ISSN 0026-2617, Microbiology, 2006, Vol. 75, No. 4, pp. 452–458. © Pleiades Publishing, Inc., 2006.
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CONFERENCE PROCEEDINGS

Involvement of Host Factors in the Regulation of the *Vibrio fischeri lux* Operon in *Escherichia coli* cells

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Received February 27, 2006

Abstract—It has been shown that the chaperonin GroEL, together with GroES co-chaperonin and Lon ATP-dependent protease are involved in the regulation of expression of the *Vibrio fischeri lux* operon in *Escherichia coli* cells. The cells of *E. coli gro*E *(pF1)*$^-$ bearing a plasmid with the complete *V. fischeri lux* regulon were weakly luminescent. The cells of *E. coli lon*A (pF1) displayed intense bioluminescence. The same effects also occurred in mutant *E. coli* strains bearing a hybrid plasmid pVFR1, where the *lux*R gene and the regulatory region of the *V. fischeri lux* operon were inserted before the *Photorhabdus luminescens lux*CDABE cassette. The *V. fischeri lux*R gene was cloned in the pGEX-KG vector with the formation of a hybrid gene *gst-lux*R. It was shown that affinity chromatography of the product of expression, the chimeric protein GST-LuxR, on a column with glutathione–agarose resulted in its copurification with the proteins GroEL and Lon. Consequently, LuxR, the transcription activator of the *lux* operon, forms complexes with these proteins. It is supposed that GroEL/GroES is responsible for the folding of the LuxR protein, and Lon protease degrades the LuxR protein either before its folding into an active globule or at denaturing.

DOI: 10.1134/S002626170604014X

Key words: *lux* operon, *Vibrio fischeri*, transcription regulation, LuxR, Lon, chaperonin, GroEL/GroES

lIn the marine bacterium *Vibrio fischeri*, the expression of *lux* genes is regulated by the LuxI–LuxR system, which determines the bioluminescence intensity in growing cells depending on their population density ("quorum sensing"): the luminescence is absent at low cell concentrations and drastically intensifies when the population reaches critical density [1, 2]. The *Lux* regulon of *V. fischeri*, *lux*RICDABEG, consists of two operons: *lux*R with promoter P_l, and *lux*ICDABEG with promoter P_r (Fig. 1). The genes *lux*I and *lux*R are regulatory. Acylsynthase LuxI synthesizes the autoinducer (AI), an acyl derivative of L-homoserine lactone, N-(3-oxo-hexanoyl) L-homoserine lactone [2], which plays the key role in bacterial "communication" since it freely diffuses across cellular membranes. The protein LuxR is a positive regulator (activator) of transcription of the operon *lux*ICDABEG. When binding with AI, LuxR acquires the ability to form a complex with the *lux* box, which is an inverted repetition of 20 bp in the region of P_r promoter, and to activate transcription of the *lux*ICDABE operon [3].

Besides the main regulators LuxI–LuxR, there are additional factors influencing the expression of *lux* genes in *V. fischeri*. For example, the CRP activator and the cAMP co-factor are involved in the regulation of *lux*R gene transcription (Fig. 1) [4]. The H-NS protein and σ subunit of RpoS RNA polymerase (σ^s) also participate in expression regulation of the *V. fischeri lux* genes at the level of transcription (Fig. 1) [5]. The H-NS protein can be classed as one of the "global" regulators influencing the transcription of different groups of genes in *E. coli*; in the case of the *lux* regulon in *V. fischeri*, it acts as a repressor inhibiting the transcription both from promoter P_l and from promoter P_r. Only the LuxR activator in complex with AI is able to overcome the repressor activity of H-NS. The contribution of the RpoS subunit to transcription of the *lux*R gene explains the well-known phenomenon of the considerable intensification of bioluminescence of the cells grown on a low-nutrient medium (the effect of "starvation"). The dark lag period significantly decreases and the bioluminescence of the cells with the *lux* regulon of *V. fischeri* intensifies on transfer to a medium with high ionic force or high sucrose content ("osmotic shock") [6]. During this process, the H-NS-induced repression is supposed to weaken due to the increase of the intracellular concentration of K^+ ions.

In addition to the factors influencing the expression of the *V. fischeri lux* system at the level of transcription, intracellular factors of a protein nature have been shown to affect the expression of *lux* genes on the post-transcriptional and posttranslational levels. For example, it has been shown that *E. coli gro*E mutant cells

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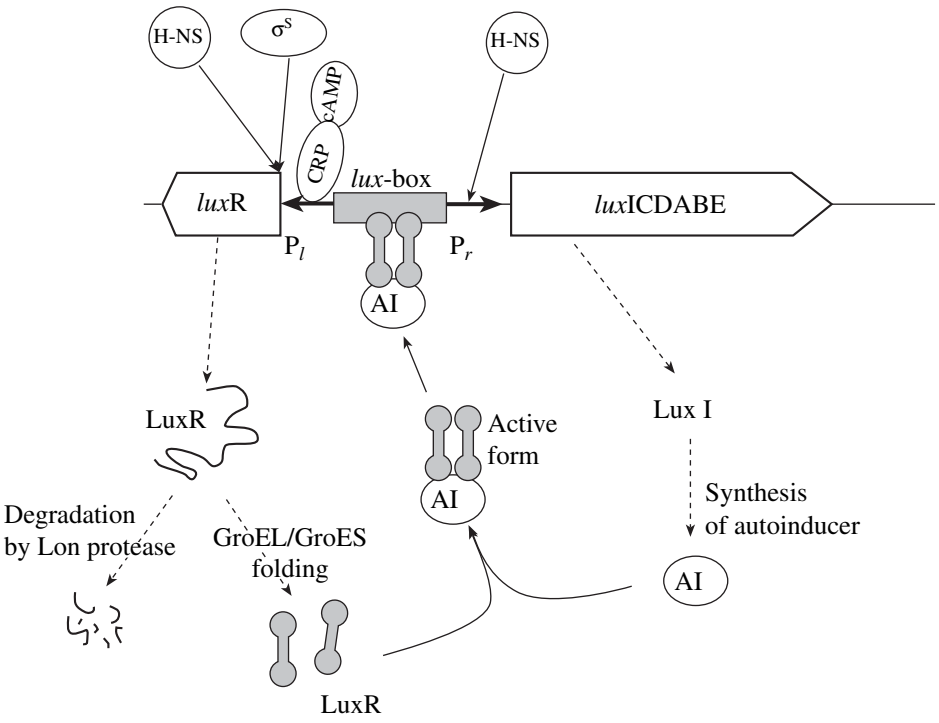

Fig. 1. Structure and transcription regulation of the *lux*-regulon of *Vibrio fischeri*.

containing a plasmid with the complete *lux* regulon of *V. fischeri* are characterized by a lower level of bioluminescence [7, 8]. It has also been revealed that *E. coli* cells with mutations in the *lon*A gene encoding the serine Lon protease and containing a plasmid with the *lux* regulon of *V. fischeri*, exhibit intensive bioluminescence even at cell concentrations significantly below the critical value [9]. It is supposed that GroEL/ES is involved in the folding of the activator protein LuxR and that LuxR is a target for Lon protease [10]. However, there is no direct evidence of the formation of LuxR complexes with proteins GroEL/ES and Lon. The goal of this work was to study the effect of GroEL/ES and Lon on expression of the genes of the *V. fischeri lux* regulon and to determine the nature of the target protein.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strain *E. coli* K12 SKB178 F⁻ *gal*E⁻ *sup gro*⁺ and its *gro*⁻ mutants: *gro*E97, *gro*EL140, and *gro*E673 have been isolated by C.P. Georgopoulos et al.; they are missense mutations with single amino acid substitutions (GroEL97 and GroEL140—the substitution S201F; GroEL673—substitutions G173D and G337D) [11]. Mutation *gro*ES30::Tn10 (strain C600 from the collection of GosNIIgenetika) was transduced to SKB178 cells. *E. coli* K12 AB1157 and AB1899 (from the collection of GosNIIgenetika): AB1157 F⁻ *thr*-1 *leu*B6 *pro*A2 *his*-4 *arg*E3 *thi*-1 *lac*Y1 *gal*K2 *ara*-14 *xyl*-5 *mtl*-1 *tsx*-33 *rps*L31 *sup*E44; AB1899 F⁻ *lon*A1, other markers as in AB1157. *E. coli* TG1 was used for production of chimeric protein GST-LuxR.

Hybrid plasmids: pF1 (vector pBR322 with the complete *lux* regulon of *V. fischeri* integrated by *Bam*HI site); pAC16 (vector pACYC184 with integrated *lux* regulon of *V. fischeri*); pF2 (vector pUC19 with integrated *lux*AB genes of *V. fischeri* coding luciferase). Vector pGEX-KG [12] was used for cloning of the *lux*R gene. The following hybrid plasmids were also used in the work: pBRlon on the basis of vector pBR327 with integrated *lon*A gene under its own promoter (provided by T. V. Rotanova, Institute of Biological Chemistry, Russian Academy of Sciences, Moscow), and pgroEL/groES on the basis of vector pACYC184 with integrated *gro* operon under its own promoter (provided by A. Vysokanov, USA). Plasmid DNA was isolated by the alkaline method.

Cultivation. Bacterial cultures were grown on a complete LB medium and minimal M9 medium. Antibiotics were used in the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 25 μg/ml; and tetracycline, 15 μg/ml. Bacteria were grown on solid media (LB + 1.7% agar) at 37°C or 30°C. Overnight cultures were used for inoculation of a liquid medium, LB or M9, followed by growth with aeration at 28°C. In the middle of the exponential growth phase, the inducer, isopropyl-β-*D*-thiogalactopyranozide (IPTG) was added to the medium and incubation was continued for 2–3 h at 28°C. The optical density (OD) of the cell suspension was determined with a KFK-2MP colorim-

eter at 590 nm. Cells were transformed by hybrid plasmids using either cell treatment by calcium ions or on an electroporator. The *gro*ES30::Tn*10* gene was transduced using bacteriophage P1vir. Expression of the *lux* genes was controlled by measuring the bioluminescence intensity of the cells.

Bioluminescence of the cells bearing hybrid plasmids was measured on a luminometer consisting of a FEU-85 photomultiplier and a B2-15 microvoltmeter. Bioluminescence intensity (**I**) was measured in special cuvettes containing 200 μl of the preparation at room temperature.

Construction of plasmid pVFR1. A fragment of *V. fischeri* DNA (about 1 kbp) was isolated using primers:

5'-CCAACCTCCCTTGCGTTTATTCG-3' and

5'-GAGCAGCTTATTCGTCAAGATTTC-3'

(plasmid pF1 was a template) and integrated in a T-vector (constructed on the basis of pUC18), and then recloned by sites *Eco*RI – *Bam*HI into the promoter-free vector pDEW201 [13] so that the regulatory region of the *lux* regulon was located immediately before the *lux*CDABE genes of *Photorhabdus luminescens*. Transcription from the right promoter P_r was initiated by addition to the medium of AI, N-(3-oxohexanoyl) L-homoserine lactone (Sigma).

Cloning of the *lux*R gene. The gene *lux*Rwas isolated from the pF1 plasmid carrying the *lux* regulon of *V. fischeri* and cloned in vector pGEX-KG. The DNA fragment with the gene *lux*R was amplified using PCR with the following primers:

LuxR/5'5'-GCTTTACTTACGTACTTAAC-3'

LuxR/3'5'-GGATAAATCGATGGGTATGA-3'

(the regions by which the fragment was inserted into vector pGEX-KG are underlined). As a result, a hybrid plasmid was obtained (marked as pGEX-LuxR). This plasmid determines the synthesis of a chimeric protein consisting of glutathione-S-transferase (GST, carrier protein) and LuxR protein connected by a linker Leu–Val–Pro–Arg–Gly–Ser–Pro–Gly which is hydrolyzed by thrombin over the Arg–Gly bond [12].

Isolation and purification of the chimeric protein GST-LuxR. Cells of the strain *E. coli* TG1 transformed by the plasmid pGEX-LuxR were grown in LB medium with ampicillin to the middle of the exponential growth phase. The promoter (P_{tac}) was induced by IPTG. The cells were centrifuged, destroyed by ultrasound, and the cell-free extract was obtained after repeated centrifugation. GST-containing proteins were further precipitated using affinity chromatography on glutathione–agarose adsorbing only GST-containing proteins, followed by elution of these proteins with reduced glutathione solution. The GST-LuxR protein was hydrolyzed by thrombin (1.5 ml of reaction mixture containing 0.5 mg of protein GST-LuxR and 6 mg (330 U act/mg) of thrombin in buffer A were incubated at 25°C for 2 h). Protein fractions were analyzed using SDS electrophoresis in polyacrylamide gel: instead of the 54 kDa band, two bands of 26 and 28 kDa were registered in the gel (data not shown). The N-terminus of the protein LuxR had a fragment of four amino acid residues: Gly–Ser–Pro–Gly.

Electrophoresis was carried out in 12% polyacrylamide gel under denaturing conditions (0.1% sodium dodecyl sulfate, SDS).

Mass-spectrometric analysis of peptides. Proteins isolated from the gel after SDS electrophoresis were identified using mass spectrometry. Samples for the mass-spectrometric analysis MALDI-TOF (matrix-assisted laser description ionization-time of flight) were prepared according to [14, 15]. Mass spectrometry of the samples was performed after trypsin treatment on a REFLEXIII instrument (Brucker) in the Center of Proteomic Research, Institute of Biomedical Chemistry, Russian Academy of Medical Sciences. The obtained mass fingerprints were identified using the SWISS-PROT database [16].

RESULTS AND DISCUSSION

Figure 2 shows the curves of the dependence of bioluminescence intensity (*I*) on the concentration (*OD*) of the cells of *E. coli* SKB178 *gro*$^+$ and of the mutants *gro*EL and *gro*ES bearing a hybrid plasmid pF1 with the *V. fischeri lux* regulon. The cells were grown in the LB medium at 28°C. In the cells mutant in the genes *gro*EL and *gro*ES, the bioluminescence intensity was considerably lower. Introduction of a hybrid plasmid pgroEL/groES into the cells of mutant strain *gro*E97 resulted in the recovery of bioluminescence intensity (Figs. 2, 5; excess of bioluminescence intensity as compared to the bioluminescence of *gro*E$^+$ cells is determined by the gene dosage). Consequently, GroEL/GroES is necessary for the synthesis of active luciferases.

In contrast to GroEl/GroES, the ATP-dependent serine protease Lon participates in the negative control of *lux* regulon expression. Figure 3 shows the curves of bioluminescence intensity dependence on concentrations of *E. coli E. coli* K12 AB1157 *lon*$^+$ and AB1899 *lon*A1 cells containing the plasmid pAC16 with the *lux* regulon of *V. fischeri*. The cultures were grown in the LB medium at 28°C. At all *OD* values, the cells with mutation in the *lon*A gene were several orders of magnitude more luminescent than the *lon*$^+$ cells. The introduction of the plasmid pBRlon (the vector pBR327 bears the active *lon*A gene under the own promoter) into AB1899 lonA1 cells resulted in a significant decrease of bioluminescence intensity at all *OD* values (Fig. 3, *3*).

The effect of mutations in the *gro*EL gene on the expression of the *V. fischeri lux* operon is restricted by the *lux*R gene and its product, the activator protein LuxR. This conclusion follows from the data obtained using the constructed plasmid pVFR1 (Fig. 4). In plas-

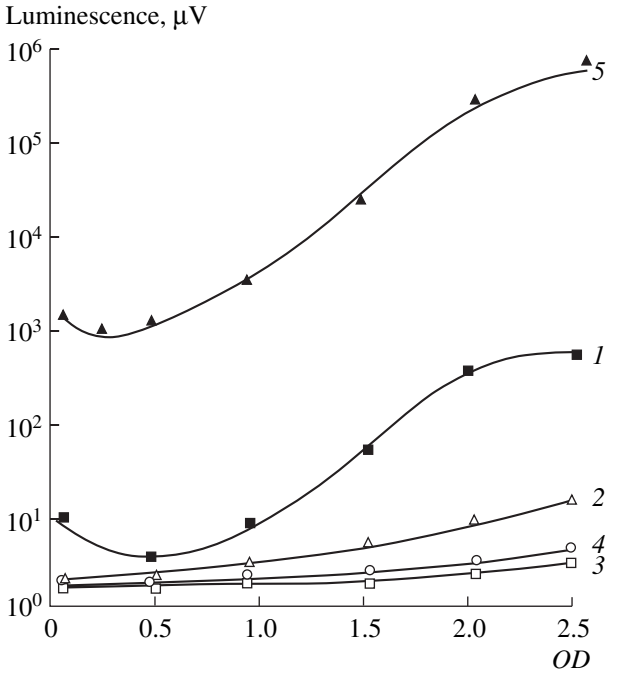

Fig. 2. Effect of mutations in the genes *gro*EL and *gro*ES on bioluminescence intensity of *E. coli* SKB178 (pF1). The curves of dependence between the bioluminescence intensity (*I*) of the cell suspension and the cell concentration (*OD*) are presented. Cells bearing plasmid pF1 were inoculated at initial *OD* = 0.01 and grown in LB medium with aeration at 28°C. *1*—SKB178 *gro*$^+$; *2*—*gro*EL97; *3*—*gro*EL673; *4*—*gro*ES::Tn*10*; *5*—*gro*EL97 (pgroEL/ES).

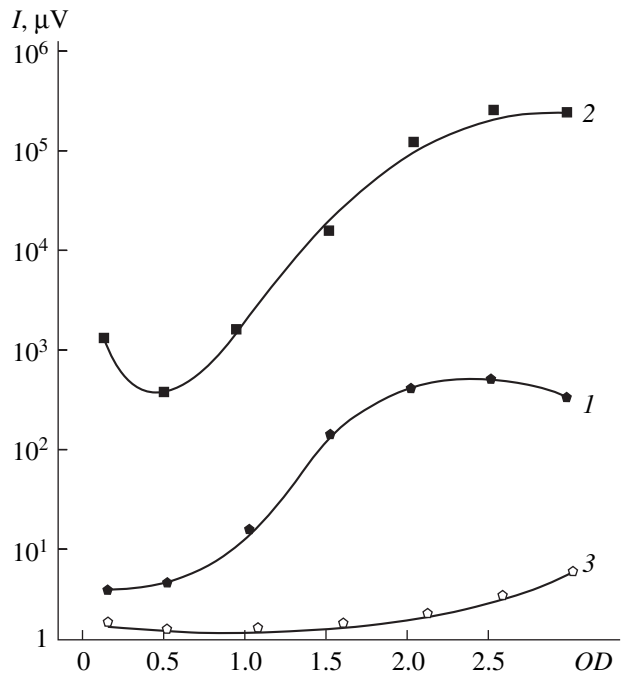

Fig. 3. Effect of mutation in the *lon*A gene on bioluminescence intensity of *E. coli* K12 AB1157 (pAC16) and AB1899 (pAC16). Curves of dependence between the bioluminescence intensity of suspension and the cell concentration (*OD*) are presented. Cells bearing plasmid pAC16 were inoculated at initial *OD* = 0.01 and grown in LB medium with aeration at 28°C. *1*—AB1157 *lon*$^+$(pAC16); *2*—AB1899 *lon*A1 (pAC16); *3*—AB1899 *lon*A1(pAC16, pBRlon).

mid pVFR1, the *lux*R gene is cloned jointly with the regulatory region of the *lux* operon with promoters P_l and P of *V. fischeri* and, instead of the genes *lux*ICDABE of *V. fischeri*, there is an integrated cassette with the *lux*CDABE *P. luminescens* genes of *P. luminescens* encoding thermostable luciferase. Transcription of the right promoter P_r and, accordingly, luciferase synthesis is initiated by the introduction of AI molecules into the medium. As can be seen, a significant decrease of bioluminescence intensity occurs in the cells of strain SKB178 *gro*EL673 bearing plasmid pVFR1 (Fig. 5a). It should be noted that the defect in the mutant gene *gro*EL673 is compensated to a certain extent by the addition of high AI concentration to the medium. Figure 5b shows the dependence of the maximal bioluminescence intensity of cells on AI concentration. While the minimal (threshold) AI concentration in the case of strain SKB178 *gro*$^+$ is approximately 10^{-9} M, the threshold AI concentration for the mutant strain SKB178 *gro*673 is approximately 10^{-6} M.

For in vitro production of the protein LuxR, we cloned the *lux*R gene in vector pGEX-KG (see Materials and Methods). As a result, we obtained a hybrid plasmid (denoted as pGEX-LuxR) carrying the *lux*R gene, the 5'-end of which was "linked" by a spacer with the 3'-end of the gene encoding the carrier protein glutathione-S-transferase (GST, molecular weight of 26 kD), with preservation of the reading frame (translational fusion). This plasmid determines the synthesis of the chimeric protein consisting of (counting from the N-terminus) GST and the protein LuxR (molecular weight 28 kDa) connected by a linker Leu-Val-Pro-Arg-Gly-Ser-Pro-Gly which is hydrolyzed by thrombin at the Arg-Gly bond.

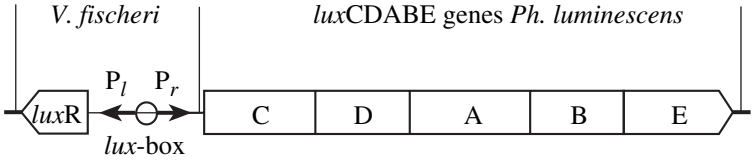

Fig. 4. Structure of plasmid pVFR1.

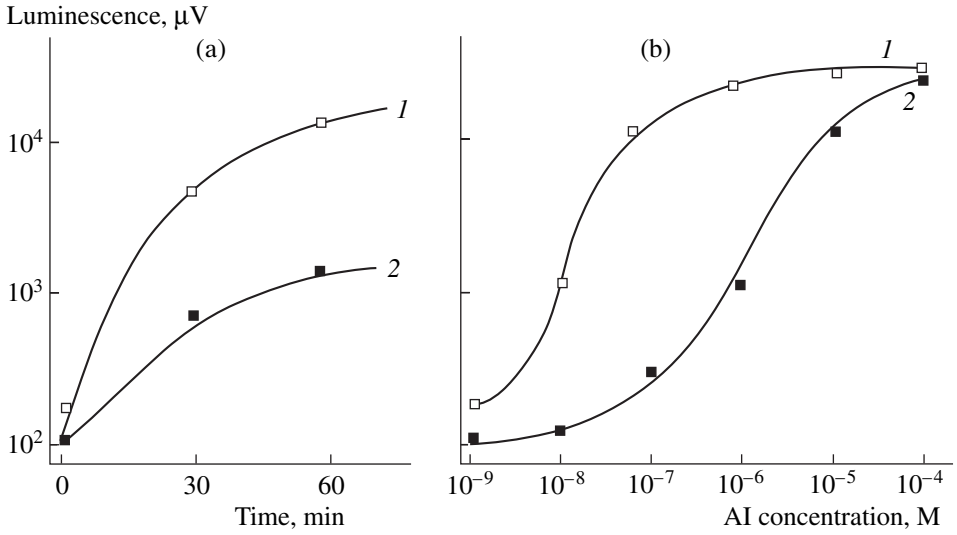

Fig. 5. Dependence between the bioluminescence intensity of cells *E. coli* SKB178 *gro* and SKB178 *gro*EL673 (bearing plasmid pVFR1) and the time of incubation at 22°C (a). The cells of overnight culture were inoculated at initial OD = 0.01 and grown in LB medium with ampicillin (100 μg/ml) with aeration at 28°C to the mid-log growth phase (OD 0.4–0.5), then incubated for 30 min at 42°C, and after AI addition, to the final concentration of 10^{-6} M, incubation was continued without agitation at 22°C. 200 μl samples were taken in fixed time intervals, and bioluminescence intensity was measured. *1*—SKB178 *gro*$^+$; *2*—*gro*EL673. (b) Dependence of maximal cell bioluminescence intensity on AI concentration: *1*—SKB178 *gro*$^+$; *2*—*gro*EL673.

Affinity purification of the chimeric protein GST-LuxR was performed as follows. *E. coli* cells bearing the plasmid pGEX-LuxR were grown in LB medium to OD 0.6–0.8. Then IPTG was added to the medium to the concentration of 1 mM and the suspension was incubated for 2 h at room temperature. Cells (200 ml) were centrifuged and transferred into buffer A (50 mM Tris-HCl, pH 7.3, 10% glycerol). Cells were lysed by ultrasound disintegration; the lysate was diluted by buffer A and centrifuged at 18000 *g* for 20 min in cold. The supernatant was twice run through a column with glutathione–agarose, prebalanced with buffer A. After adsorption, the column was washed with buffer A to remove unbound proteins. The chimeric protein GST-LuxR was eluted with buffer A containing 10 mM reduced glutathione. The molecular weights of proteins obtained as a result of affinity purification were assessed by SDS electrophoresis in polyacrylamide gel (12%). Figure 6 shows the separation of proteins in polyacrylamide gel. Besides the band of the main protein GST-LuxR (mol. weight 54 kDa), two additional bands can be seen: an intensive band in the 60 kDa region and a weak one in the 90 kDa region.

Mass spectrometric analysis of peptides was performed as follows. The proteins isolated from the gel after SDS electrophoresis were prepared for MALDI-TOF mass-spectrometric analysis. Mass spectrometry of the samples was performed after the treatment with trypsin on a REFLEXIII instrument (Brucker). The resulting mass fingerprints were identified according to the SWISS-PORT database [16]. Mass-spectrometric protein analysis showed that the proteins isolated from 60 and 90 kDa bands, judging from the composition and distribution of peptide fingerprints, were chaperonin GroEL (60 kDa) and protease Lon (90kDa) according to the SWISS-PORT database. Thus, it can be concluded that the protein activator of *lux* operon transcription, LuxR, forms complexes both with chaperonin GroEl and with protease Lon.

It should be noted that, according to the data presented in Figure 6, protein GroEL is excreted together with the chimeric protein GST-LuxR not only from wild type cells but also from mutant *gro*$^-$ cells. Consequently, the *gro*$^-$ mutants belonging to the group of missense mutants, which are used in this work, do not lose the ability to bind target proteins but are unable to fold them correctly. For strain SKB178 *gro*EL140 (and, consequently, for strain SKB178 *gro*EL97 identical to it), such a conclusion was made on the basis of measurement of in-vitro-formed complexes of GroEL140 with denatured protein substrate, Rbu-P2-carboxylase [17].

The findings of this work concerning the formation in vivo (in *E. coli* cells) of the complexes of transcription activator of the *lux* genes of *V. fischeri*, LuxR, with proteins GroEL and Lon suggest that Lon protease degrades the LuxR protein, and GroEL/GroES performs its correct folding during biosynthesis. As a result, the quantity of molecules of LuxR transcription activator in *E. coli lon*$^-$ cells is higher than in *lon*$^+$ cells, which results in a significant increase of cell bioluminescence during growth. *E. coli* cells with mutations in the genes *gro*EL-*gro*ES have much lower effectiveness of correct LuxR folding, which results in a drastic drop in bioluminescence intensity.

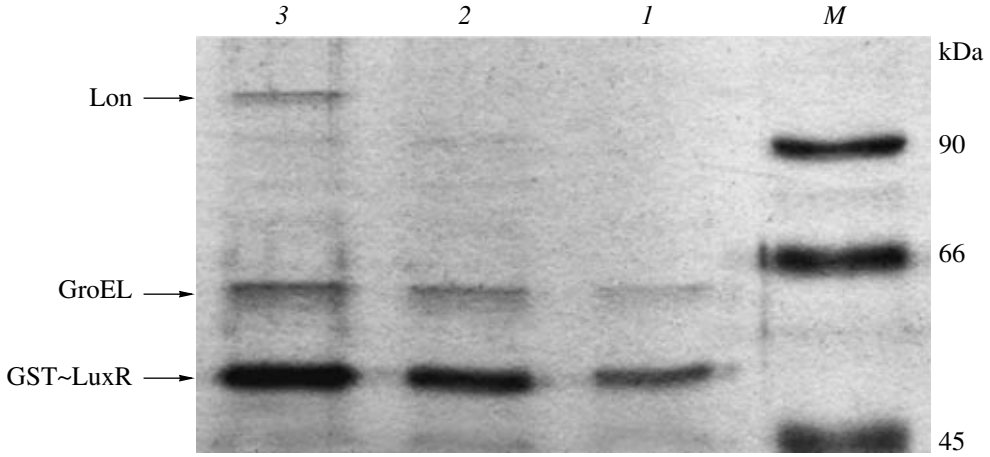

Fig. 6. SDS electrophoresis in polyacrylamide gel (12%) of the protein fraction obtained as a result of affinity chromatography on a column with glutathione–agarose. Protein fraction was isolated from cells of the following strains (all strains bearing plasmid pGEX-LuxR): *1*—*E. coli* SKB178 *gro*$^+$; *2*—*E. coli gro*EL97; *3*—*E. coli gro*EL673; *M*—marker proteins.

Apparently, cell proteases are widely used as negative regulators of expression of the genes which are controlled by systems of the "quorum sensing" type. For example, it is shown that the proteases Clp and Lon cause in vivo degradation of the protein TraR in *E. coli* cells [18]. TraR, a transcription activator of the genes of conjugative transfer in *Agrobacterium tumefaciens* Ti plasmid, belongs to the LuxR family [19].

In this work, the effect of proteins GroEL/GroES and Lon on expression of the *lux* system of *V. fischeri* is demonstrated in *E. coli* cells. However, one can be quite certain also of the involvement of these proteins in marine bacteria *V. fischeri*, because the recent analysis of the genomic nucleotide sequence of *V. fischeri* revealed the presence of the genes *gro*EL/*gro*ES and *lon*, which were highly homologous to the corresponding genes of *E. coli* [20].

ACKNOWLEDGMENTS

We are grateful to S.Z. Mindlin (Institute of Molecular Genetics, Russian Academy of Sciences, Moscow) for strains SKB178 *gro*$^-$; to T.V. Rotanova and N.N. Starkova (Institute of Biological Chemistry, Russian Academy of Sciences, Moscow) for plasmid pBRlon; to T. K. Van Dyk (USA) for plasmid pDEW201; and to A. Vysokanov (USA) for plasmid pgroEL/ES.

The work was supported by the Russian Foundation for Basic Research (grants no. 05-04-49341 and 05-04-08033).

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