were grown in L broth (6) containing antibiotics (80 \(\mu\)g of ampicillin or 50 \(\mu\)g of chloramphenicol per ml) with shaking at 37°C. Cells from which the luxR gene product was purified were grown in 100-ml batches to an optical density at 550 nm of about 0.8; the inducer of the tac promoter, isopropyl \(\beta\)-D-thiogalactoside (IPTG), was added to a final concentration of 1 mM; and the cultures were incubated a further 3 hr.

Plasmid Purification and Constructions. Plasmids were purified as described (15). Manipulations of plasmid DNA were performed by following the procedures of Maniatis et al. (16) and Struhl (17). The transformation procedure used was that described by Hanahan (18).

For identifying ColE1 replicons containing luxR under control of new promoters, the other lux genes were supplied on a compatible replicon, and colonies on plates incubated at 30°C were screened for luminescence. These lux genes from a ColE1 replicon were subcloned into pACYC184 (a P1S3A replicon) by digestion of plE455 with SalI and ligation of a fragment of about 19 kilobases (kb) into the SalI site of pACYC184. The resulting plasmid, pHK555, contained luxI, C,D,A,B,E, a luxR deletion mutation, and some mini-Mu DNA.

A plasmid containing luxR under its own control, pHK737, was constructed as a source of lux regulatory regions for use in DNA-binding assays. This plasmid was also used to identify the luxR gene product by in vitro transcription--
Table 1. Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant characteristics</th>
<th>Source (reference)</th>
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<tr>
<td>pACYC184</td>
<td>P15A replicon, Cm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>B. Brahamsrsha (13)</td>
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<td>pDR720</td>
<td>ColE1 replicon, Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Pharmacia</td>
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<tr>
<td>pUC8</td>
<td>ColE1 replicon, Ap&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>lac promoter vector, Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>M. Weiner (12)</td>
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<td>pJE455</td>
<td>lux&lt;sup&gt;r&lt;/sup&gt;::mini-Mu, luxIC,D,A,B,E&lt;sup&gt;+&lt;/sup&gt;, Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>J. Engerbrecht&lt;sup&gt;l&lt;/sup&gt;</td>
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<tr>
<td>pJE737</td>
<td>lux&lt;sup&gt;r&lt;/sup&gt;<em>, lux&lt;sup&gt;i&lt;/sup&gt;</em>, Cm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>J. Engerbrecht (10)</td>
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<tr>
<td>pHK555</td>
<td>lux&lt;sup&gt;r&lt;/sup&gt;*, luxIC,D,A,B,E&lt;sup&gt;+&lt;/sup&gt;, Cm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<td>pHK724</td>
<td>lux&lt;sup&gt;r&lt;/sup&gt;*, Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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Cm<sup>+</sup> and Ap<sup>+</sup>, chloramphenicol and ampicillin resistance.

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translation analysis. A HincII fragment from pJE737 of about 1.5 kb containing lux<sup>r</sup> and a small amount of lux<sup>i</sup> was cloned into the Sma site of pDR720 by P. Dunlap in our laboratory, resulting in the recombinant plasmid pPD737. To produce pHK737, the lux DNA was removed from pPD737 by digestion with EcoRI and Sal I and then inserted into pUC8.

Polycrylamide Gel Electrophoresis. The procedure of Laemmli (19) for NaDodSO<sub>4</sub>/PAGE was used as described (20). The relative amount of the lux<sup>r</sup> gene product was determined with scanning densitometry. Standard fluorography procedures were used with molecular weight markers (Sigma) prestained with Remazol brilliant blue R (21). Protein concentrations were determined by the method of Bradford (22).

The charge of the lux<sup>r</sup> gene product was estimated by the nonequilibrium pH gradient electrophoresis technique of O’Farrell et al. (23). Samples were prepared as described (20) except that NaDodSO<sub>4</sub> was omitted. The standards used were broad- and low-pI calibration kit standards (Pharmacia).

Purification of the lux<sup>r</sup> Gene Product from E. coli (pHK724). The procedure for the purification of the lux<sup>r</sup> gene product was similar to that for other overproduced proteins that precipitate in cells (24, 25). Cells induced to overexpress lux<sup>r</sup> were harvested, the supernatant fluid was decanted, and the cell pellet was weighed (usually 0.6 g). The pellet was suspended in 2.4 ml of R buffer (50 mM Tris, pH 7.8 at 5°C/200 mM NaCl/0.1 mM EDTA/0.1 mM dithiothreitol/5% glycerol). A 0.24-m1 volume of 50 mM EDTA in 10% Triton X-100 was added to the suspension, and then a fresh solution of lysozyme was added to a final concentration of 150 µg/ml.

The suspension was dissolved for 45 min, sonicated, and then centrifuged at 100,000 × g for 30 min. The resulting supernatant fluid was removed, and the pellet was suspended in 2.4 ml of 6 M guanidine hydrochloride in R buffer and incubated for 20 min. This was followed by centrifugation for 30 min at 100,000 × g. The resulting supernatant fluid was diluted to 1 M guanidine hydrochloride by a dropwise addition of R buffer (R buffer containing 1 M NaCl and 50% glycerol), dialyzed in ice for two steps against R buffer containing decreasing concentrations of guanidine hydrochloride (0.5 M and 0.25 M), and then dialyzed twice against RS buffer without guanidine hydrochloride. All manipulations were at 4°C or on ice. The protein was stored at −70°C at a concentration of 0.8 mg/ml. For the amino-terminal sequence analysis (performed by the Cornell Biotechnology Institute Protein Sequencing Facility), the purified lux<sup>r</sup> gene product was dialyzed for 16 hr against 10 mM HCl.

DNA-Binding Assays. A membrane filter binding assay based on a previously described method (26, 27) was used to measure DNA-binding activity. The lux<sup>r</sup> gene product in RS buffer was diluted to 7.5 µg/ml in the buffer described by Riggs et al. (26) and was then added to a reaction mixture containing 200 nM autoinducer and 2–250 ng of [35S]pHK737 (specific activity was adjusted so that 1000 cpm/min was added to each reaction mixture) in Riggs buffer (final volume, 1 ml). These reaction mixtures were incubated at 25°C for about 20 min. Samples (0.4 ml) were filtered and washed as described elsewhere (26), except that 25-mm mixed nitrocellulose-cellulose acetate filters, 0.45-µm pore size, (Millipore) were used. The amount of radiolabel retained on each filter was determined. The data have been corrected for background counts (the counts retained on filters in the absence of lux<sup>r</sup> gene product). The [35S]pHK737 was prepared by digesting pHK737 with EcoRI and end-labeling with reverse transcriptase and [α-32P]dATP (specific activity, 3000 Ci/mmol; 1 Ci = 37 GBq).

Autoinducer-Binding Assays. One of the methods used to measure autoinducer binding to the purified lux<sup>r</sup> gene product was equilibrium dialysis (28, 29). The procedure used two Plexiglas chambers separated by dialysis membrane. One side of the membrane contained ED buffer (10 mM Tris, pH 7.8 at 5°C/100 mM KCl/10 mM magnesium acetate/0.1 mM EDTA and 0.1 mM dithiothreitol), 50 µg (ca. 2 µM) of lux<sup>r</sup> gene product or 50 µg (ca. 2 µM) of bovine serum albumin without or with 5 µg (0.01 µM) of closed circular pHK737 as indicated. The other side contained ED buffer with 0.1 µCi of tritiated autoinducer (30) at a concentration of 13 nM or 100 nM. Incubation was at 4°C with rotation at 25 rpm for 3 hr; equilibration was reached within this time. The amount of radioactivity in each chamber was determined.

The other procedure used to measure autoinducer binding was the membrane filter assay described above. However, for these assays tritiated autoinducer was present in the reaction mixtures at concentrations ranging from 0.02 nM (1 nCi) to 2 nM (100 nCi), and the amount of tritium retained on the filters was determined.

RESULTS

Construction of the lux<sup>r</sup> Expression Vector pHK724 and Overproduction of the lux<sup>r</sup> Gene Product. To overproduce the lux<sup>r</sup> gene product, a series of manipulations of the lux<sup>r</sup> DNA was performed (Fig. 1). A plasmid (pJE737) obtained from J. Engerbrecht containing lux<sup>r</sup> and part of lux<sup>i</sup> was used as a source of lux<sup>r</sup>. Expression of lux<sup>r</sup> is low in cells containing pJE737 in part because of the presence of lux<sup>i</sup> or its truncated gene product (J. Engerbrecht, personal communication). To increase expression of lux<sup>r</sup>, it was initially subcloned into pUC18 (Fig. 1). First, lux<sup>i</sup> and the regulatory region of the lux<sup>r</sup> gene were removed by digestion of pJE737 with BAL-31 nuclease. The DNA was then inserted into pUC18 and used to transform E. coli JM109 (pHK555). Clones were selected for ampicillin and chloramphenicol resistance and screened for luminescence in the presence of IPTG. Luminescence was due to a functional lux<sup>r</sup> gene product and the intact lux<sup>i</sup>, C,D,A,B,E on pHK555. To ensure that lux<sup>r</sup> was under control of the lac promoter, luminous clones were then screened to obtain those that did not make light in the absence of IPTG. NaDodSO<sub>4</sub>/PAGE of IPTG-induced E. coli containing one such plasmid, pHK705 (data not shown), and of in vitro transcription–translation products of pHK705 (Fig. 2) indicated, respectively, that expression of the lux<sup>r</sup> gene was poor (it could not be visualized on Coomassie blue-stained gels) and that a 29-kDa fusion product was synthesized.

To obtain a clone that did not encode a fusion protein, lux<sup>r</sup> was further subcloned into pKK223-3 (P. V. Dunlap and...
Fig. 1. The construction of pHK724. Restriction sites: B, BamH1; E, EcoR1; H, HindII; K, Kpn I; S, SalI; Sm, Sma I. P_{lac} and P_{acs} are the lac promoter and the tac promoter, respectively. SD is the Shine–Dalgarno sequence (ribosome binding site).

E.P.G., manuscript in preparation). The luxR gene was excised from pHK705, removing the lac ribosome binding site and translation initiation sequence (ATG), and was cloned into a site downstream of the pKK223-3 tac promoter (a hybrid of the lac and trp promoters that is regulated by IPTG and the lac repressor; ref. 31), resulting in pPD723 (Fig. 1). Because pKK223-3 contains a ribosome binding site (a Shine–Dalgarno sequence) but lacks a translation initiation sequence, translation of luxR in pPD723 requires a luxR initiation sequence. Clones containing pPD723 together with pHK555 produced light under control of IPTG; however, expression of the luxR gene was low as measured by NaDodSO4/PAGE of IPTG-induced E. coli (pPD723) (data not shown) in the presence or absence of pHK555. In vitro transcription–translation of pPD723 showed that the apparent monomeric molecular weight of the luxR gene product was indistinguishable from that of the gene product encoded by pHK737 (data not shown).

Sequence information for the DNA between the ribosome binding site on the vector and the DNA cloned from pHK705 indicated that 26 base pairs (bp) of vector (pKK223-3 and pUC18) were present. To increase the efficiency of translation, 16 bp from this region were removed (Fig. 1). The resulting molecules were ligated and transformed into E. coli (pHH55). Clones recovered were more highly luminescent in the presence of IPTG than those containing pPD723 and produced relatively high levels of light in the absence of IPTG. When IPTG-induced cultures of E. coli containing one resulting plasmid, pHK724, and pHK555 were monitored for luminescence, an autoinducer response was observed (H.B.K., unpublished data). The monomeric molecular weight of the in vitro transcription–translation product of luxR on pHK724 was indistinguishable from that encoded by pHK737 (Fig. 2).

Overproduction of the luxR gene product was not observed by NaDodSO4/PAGE of these luminescent cells containing both pHK724 and pHK555 when grown in the presence of IPTG. However, when IPTG-induced E. coli (pHK724) lacking pHK555 was analyzed by NaDodSO4/PAGE, the luxR gene product was found to constitute ~10% of the total cellular protein (Fig. 3) and synthesis of the luxR gene product was unaffected by the presence of pACYC184. It has been demonstrated previously that the synthesis of the luxR gene product is suppressed by the presence of luxI (10). Here we demonstrate suppression even when luxI is in trans and when luxR is not under control of its own promoter. Although the luxI gene product is involved in autoinducer synthesis (9, 10), addition of pure autoinducer (200 nM) together with IPTG to E. coli (pHK724) did not decrease synthesis of the luxR gene product (D. Kolbachuk and E.P.G., unpublished data).

Purification of the luxR Gene Product and Chemical Characteristics of the Purified Protein. Granules similar in appearance to cytoplasmic inclusion bodies were observed in cells of IPTG-induced E. coli (pHK724). Cytoplasmic inclusion bodies are insoluble precipitates of overproduced proteins sometimes observed in cells (25, 32). A characteristic of some of these insoluble overproduced proteins is their solubility in high concentrations of guanidine hydrochloride and poor solubility in detergents (24, 25). The overproduced luxR gene product exhibited these characteristics. The selective solubilization of the luxR gene product in 6 M guanidine hydro-
chloride from the material pelleted by centrifugation of sonicated detergent-treated cells formed the basis of our purification. Removal of the guanidine hydrochloride by slow dilution and dialysis in RS buffer resulted in a soluble, purified preparation of the luxR gene product that contained other polypeptides in minor amounts only (Fig. 3). Nonequilibrium pH gradient electrophoresis of the luxR gene product resulted in a band that migrated to the same relative position as the lentil lectin protein middle band (pI, 8.45). The amino-terminal sequence through residue 15 is shown in Fig. 4. This amino-terminal sequence is in agreement with that predicted by the DNA sequence of luxR (J. Devine, C. Countryman, and T. Baldwin, personal communication).

**DNA and Autoinducer Binding Studies.** Membrane filter binding assays were used to demonstrate that the purified luxR gene product bound to DNA; binding of this protein to DNA was saturable (Fig. 5), as was the binding of DNA to this protein (Fig. 6). Binding of DNA as a function of either DNA concentration or protein concentration was not reproducibly affected by autoinducer over a concentration range of 0–200 nM (data not shown). Competition experiments, in which increasing amounts of unlabeled vector, pUC8, or recombinant plasmid pHK737 (providing lux regulatory DNA) were added to the reaction mixture, indicated that, under the conditions used, binding was not lux DNA specific (data not shown).

Filter binding assays with various concentrations of tritiated autoinducer in the presence of the luxR gene product or the luxR gene product and lux regulatory DNA (provided on pHK737) did not reveal any binding of the luxR gene product to autoinducer. In addition, binding of the luxR gene product to autoinducer in the presence or absence of lux regulatory DNA (provided on pHK737) could not be detected by equilibrium dialysis. Either the purified protein had lost its ability to bind autoinducer, the experimental conditions were not appropriate for binding, the affinity constant was too low to detect binding in these assays, or the luxR gene product alone does not bind autoinducer.

**DISCUSSION**

The product of the luxR gene has been overproduced in E. coli (Fig. 3). This was accomplished by placing luxR under control of the tac promoter on pHK724 (Fig. 1). The overproduced protein was active in vivo, and the apparent monomeric molecular weight was indistinguishable from that produced by an unaltered luxR (Fig. 2). The cloning of luxR first involved transformation of E. coli containing the other lux genes on a compatible plasmid and screening for colonies that were luminous only when IPTG was present. Once a plasmid containing luxR under IPTG control was obtained, expression of luxR was optimized by construction of pHK724. luxR remained under IPTG control (Fig. 3); however, cells containing both pHK724 and pHK555 were luminous even in the absence of IPTG. The basal level of luxR synthesis directed by pHK724 was sufficient for in vivo

![FIG. 5. DNA binding as a function of the amount of luxR gene product. Each reaction mixture contained 50 ng (0.02 nM) of [32P]pHK737 and 200 nM autoinducer. Each point represents the average from duplicate filtrations, and the marker bars represent the range between the values.](image-url)

![FIG. 6. Dependence of DNA binding on the concentration of [32P]pHK737 DNA. Each reaction mixture contained 20 nM luxR gene product and 200 nM autoinducer. Bound DNA (relative units) was calculated from the specific activity of the DNA in each reaction mixture and the number of counts retained on the filters. Each point represents the average from duplicate filtrations, and the marker bars represent the range between the values.](image-url)
activity, presumably because this protein functions at very low concentrations. This sort of scheme, involving a screen for regulated activity in vivo, followed by optimization of expression, may be useful in attempts to produce large quantities of other transcriptional activators.

The tac-luxR construct in pHK724 directed the synthesis of large quantities of the luxR gene product in IPTG-induced E. coli lacking other lux genes (Fig. 3). In E. coli containing both this construct and pHK555 (luxI,C,D,A,B,E), the luxR gene product was not abundant enough to be visualized by gel electrophoresis. This is consistent with the conclusion of Engebrecht and Silverman (10), that the expression of luxR is low in the presence of luxI. We observed that the presence of luxI,C,D,A,B,E in low-copy number suppressed synthesis of the luxR gene product when luxR in high-copy number was controlled by the tac promoter and was not adjacent to luxI. These results suggest that the effector of luxR protein synthesis acts on a site within the luxR structural gene or it acts posttranscriptionally.

The overproduced luxR gene product was purified (Fig. 3) and used for an amino terminus amino acid analysis (Fig. 4). It was determined that the luxR gene product is basic, as is true of many other proteins that affect transcription (33). The purified luxR gene product exhibited DNA-binding activity (Figs. 5 and 6); however, specificity for lux DNA was not detected. Since no specific affinity for lux DNA was observed, it may be that the renaturation procedure used did not lead to correct refolding of the protein or that only a small percentage of the molecules folded correctly. However, no or low specificity of DNA binding, as measured by filter binding assays, is a problem not uncommon with purified transcriptional activators (26, 34, 35). It is also possible that the conditions used were not optimal for measurement of DNA-binding activity. Within this context, it is possible that other proteins are required for activity, perhaps RNA polymerase, as has been shown for cII protein of phage λ (36). Further characterization of the luxR gene product and its DNA-binding capacity will require an increase in its activity. In addition, genetic analyses can provide more precise information about binding sites for the LuxR protein, facilitating development of a specific DNA-binding assay.

The fact that no direct association of autoinducer with the luxR gene product in vivo was detected, either by filter binding assays or equilibrium dialysis in the presence or absence of DNA, provides further evidence to suggest that the native state of the protein was not recreated or perhaps conditions of the assays were not appropriate for the measurement of such interactions. It is also possible that the current model (9) in which the luxI,C,D,A,B,E operon is regulated by the direct interaction of autoinducer bound to the luxR gene product is incorrect.

The development of this rapid method for purification of large quantities of the luxR gene product has allowed a basic characterization of this protein and enabled us to demonstrate that this is a DNA-binding protein. It should now be possible to characterize the interaction of autoinducer and the luxR gene product biochemically and to determine the molecular mechanisms by which they serve to activate transcription.

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