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Cloning and Expression of Mycobacterium Tuberculosis Rv0285 (Pe5) Gene

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Background: PE5 is a member of the PE protein family whose precise function is yet to be understood. There are about 100 members of the PE family proteins in mycobacterium tuberculosis. These glycine- and alanine-rich proteins consist of Proline-Glutamate motifs at their N-termini and may be implicated in pathogenesis of the bacilli.
Objectives: We aimed to clone and over-express the Rv0285 coding region in the BL21 (DE3) *Escherichia coli* strain for the future functional investigations.
Materials and Methods: The PE5 coding region was cloned into a specific vector containing N-terminal GST tag using ligation independent cloning (LIC) method and then the recombinant vector was transferred into the competent TOP10 *E. coli* strain. The positive colonies were screened by the colony PCR approach and finally integration of the constructed expression vector was assessed by DNA sequencing. The vectors were then transferred and expressed in *E. coli* BL21 (DE3) strain and finally the protein expression level was analyzed using SDS-PAGE.
Results: In this study, the PE5 (Rv0285) coding region was amplified as a 309 bp DNA fragment from the mycobacterium tuberculosis H37Rv chromosome using specific sets of primers. The amplicon was then cloned into pLEICS-02 and then confirmed by DNA sequencing. The recombinant protein was over-expressed as a ~ 40 kDa tagged protein in *E. coli*, and finally confirmed by SDS-PAGE analysis.
Conclusions: Our data showed that recombinant PE5 coding region was successfully cloned in pLEICS-02 and expressed in BL21 (DE3) *E. coli* strain as host.

Keywords: Mycobacterium Tuberculosis; Genes

1. Background

Tuberculosis (TB), once thought to be controlled, is now resurgent in many parts of the world, claiming more lives annually than any other infectious disease. Every second someone in the world is newly infected with TB Bacilli and five to ten percent of the infected individuals become infectious at some time during their life. An estimated two billion people, about one third of world's population, is currently infected with TB Bacilli, resulting in about two million deaths annually (1, 2). The major burden of TB is borne by developing countries, particularly in Africa and South-East Asia, mainly because of the reduced socio-economic status, lack of proper public healthcare system, emergence of both multi-drug resistant (MDR) and extensively drug resistant (XDR) tuberculosis and spread of HIV/AIDS (3). However, in economically favored countries with outstanding clinical facilities and proper public healthcare system, other risk factors such as, stressful life, alcohol consumption and particularly increased number of immigrants from high burden counties have resulted in an increase in the number of reported TB cases (2, 4).

The *M. tuberculosis* genome was sequenced completely about 14 years ago (5). The singular GC rich circular chromosome consists of 4.4 Mbp and contains 4006 functional protein genes (6). About 52% of these proteins have been assigned a precise or predicted function (7). An unusual characteristic of the genome is the presence of 167 genes encoding the two unrelated, acidic protein families, known as the PE and PPE proteins (8). These two protein families are recognized by their N-terminal proline-glutamic acid and proline-proline-glutamic acid motifs and it has been suggested that at least some of these proteins are surface exposed and play a role in adhesion, immune modulation and antigenic variation (9, 10). PE5, a member of the PE family proteins, is encoded by the RV0285 gene (11). This 306 bp coding region encodes a polypeptide, containing 102 amino acids (12). Given the possibility that this polypeptide acts as virulence factor, we cloned and then expressed the Rv0285 coding region in the BL21 (DE3) *E. coli* strain for the future functional investigations.

Implication for health policy/practice/research/medical education:
It helps to clone and over-express the Rv0285 coding region in the BL21 (DE3) Escherichia coli strain for the future functional investigations.

2. Objectives

We aimed to clone and over-express the Rv0285 coding region in the BL21 (DE3) *E. coli* strain for the future functional investigations.

3. Materials and Methods

3.1. Primer Design and Polymerase Chain Reactions

In the present study, the m. tuberculosis H37Rv chromosome we used as a template for PCR. The coding regions were amplified by PCR from the DNA template using Pfx DNA polymerase (Invitrogen), and the following primers (Table 1):

Table 1. The sets of Forward and Reverse Primes Used to Amplify Rv0285 Coding Region [a]

Primer Names	The Sequences (5'-3')
F1	TGGTGAAACCCAACAGAA
R1	GCGAAGCCATCCAGATGG
F2	TACTTCCAATCCATGACGTTGCGAGTGGTT
R2	TATCCACCTTTACTGTCAGCCGCCCACGACCCC

[a] Abbreviations: F, forward; R, reverse.

The second set of forward and reverse primers (F2 and R2) contain a 5' extension (the underlined fragments) which is recognized by a 3'-5' exonuclease. The PCR reactions were performed according to the manufacturer's protocols, using the TECHNE thermal cycling system (TECHGENE). The PCR products were finally identified by electrophoresis using 1% (w/v) agarose gels, and purified using the QIA quick PCR purification kit. PCRs were accomplished in a 50 μL reaction volume and the protocol consisted of a five minute initial denaturation at 94°C followed by 30 cycles of one minute at 94°C, one minute at 50°C for annealing and one minutes at 68°C for extension. The final extension cycle was five minutes at 68°C.

3.2. DNA Gel Electrophoresis

PCR products were identified by horizontal electrophoresis using 1% (w/v) agarose gels run at a constant 100 V for 40 minutes. Ethidium bromide was used to visualize the location of the plasmid DNA under ultra violet light.

3.3. Ligase Independent Cloning

pLEICS-02 expression vector was digested by the restriction enzyme BseRI to remove the inserted lethal *SacB* gene (the *Bacillus subtilis SacB* gene encodes levansucrase that induces lethality upon exposure to 5% (wt/vol) sucrose in the growth medium) (13). The freeze-dried 3'-5' exonuclease supplied by In-fusion™ PCR cloning Kit (Clontech) was then thoroughly dissolved in 5 μL dH_2O. 2 μL of dissolved enzyme, the linearized vector and the purified PCR product were mixed together and the mixture was finally subjected to a two-step incubation procedure at 30°C and room temperature respectively (14).

3.4. Transformation the Competent E. Coli Strains

An aliquot of 50 μL competent cells was mixed with 4 μL of plasmid mixture and then mixed gently. The mixture was incubated on ice for 30 minutes and then heat shocked at 42°C for 45 seconds. Following the heat shock, the sample was left on ice for five minutes. 300 μL LB broth was then added to the ice-cold sample and incubated for 45 minutes at 37°C and 200 rpm, with the tubes placed horizontally in the incubator. The incubated sample was plated out in 50, 100, 200 μL aliquots onto LB agar containing 100 μM ampicillin and finally incubate at 37°C overnight.

3.5. Colony PCR Screening and DNA Sequencing

Ten colonies were picked and transferred separately into 10 PCR tubes containing 20 μL of sterile water. In order to trace out the positive transformants, 5 μL of each inoculated sample was used to inoculate 10 mL LB broth containing 100 μg/mL ampicillin. The 10 mL inoculated cultures were then incubated at 37°C/200 rpm for overnight to be subjected for miniprep (Promega). The remaining samples (15 μL) were then utilized for the analytical PCR using F2 and R2 primers. The PCR products were then analyzed by DNA electrophoresis. The positive constructs identified by the colony PCR screening were finally checked by sequencing which was performed at the Reference lab, Qazvin University of Medical Sciences.

3.6. Protein Expression Trials

Time-course expression trials were carried out using BL21 (DE3) transformed by the respective construct. LB starter cultures (50 mL) containing 100 μg/mL Ampicillin was inoculated with one colony of each selected transformant and grown overnight at 37°C, and 200 rpm in a shaker. The cells centrifuged for 10 minutes at 5000 rpm. The pellets were resuspended in 1mL fresh LB (without antibiotic) and subcultured into 50 mL LB broth containing 100μg/ml Ampicillin to final OD 600 nm of about 0.1. The cultures were grown at 37°C until mid-log phase (OD600 nm 0.6-0.7) and 1 mL of each culture removed and stored at -20°C for SDS-PAGE analysis. The cells were induced by addition of 1 mM IPTG and 1 mL of the cells were harvested every hour post-induction for 4 hours. Normalized pre- and post-induction samples were analyzed by SDS-PAGE for protein production.

4. Results and Discussion

4.1. Specific Primers Were Designed to Amply the Target Rv0285 Coding Region

Our blast analysis unmasked that there were many cod-

ing regions in the bacilli chromosome very similar to that of RV0285. This made us design two sets of forward and reverse primers to circumvent amplification of unwanted genes. In fact, we used the bacterial chromosome as template and applied the first set of forward and reverse primers (F1 and R1) to amplify the Rv0285 coding region with 5' and 3' flanking regions. The resulting PCR products were then utilized as secondary template to amplify only the Rv0285 coding region using the second set of primers, i.e. F2 and R2. The amplicon was then analyzed and confirmed using DNA electrophoresis as shown in the Figure 1.

4.2. Ligase Independent Cloning Were Used to Clone Rv0285 in pLEICS-02

In order to produce Rv0285 protein with removable glutathion-S-transferase-tag, the corresponding coding regions were cloned into the pLEICS-02 expression vectors using LIC-PCR method (14). Ligation-independent cloning (also known as enzyme-free cloning) increases both the speed and efficiency of cloning of PCR products. LIC-PCR also eliminates the need to ligate PCR products to a vector, and does not rely on restriction sites (14). In this method, overlapping sequences are designed into the vector and PCR primers used to amplify the target DNA. Controlled digestion of the PCR product and the vector with a 3'-5' exonuclease such as exonuclease III is then used to create complementary protruding 3' ends. When the insert and the vector are mixed, the PCR products are annealed to the prepared vector. The chimeric molecule is then used to transform E. coli, and the ligase of the bacterial cells finally seals the single-stranded nicks and generates a covalently closed circular molecule (14).

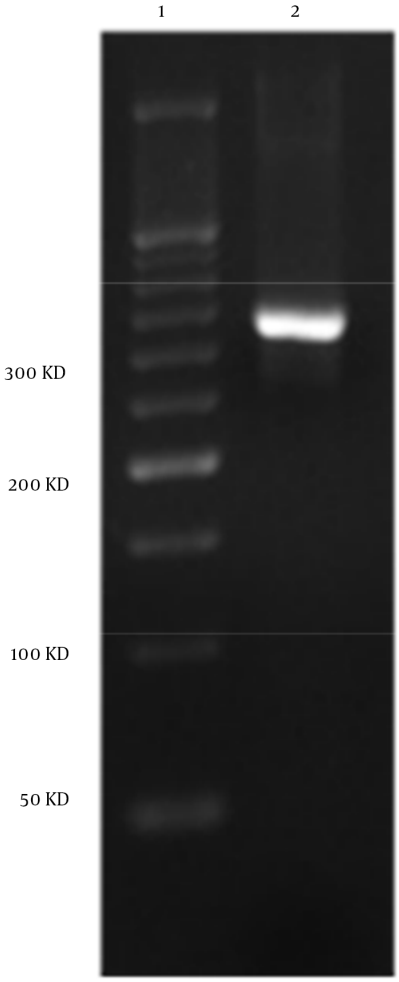
4.3. Colony PCR Screening and DNA Sequencing Were Used to Confirmed the Constructed pLECS-02 Plasmid

Following cloning of the Rv0285 amplicon into pLECS-02 expression vector, we used colony PCR screening to trace out the colonies carrying the chimeric plasmid. As expected, the method of cloning performed was quite efficient. This is evidenced by the fact that all 10 colonies selected for colony PCR screening were positive (Figure 2). To double check if we had the right colons at hands, the recombinant vector was extracted by miniprep from the selected transformants mentioned above and then sent for DNA sequencing. Finally, our sequencing data confirmed that the right coding region had been cloned in the pLECS-02 expression vector.

4.4. Expression Trails Showed That the PE5 Polypeptide Can be Expressed in BL21 E. coli Strain

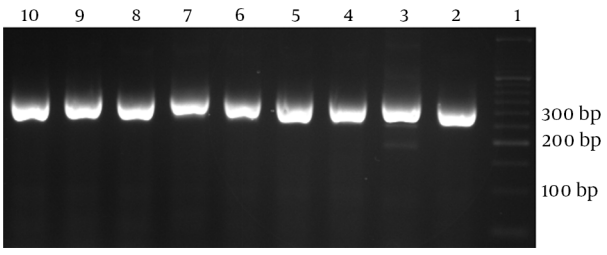
Our expression trials clearly demonstrated that the GST-tagged protein was expressed in the standard BL21 (DE3)

Figure 1. PCR Amplification of the Rv0285 Coding Region



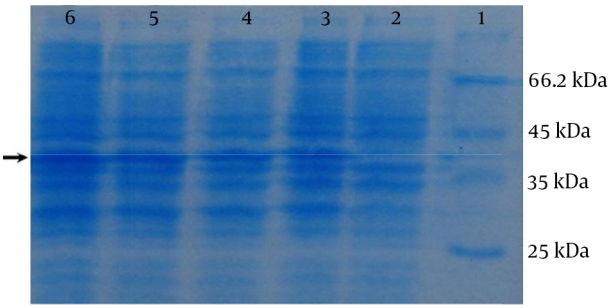
The gel is a 1% w/v agarose gel viewed under UV light with ethidium bromide used to visualize the products of the PCR reactions. Lanes 1 and 2 correspond to a 100 bp DNA ladder and the PCR products of Rv0285 coding region, respectively.

Figure 2. Colony PCR Screening of Transformants



The gel shows the results of colony PCR screening for the transformants carrying of the right Rv0285-pLEICS-02 (lanes 2-10) construct. Lane one contains a 100 bp DNA marker.

Figure 3. SDS-PAGE Gel of Expression Trails of the Recombinant Rv0285



Gel shows the expression trials of the GST-Rv0285 in BL21 (DE3) *E. coli* cells. Lanes 1, 2, 3, 4, 5 and 6 contain a high molecular weight protein marker, the pre-induction cell lysate, 1, 2, 3, and four hour post-induction fractions (whole cell lysate). The Position of the GST-Rv028s band on the gel is shown by the arrow on lanes 6.

cells. Indeed, comparison of the pre-induced cell lysate with 1 hour-, 2 hour-, 3 hour- and 4 hour-post induction lysates by SDS-PAGE analysis showed that a protein band with molecular weight of about 40 kDa was appeared in all but the lane corresponding to the pre-induced sample (Figure 3), suggesting that the GST-PE5 polypeptide (Mw: 40 kDa) was expressed upon induction of the transformants with IPTG.

Collectively, our data reveal that the Rv0285 coding region was successfully cloned in the pLECS-02 expression vector using efficient, rapid and inexpensive ligase independent cloning approach and was expressed in the standard BL21 (DE3) *E. coli* strain. It is noteworthy that we are planning to purify the expressed GST-PE5 protein using GST affinity chromatography and further confirm it using mass spectroscopy.

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Author's Contribution

Sirati-Sabet conducted cloning and expression of Rv0285, and helped with manuscript preparation; Sahmani, Najafipour, Alizadeh, Piri, Abdolvahabi and Khabbaz were involved in solution preparations, miniprep, competent cell preparation, and accomplishment of experiments; Ilghari designed the experiment, wrote the manuscript, and rewarded the grant.

Financial Disclosure

There is no conflict of interest.

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