Rare Codons Optimizer Host Strain of *E. coli* Improves Expression of *Clostridium septicum* Alpha Toxin Gene

Reza Pilehchian Langroudi1*

1Department of Anaerobic Vaccine Research and Production, Razi Vaccine and Serum Research Institute, Alborz, Karaj, Iran.

Author’s contribution

The sole author designed, analyzed and interpreted and prepared the manuscript.

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ABSTRACT

**Aims:** *Clostridium septicum* is an anaerobic, gram positive bacterium that is able to form resistant spores. It produces numerous toxins that cause many diseases such as gas gangrene in humans and braxy in farm animals. In the present study, an attempt was done to express an active fragment of alpha toxin gene of *C. septicum* vaccine strain, in *E. coli* strains BL21 (DE3) and Rosetta (DE3) strains.

**Methodology:** In the present study, the active fragment of alpha toxin gene of *C. septicum* vaccine strain was amplified and ligated with pJET1.2blunt cloning vector and after cloning was extracted from the pJETαsep recombinant cloning vector. After purification and evaluation, it was ligated with pET22b(+) expression vector and the pET22αsep was transformed into *E. coli* strains BL21(DE3) and Rosetta(DE3) strains.

**Results:** Results showed that a recombinant protein was expressed as a soluble protein after IPTG induction in Rosetta (DE3) rare codons optimizer host strain, but not in BL21 (DE3). Further optimization on expression conditions of the recombinant alpha toxin protein was achieved by incubating the culture of recombinant *E. coli* Rosetta (DE3[pET22αsep]) cells in 37°C and induction...
with 0.5, 1.0 and 1.5 mM IPTG for 3-6 h. Protein expression was evaluated by SDS-PAGE and the recombinant alpha toxin protein was purified using Ni-NTA resin and for its reconfirmation was analysed by Western blot.

Conclusions: We concluded that, *E. coli* strain Rosetta (DE3) is a suitable expression host for production of *C. septicum* alpha toxin and the obtained recombinant plasmid could be used for further research on production of recombinant vaccine against braxy disease.

Keywords: Alpha toxin; expression; rare codons; Rosetta.

1. INTRODUCTION

*Clostridium septicum* is a large anaerobic, Gram positive, rod shaped and fermentative bacterium. Peritrichous flagella enable the bacterium to be motile and its drumstick-like shape is because of its terminal spore. It is a member of the normal gut flora in humans as well as farm animals [1]. *C. septicum* can produce and secrete a number of toxic proteins such as beta, gamma, delta and alpha which are hemolytic, lethal and necrotizing toxins. There are several reports about *C. septicum* which is responsible for some diseases, the most serious are “frequently fatal non-traumatic gas gangrene” and “cancer” [2,3] in human and braxy, malignant oedema and black quarter in farm animals. Immunity to these diseases is mediated mainly by antitoxin raised against *C. septicum* alpha toxin [4].

The activity of *C. septicum* alpha-toxin had been determined in erythrocytes of various animals and it had been cleared that the sensitivity is in the order of mouse, rat, canine, equine, rabbit, chicken, bovine, swine and ovine. This report concluded that, alpha-toxin associates with specific erythrocyte membrane proteins in any animal species, and is subsets of glycosylphosphatidylinositol-anchored proteins in various animal species [5]. Alpha-toxin is functionally similar to aerolysin and is a beta-barrel pore-forming cytolyisin. Those residues which are important in receptor binding, oligomerization, and pore formation, have been identified. Although it is obvious that alpha toxin is essential for disease, but little is known about its activity in an infection [6].

Alpha toxin is a cytolytic protein with a molecular mass of approximately 48 kDa [7]. Alpha-toxin gene of *C. septicum* BX96 was cloned and expressed in *E. coli*. The toxin had 443 amino acids in length which its 31-residue signal peptide was removed from the toxin during secretion. There were no extended hydrophobic regions in the mature toxin sequence. It was determined that the alpha-toxin proteolytic activation was located on the carboxyl-terminal side of arginine 398 [8]. Another report revealed that eukaryotic protease; furin is involved in the activation of *C. septicum* alpha-toxin on the cell surface but that alternate eukaryotic proteases such as trypsin can also activate the toxin. Regardless of the activating protease, the furin consensus site appears to be essential for the activation of alpha-toxin on the cell surface [9].

In 1994 one fragment of DNA (2293 bp) as *C. septicum* alpha toxin gene was reported and deposited in GenBank with accession number D17668.1 [10]. Further studies on this fragment showed that the fragment consists of nucleotide numbers 561 up to 1982 (1332 bp) was active alpha toxin gene [8]. In the present study, an attempt was done to express active fragment of alpha toxin gene of *C. septicum* vaccine strain, in *E. coli* strains BL21 (DE3) and Rosetta (DE3). Purified protein was analysed to show the efficacy of these hosts for this purpose.

2. MATERIALS AND METHODS

Linearized pJET1.2/blunt cloning vector, *Pfu* DNA polymerase, dNTPs, T4 DNA ligase, NdeI and XhoI restriction enzymes and protein weight markers (PageRuler™), 100 bp Plus DNA size markers (GeneRuler™), and plasmid extraction kit were purchased from Fermentas. High Pure PCR Product Purification Kit for DNA fragments recovery was purchased from Vivantis. Sheep primary antibody and conjugate anti-sheep HRP were purchased from DAKO Company (Denmark).

2.1 Cloning

*C. septicum* vaccine strain (CN913), *E. coli* strain TOP10 (cloning host) and strain (BL21 (DE3) and Rosetta (DE3)) as expression host were prepared from Razi Institute. *C. septicum* was cultured anaerobically using anaerobic chambers in liver extraction media at 37°C during overnight. After centrifugation supernatant was discarded.
and whole genomic DNA was extracted from pellet by phenol-chloroform method. Alpha toxin gene was amplified using one pair of specific primers consisting of NdeI at the 5' end of forward and XhoI at the 3' end of reverse primers. These primers produce 1332 bp fragments of DNA that is only consisting of activated fragment of C. septicum alpha toxin. Pfu DNA polymerase was used for amplification of blunt-end PCR product. After ligation, pJETasep recombinant vector was transformed into E. coli / Top 10 competent cell and screening of recombinant clones was done by antibiotic resistance and Colony PCR and nucleotide sequencing was carried out. The whole procedure was done as described previously [11].

2.2 Sub Cloning

pJETasep was extracted from E. coli TOP 10/ pJETasep clone and purified, then was digested by NdeI and XhoI to produce sticky-ends alpha toxin gene. After digestion and electrophoresis, the csa was extracted from the gel and purified. pET22b(+) as expression vector was digested using the same enzymes, then was purified and ligated with csa active fragment.

E. coli strains BL21 and Rosetta were selected as expression host. After preparing of competent cells, pET22asep was transformed into competent cells, so E. coli / BL21 / pET22asep and E. coli / Rosetta / pET22asep were provided. At the next step pET22asep recombinant expression vector was purified and digested using the same restriction enzymes.

2.3 Expression of Alpha Toxin

Confirmed recombinant cells were cultured in LB-Amp media and incubated at 37°C to OD₆₀₀=0.6-0.7. 0.5 mM IPTG was added to induce the protein expression and growth was continued for 18 h. Different concentration of IPTG (0.5, 1.0 and 1.5mM) was used for optimization of protein expression. Protein analysis was performed by SDS-PAGE.

2.4 Purification of the CSA

The recombinant CSA, which contains a 6-His tag at C-terminus, was purified by Ni-NTA resin. For this purpose the culture of recombinant cell was centrifuged and the pellet was suspended in lysis buffer and the cells were lysed by 6 times sonication on ice for 5 min. The cell lysate was centrifuged at 13,680×g and the clarified supernatant was loaded on Ni-NTA resin at the flow rate of 1 ml/min. The column was washed with 5 volumes of wash buffer and finally the protein was eluted by adding elution buffer as described previously [12]. The purified protein was analysed using SDS-PAGE and Western blot. The protein concentration was determined using a standard procedure [13].

3. RESULTS

Electrophoresis result showed that genomic DNA is extracted and PCR analysis revealed that blunt end csa is amplified. Recombinant pJETasep was produced and successfully transformed into E. coli/TOP10 competent cell. pJETasep was extracted from E. coli / TOP 10 / pJETasep cells and was digested by NdeI, XhoI enzymes so sticky-end csa was produced.

pET22b(+) was digested by the same restriction enzymes to produce sticky ends and was ligated with sticky ended csa. pET22asep recombinant expression vector was successfully transformed into E. coli strains BL21 and Rosetta. Recombinant E. coli strains were grown on LB-Amp agar and positive colonies were screened. Colony PCR was done to show the presence of recombinant pET22asep vector into both recombinant E. coli colonies. Those colonies which showed csa (Fig. 1) were selected for the next step. pET22asep was extracted from both recombinant expression hosts, then was digested and recombinant csa was shown (Fig. 2). Sequencing also revealed that insert gene is consistent with csa in GenBank. After expression, SDS-PAGE analysis showed that recombinant protein is well expressed 4 h after induction with 0.5 mM IPTG in recombinant E. coli/Rosetta (Fig. 3), cells but not in E. coli/BL21 (Fig. 4). Different concentrations of IPTG had no significant effect on protein expression (Fig. 5). Recombinant protein was purified by Ni-NTA resin and the result was shown on SDS-PAGE. Purified protein appeared as about 41.5 KDa protein band on SDS-PAGE and Western blot using sheep primary antibody and conjugate anti-sheep HRP (Fig. 6).

4. DISCUSSION

C. septicum produces lethal antigen only in low titer and in a variable way. In addition, the immunogenicity of native toxic filtrates is weak, which results in poor antibody response in animals.
Fig. 1. Colony PCR analysis of two different recombinant strains of *E. coli*
Lanes 1-4; *E. coli*/Rosetta/pET22αsep, lanes 6-9; *E. coli*/BL21/pET22αsep. Alpha toxin gene (about 1332 bp) could be seen in lanes 1, 3, 4 and 8. Lane 5; 100 bp plus DNA molecular size markers

Fig. 2. Agarose gel electrophoresis of pET22αsep recombinant expression vector digestion
Lane 1; digested pET22αsep showing alpha toxin gene (csa), lane 2; 100 bp plus DNA molecular size markers, lane 3; undigested pET22αsep

CSA that is the lethal cytolytic and the major virulence factor, appears to be its immunodominant extracellular antigen [14]. Most commercial vaccines are inactivated and may contain combinations of clostridial bacterin, toxoid or bacterin/toxoid. Enough time is essential for provision of maximal protection at the most likely age of susceptibility. Genetic engineering helps us to produce recombinant protein that would be a good alternative to native toxins of *C. septicum* [15]. This bacterium produces several extracellular factors, but CSA is the only toxin that is secreted as a single lethal extracellular toxin [16].

In 1995 expression of alpha-toxin in *E. coli* BL21 resulted in the production of a 46.5 kDa inactive prototoxin (ATpro) of, which was identical to *C. septicum* native toxin and requires proteolytic activation to yield a cytolytically active form (ATact) 41.3-kDa. This report showed that it respect to activity and activation[8]. The activation also could be done *in vitro* using trypsin, that nicks the toxin at R367, near the C-terminus [7]. Another report demonstrated that the activation leads to oligomerization into a prepore complex [17], which subsequently inserts into the membrane to form the beta barrel pore. The pore has been estimated to be approximately 1.6 nm in diameter [7].

Based on this information, we decided to clone and express a restricted fragment of the csa that could lead to the 41.5 kDa recombinant active CSA toxin.
Fig. 3. SDS-PAGE analysis of alpha toxin expression
Lane 1: protein weight markers (PageRuler), Lane 2; *E. coli*/Rosetta/pET22b(+) (Negative control). Lane 3; *E. coli*/Rosetta/pET22αsep without induction, Lane 4; *E. coli*/Rosetta/pET22αsep 4 h after induction with 0.5 mM IPTG, arrow shows recombinant alpha toxin protein (CSA).

Fig 4. Comparison of alpha toxin expression in *E. coli*/BL21/pET22β and *E. coli*/Rosetta/pET22β
Lanes 1 and 2; *E. coli*/BL21/pET22b(+), lanes 4 and 5; *E. coli*/Rosetta/pET22β, 4 h after induction with 0.5 mM IPTG, arrow shows recombinant alpha toxin protein (CSA). Lane 3 protein weight markers (PageRuler)

In the case of *E. coli*, a clear codon bias exists among the 61 amino acid codons found within the population of mRNA molecules, so this situation can cause serious problems after the initiation of transcription of a cloned heterologous gene in the *E. coli* host. Some reports suggest that clusters of AGG/AGA, CUA, AUA, CGA or CCC codons can reduce both the quantity and quality of the synthesized protein. An excess of any of these codons, may create translational problems. *E. coli* strain Rosetta is a good expression host for alpha toxin because it is designed to enhance the expression of eukaryotic proteins that contains codons rarely used in *E. coli* and supplies tRNAs for above mentioned codons, on a compatible chloramphenicol-resistant plasmid [18].

In the present study, recombinant plasmid pET22b(+) carrying *C. septicum csa* was transformed into *E. coli* strain Rosetta. Sequencing result revealed that insert gene was 1332 bp. However our study showed that BL21 cannot express *C. septicum* CN913.alpha toxin with its rare codons. pET22b (+) proved to be a suitable vector for expression of alpha toxin.
In our work, recombinant toxin expression was started 30 min. after induction with IPTG and continued for 18 h. After considering of different concentrations of IPTG and different temperatures it was revealed that different concentrations of IPTG have no obvious effect on protein expression level. Although we could not find any report on IPTG concentrations effects on csa expression, but a previous study showed that IPTG gradient has no effect on expression of epsilon-beta fusion gene [12]. We also fund that optimal thermal condition for alpha toxin protein expression is 37ºC. At present, accessing recombinant bacterium and manufacturing of recombinant vaccine is possible. Therefore prevention of clostridial diseases would happen by manufacturing of vaccine in safe conditions and with less cost.

5. CONCLUSIONS

Finally we concluded that, *E. coli / Rosetta* is a suitable expression host for production of *C. septicum* alpha toxin and this clone could be used for further studies on production of braxy recombinant vaccine.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

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