

Full Length Research Paper

Molecular cloning and expression of the luciferase coding genes of *Vibrio fischeri*

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Vibrio fischeri is a symbiotic marine bacterium and a nonpathogenic member of the Vibrionaceae which produces luminescence by expressing the lux operon. The lux operon encoding luciferase (*luxAB*) and proteins required to synthesize the aldehyde substrate (*luxCDE*), is controlled with *luxR* and *luxI*. In this study, we amplified the chromosomal fragment contains *luxAB* of *V. fischeri*, amplified fragment cloned into the pTZ57R vector and sequencing to confirmed the fragment. The sub cloning of *luxAB* gene was carried out in the pETDuet-1 expression vector and expression procedures were performed in *Escherichia coli* strain Nova blue. As a result, a 2046 bp fragment which contains the whole fragment of luciferase coding genes and intergenic sequences were cloned in pETDuet-1 expression vector. pETDuet *luxAB* recombinant plasmid was confirmed by restriction analysis; subsequently 76 kD expressed protein was detected using SDS-PAGE and western blot using specific polyclonal antibody. In this study, cloning of the luciferase coding genes was performed successfully, in which the synthesized construct can be applied as a reporter cassette in prokaryotic systems and as a marker or tag in the manipulation, and the control of gene expression in the fields of research, production, control of microorganism and other biotechnological applications.

Key words: *Vibrio fischeri*, lux operon, luciferase, luminescence.

INTRODUCTION

Vibrio fischeri is a marine bioluminescence bacterium and a nonpathogenic member of the Vibrionaceae, a large family of marine γ -proteobacteria, (Thompson et al., 2004) it lives both as a free living organism and also as a symbiotic in the light-emitting organs of the Hawaiian bobtail squid, *Euprymna scolopes*, where it produces luminescence by expressing the lux operon (Nyholm and Mcfall-Ngai, 2004; Ruby, 1999; Visick and Mcfall-Ngai, 2000). In seawater and free-living form, *V. fischeri* exists at low cell densities and appear to be non-luminescent, while in light organ symbiosis with fish and squid, where the density of *V. fischeri* cells is high, it is usually luminescent (Sitnikov et al., 1995). In the best studied luminous bacterium, *V. fischeri*, there are at least eight lux genes encoding the proteins essential for luminescence. The lux regulon is organized in two operons with a divergent transcription pattern. The seven genes (*luxICDABEG*), followed by a transcriptional terminator comprise the rightward operon, which encode the enzymes required for the synthesis of the autoinducer (*luxI*) and the alpha (*luxA*) and beta (*luxB*) subunits of the enzyme luciferase, the *luxC*, *luxD* and *luxE* genes encode the enzymes participating in the formation of the long-chain aldehyde, *luxG* is not essential for luminescence but is believed to increase the capacity of the cell to synthesize flavin mononucleotide (FMN). The leftward operon consists of a single gene, *LuxR*, encoding the transcriptional regulatory protein (Figure 1) (Visick et al., 2000). Bacterial luciferase is a heterodimeric enzyme of 77 kDa comprising α and β

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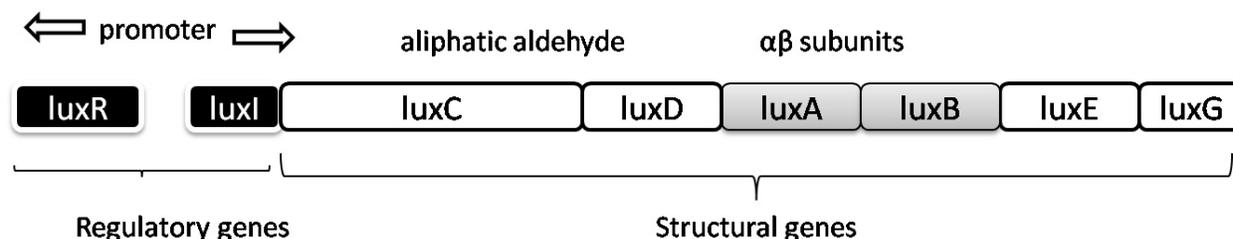


Figure 1. Luciferase gene organization in *V. fischeri*: Structural genes contain, luxA and luxB, luxC, luxD, luxE and regulatory genes contain, luxI and luxR.



Figure 2. The reaction of bacterial luciferase.

subunits with approximate molecular size of 40 and 37 kDa, respectively. Amino acid sequence alignment between the two subunits reveals that, they share 32% sequence identity. The α subunit contains 29 additional amino acid residues inserted between residues 258 and 259 of the β subunit, indicating that the two genes have arisen by gene duplication through the course of evolution. There is a single active center in the luciferase heterodimer that resides on α subunit and binds one reduced flavin molecule. It seems that, β subunit is essential for a high quantum yield reaction (Baldwin et al., 1995; Fisher et al., 1996).

In the luminescence reaction, luciferase converts aliphatic-aldehyde substrate, oxygen and reduced FMN (FMNH_2) into the corresponding aliphatic acid, water and FMN, with the concomitant production of light (Figure 2). In the absence of the aldehyde substrate, luciferase catalyzes a reaction that yields no light and produces oxygen radicals rather than water (Nelson et al., 2007).

Luminescence in *V. fischeri* is controlled by a population density- responsive regulatory mechanism called quorum sensing (Dunlap, 1999). At low cell population density, *LuxI* synthesizes only basal levels of the signal, 3-oxohexanoyl L-homoserine lactone (N3-oxo-C6-HSL); however, with increasing cell density the signal molecule accumulates to reach a threshold concentration that can bind to *LuxR*. The LuxR-AHL complex directly activates transcription of the *luxICDABEG* genes and resulting light production. *V. fischeri* cells also synthesize a second autoinducer molecule, octanoyl L-homoserine lactone (C8- HSL), that under some conditions it may stimulate transcription of the lux genes (Fuqua et al., 1994; Milton, 2006; Ulitzur, 1998; Waters and Bassler, 2005). The *V. fischeri* transcriptional regulator, *LitR* also participates in luminescence regulation by inducing the transcription of *luxR*, particularly at low cell densities (Lupp et al., 2003).

The aim of the current study was to clone the alpha (*luxA*) and beta (*luxB*) subunits of the enzyme luciferase

gene in a suitable prokaryotic expression vector in order to express and produce the desired protein in *Escherichia coli* and it can be used as a reporter gene construct for further biological and biotechnological applications. Moreover, purification and testing the biological activities along with comparison with other reporter gene systems or other constructs which carry luciferase genes will be the next goals of this research work.

MATERIALS AND METHODS

Bacterial strains and plasmids

V. fischeri strain, ATCC 7744 was kindly provided by Iranian Research Organization for Science and Technology (IROST) which was grown at 20 to 25°C. Nutrient media used for the growth of *V. fischeri* was DSMZ medium 246 that contained artificial seawater (750.0 ml), peptone (10.0 g), beef extract (10.0 g) and water (250.0 ml). The ingredients of artificial sea water were NaCl (28.13 g), KCl (0.77 g), $\text{CaCl}_2 \times 2\text{H}_2\text{O}$ (1.60 g), $\text{MgCl}_2 \times 6\text{H}_2\text{O}$ (4.80 g), NaHCO_3 (0.11 g), $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ (3.50 g) in 1000.0 ml distilled water. Cloning vector pTZ57R (2.8 kb) and expression vector pETDuet-1 (5.4 kb) were obtained from Fermentase and Novagene, respectively.

Genomic DNA extraction, PCR and sequencing

Genomic DNA extraction was carried out by Phenol-chloroform procedure and precipitated in ethanol (Sambrook and Russell, 2001). Sense and antisense oligonucleotide primers were designed (Table 1) based on the nucleotide sequence data of *luxA* and *luxB* obtained from Gene Bank (NC_006841.1 for *luxA* and NC_006841.2 for *luxB*). PCR reaction mixture (30 μ l) contained, 1 μ g DNA 0.1 mM dNTPs, 1.5 mM MgCl_2 , 1X PCR buffer, 20 pmol of each primers and 1.25 units Taq DNA polymerase (CinnaGen, Iran).

PCR amplification was performed under the following condition: denaturation step; 5 min at 94°C followed by 30 cycles of 40 s at 94°C, 60 s at 51°C in the annealing step and 60 s at 72°C for the extension step. The resulting PCR product was extended for a further 5 min at 72°C. The PCR product was analyzed on a 1.5% agarose gel and purified by using DNA extraction kit (Fermentase Lithuania) according to the manufacturer's instructions and

Table 1. Primers for amplifying *lux* genes by PCR.

| <i>LuxAB</i> | Primer |
|-----------------|-----------------------------------|
| Forward (FluxA) | <u>GGATCCATGAAGTTTGGAAATATTTG</u> |
| Reverse (RluxB) | <u>GGTACCTTAAGGCAGATTCTTTTC</u> |

The primers are presented from 5' to 3' ends; the introduced restriction sites are underlined.

subjected to sequencing using dideoxynucleotide chain termination method.

Construction of recombinant plasmids pETDuet-*luxAB*

To construct luminescent *E. coli*, purified PCR product of *luxAB* gene was ligated to a 3' T tailed EcoRV digested pTZ57R (Gaastra and Hansen, 1984) and transformed in *E. coli* TOP10 competent cells as described previously (Hanahan, 1983). Recombinant clones were confirmed by universal PCR and restriction enzyme digestion analysis. The *luxAB* gene was released by BamH1 and Kpn1 digestion and subcloned in to the pETDuet-1 expression vector.

Protein expression

PETDuet-*luxAB* was transformed in *E. coli* Nova blue strain and selected on LB agar containing 50 µg/lm of ampicillin. A bacterial colony was inoculated into a medium (1.2% Bacto tryptone, 2.4% yeast extract, 0.04% glycerol, 1% M9 salts (6.4% Na₂HPO₄·7H₂O, 1.5% KH₂PO₄, 0.025% NaCl, 0.05% NH₄Cl)) and incubated overnight at 37°C in a shaker incubator at 200 rpm. The overnight cultured bacteria was inoculated into a 50 ml flask and incubated at 37°C on orbital shaker incubator at 200 rpm. Cultures in logarithmic phase (at OD₆₀₀ of 0.6) were induced with IPTG (0.5 mM) and samples were collected before and after induction (0, 3 and 5 h), cells were lysed in 2x sample buffer (100 mM Tris-HCl pH 8, 20% glycerol, 4% SDS, 2% beta mercaptoethanol, 0.2% bromo phenol blue) and separated by 12% SDS-PAGE. The gel was stained by Coomassie brilliant blue R250 and gene expression analyzed in comparison with uninduced control samples in parallel (Smith, 1984).

Western blot analysis using antibody

For western blot analysis, cell lysate was separated on 12% (v/v) SDS-PAGE and electrophoretically transferred onto nitrocellulose membrane, after UV cross linking for protein fixation; the membrane was blocked with 3% BSA at room temperature. The bacterial luciferase antibody (HRP) (Abcam, UK) was used as specific antibody in 1:1000 of the protein band was performed by DAB (Diamino benzoic acid) and H₂O₂ (Shewry and Fido, 1998).

RESULTS

A DNA fragment encoding the *luxAB* part of lux operon was amplified by PCR using sense and antisense primers, specific restriction sites for BamH1 and Kpn1 were introduced into 5' end of forward and reverse primer, respectively. The PCR product was analyzed in 1.5% agarose gel after electrophoresis (Figure 3).

The PCR product of *luxAB* was purified from agarose gel using Fermentase kit and ligated into cleaved pTZ57R cloning vector. Following the confirmation of the cloned luciferase coding genes by sequencing, using BamH1, Kpn1 restriction enzymes pTZ57R/*luxAB* plasmid was digested, a 2046 bp DNA fragment purified, ligated into the BamH1, Kpn1 sites of an expression vector (pETDuet-1) and transformed into *E. coli* TOP10; recombinant plasmid was confirmed through restriction digestion using BamH1 and Kpn1 enzymes and named pETDuet -1/*luxAB*, (Figure 4); subsequently, transformation procedure continued into *E. coli* expression strains including BL21, Nova blue and JM109.

After induction with IPTG (1 mM), produced proteins were run onto 12% SDS- PAGE gel, the three protein bands 37, 40 and 76 kD were detected, which indicates protein expression of *luxB*, *luxA* and *luxAB* genes, respectively as shown in Figure 5. The best protein expression results was obtained after transformation of the recombinant plasmid onto Nova blue cells in comparison with two other *E. coli* strains JM109 and BL21. The gene segment was under the control of T7 promoter in the expression vector and the expressed proteins were accumulated in the cytoplasm.

The protein identity was verified by western blot analysis. The assay revealed specifically recognition of luciferase coding genes (*luxAB*) by bacterial luciferase antibody (a rabbit polyclonal antibody to bacterial luciferase, IgG HRP conjugated), whereas no reactivity was observed in control sample (Figure 6).

DISCUSSION

Gene screening with an easily assayable product, reporter genes, amplify the signal from the cell surface to produce a rapid, highly sensitive, reproducible and easily detectable response. The variety of reporter genes available including β-galactosidase (*lacZ*), chloramphenicol acetyltransferase (*CAT*), insect luciferase (*luc*), bacterial luciferase (*lux*), alkaline phosphates (*phoA*), β-lactamase (*bla*), β-glucuronidase *uidA* (*gusA*, *gurA*), green fluorescent protein (*GFP*) and their applications are very broad in both *in vitro* and *in vivo* assays (Jiang et al., 2008; Köhler et al., 2000; Naylor, 1999). Among all studied reporter genes, bacterial luciferase are the most abundant, widely distributed, extensively studied and the best understood of all types of bioluminescent genes. It is

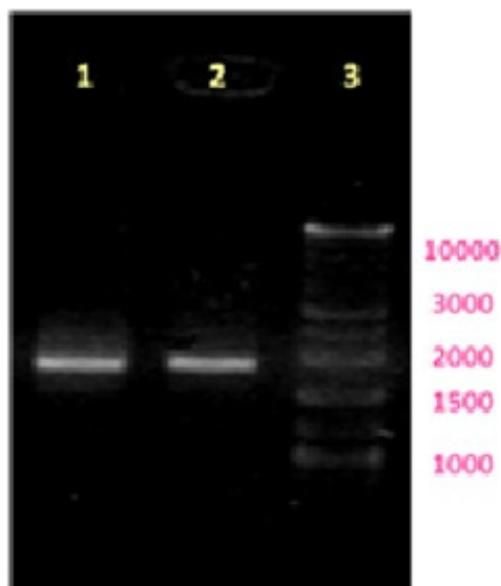


Figure 3. PCR amplification of the *luxAB* coding region using FluxA and RluxB primers. Lane 1 and 2 are the same PCR products; lane 3 is a DNA ladder marker.

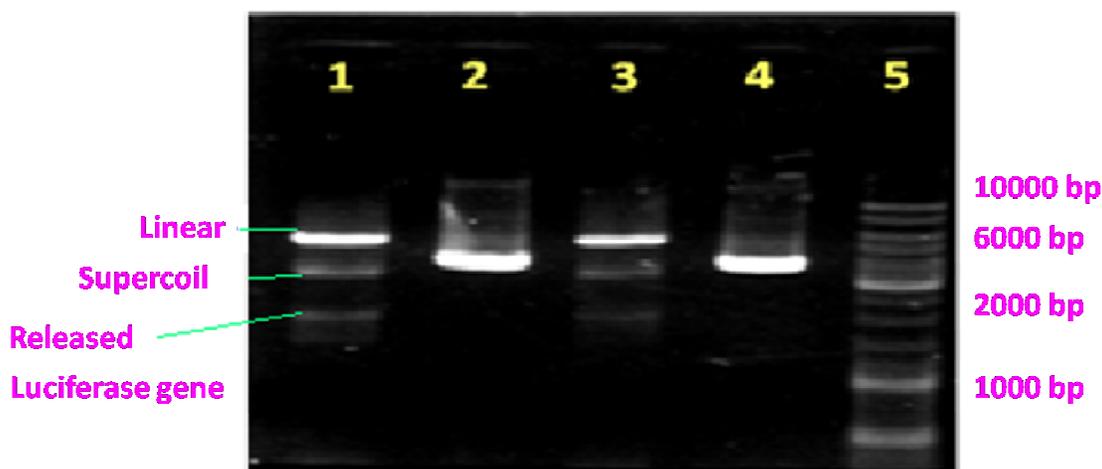


Figure 4. lane 1 and 3; the products of digestion with BamH1 and Kpn1; lane 2 and 4 uncut recombinant plasmid; lane 5 molecular size marker 10000, the released fragment migrated below the 2500 bp fragment of the DNA ladder.

an α/β heterodimer, a flavine monooxygenase which is homologous in all bacterial luciferase, catalyzes the oxidation of a long-chain aldehyde and releases energy in the form of visible light. Yet luciferase coding genes have been isolated and cloned from different bacterial strains like *Vibrio harveyi*, *V. fischeri*, *Photobacterium phosphoreum*, *Photobacterium leiognathi* and *Xenorhabdus luminescens* in separate or fusion forms (Meighen, 1994; Wilson and Hasting, 1998). The lux

genes have been transferred into *E. coli* and a multitude of different prokaryotic species by transformation, transduction, conjugation or even bacterial genome integration, using a variety of different plasmid vectors (Meighen, 1991).

Previous studies indicate that, the genes encoding luciferase subunits can generate heterodimers in various forms (AB1, AB2, BA1, BA2 and A+ B), but the enzyme activity is decreased in comparison with wild type binary

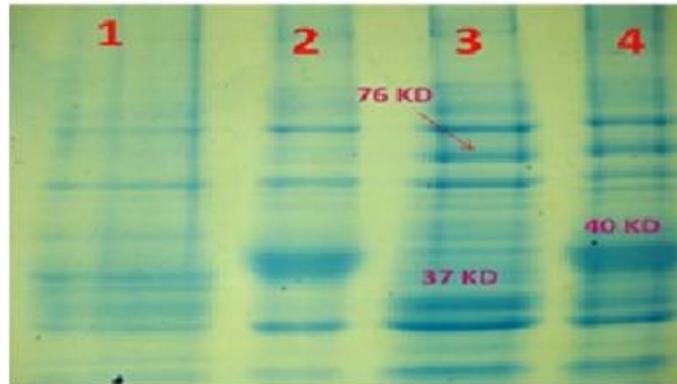


Figure 5. SDS PAGE analysis, lane 1 control cell lysate without recombinant plasmid; Lane 3 and 4 cell lysate of *E. coli* Nova blue containing pETDuet-1/*luxAB* in different sampling times (3 and 5 h, respectively) after induction with IPTG; lane 2 before induction with IPTG.

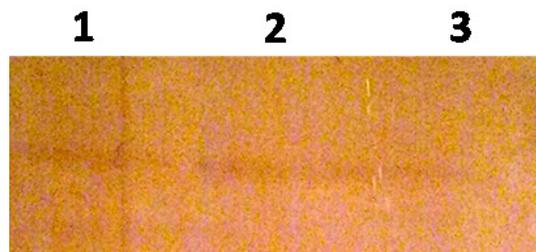


Figure 6. Western blot analysis of *luxAB* protein. Lane 1 induced culture containing Nova blue bacteria harboring pETDuet-1 recombinant plasmids after 3 h induction; lane 2 after 5 h induction; lane 3 extracted protein from culture containing Nova blue strain, as control.

A+B construct, especially in the case of BA1 and BA2 forms which their activity reported to be about 2% of the wild type (Olsson et al., 1989). In contrast, the current synthesized construct pETDuet-1/*luxAB* not only simplify the use of lux system as a reporter enzyme in prokaryotic cells, but also can provide a special condition in which both lux genes express simultaneously, from a same promoter (T7). Furthermore, it has been shown that obtaining the high expression of the lux genes requires a strong promoter and ribosome binding site (RBS) on the expression plasmid that can increase the amount of product, in which both are available in this effort using pETDuet-1 vector.

In the previous researches, expression of luciferase (*luxAB*) component of the lux system using multiple plasmids (PB, pGMC12, pFIT001, Plx, pRS1105, pCVG) about *V. harveyi* and in the fusion form (pCK218) about *V. fischeri* have been reported (Greer and Szalay, 2002), but in this study, we constructed a polycistronic lux system by cloning the luxA, luxB and intergenic sequence

as a 2046 bp fragment into a TA cloning vector (pTZ57R), followed by subcloning the fragment into the expression vector pETDuet-1 and successfully expressed in Nova blue cells that accordingly, not have already been expressed and reported. In addition to *luxAB* protein (76 kD), free luxA and luxB subunits (40 and 37 kD) were also detected in different extracts. The amount of free subunits, moreover the 76 kD protein, increased with IPTG induction which confirm that these subunits were also translated from the lux transcripts.

Finally, pETDuet-1/*luxAB* construct can now be applied as a reporter cassette in prokaryotic systems, resulting in luminous phenotype on addition of a fatty aldehyde (decanal) to the cells. The expression of the lux genes in different bacterial species provided a simple and sensitive system for monitoring the growth and distribution of the bacteria in the environment. By using the lux genes as reporters of gene expression, the strength and regulation of transcription from various promoters can be readily monitored and also light emission can easily be

detected and measured. Furthermore, this reporter cassette can be used as a marker or tag in the manipulation and the control of gene expression in the fields of research, production, control of microorganism and other biotechnological applications.

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