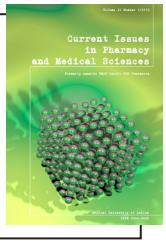
DOI: 10.2478/cipms-2019-0024

Curr. Issues Pharm. Med. Sci., Vol. 32, No. 3, Pages 130-133

Current Issues in Pharmacy and Medical Sciences

Formerly ANNALES UNIVERSITATIS MARIAE CURIE-SKLODOWSKA, SECTIO DDD, PHARMACIA

journal homepage: http://www.curipms.umlub.pl/



Obtaining the tbf gene which encodes immunodominant epitopes of pathogenic cholera strains

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ARTICLE INFO

Received 06 November 2018
Accepted 08 February 2019

Keywords:
candidate cholera vaccine,
hybrid tbf gene,
Sanger sequencing,
protein design,
TBF recombinant protein,
immunodominant epitopes,
Vibrio cholerae.

ABSTRACT

We experimentally carried out the synthesis of the tbf gene that encodes highly-immunogenic epitopes of pathogenic cholera strains, through the cloning of the tbf gene in pGEM-T Easy plasmid. Moreover, we tested the hybrid gene sequence for absence of mutations, using the Sanger sequencing. We also calculated the nucleatic sequence of the tbf gene. The obtained results have both scientific and practical significance.

INTRODUCTION

Cholera is an intestinal infection disease that rapidly dehydrates the human organism. It is caused by the bacterium of the *Vibrio cholerae* species, namely, toxigenic serogroup O1 and less commonly, O139. Cholera is characterized by a fecal-oral transmission route (more rarely human-to-human direct transmission) affecting the small bowel, and symptoms that include watery diarrhea, vomiting, rapid loss of water and electrolytes with various ranges of dehydration up to hypovolemic shock.

The cholera toxin (exotoxin) is responsible for the disease symptoms and its rapid evolution, and is a key factor of its pathogenicity.

As of today, scientists have come to a conclusion that oral immunization is the most efficient immune protection from cholera. Along with the developed attenuated and inactivated oral anti-cholera vaccines, recombinant vaccines based on epitopes of bacterial antigens occupy leadership positions for cholera treatment. These vaccines induce a pronounced immune response and depress the bacterial transcytosis, which is a key factor for the disease evolution.

The object under study in this work is the *tbf* gene that encodes immunodominant epitopes of proteins of pathogenic cholera strains TcpA and B(rBS), as well as an area which provides antigen penetration through the epithelium of the stomach wall.

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The TcpA protein is a component of a toxin-coregulated adhesion pilus, and forms polymers from subunits of 20.5 kDa [1]. This protein is a factor in pathogen colonization [2]. Toxin-coregulated piles are important protective antigens, which possess well-pronounced protective properties [3].

The subunit of cholera exotoxin B (rBS) is a pentamer, each sub-subunit of which is presented as a polypeptide chain consisting of 103 amino acid residues [4]. The protein contains antigenic determinants [5], which induce neutralization antibodies.

The neonatal FcRn-receptor is a receptor of the Fc fragment that forms a heterodimer by non-covalent interaction of alpha-chain with beta-2 microglobulin [6]. At oxygen hydrolysis, the pH receptor bounds the segment that connects CH2- and CH3-domains of antibodies of the IgG class [7]. After antibody bonding, the receptors activate a cascade of reactions for pathogen elimination by antibody-dependent phagocytosis [8].

Thus, the vaccine, which contains purified immunogenic protein, is stable and safe, its chemical properties are well-studied, and it does not contain any additional proteins and nucleic acids that might cause undesirable effects in the host organism.

The aim of this work is to inform readers of a means of replicating the *tbf* gene that encodes immunodominant epitopes of pathogenic cholera strains.

The work was performed at Laboratory No. 6 "Genetic engineering of vaccines" of the State Research Institute of

Highly Pure Biopreparations of the Russian Federal Medical-Biological Agency (FMBA), Saint-Petersburg, Russia.

MATERIALS AND METHODS

Objects under study

The gene, which encodes TBF recombinant protein, was obtained through chemical synthesis. Synthesis of sequence of the calculated gene that encodes the TBF protein, was performed by applying the polymerase chain reaction technique, using overlapping oligonucleotides. These oligonucleotides were synthesized by by employing the DNA synthesizer ASM-800 (BIOSSET, Russia). The main requirements for primers were the following: length not more than 60 nucleotides, and hybridization segments not more than 20 nucleotides. Beyond the aforementioned, there should not be long segments with repeated G or C. In total, for the synthesis of the gene that encodes the TBF protein of 2085 nucleotide pairs, we used 77 primers. The synthesized sequence was obtained from agarose gel, by means of electrophoresis, and cloned in the plasmid vector pGEM-T Easy. We performed the blunt-end cloning. After sequencing utilizing the capillary sequencer Applied Biosystems 3500/3500xL Genetic Analyzer (Applied Biosystems, USA), the fragments were amplified in a thermocycler for amplification – C1000 ThermalCycler (Bio-Rad, USA). In the terminal gene segments, we included restriction sites XhoI and NdeI for further cloning in plasmid pET28a (+).

The vector map is presented in Figure 1.

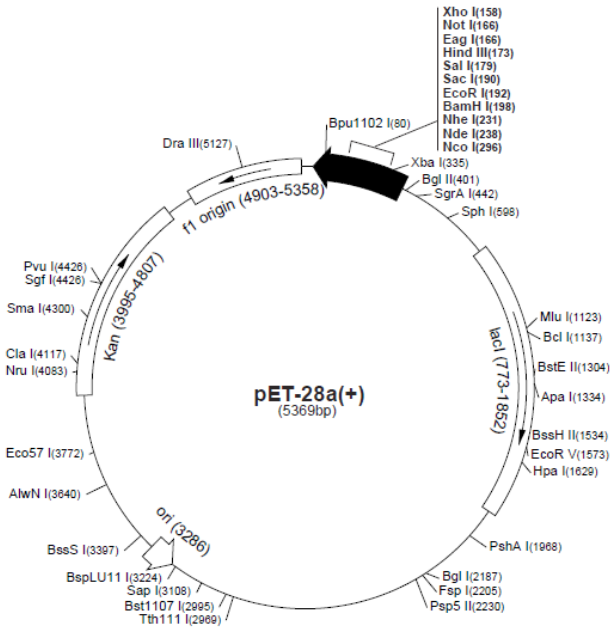

Figure 1. Circular pET28a (+) plasmid map [9]

The expression plasmid comprises the replication origin, promoter of T7 phage polymerase, lac-operator, kanamycin-resistance gene, start codon for translation of cloned fragments and a fragment which encodes polyhistidine located in the reading frame at the N-end of the sequence. Hence, any nucleatic sequence cloned in the vector is expressed as a polyhistidine-tagged protein as a matter of convenience for its further purification by means of immobilized metal affinity chromatography. For lac-operon operation, the plasmid contains a fragment that encodes the lactose repressor lacI.

The gene cloning in the vector was performed via the XhoI and NdeI restriction sites.

For the genetic engineering, we used *E. coli* DH10B/R (Gibko BRL, USA) cells with F-mcrA Δ(mrr-hsdRMS-mcrBC) φ80dlacZΔM 15 ΔlacX74 deoR recA1 endA1 araD139 Δ(ara,leu)769 galUgalKλ- rpsLnupG genotype.

For the expression of the gene that encodes the TBF protein, we employed *E.coli* BL21 Star (DE3) cells with the F- ompThsdSB (rB-mB-) galdcm rne131 (DE3) genotype that contains in its genome, λDe3 lysogen and *rne131* mutation. The mutated gene *rne* (*rne131*) encodes reduced RNAase E, which decreases the intracellular destruction of mRNA. This results in the enhancement of its fermentation stability. Of note, lon- and ompT- mutations in protease genes allow the possibility to obtain large amounts of non-proteolyzed recombinant proteins.

Research methods

Synthesis of sequence of the calculated gene that encodes TBF protein, was performed by means of the polymerase chain reaction technique and the use of overlapping oligonucleotides. These oligonucleotides were synthesized by means of the DNA synthesizer ASM-800 (BIOSSET, Russia). The main requirements for the primers were the following: length not more than 60 nucleotides and hybridization segments not more than 20 nucleotides. Moreover, there should not be long segments with repeated G or C. In total, for the synthesis of gene that encodes the TBF protein of 2085 nucleotide pairs, we used 77 primers. The synthesized sequence was obtained from agarose gel, through electrophoresis, and cloned in the plasmid vector pGEM-T Easy. We performed the blunt-end cloning. After sequencing via the capillary sequencer Applied Biosystems 3500/3500xL Genetic Analyzer (Applied Biosystems, USA), the fragments were amplified in a thermocycler for amplification C1000 ThermalCycler (Bio-Rad, USA). In the terminal gene segments, we included restriction sites XhoI and NdeI for further cloning in plasmid pET28a(+).

For the design of TBF recombinant protein, we accessed NCBI databases (www.ncbi.nlm.nih.gov).

RESULTS

For the design of the TBF recombinant protein, we used the most protective epitopes of bacterial antigens: subunit of B cholera exotoxin, protein of cholera A pilus, and domain, which is a ligand to Fc-receptors at the stomach wall. The selected consensus segments, common for *V. cholerae* pathogenic strains, were analyzed for T and B cell epitopes. During the analysis, we employed the following software: Bepipred Linear Epitope Prediction 2.0 and IEDB Analysis Resource. The obtained amino acid sequence of TBF recombinant protein (695 amino acid residues) is presented in Figure 2.

```
10         20         30         40         50         60
MQLLKQLFKK KFVKEEHDKK TGQEGMTLLE VIIVLGIMGV VSAGVVTLAQ RAIDSQIMTK

        70         80         90        100        110        120
AAQSLNSIQV ALTQTYRGLG NYPATADATA ASKLTSGLVS LGKISSDEAK NPFNGTNMNI

       130        140        150        160        170        180
FSFPRNAAAN KAFAISVDGL TQAQCKTLIT SVGDMFPYIA IKAGGAVALA DLGDFENSAA

       190        200        210        220        230        240
AAETGVGVIK SIAPASKNLD LTNITHVEKL CKGTAPFGVA FGNSGGGGGG GGMIKLKFGV

       250        260        270        280        290        300
FFTVLLSSAY AHGTPQNITD LCAEYHNTQI YTLNDKIFSY TESLAGKREM AIITFKNGAI

       310        320        330        340        350        360
FQVEVPGSQH IDSQKKAIER MKDTLRIAYL TEAKVEKLCV WNNKTPHAIA AISMANGGGG

       370        380        390        400        410        420
GGGGGASTKG PSVFPLAPSS KSTSGGTAAL GCLVKDYFPE PVTVSWNSGA LTSGVHTFPA

       430        440        450        460        470        480
VLQSSGLYSL SSVVTVPSSS LGTQTYICNV NHKPSNTKVD KKVEPKSCDK THTCPPCPAP

       490        500        510        520        530        540
ELLGGPSVFL FPPKPKDTLM ISRTPEVTCV VVDVSHEDPE VKFNWYVDGV EVHNAKTKPR

       550        560        570        580        590        600
EEQYNSTYRV VSVLTVLHQD WLNGKEYKCK VSNKALPAPI EKTISKAKGQ PREPQVYTLP

       610        620        630        640        650        660
PSRDELTKNQ VSLTCLVKGF YPSDIAVEWE SNGQPENNYK TTPPVLDSDG SFFLYSKLTV

       670        680        690
DKSRWQQGNV FSCSVMHEAL HNHYTQKSLS LSPGK
```

Figure 2. TBF protein amino acid sequence

The nucleatic sequence that encodes the TBF protein (with length of 2085 nucleotide pairs) was obtained via calculation (Fig. 3).

```
10         20         30         40         50         60
ATGCAGCTGC TGAAACAGCT GTTTAAAAAA AAATTTGTGA AAGAAGAACA TGATAAAAAA

        70         80         90        100        110        120
ACCGGCCAGG AAGGCATGAC CCTGCTGGAA GTGATTATTG TGCTGGGCAT TATGGGCGTG

       130        140        150        160        170        180
GTGAGCGCGG GCGTGGTGAC CCTGGCGCAG CGCGCGATTG ATAGCCAGAT TATGACCAAA

       190        200        210        220        230        240
GCGGCGCAGA GCCTGAACAG CATTCAGGTG GCGCTGACCC AGACCTATCG CGGCCTGGGC

       250        260        270        280        290        300
AACTATCCGG CGACCGCGGA TGCGACCGCG GCGAGCAAAC TGACCAGCGG CCTGGTGAGC

       310        320        330        340        350        360
CTGGGCAAAA TTAGCAGCGA TGAAGCGAAA AACCCGTTTA ACGGCACCAA CATGAACATT

       370        380        390        400        410        420
TTTAGCTTTC CGCGCAACGC GGCGGCGAAC AAAGCGTTTG CGATTAGCGT GGATGGCCTG

       430        440        450        460        470        480
ACCCAGGCGC AGTGCAAAAC CCTGATTACC AGCGTGGGCG ATATGTTTCC GTATATTGCG

       490        500        510        520        530        540
ATTAAAGCGG GCGGCGCGGT GGCGCTGGCG GATCTGGGCG ATTTTGAAAA CAGCGCGGCG

       550        560        570        580        590        600
GCGGCGGAAA CCGGCGTGGG CGTGATTAAA AGCATTGCGC CGGCGAGCAA AAACCTGGAT

       610        620        630        640        650        660
CTGACCAACA TTACCCATGT GGAAAAACTG TGCAAAGGCA CCGCGCCGTT TGGCGTGGCG

       670        680        690        700        710        720
TTTGGCAACA GCGGCGGCGG CGGCGGCGGC GGCGGCATGA TTAAACTGAA ATTTGGCGTG

       730        740        750        760        770        780
TTTTTTACCG TGCTGCTGAG CAGCGCGTAT GCGCATGGCA CCCCGCAGAA CATTACCGAT

       790        800        810        820        830        840
CTGTGCGCGG AATATCATAA CACCCAGATT TATACCCTGA ACGATAAAAT TTTTAGCTAT

       850        860        870        880        890        900
ACCGAAAGCC TGGCGGGCAA ACGCGAAATG GCGATTATTA CCTTTAAAAA CGGCGCGATT

       910        920        930        940        950        960
TTTCAGGTGG AAGTGCCGGG CAGCCAGCAT ATTGATAGCC AGAAAAAAGC GATTGAACGC

       970        980        990       1000       1010       1020
ATGAAAGATA CCCTGCGCAT TGCGTATCTG ACCGAAGCGA AAGTGGAAAA ACTGTGCGTG

      1030       1040       1050       1060       1070       1080
TGGAACAACA AAACCCCGCA TGCGATTGCG GCGATTAGCA TGGCGAACGG CGGCGGCGGC

      1090       1100       1110       1120       1130       1140
GGCGGCGGCG GCGGCGCGAG CACCAAAGGC CCGAGCGTGT TTCCGCTGGC GCCGAGCAGC

      1150       1160       1170       1180       1190       1200
AAAAGCACCA GCGGCGGCAC CGCGGCGCTG GGCTGCCTGG TGAAAGATTA TTTTCCGGAA

      1210       1220       1230       1240       1250       1260
CCGGTGACCG TGAGCTGGAA CAGCGGCGCG CTGACCAGCG GCGTGCATAC CTTTCCGGCG

      1270       1280       1290       1300       1310       1320
GTGCTGCAGA GCAGCGGCCT GTATAGCCTG AGCAGCGTGG TGACCGTGCC GAGCAGCAGC

      1330       1340       1350       1360       1370       1380
CTGGGCACCC AGACCTATAT TTGCAACGTG AACCATAAAC CGAGCAACAC CAAAGTGGAT

      1390       1400       1410       1420       1430       1440
AAAAAAGTGG AACCGAAAAG CTGCGATAAA ACCCATACCT GCCCGCCGTG CCCGGCGCCG

      1450       1460       1470       1480       1490       1500
GAACTGCTGG GCGGCCCGAG CGTGTTTCTG TTTCCGCCGA AACCGAAAGA TACCCTGATG

      1510       1520       1530       1540       1550       1560
ATTAGCCGCA CCCCGGAAGT GACCTGCGTG GTGGTGGATG TGAGCCATGA AGATCCGGAA

      1570       1580       1590       1600       1610       1620
GTGAAATTTA ACTGGTATGT GGATGGCGTG GAAGTGCATA ACGCGAAAAC CAAACCGCGC

      1630       1640       1650       1660       1670       1680
GAAGAACAGT ATAACAGCAC CTATCGCGTG GTGAGCGTGC TGACCGTGCT GCATCAGGAT

      1690       1700       1710       1720       1730       1740
TGGCTGAACG GCAAAGAATA TAAATGCAAA GTGAGCAACA AAGCGCTGCC GGCGCCGATT

      1750       1760       1770       1780       1790       1800
GAAAAAACCA TTAGCAAAGC GAAAGGCCAG CCGCGCGAAC CGCAGGTGTA TACCCTGCCG

      1810       1820       1830       1840       1850       1860
CCGAGCCGCG ATGAACTGAC CAAAAACCAG GTGAGCCTGA CCTGCCTGGT GAAAGGCTTT

      1870       1880       1890       1900       1910       1920
TATCCGAGCG ATATTGCGGT GGAATGGGAA AGCAACGGCC AGCCGGAAAA CAACTATAAA

      1930       1940       1950       1960       1970       1980
ACCACCCCGC CGGTGCTGGA TAGCGATGGC AGCTTTTTTC TGTATAGCAA ACTGACCGTG

      1990       2000       2010       2020       2030       2040
GATAAAAGCC GCTGGCAGCA GGGCAACGTG TTTAGCTGCA GCGTGATGCA TGAAGCGCTG

      2050       2060       2070       2080
CATAACCATT ATACCCAGAA AAGCCTGAGC CTGAGCCCGG GCAAA
```

Figure 3. *tbf* gene nucleatic sequence

DISCUSSION

In the study, we carried out the synthesis of a calculation model of *tbf* gene sequences with length of 2085 nucleotide pairs by means of the PCR technique and overlapping oligonucleotides that were synthesized via the DNA synthesizer ASM-800 (BIOSSET, Russia). The absence of mutations in the sequence of hybrid gene was indicated through applying the sequencing technique (Fig. 4).

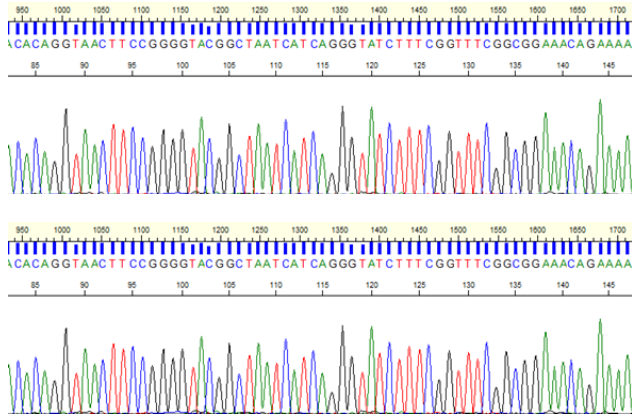

Figure 4. A *tbf* gene chromatogram fragment. Interpretation according to the direct-primer Sanger technique (TAATACGACTCACTATAGGG)

Sequencing of amplified DNA segments was performed according to the Sanger technique.

CONCLUSIONS

Our study was the first to calculate the sequence of nucleotide pairs forming part of *tbf* gene that encodes immunodominant epitopes of pathogenic cholera strains and to synthesized it.

We cloned the *tbf* gene in plasmid pGEM-T Easy and examined the hybrid gene sequence for the absence of mutations, using the Sanger sequencing technique.

The obtained results are of interest for further development of stable and safe recombinant vaccines that contains subunits of cholera toxin B (rBS), protein of cholera A piles (TcpA), as well as the domain that is a ligand to Fc-receptors at the stomach wall (FcL).

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