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Original Article

In vitro elaboration Mutagenesis and cloning of the *PA* gene in *Bacillus subtilis*

Mohammad Abootaleb*, Narjes Mohammadi Bandari

Department of Biology, Qom Branch, Islamic Azad University, Qom, Iran

*Corresponding Author E-mail: mohammad_abootaleb@yahoo.com

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ABSTRACT

Background: The immune antigen of *Bacillus anthracis* is a protein that can attach to the surface receptor of all human cells. At the surface of cancer cells, there is a receptor that activates the uPA (Urokinase plasminogen) that do not exist in normal human cells.
Objectives: The aim of this study was changing the location of the attachment of the *PA* gene by a directed mutation in order to attach only to the cancer cells.
Methods: *PA* gene was extracted from the *pMNA1* plasmid. The mutation on the *PA* gene was made by Overlap Extension PCR. The mutated segment was transferred to DH5*α*; the strain of *Escherichia coli*. With *TA coning* carrier. By restriction enzymes *Hind III* and *BamH I* the mutated *PA* gene was extracted and transferred to *pWB980* and by electroporation method, it was transferred to the *WB600* strain.
Results: In this study, the mutation occurred in sequences of *PA* gene by SOE PCR method resulting in a change in the genetic code of amino acid 194. The occurrence of mutation was confirmed by determining base sequences.
Conclusion: Cancer is a severe disease that has a major impact on large groups of people, about whom the problem of cancer is a leading cause of death across the world. One of the treatment methods of cancer is bacterial toxins if only cancer cells receive them. Therefore, these mutated *PA* proteins can be effective as novel therapeutic agents for the treatment of cancer.

Key words: Protective antigen, Overlap Extension PCR, Immune antigen, Urokinase Plasminogen Activator, *B. subtilis*

Introduction

Bacillus anthracis the causative agent of anthrax is the Gram-positive, endospore-forming aerobic or facultatively anaerobic and rod-shaped bacterium (Helgason *et al.*, 2011). *Anthrax* toxins are composed of three proteins components the protective antigen (*PA)*, edema factor (*EF*), and lethal factor (*LF*). These proteins are encoded by *Pag*, *Lef*, and *Cyo* genes. *LF* and *EF* are associated with *PA* causing different pathogenic responses (Ivanova *et al.*, 2003).

The *PA* protein with a molecular weight of 83 kD and consists of four domains that are connected to cell surface receptors such as uPA[1] through their carboxyl-terminal residues. In the following process, after creating a pore in the cell, subunits *LF* and *EF* of toxin bind to the *PA* subunit and transfer into the cell (Cunningham *et al*., 2002; Liu *et al*., 2009). uPA is a serine protease present in humans and animals. uPA has an important role in extracellular matrix degradation during cell proliferation. The genes are assembled in syntenic domains on human chromosomes 8, 19, 6 (Casey *et al*., 1994; Zhang *et al*., 2011).
The high expression of uPA and its receptor (uPAR[2]) correlate with various tumors in cells of the body, decomposition inhibitor for extracellular matrix, cell adhesion, motility and invasion (Abi-Habib *et al*., 2006; Deryugina *et al*., 2012; Wang *et al*., 2001). SDM[3] is an invaluable research tool used in genetic engineering and molecular biology. This method is often performed by PCR[4] technique, especially SOE PCR[5] which is a simple, accomplished technique for SDM and gene splicing. The purpose of this study is to generate Mutations in the *PA* gene by SOE PCR and cloning it's into the *B. subtilis* by using the *pWB980* expression vector.

Experimental

Methods

Bacterial strains and plasmids

E. coli strain *Top10* and *B. subtilis* strain *wB600* were obtained from a gene bank in the Pasteur Institute of Iran, plasmids used in this study, plasmid *TA Cloning* Vector as a *T. vector* and expressive plasmid *pWB980* as a carrier.

Media

Strains were maintained in LB medium bottle and LB agar plate (SRL, India) containing ampicillin 100 µg/mL and Kanamycin 10 µg/mL at 37 °C.

Plasmid isolation

In this study, *B. subtilis* strain *wB600* was used containing *pMNA1* plasmid that contained *PA* for extracting *PA* Purification of plasmid DNA from *B. subtilis* by using phenol/chloroform extraction method.

PCR reaction and DNA manipulations

The primers (F1 and R2) used for the amplification of the *PA* genes and the primers (F1 and R2) produced the first intermediate segment by 600 bp size and the primers (F2 and R1) produced the second intermediate fragment 1800 bp size. To reproduce intermediate fragments, *Ex Taq DNA Polymerase* enzyme was used. The final fragment of mutated *PA* was produced by overlap extension PCR using overlapping supplementary fragments 17 bp. Finally, to duplicate mutated *PA* was used to F2, R2 primers and *Ex Taq DNA Polymerase* enzyme (Table 1 shows the primer sequences).

1. Urokinase Plasminogen Activator
2. Urokinase Plasminogen Activator Receptor
3. *Site-directed mutagenesis*
4. *Polymerase chain reaction*
5. *Splicing* by *overlap extension* by pcr

Table 1. Primers used in reactions

Primers	Conditions	Conditions	PCR volume (25 **µl**)
F1: 5´-GTAGGATCCTAAAAGGAGAACGTATATGA-3´ R1: 5´-TAAAGCTTTGTTTAAAACATACTCTCCTTG-3´ F2: 5´-pTGGTGAGTTCGAAGATTTTTGTTTTAATTCTGG-3´ R2:5´-pGGAAGTGGAAGATCAGCAAGTACAAGTGCTGGACCTACGGTTCCAG-3´	1 cycle 25 °C.....10 min 47 °C.....60 min 70 °C.....10 min	1 cycle 95 °C.....5 min 40 cycle: 95 °C.....30 s 58 °C.....30 s 72 °C.....45 s 1 cycle: 95 °C.....10 min	10X PCR Buffer: 2.5 µl 10 mM dNTPs: 0.5 µl 10 mM $MgCl_2$: 0.75 µl 10 pmol F+R Primer: 1.25 µl Taq DNA polymerase (5u $µl^{-}$): 0.2 µl Template DNA: 1 µl H_2O up to 25 µl

Cloning and expression of the PA gene in *E. coli* and *B. subtilis*

The ligation was established between PCR product 500 ng and *T. vector* 200 ng for providing *E. coli* containing *PA*, then the ligation mixture was transformed into *E. coli* by the standard method ($CaCl_2$ method). 100 µl of transform solutions was transported to LB Agar plate containing ampicillin antibiotic (100 µg/mL), X-gal (30 µg/mL) and IPTG (2 mM). It was then incubated at 37 °C. White colonies were cultured, separated and used for colony PCR and plasmid DNA containing the *PA* gene was detected by enzymatic digestion after sequencing by Bioscience company. Finally, the mutated *PA* gene was detected and separated. In the following process, for providing *B. subtilis* containing mutated *PA.*, the expression vector *pWB980* was digested with restriction enzymes *Hind III* and *BamH I* followed by DNA being purified by DNA Purification Kit (Viogen, Korea). The ligation was established between plasmid 1 µg and fragment of gene 3 µg. Next, electroporation was performed in 1000 v/cm and 8.5 msec. 200 µl of transform solutions were transported to the LB Agar plate containing Kanamycin antibiotic (10 µg/mL). Then it was incubated for 48 h at 37 °C.

Statistical analysis

To analyze the obtained data, SPSS version 23.0 (SPSS, Chicago, IL) was used. *T*-test and Pearson's chi-square test were employed to interpret the correlation between parameters. The level of significance in the current study was <0.05.

Results

At first, a *pMNA1* plasmid containing the *PA* gene of *B. subtilis* was isolated. Then, the PCR technique was performed and 2.4 kbp band was observed (Figure 1). Secondly, intermediated fragments were achieved (600 bp and 1800 bp) by PCR1 and PCR2; these fragments have to overlap with each other (Figure 2). The third, two fragments were used with an equal molar

concentration in the other PCR reaction (PCR3) and then ligated together. This fusion causes mutated *PA* gene 2.4 kb size (Figure 2). *T*-vector cloning products grew up on the LB agar plate containing ampicillin antibiotic, X-gal and IPTG and the colonies screening by blue-white color, so the presence of *PA* was verified with colony PCR in (Figure 2).
Finally, the appearance of mutation in *PA* was approved by sequencing. The mutated *PA* gene was separated from *TA Vector* with *BamH I* and *Hind III* enzymes (Figure 3). Then it was inserted to *pW980* by ligation and solution used for electroporation. The result was verified with single digestion by *BamH I* and PCR.

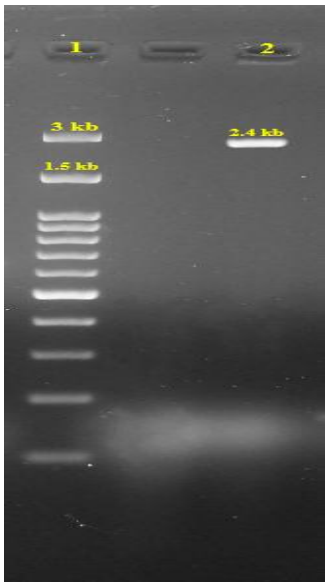

Figure 1. Gel electrophoresis shows the PCR product of *PA*
Lane 1. Gene ruler 1kb DNA Ladder, Lane 2. PCR product of *PA* on *pW980* plasmid.

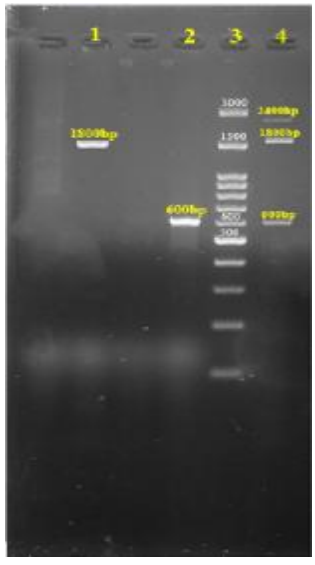

Figure 2. A. Gel electrophoresis shows the pcr1 product
Lane 1. PCR2 product of F1 and R2 primers, Lane 2. PCR1 product of F1 and R1 primers, Lane 3. Gene ruler 100 bp DNA Ladder, Lane 4. Gel electrophoresis shows the PCR3 product

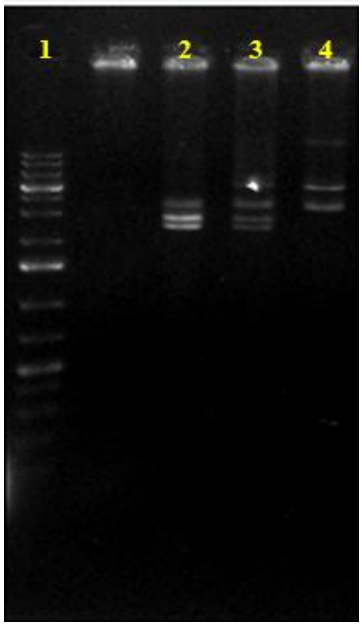

Figure 3. Gel electrophoresis shows the digestion of *TA* vector with *BamH I* and *Hind III* restriction endonuclease
Lane 1. Gene ruler 1 kb DNA ladder, Lanes 2, 3. digestin enzyme, Lane 4. *TA vector* plasmid

Discussion

Anthrax toxin is one of the best-characterized protein delivery systems for tumor targeting. The *PA* protein can be altered in numerous ways to obtain high specificity for tumors (Wein *et al*., 2015). Remarkable progress has been achieved in recent years towards the characterization of the structure and function of anthrax toxin that can be manipulated for cancer therapy (Liu *et al*., 2003).

The current study was performed by designing terminated primers and phosphorylated for a mutation, in the *PA* gene which is in agreement with the results of (Liu *et al*., 2003). They developed mutant anthrax toxin and analyzed the anti-tumor effects of the mutated toxin. They used phosphorylated primers and utilized splicing by overlap extension PCR for developing a mutation in the *PA* gene. In a similar study, (Wein *et al*., 2015), using an anthrax toxin variant in tumor targeting, have demonstrated the new combination of *PA* has higher anti-tumor activity and low toxicity in comparison to the performance of the original combination.

In another study, (Rogers *et al*., 2007) utilized mutant anthrax toxin (protective antigen) for inhibitions angiogenesis and aumor growth. They reported that the anthrax toxin receptor (ATR) ligands, such as *PA* are angiogenesis inhibitors and that ATRs are useful targets for antiangiogenic therapy.

In our study, the plasmid isolation from *B. subtilis* was performed using alkaline lysis and some changes, especially in the use of lysozyme, which was easier and less costly than the CsCl-ethidium bromide gradient method. The finding was dissmiliar to that of (Sullivan *et al*., 2011). They performed plasmid isolation from *Bacillus* based on CsCl-ethidium bromide gradient and also by boiling method.

The transformation efficacy of the ligated plasmid DNA into the competent Bacillus subtilis cells is highly dependent on the ratio of plasmid oligomers in the plasmid DNA solution and compared to the shock-induced cells of *E. coli* at low. In this study, we used electroporation to transfer plasmid DNA, which is a rapid and effective method for the transformation of *Bacillus*.

Conclusion

Due to the high prevalence of cancer in the world, it is necessary to have an effective and efficient drug. The result of this study seems to be promising in treating cancer.

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