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Prokaryotic expression, identification and bioinformatics analysis of *fbpB-esxA* fusing gene from *Mycobacterium tuberculosis*

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ABSTRACT

Objective: To obtain *fbpB-esxA* fusing gene of *Mycobacterium tuberculosis* (MTB), express the encoded fusing protein in *Escherichia coli* (*E. coli*), identify protein acquired, and predict the structure and function of the protein utilizing methods of bioinformatics. **Methods:** *fbpB* and *esxA* gene were amplified from genome of MTB H37Rv by PCR. The *fbpB-esxA* fusing gene ligated by $(Gly_4Ser)_3$ linker was gained by means of Gene Splicing by Overlapping Extension PCR (SOE-PCR), and fusing gene was cloned into expression vector pET-30a. The recombinant plasmid was sequenced and expressed in *E. coli* BL21(DE3). The protein was identified by Western blot using anti-HIS antibody. Secondary structure and antigenic epitopes of the protein were predicting using tools of bioinformatics. **Results:** The DNA sequences of *fbpB-esxA* were identical with that published by GenBank. The Ag85B-ESAT-6 fusion protein about 50 kDa comprised 485 amino acids was efficiently produced from expression system in *E. coli* BL21(DE3) under the induction of IPTG. Bioinformatics analysis showed the protein contained one transmembrane region and fourteen potential antigenic epitopes. **Conclusions:** The Ag85B-ESAT-6 fusion protein is successfully expressed with N-terminal HIS-tag. Gel filtration demonstrated that it exists as insoluble inclusion bodies mainly. The existence of linker doesn't affect immunogenicity of Ag85B and ESAT-6. It will allow for characterization *in vitro* and establish a foundation of further function research such as vaccine or diagnostic reagent.

1. Introduction

Tuberculosis (TB) is a major social and economic problem around the world, mostly in developing countries, especially in the South-East Asia, African and Western Pacific regions[1]. According to reports of World Health Organization (WHO), over a third of the world's population are infected with TB, and annually about 9 million TB cases is reported to be responsible for adult deaths every year, more than any other single infectious disease[2].

Mycobacterium bovis Bacille Calmette-Guérin (BCG) vaccine has been used for about 80 years as currently the sole available administered vaccine against TB[3]. Though vaccination of infants and children with BCG has reduced several serious TB diseases, unfortunately its efficacy against adult pulmonary TB disease is highly unstable. Large numbers of field trails have shown that efficacy of BCG vary from 0% to approximately 80%[4]. It can also cause pathogenicity in immunocompromised patients and prevent the use of tuberculin in TB diagnosis[5]. Since TB represents a severe danger to public health in spite of the widespread use of BCG vaccine, more new effective vaccines or vaccination strategies are urgently needed[6]. Considerable

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efforts are aiming at the development vaccine against TB especially using molecular techniques. They include recombinant BCG vaccines, live attenuated vaccines, non-mycobacterial vaccine vectors, DNA vaccines, and subunit vaccines, *etc*. Culture filtrate proteins isolated from MTB are mainly using for vaccine candidates[7]. *fbpB* and *esxA* genes encode major secretory protein antigen 85B (Ag85B) and early secretory antigenic target-6 (ESAT-6), respectively. Both Ag85B and ESAT-6 are major co-secreted proteins that exist in culture filtrates[8,9]. Ag85B which participates in cell wall mycolic acid synthesis is the most dominant protein of MTB, and is a potent immuno-protective antigen as well as a leading drug target[10]. Immunization with Ag85B and ESAT-6 protein or DNA induces a strong IFN-γ response and antigen-specific $CD4^+$ Th1 cells and $CD8^+$ T cells, and protected against TB[11,12].

Early and rapid diagnosis plays an important role in TB disease control, but the microscopic examination is still the unique global available diagnostic tool for identifying TB. Therefore, the development of simple, sensitive and specific methods for diagnosis of TB infections is critical needed[13]. Serological test appears to be a promising approach for the diagnosis of pulmonary as well as extra-pulmonary TB[14]. Ag85B and ESAT-6 are demonstrated as specific antigens of MTB as they are absent in most of the nonpathogenic mycobacteria and in BCG. They are recognized by immunity system of infected host. Thus they could be strong superior serodiagnostic antigens to distinguish infected and BCG-vaccinated individuals with TB[15]. Enzyme-linked immunosorbent assay based Ag85B and ESAT-6 is high degree of sensitivity (84.1% and 64.9%, respectively) and specificity (about 90%) for TB diagnosis, Ag85B-ESAT-6 fusion protein could have higher sensitivity than single antigen accordingly[16].

In the search for more safer and potent TB vaccines, and evaluate specific antigen potential as diagnostic markers, we expressed and identified the *fbpB-esxA* fusing gene in *Escherichia coli (E. coli)* strain and predicted the structure and characteristies of recombinant fusion protein by bioinformatics methods.

2. Materials and methods

2.1. Bacterial strains, plasmids, enzymes and culture conditions

Genomic DNA of *Mycobacterium tuberculosis* (MTB) H37Rv was kindly provided by Lab Director Zhu ZY of Affiliated Xinhua Hospital of Hainan Medical University. *E. coli* DH5α (TaKaRa Biotechnology, China) and *E. coli* BL21 (DE3) (Merck-Novagen, Germany) were used as the recipient of recombinant plasmids and the host of expressing protein. All *E. coli* strains were grown aerobically on a rotary shaker in liquid or on solid (1.5% agar) Luria-Bertrani (LB) medium at 37 ℃. When needed, kanamycin (50 μg/mL) was used for selection of recombinant strains. Plasmid pET30-a (Merck-Novagen, Germany) were used as expression vector respectively. Restriction enzymes (*Bam*HⅠ, *Sac*Ⅰ), PrimeSTAR™ HS DNA Polymerase and T4 ligase were purchased from TaKaRa Biotechnology, China. High Fidelity PCR Enzyme Mix was purchased from Fermentas Canada. All other chemicals were of an analytical grade.

2.2. Amplification of *fbpB* and *esxA* genes

Nucleotide sequences of *fbpB* and *esxA* genes were retrieved from MTB H37Rv genome (GenBank Accession No.NC_000962, http://www.ncbi.nlm.nih.gov). PCR was performed using MTB H37Rv Genomic DNA as the template with following primers: *fbpB*-F and *fbpB*-R were used for amplification of *fbpB* gene (reverse direction), and primers *esxA*-F and *esxA*-R were used for amplification of *esxA* gene (Table 1). Two sets of primers contained *Bam*HⅠ and *Sac*Ⅰ endonuclease restriction sites. PCR was performed by using of High Fidelity PCR Enzyme Mix in a volume of 50 μL as follow cycling temperatures: 94 ℃ for 5 min, 10 cycles (94 ℃ 30 s; 60 ℃ 40 s; 72 ℃ 1 min), 25 cycles (94 ℃ 30 s; 65 ℃ 40 s; 72 ℃ 1 min), and then 72 ℃ for 7 min. The PCR products were visualized on a 1% agarose gel stained with ethidium bromide.

Table 1
Primers used in this study.

Primer name	Sequence
fbpB-F	5′-CGCGGATCCATGACAGACGTGAGCCGAAAGATTC-3′
fbpB-R	5′-TCCGAGCTCTCAGCCGGCGCCTAACGAACTC-3′
esxA-F	5′-CGCGGATCCATGACAGAGCAGCAGTGGAATTTCG-3′
esxA-R	5′-TCCGAGCTCCTATGCGAACATCCCAGTGACGTTG-3′
fbpB-linker	5′-GCCGCTTCCGCCACCGCCGCTTCCACCGCCACCGCCGGCGCCTAACGAACTCTGC-3′
esxA-linker	5′-AGCGGCGGTGGCGGAAGCGGCGGTGGCGGCAGCATGACAGAGCAGCAGTGGAATTTCG-3′

2.3. Assembly of the *fbpB-esxA* fusing gene by PCR

After the first round of PCR, DNA fragments of amplification were isolated from agarose gels by using TIANgel midi purification kit (TIANGEN, China), according to the manufacturer protocol. Linking *fbpB* and *esxA* gene with $(Gly_4Ser)_3$ linker by the use of Gene Splicing by Overlapping Extension PCR (SOE-PCR). The fragments encoding the *fbpB*+$(Gly_4Ser)_3$ and $(Gly_4Ser)_3$+*esxA* was amplified by second round PCR using the primers *fbpB*-F and *fbpB*-linker, the primers *esxA*-linker and *esxA*-R, respectively (Table 1). We deleted stop codon of the *fbpB* gene in this study. Finally, using the purified fragments of *fbpB*+$(Gly_4Ser)_3$ and $(Gly_4Ser)_3$+*esxA* as template, the third round of PCR was performed using PrimeSTAR™ HS DNA Polymerase as follow conditions: 7 cycles (98 ℃ 10 s; 68 ℃ 60 s), after 98 ℃ 20 s, primers *fbpB*-F and *esxA*-R were added, 25 cycles (98 ℃ 10 s; 68 ℃ 60 s).

2.4. Construction of expression plasmid

PCR products of assembled *fbpB-esxA* were gel purified and digested with *Bam*H Ⅰ and *Sac* Ⅰ restriction enzymes for 16h at 37 ℃, and then was ligated into prokaryotic expression vector pET30-a (digested with these same enzymes) at 16 ℃ for overnight resulting in recombinant plasmid pET30-*fbpB-esxA*, and chemically transformed into the *E. coli* DH5 α cells using standard procedures. Colonies were screened by PCR (using primers *fbpB*-F and *esxA*-R) and restriction endonucleases, and then sequenced to confirm that no base changes had been introduced during amplification of the DNA.

2.5. Expression of fbpB-esxA fusing gene in E. coli

The *E. coli* BL21 (DE3) was employed to express the fusion proteins. The recombinant plasmid pET30-*fbpB-esxA* was transformed into competent *E. coli* BL21 (DE3) cells and selected according to the manufacturer's direction. One engineering strain was incubated in the standard conditions as described by the manufacturer (Merck-Novagen, Germany). One mL overnight culture was subcultured in 100 mL fresh LB medium at 37 ℃ with 250 rpm shaking until they reached an A_{600} of 0.5. isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 0.5 mM for various induction periods of 1, 2 and 3 h at 37 ℃. After induction, cells were incubated on ice and harvested with 5 min of 10 000 *g*, and then total proteins of the induced and control cells were resolved on 12% SDS-PAGE with a 1.0-mm-thick gel in a Bio-Rad Mini-Protein Ⅱ Electrophoresis System (Bio-Rad, USA). After separation, proteins were visualized by 2.5 g/L Coomassie brilliant blue G-250 staining, and then destained with 10% acetic acid and 25% isopropanol. In order to examine the extent of aggregation of the produced, induced cells were disrupted by sonication for 5 min on ice and soluble as well as insoluble fractions were analyzed using SDS-PAGE as described previously.

2.6. Western blot analysis

Whole-cell proteins were run on 12% SDS-PAGE and transferred to PVDF membrane. After blocking with 5% nonfat dry milk, the membrane was probed by incubating the membrane with 1∶5000-dilution of mouse anti-polyhistidine monoclonal HIS-1 antibody (Sigma, USA) followed by 1∶7000-dilution of HRP-conjugated goat anti-mouse IgG antibody (Sigma, USA). The immunoreactive protein was visualized by DAB (TIANGEN, China).

2.7. Bioinformatics analysis of Ag85B-ESAT-6 fusion protein

All of amino acid sequences were from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) in this study. Basic physical and chemical properties were analyzed using Vector NTI Advance 11.0 software (Invitrogen, USA). Signal peptides, cleavage sites and subcellular location were predicted by the SignalP program, TargetP program and TMHMM program, respectively (http://www.cbs.dtu.dk/). Antigenic epitopes were predicted by Abie Pro 3.0 program (http://www.changbioscience.com/abie/abie.html) and Predicting Antigenic Peptides program (http://bio.dfci.harvard.edu/Tools/antigenic.html). Secondary structure was predicted by PredictProtein program (http://www. Predictprotein.org/), COILS program (http://www.ch.embnet.org/software/COILS_form.html), ANTHEPROT 2000 software (http://antheprot-pbil.ibcp.fr/), then displayed using Geneious Pro 4.8.5 software (Biomatters, USA).

3. Results

3.1. PCR amplification of fbpB and esxA genes

fbpB and *esxA* genes were amplified by PCR (Figure 1). DMSO has been added to PCR reaction buffer to enhance denaturation of GC rich template DNA and to aid polymerase extension through the secondary structures in this study. Amplification of *fbpB* generated a major DNA fragment with the expected length about 996 bp, while *esxA* generated an expected 306 bp.

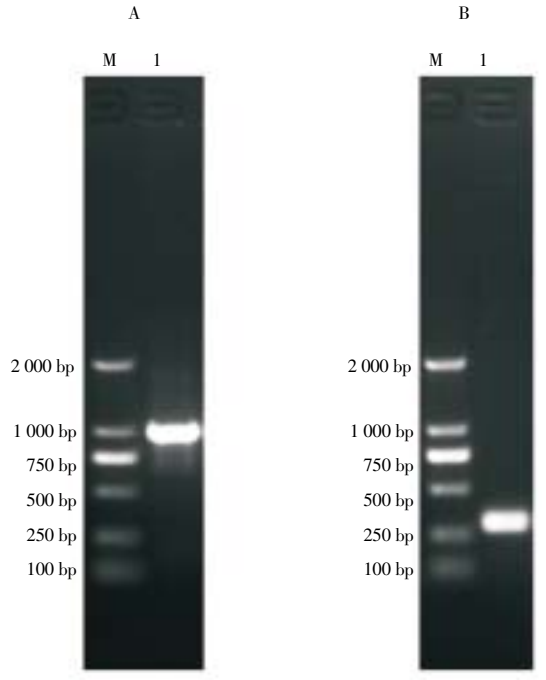

Figure 1. Agarose gel electrophoresis of PCR products of *fbpB* and *esxA* genes.
(A) Lane M, the molecular mass markers; lane 1, PCR product of *fbpB* gene. (B) Lane M, the molecular mass markers; lane 1, PCR product of *esxA* gene.

3.2. Construction of the fbpB-esxA fusing gene and expression plasmid

We gained the fusion gene that contains 1 326 bp by SOE-PCR (Figure 2A). The *fbpB-esxA* fusing gene was then cloned into pET30-a. The recombinant plasmid pET30-*fbpB-esxA* was identified by PCR and restriction endonuclease (Figure 2B). The sequencing results demonstrated that the cloned *fbpB-esxA* gene was identical to the published sequence (GenBank Accession No. BX842578, GenBank Accession No. BX842584) and there was no any change of base pair.

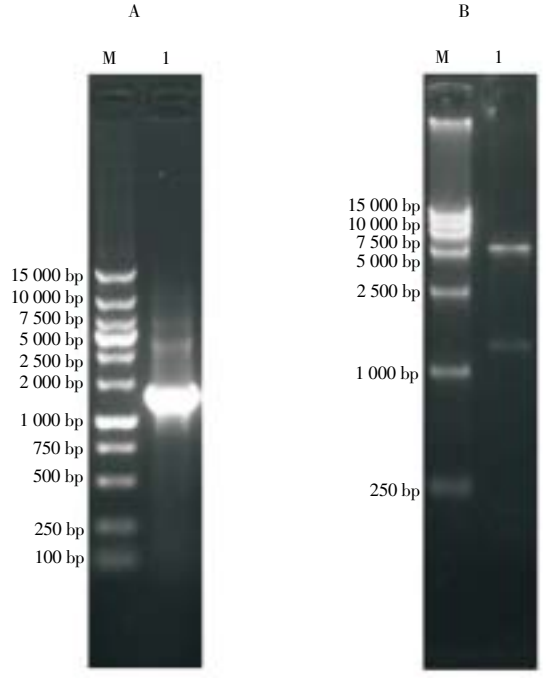

Figure 2. Agarose gel electrophoresis of PCR products of the *fbpB–esxA* fusing gene and restriction endonuclease analysis of recombinant plasmid pET30–*fbpB–esxA*.
(A) Lane M, the molecular mass markers; lane 1, PCR product of *fbpB–esxA* fusing gene. (B) Recombinant plasmid pET30–*fbpB–esxA* was identified by restriction endonuclease. Lane M, the molecular mass markers; Lane 1, pET30–*fbpB–esxA* was digested by *Bam*H Ⅰ and *Sac* Ⅰ.

3.3. Expression and identification of Ag85B–ESAT–6 fusing proteins

The Ag85B–ESAT–6 fusing proteins were produced in *E. coli* BL21 (DE3) cells carrying pET30–*fbpB–esxA* plasmid. We attempted to induce the protein at a lower incubation temperature (28℃), lower concentration of IPTG (0.1 mM), in order to avoid inclusion bodies formation, but it was not successful. The Ag85B–ESAT–6 fusing protein was observed on Coomassie blue G–250 stained gel as expected about 50 kDa band (Figure 3A). Analysis of the total and soluble fractions of the expressed Ag85B–ESAT–6 fusing protein by SDS–PAGE demonstrated that the protein was localized mainly in the insoluble fraction (Figure 3B). Western blot analysis of the total cells proteins confirmed that the observed protein band reacted with the specific antibodies (Figure 3C).

3.4. Characteristics and structures of Ag85B–ESAT–6 fusing protein from bioinformatics analysis

The Ag85B–ESAT–6 fusing protein composed 485 amino acids with an estimated molecular mass of 50.8 kDa and a pI value of 5.36. This fusing protein has a transmembrane region (LMIGTAAAVVLPGLVGLAGGA) and no signal peptide, locates outside of membrane mostly. Secondary structure analysis revealed that the protein contained alpha helix (22.1%), beta strand (26.9%), random coil (31.4%) and turn (19.6%), respectively. It possessed fourteen potential antigenic epitopes larger than six amino acids (Figure 4).

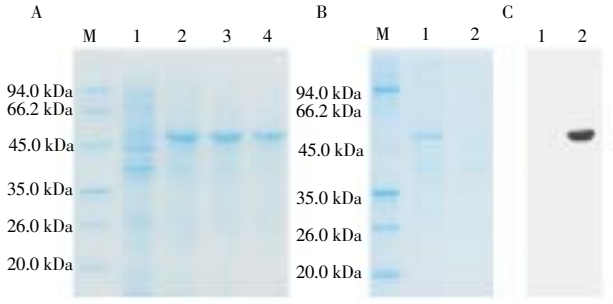

Figure 3. SDS–PAGE analysis and Western blot identification of the Ag85B–ESAT–6 fusing protein expressed in *E. coli* BL21 (DE3) cells.
(A) SDS–PAGE analysis of total proteins expressed. Lane M, protein molecular mass standards; lane 1, cell lysate of bacteria transformed with pET30–*fbpB–esxA* without induction; lane 2–4, cell lysate of bacteria transformed with pET30–*fbpB–esxA* under 0.5 mM IPTG induction for 1 h, 2h, 3h, respectively. (B) SDS–PAGE analysis of soluble and insoluble fractions proteins expressed. Lane M, protein molecular mass standards; lane 1, induced pET30– *fbpB–esxA* by 0.5 mM IPTG for 1 h, pellet; lane 2, induced pET30–*fbpB–esxA* by 0.5 mM IPTG for 1 h, supernatant. (C) Western blot analysis of the Ag85B–ESAT–6 fusing protein. lane 1, negative control; lane 2, the Ag85B–ESAT–6 fusing protein.

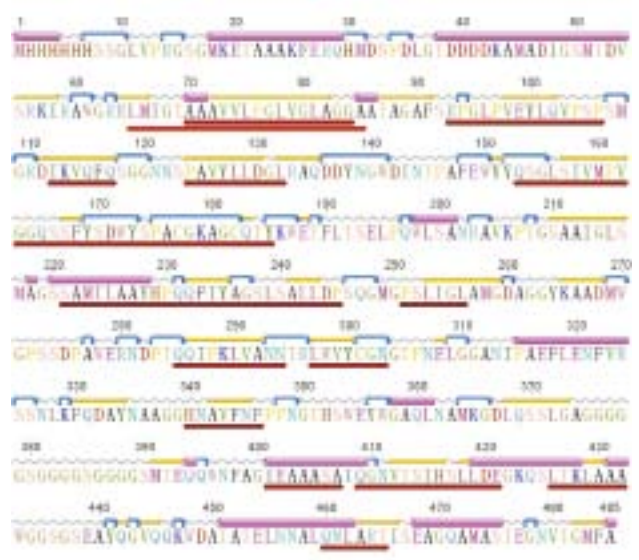

Figure 4. Prediction of secondary structure, subcellular location and possible epitopes of the Ag85B–ESAT–6 fusion protein.
: alpha helix, : beta strand, : random coil, : turn, : transmembrane region, : antigenic region.

4. Discussion

TB is a serious public health problem throughout the world due to the occurrence of multi–drug resistant–TB, and due to its association with human immunodeficiency infection. Treatment, diagnosis, and prevention for TB are out of date and inadequate[17,18]. Most attempts to develop TB vaccines have resulted in low activity in clinical application. The strategy based on fusion TB antigen proteins for developing

novel subunit vaccines could promote efficient protection[19]. IFN-γ produces CD4 T cells and protective immunity, and may induce powerful cell-mediated response. So the fusion protein consisting of Ag85B and ESAT-6 is produced and evaluated as a potential tuberculosis subunit vaccine[20]. Both Ag85B and ESAT-6 are deleted from BCG, and therefore are particularly promising candidates for development of serodiagnostic assays to detect active TB[21-24].

In the present work, we used SOE-PCR to obtain the *fbpB-esxA* fusing gene. SOE-PCR is a technique that DNA fragments are fused together by PCR without restriction digestion. Primer for each fragment is designed to overlap sequences on the other fragment. Mix PCR products of the two fragments, and amplification using outer primers result in the production of fusion DNA. Contrasting with other methods, it is simple, quick and convenient[25].

To study the possible function of Ag85B-ESAT-6 fusing protein, pQE-30 vector (Qiagen, Germany) was first used for the expression of the fusing protein in *E. coli* M15. However, this approach is not successful in our study. Therefore, plasmid pET30-a was chosen because it provides the N-terminal 6×His Tag and S- Tag sequence to the expressed proteins, which could facilitate protein purification by affinity purification methods. Tag-encoded protein sequences can be removed from the resulting fusion proteins by cleavage with enterokinase[26]. The Ag85B-ESAT-6 fusing protein band of interest is absent from the soluble fraction by SDS-PAGE, but is obviously detected in the insoluble fraction. It is thus not possible to purify the protein in a soluble form, so we could change conditions for expression or refolding of protein from the insoluble fraction in the future.

We predicted potential antigenic epitopes of the Ag85B-ESAT-6 fusing protein by using of bioinformatics methods, and demonstrated that the protein reserved virgin immunogenicity of single antigen. Therefore, the Ag85B-ESAT-6 fusing protein is a potential and suitable molecular target to develop new TB vaccines and diagnostic reagents.

Conflict of interest statement

We declare that we have no conflict of interest.

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