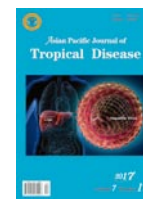


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Rare codons effect on expression of recombinant gene cassette in *Escherichia coli* BL21(DE3)Aghil Esmacili-Bandboni^{1*}, Hosein Honari², Sadegh Safaei³¹Department of Medical Biotechnology, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran²Department of Biology, Imam Hossein University, Tehran, Iran³Department of Molecular Medicine, Faculty of Advanced Technologies in Medicine, Iran University of Medical Sciences, Tehran, Iran

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ABSTRACT

Objective: To demonstrate the sensitivity of expression of fusion genes to existence of a large number of rare codons in recombinant gene sequenced.**Methods:** Primers for amplification of *cholera toxin B*, *Shiga toxin B* and *gfp* genes were designed by Primer3 software and synthesized. All of these 3 genes were cloned. Then the genes were fused together by restriction sites and enzymatic method. Two linkers were used as a flexible bridge in connection of these genes.**Results:** Cloning and fusion of *cholera toxin B*, *Shiga toxin B* and *gfp* genes were done correctly. After that, expression of the recombinant gene construction was surveyed.**Conclusions:** According to what was seen, because of the accumulation of 12 rare codons of *Shiga toxin B* and 19 rare codons of *cholera toxin B* in this gene cassette, the expression of the recombinant gene cassette, in *Escherichia coli* BL21, failed.

1. Introduction

Oral vaccines are one of the most immunogenic materials for the body. An important challenge in the field of treatment is the cost of the drug in the developed countries and their shortage in the developing countries. For example, the cost of interferon (IFN)- α in the United States, which is used in treatment of cancer and hepatitis C, is about 26,000 \$ for a man per 4 months while the median income of one third of people in this country are 2 \$ daily. Synergic effects of oral drug (or vaccine) and utilization of the chimeric recombinant proteins that possess a part of drugs (or vaccines) can lessen final cost and used dose of drugs[1,2].

For this purpose, adjuvants are considered by scientists. It is interesting; there are some of adjuvant in bacterial toxins[3].

Cholera toxin (produced by the *Vibrio cholera*) is sensitive to heat. Its molecular weight is about 84 kD and is a member in two-part toxins family that is composed of cholera toxin A (sub-unit A as named CTA) and cholera toxin B (sub-unit B as named CTB). The part "A" in the AB toxins is toxic. The CTB-CTA connection is by non-covalent linkage. Action of this toxin into cells is done by connecting to its receptor in the mucous cells that are recognized by part B and then CTA enters into cells[4,5]. CTB have a homopentameric (5 same subunits) and non-toxic structure. Each subunit is composed of 124 amino acids. The genome of *Vibrio cholera* has two chromosomes, small and large, with 4,033,460 base pairs that CTB generating gene is on the large one. Each homopentamer is a mixture of 5 same monomers (with molecular weight about 11.6 kD). There are 6 β -sheet in external surface and 2 α -helix in center in 3D structure of each monomer. The cell surface receptor is a type of pentasaccharide ganglioside that is named GM1 and expressed on many nucleated

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cells in the body such as mucous epithelial cells, lymphoid cells and antigen processing cells. The maintenance of the pentameric form of CTB is essential for its connecting to GM1[6]. *Shigella* can infect the digestive tract and cause a large range of infections that called shigellosis. These bacteria also contain 2 subunits of shiga toxin. Shiga toxin part B (STB) just like CTB is non-toxic and has homopentameric (5 subunits) structure. Each monomer of STB has 89 aa, with molecular weight about 7.7 kDa, and has one α -helix and 6 β -sheet. STB folding is very like to CTB. STB-STA connection is by non-covalent linkage[4,7]. There are several roles for STB and CTB and other transferor genes, including induction of tolerance, induction of immune response and also the adjuvant role. In addition, STB is an appropriate factor to transfer drug inside the cancer cells, because of high expression of its receptor on the surface of cancer cells. Also the labeled STB has been used for tumor imaging and studied on intracellular pathways[8,9].

Green fluorescent protein (GFP) – as a reporter protein – has 238 aa and its molecular weight is 26.9 kDa. It can emit green light under UV radiation. GFP is first time extracted from starfish *Victoria*, albeit another marine organisms have the GFP similar to this. Generally, reporter genes are used to survey the performance of fusion genes and gene transfer systems, determine the intracellular location of a gene product, investigate events related to signaling and performance trigger signals and molecular cloning[10].

In this study, preservation of chimeric proteins 3D structure, an interval sequence, known linker, is placed between fusion genes. Linkers are used as a flexible bridge in the connections of biological toxic ligands. Generally, linkers have 5 to 10 neutral amino acid (such as alanine, glycine, serin) and each of them is repeated 2 to 3 times. Linker engineering is the process that organizes the linker structure, flexibility and creates distance between the protein domains and lastly creates multi-functional artificial proteins. Length of the linker, structure and stability of linker are a number of key parameters for designing linker sequences. In the example for reduction of immunization, length of linker must be very short[11,12]. In this experiment, we used furin linker (Pro-Arg-Ala-Arg-Arg). Furin is a type of protease and able to cut in R-X-[K/R]-R site in the endoplasmic network and Golgi apparatus[13].

The purpose of this study was to design and construct *cholera toxin B-gfp-Shiga toxin B* gene cassette and investigate the expression in order to use in diagnostic studies and drug construction and produce chimeric recombinant vaccine in future.

2. Materials and methods

2.1. Primers design

Initially, the complete sequences of *cholera toxin B*, and *Shiga toxin B* genes were extracted from NCBI GenBank in order to design primers. Then, the primers were designed by using Primer3 software. In 5' of forward primer of *Shiga toxin B*, recognition site

for XhoI, and BamHI restriction enzymes were set and distance was created using by a furin linker. Also, in 5' of reverse primer, recognition site for HindIII restriction enzyme was set .

Shiga toxin B – For: 5'GGATCCCCAAGAGCACCGCCTCTCG AGATGAAAAAACATTATTAAT3'

Shiga toxin B – Rev: 5'AAGCTTATTATCAACGAAAAATAACT TC3'

In forward primer of *cholera toxin B* gene, recognition site was not set for restriction enzymes, but in its reverse primer, two recognition sites were set for HindIII, BamHI restriction enzymes.

Cholera toxin B – For: 5'GGAGGCTTTATGATTAATTAATAAT TTG3'

Cholera toxin B – Rev: 5'AAGCTTATTAGGATCCCCAAATTTG CCATACTAA3'

The complete sequence of *gfp* gene was extracted from data sheet of pEGFP-C1 synthetic vector for designing primers. In forward primer a recognition site was set for BamHI restriction enzyme and in reverse primer recognition site was set for XhoI restriction enzyme.

GFP-For: 5'GGATCCCCAAGAGCACCGCCTCCTCGCGCCCG CCGCGTGAGCAAGGGCGAG3'

GFP-Rev: 5'AGCTCGAGAGGCGGTGCTCTTGGACACAGCT CGTCCATGCCGAG3'

The recognition sites were determined using by Biolabs NEBcutter.

2.2. Amplification with PCR method

Vibrio cholera O1 and *Shigella* dysentery serotype 1 strains were provided from Baqiyatallah Research Center. Then, the bacteria were cultured in lysogeny broth liquid culture for 24 h at 37 °C.

The bacteria genome and plasmids were extracted by using CTAB-NaCl method and were used as template in PCR.

The PCR reaction for amplification of *cholera toxin B* gene was optimized with *Taq* polymerase enzyme (Cinagene, Fermentas) in 2 mM/L MgCl₂ concentration, 0.4 pmol/L of each primer, and 0.2 mmol/L deoxyribonucleotide triphosphates at annealing temperature 55 °C. Avoiding unwanted mutations, the final amplification of gene was done by using Pfu polymerase (Fermentas) in 25 μ L reaction volume.

A total of 25 μ L reaction volume contained: 0.5 pmol/L of each primer, 0.25 mmol/L deoxyribonucleotide triphosphates, 1.5 units Pfu DNA polymerase enzyme, 2.5 μ L 10 \times PCR buffer, 2.5 mmol/L MgSO₄ final concentration, and 70 ng genome extracted from *Vibrio cholera*.

PCR program consisted of the following steps: initial denaturation for 3 min at 95 °C, 35 cycles denaturation for 40 s at 95 °C, annealing for 40 s at 55 °C, extension for 1 min at 72 °C and final extension for 8 min at 72 °C.

Shiga toxin B and *gfp* genes (included in pEGFP-C1 vector and provided by Tarbiat Modares University) were amplified using the

mentioned method. Whereas, there was a difference in annealing temperature between 2 genes, which means *Shiga toxin B* and *gfp* genes were amplified at 59 °C and 61 °C, respectively.

2.3. Cloning of PCR fragments

In order to clone, we added a nucleotide to the end of fragments after confirmation of PCR products on agarose gel. Then, we added the following materials in a 0.2 mL Eppendorf tube: 2 µL PCR purified products, 1 µL MgCl₂ solution, 1 µL PCR buffer, 1 µL dATP with 0.2 mmol/L final concentration, 1 µL *Taq* DNA polymerase enzyme and then we added enough water to make the final volume of the sample to 10 µL. Then, heating was done by thermal cycler for 30 min at 70 °C.

In order to clone, we used pGEM-T Easy Vector (Promega Company) and susceptible cells of *Escherichia coli* (*E. coli*) strain DH5. Clones, that contained desired fragments, were confirmed by PCR and enzymatic digestion. The sites of enzymatic digestion in the clones are in accordance with Figure 1.

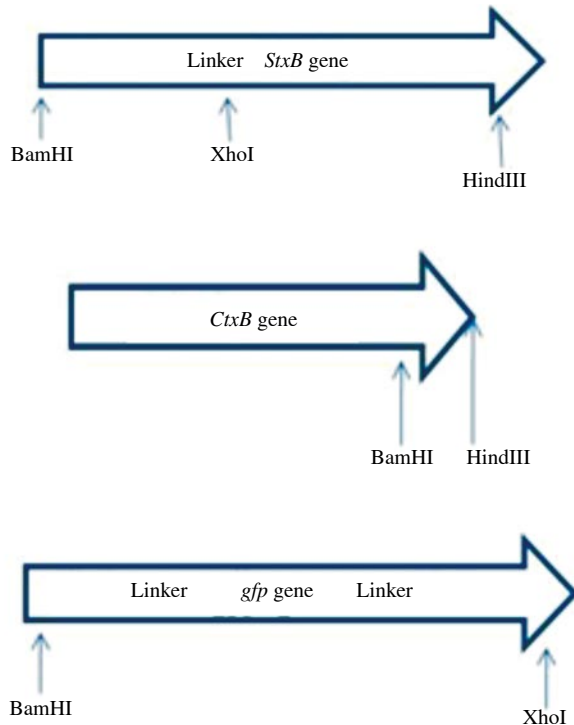


Figure 1. The schematic representation of *Shiga toxin B*, *cholera toxin B*, and *gfp* clones.

2.4. Fusion of *Shiga toxin B* and *cholera toxin B* genes

Vector containing *Shiga toxin B* gene fragment was separated and purified by BamHI and HindIII cutting enzymes. Also, pGEM-*Cholera toxin B* vector had been cut by these 2 restriction enzymes. Then, *Shiga toxin B* gene fragment was included in vector by T4 ligase enzyme. Finally, *cholera toxin B* – *Shiga toxin B* gene cassette was constructed (Figure 2).

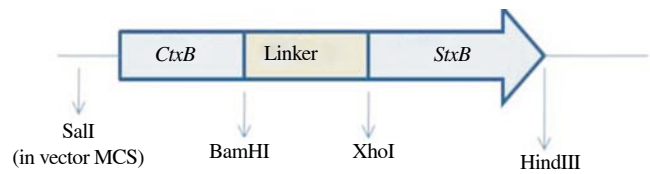


Figure 2. Schematic representation of the fused *cholera toxin B* and *Shiga toxin B* genes as a one open reading frame (~600 bp).

MCS: Multiple cloning site.

2.5. Construction of *cholera toxin B*–*gfp*–*Shiga toxin B* gene cassette

pGEM-*cholera toxin B*–*Shiga toxin B* vector and vector containing *gfp* gene fragment were digested and purified by XhoI and BamHI cutting enzymes.

Gfp gene fragment was cloned into pGEM-*cholera toxin B*–*Shiga toxin B* vector by T4 ligase enzyme. Finally, *cholera toxin B*–*gfp*–*Shiga toxin B* gene cassette was constructed (Figure 3).

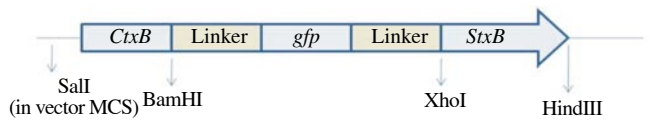


Figure 3. Schematic representation of the fused *cholera toxin B* and *gfp* and *Shiga toxin B* genes as a one open reading frame (~1350 bp).

MCS: Multiple cloning site.

Sub-cloning recombinant fragment of *cholera toxin B*–*gfp*–*Shiga toxin B* in pET-28a(+) expression vector was needed for expression of target. In order to sub-clone, *cholera toxin B*–*gfp*–*Shiga toxin B* gene cassette was constructed by using enzymatic digestion of pGEM-*cholera toxin B*–*gfp*–*Shiga toxin B* vector by HindIII and SalI enzymes. Then, gene cassette was sub-cloned in pET-28a(+) expression vector that has been cut by those enzymes. pET-28a(+)-*cholera toxin B*–*gfp*–*Shiga toxin B* recombinant plasmid was transformed into *E. coli* BL21(DE3) to amplify.

To final confirmation, 20 µL of plasmid was sent to Human Genetic Laboratory (Nasl-e-Omid, Tehran) for sequencing. Sequencing was done by using specific primers including forward primer of *cholera toxin B* and reverse primer of *Shiga toxin B*.

2.6. Investigating expression of *cholera toxin B*–*gfp*–*Shiga toxin B* gene cassette

After an overnight inoculation of 100 µL of BL21 containing vector, it was grown to the new culture that contained 50 µg/mL kanamycin. The expression of pET-28a(+)-*cholera toxin B*–*gfp*–*Shiga toxin B* recombinant vector was induced in *E. coli* BL21(DE3) strain in 1 mmol/L isopropyl β-D-1-thiogalactopyranoside concentration for 24 h at 37 °C. Expression of gene was investigated by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

2.7. SDS-PAGE

The electrophoresis of protein samples was under the denaturing

condition and accompanied with SM0671 protein marker on polyacrylamide with 12% concentration and 25 mA constant current for 24 h.

3. Results

3.1. PCR

The target fragments of *cholera toxin B*, *Shiga toxin B* and *gfp* genes with the expected sizes (220 bp, 375 bp, 750 bp respectively) were confirmed by the PCR method and then electrophoresis of 1% (w/v) agarose gel was used, comparing with DNA size marker.

3.2. Construction and analysis of cholera toxin B–Shiga toxin B recombinant fusion plasmid

pGEM-*cholera toxin B* and pGEM-*Shiga toxin B* vectors were double digested with HindIII and BamHI enzymes after purification and their confirmation on agarose gel. Then *Shiga toxin B* was ligated in the pGEM-*cholera toxin B* vector. After ligation, the size of generated fragment (600 bp) was confirmed using PCR and digestion methods and by the electrophoresis of 1% (w/v) agarose gel, comparing with DNA size marker.

3.3. Construction and analysis of cholera toxin B–gfp–Shiga toxin B recombinant fusion plasmid

pGEM-*cholera toxin B–Shiga toxin B* and pGEM-*gfp* vectors were double digested with XhoI and BamHI enzymes. Then *gfp* was ligated in the pGEM-*cholera toxin B–Shiga toxin B* vector. After ligation, the size of generated fragment (1350 bp) was confirmed by PCR, nested PCR and digestion methods and the electrophoresis of 1% (w/v) agarose gel, comparing with DNA size marker.

3.4. Sub-cloning of cholera toxin B–gfp–Shiga toxin B gene cassette

The pGEM-*cholera toxin B–gfp–Shiga toxin B* vector were double digested with SalI and HindIII enzymes. Then *cholera toxin B–gfp–Shiga toxin B* was cloned in pET-28a(+) expression vector. Sub-cloning was confirmed by digestion method and the electrophoresis of 1% (w/v) agarose gel, comparing with DNA size marker.

3.5. Analysis of expression of cholera toxin B–gfp–Shiga toxin B recombinant protein

Induction of the expression by isopropyl β -D-1-thiogalactoside in *E. coli* B121(DE3) cells transformed with the pET-28a(+)-*cholera toxin B–gfp–Shiga toxin B* plasmid had not any result in the production of recombinant protein. This was confirmed by SDS-PAGE.

4. Discussion

Oral delivery mediated by receptor across the intestine can be a way for transporting vaccines and biopharmaceutical proteins. For facilitated entry of these proteins into the intestine, GM1 and globotriaosylceramide receptors on the intestinal epithelial cells have been used by several pathogens such as *Vibrio cholerae* and *Shigella dysenteriae*[14,15].

High cost of instruction drugs in the developed countries and their low availability in the developing countries are the most difficult troubles of human health management. In the example, IFN alpha 2b is useful for curing hepatitis C and some other viral diseases and some cancers. But insurance of many of Americans and more people in developing countries for treatment by IFN for four months is not enough[2,16]. As a solution for this problem, transgenic plants have many benefits including: ability to deliver strange proteins via oral system, stability against heat, low level of cost in production, storage and transportation, protection by bioencapsulation, lessening of the requirement for costly and difficult purification, *in vitro* handing out and sterile injections. Also other advantages are the making of systemic and mucosal immunity or induction of oral tolerance, enhanced safety, and lack of human pathogens. Because of reduced permeability across the intestinal epithelial layer, a major drawback, despite these advantages, there is the efficient delivery across the intestinal mucus membrane of therapeutic proteins expressed by plant[2,17]. Furin, a member of prohormone-proprotein convertases, is a ubiquitously expressed protein found in the trans-Golgi network, endosomes, plasma membrane and extracellular space. This enzyme can cleave Arg-Xaa-Lys/Arg-Arg-like motifs in protein precursors specifically[18]. In Limaye *et al.* study, the furin cleavage site between CTB and GFP facilitated intracellular cleavage of the target protein (GFP). In Limaye *et al.* study, three groups of mice ate plant leaves that expressed CTB-GFP, IFN alpha5-GFP (IFN-GFP) and wild-type respectively[2]. Fluorescence microscopy in mice that had eaten plant leaves with expressed CTB-GFP, showed the existence of GFP in intestinal mucosa, submucosa and the hepatocytes of the liver and also in diverse cells of the spleen. They did not observe GFP fluorescence in the mice that had eaten wild-type leaves. Also they did not observe GFP fluorescence in mice that had eaten IFN-GFP leaves. But they observed GFP fluorescence in the liver or spleen of mice that had eaten *cholera toxin B–gfp* leaves. These observations demonstrated that the delivery of the protein across the intestinal lumen was successful and for delivery of sufficient amount of protein across the intestinal lumen into the systemic circulation, a carrier protein such as CTB is needed[2]. For the production of efficient fusion proteins, a suitable linker sequence is required. Several studies have surveyed the linker selection. These studies recommended mainly that the main properties of the linkers for remaining functions of the fusion protein domains are flexibility and hydrophilicity[11,12].

In the present study, *cholera toxin B–gfp–Shiga toxin B* fusion genes were obtained by using enzymatic method and its expression

was surveyed in *E. coli* BL21(DE3). The expression was not observed for this gene cassette in *E. coli*. Because in *E. coli* there is an obvious codon bias and the level of cognate tRNA is directly proportional to the rate of codon usage. In this condition, there are the translational problems because of existence of very rare tRNA codons in many mRNA specially for expression of heterologous genes in the *E. coli* host[19]. Zhang *et al.* defined codon usage in 1999. Codon usage is frequency of a translated codon per unit time in the cell of an organism[20]. Indeed, a rare codon would be certainly a low-usage codon. *E. coli* has about 30 low-usage codons, but only 20 of them were confirmed as rare codons: AGG, AGA, CGA, CUA, AUA, CCC and CGG in frequency of < 0.5% and ACA, CCU, UCA, GGA, AGU, UCG, CCA, UCC, GGG, CUC, CUU, UCU and UUA in frequency of > 0.5%.

All the rare codons in first set and the first 6 rare codons in second set are able to effect translation in *E. coli*[21].

In this study, we found that the *cholera toxin B* and *Shiga toxin B* genes, respectively from *Vibrio Cholera* and *Shigella dysenteriae*, possess a large number of *E. coli* low-usage codons (12 rare codons in *Shiga toxin B* gene and 19 rare codons in *cholera toxin B* gene). Then plasmid containing *cholera toxin B-gfp-Shiga toxin B* failed to express in *E. coli* BL21(DE3). However, in *E. coli*, high expression of recombinant *cholera toxin B-gfp-Shiga toxin B* required modification of the native genes and synonymous substitution of the rare codons.

However, it seems that in future, expression of this recombinant gene cassette can be considered as improvement in production of novel vaccines for drug delivery.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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