Rv1886c Gene Expression for the Production of *Mycobacterium tuberculosis* Major Secreted Protein Antigen 85B in *Escherichia coli*

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ABSTRACT

Tuberculosis (TB) is one of the major infectious diseases that cause health problems worldwide. Antigen 85B is a secreted protein of the infectious strain *Mycobacterium tuberculosis.* Our main focus is on production of proteins as a booster vaccine to replace the traditional *Mycobacterium bovis* bacillus Calmetter-Guerin (BCG) vaccine formula. The main challenge is to express a high yield of an active recombinant protein in native soluble form. To the extent of our knowledge, the cultivation conditions, such as optimal temperature, for overexpressing soluble Ag85B protein of the gene *Rv1886c,* have never been reported. This study showed for the first time the optimizing culturing conditions for inducing expression of soluble Ag85B protein by isopropyl β-D-1-thiogalactopyranoside (IPTG) in *Escherichia coli* BL21 (DE3) pLysS. Protein yields were higher at a low temperature of 25 °C (for 12 h), compared to those at a high temperature of 37 °C (for 5 h). To conclude, low temperature is associated with slow expression which allows the protein to adopt a well-folded conformation and provides a high yield of soluble recombinant protein.

Keywords: *M. tuberculosis* Ag85B; Bacterial expression; Recombinant protein; *Escherichia coli*; Induction parameter

Introduction

Tuberculosis (TB) is one of the leading causes of mortality in humans, especially in third world countries. The disease caused over 1.8 million deaths worldwide in the year 2015 and remains a major public health problem [1]. *Mycobacterium tuberculosis* (*Mtb*) is the main causative agent in humans. Currently, the only licensed vaccine against TB is derived from *Mycobacterium bovis* bacillus Calmetter Guerin (BCG), which has been made since 1920. However, the efficacy of this vaccine is limited. The BCG vaccine fails to protect against pulmonary TB in adults and does not persist long enough to generate adequate long-term immunity. Over

the past decades, the BCG vaccine has lost sensitivity to new evolving multidrug-resistant *M. tuberculosis* strains [2]. An immediate search for a reliable and effective vaccine against TB is required. Recent studies have confirmed that proteins obtained from *M. tuberculosis,* including Ag85 [3], ESAT-6 [4], TB10.4 [5], and HspX [6, 7], can be used as a booster vaccine or combined with the conventional BCG vaccine to enhance the immune system against TB [8, 9].

Antigen 85 (Ag85) protein complexes (Ag85A, Ag85B, Ag85C) are major secretory proteins of actively replicating *M. tuberculosis*. The protein complexes participate in the biosynthesis of the mycobacterial cell wall [3, 10]. The three antigens - Ag85B:Ag85A:Ag85C are expressed at a ratio of 3:2:1 [11]. The proteins are the most abundant in *M. tuberculosis* during the primary phase of illness. Ag85B was capable of inducing the highest antigen-specific humoral responses [10]. The protective efficacy of Ag85B as a vaccine agent has been increasingly explored [6, 12, 13]. However, the overexpression of Ag85B in other studies was found to accumulate mostly in the inactive insoluble form [10, 11]. Aghababa H. *et al.*, cloned Ag85B gene PJET1.2 plasmid (Fermentas) in *E. coli* GM2163 (Fermentas) and transformed the expression vector pET32a(+) (Novagen) in *E. coli* Rosetta gami (Novagen). The recombinant plasmids were induced by 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37 °C. The heterogeneous host lacks a specific chaperone system, resulting in an incomplete protein folding and a high yield of intracellular inclusion [10]. Zarif R. *et al.*, transformed pET101/D Ag85B into *E. coli* BL21 (DE3) and induced the protein expression by adding 2 mM IPTG at 37 °C. The expressed recombinant proteins were found entirely in the inclusion body [11]. Overexpression often results in improper folding machinery of the proteins and leads to insoluble aggregation [14]. For functional and structural analysis, water soluble proteins are required. In order to recover the proteins in an active form, the inclusion bodies have to be solubilized in denaturing agents, including urea and guanidinium chloride. The presence of these denaturing agents causes proteins to have poorer immunogenicity compared to native antigens [15]. To the best of our knowledge, the cultivation conditions for expressing a high yield Ag85B recombinant protein of the gene *Rv1886c* have never been reported.

Although it is possible to express Ag85B directly from *M. tuberculosis*, it is safer and more efficient to express the protein in a heterologous prokaryotic host, such as *Escherichia coli*. Prokaryotic hosts are generally preferred due to their short doubling time (20 min) compared to eukaryotic hosts (18-24 h) [16]. The gram negative bacterium *E. coli* has been widely employed as a host due to its genetic simplicity, fast growth rate, ability to host foreign DNA, ability to express high levels of heterologous proteins, and the advanced knowledge of its protein expression mechanism. *E. coli* BL21 (DE3) pLysS is a common *E. coli* strain used for gene expression. The bacterial strain contains the T7 RNA polymerase gene which induces a high level of protein production. In addition, the presence of the T7 lysozyme inactivates the T7 promoter and increases cell tolerance for toxicity. BL21 and its derivatives were also modified to be deficient in Ion and OmpT proteases to increase the protein stability [17].

The present study focuses on expressing these antigens in a native soluble form by *E. coli* cells. Various factors need to be optimized for enhancing protein solubility. Inducing protein expression at lower temperature demonstrated success in increasing the intracellular concentration of molecular chaperones [18, 19]. Isopropyl β-D-1-thiogalactopyranoside (IPTG), is a common chemical inducer of the lac

promoter [18]. This study aims to optimize the induction parameters for high *M. tuberculosis* Ag85B protein expression in *E. coli*. The results from this study can be applied to achieve the production of a high yield of functional soluble recombinant protein of the gene *Rv1886c*.

## Materials and Methods
Strains and medium

Genetically engineered bacteria, *E. coli* BL21 (DE3) pLysS (Invitrogen), were transformed with the recombinant plasmid pMRLB.47 (Rv1886c-ss.pET32b; BEI Resource), containing the *Rv1886c* gene of *M. tuberculosis* strain H37Rv (Fig. 1). The lactose operon, including the T7 promoter and T7 terminator, is responsible for regulating the protein transcription. f1 origin marks the origin of the DNA replication. The plasmid contains an ampicillin resistant gene (Ap^r) which was used as the selective media. The expressed protein (32 kDa) was histidine-tagged for simple specific protein detection with the antibodies. The recombinant plasmid was transformed into *E. coli* by the heat shock method. The colony that produced the highest induced over un-induced protein ratio was selected as the seeds.

Genetically engineered bacteria *E. coli* were cultivated in a rich medium, Luria Bertani broth (LB Broth, Miller) medium (Sisco Research Laboratories). LB broth is a widely used rich medium for culturing *E. coli* as it permits fast growth rate and good productivity. The medium contains the essential components, including tryptone (10 g/L), yeast extract (10 g/L), and NaCl (10 g/L), for growing high density active bacteria. All media were prepared with ultrapure (Type 1) water and were autoclaved (121 °C, 20 min). Ampicillin stock solution (100 g/L) was added at a final concentration of 130 µg/mL.

Cultivation methods

The recombinant protein production protocol was adopted from Dobos laboratory's protocol, Colorado State University.
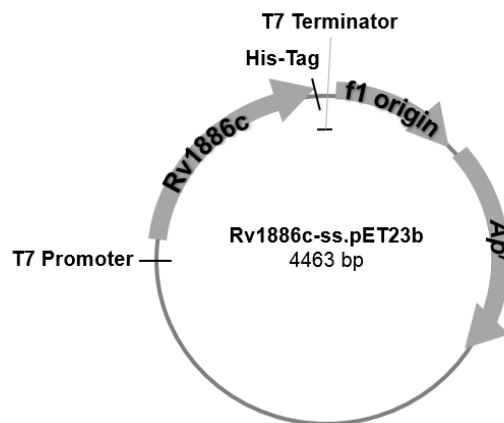
Fig. 1. Simplified recombinant plasmid map of pMRLB.47 containing the gene *Rv1886c* of *M. tuberculosis* H37Rv (BEI Resource).

Seed culture

The seeds in frozen glycerol stock (100 µL) were transferred into 10 mL of LB broth supplemented with ampicillin in a shaker, at 37 °C and 200 rpm. The microorganisms were allowed to adapt and grow overnight for 12 h.

Flask culture

The active seed culture (200 µL) was transferred into 10 mL inoculum containing LB broth supplemented with ampicillin and incubated in a shaker, at 37 °C and 200 rpm until OD_{600} = 1.0. The incubation time was approximately 45 min.

Protein expression

The inoculum containing a high cell density (10 mL) was inoculated into 100 mL LB broth, supplemented with ampicillin and incubated in a shaker, at 37 °C and 200 rpm. Two induction protocols, including low- temperature induction at 25 °C (for 12 h) and high-temperature induction at 37 °C (for 5 h), were performed. The two induction parameters were modified from Schedl laboratory's protocol, Washington University, St. Louis, U.S.A. IPTG (Merck) was added to a final concentration of 0.5 mM

for inducing protein expression after OD_{600} reached 0.5. The recommended conditions for adding the IPTG is at the mid-log phase of *E. coli* growth (typically $OD_{600} = 0.6$) [19, 20]. The exponential phase of *E. coli* growing in LB broth was reached when OD_{600} was between 0.6-1 [21]. Therefore, the inoculum was grown until $OD_{600} = 1$ to obtain a high cell density, and IPTG was added in the high cell density broth when $OD_{600} = 0.5$. The incubation time required to reach the desired OD_{600} was approximately 45 min.

Protein extraction

Protein extraction was performed to obtain both the soluble and insoluble protein fractions. Pre-induced and post-induced cultures were collected in a 1.5 mL microcentrifuge tubes by centrifugation (5,000 rpm, 5 min, 4 °C). The samples were adjusted to obtain the same number of cells by the OD_{600} measurement. Bacterial cells were dissolved in lysis buffer (pH 7.9) containing Tris-HCl (tris-hydroxymethyl aminomethane hydrogen chloride, 20 mM, Merck), NaCl (0.5 M, Merck), PMSF (phenylmethane sulfonyl fluoride, 1 mM, Merck), and lysozyme (0.75 mg/mL, Sigma Aldrich). The lysate was sonicated using an ultra-homogenizer (Sonics Vibra-cell) at 20% amplitude with 10 s pulse, followed by 30 s rest for 10-20 cycles. Both the supernatant (soluble protein fraction) and cell pellets (insoluble protein fraction) were collected by centrifugation at 12,000 rpm for 5 min, at 4 °C. The collected cell pellets were resuspended in the lysis buffer described above with the same volume as the collected supernatant. The collected supernatant and pellet samples were stored at -20 °C.

Analysis method

Western blot analysis was applied to measure the amount of proteins. The samples were heated (100 °C, 5 min) in the presence of SDS-PAGE sample buffer, centrifuged, and loaded onto 12% SDS-PAGE gel. The specific protein band was detected by using primary antibody (1:5000 mouse anti his-tag;

Merck). The signal was detected by enhanced chemiluminescence (ECL). The intensity of the specific protein band (32 kDa) was measured with ImageJ software (National Institutes of Health, NIH, USA). The fluorescence intensity of each protein fraction was measured and reported as a percentage of fluorescence intensity (arbitrary units).

Results and Discussion

The use of overexpression conditions for protein production often results in a low yield of soluble proteins [5]. Under these conditions, proteins are often accumulated in a cell as inactive inclusion bodies which require denaturing agents to dissolve the proteins. These denaturing agents damage living cells and limit the biological use of the solubilized protein [10]. The optimization of the induction temperature for protein expression is an approach for enhancing protein solubility and activity. Slow inducible expression systems under lower temperature conditions were found to increase the intracellular concentration of molecular chaperones [19]. Low and high-temperature induction protocols for overexpression of *M. tuberculosis* Ag85B protein were conducted side by side in this study, and the amounts of proteins were compared.

The recombinant plasmid pMRLB.47 (Rv1886c-ss.pET32b) contains the gene *Rv1886c* which is a precursor of 85B antigen. The gene was cloned without a signal sequence (-ss). The gene encodes the truncated form of Ag85B, in which an ER signal sequence is absent. The solubility of a truncated protein is higher than the native form in the cytosol. This prevents the expressed protein from aggregation as inclusion bodies [22]. The protein expression with the low-temperature induction conditions at 25 °C (for 12 h) and the high-temperature induction conditions at 37 °C (for 5 h), were quantitatively measured. Induction at low temperature in the range of

15-23 °C was found to express significant functional proteins [17], however, induction at 25 °C (room temperature) was evaluated in order to reduce the electrical load on the cooling system.

The levels of protein expression from two induction parameters were analyzed by Western blot analysis (Fig. 2). Results showed a significant increase in the soluble protein yield after induction by 3 fold (Fig. 3). The percentage of fluorescence intensity of other protein fractions, including pellet un-induced (PU), pellet induced (PI), supernatant un-induced (SU), and supernatant induced (SI) are shown in Fig. 4. The protein was expressed at the highest level in the supernatant induced fraction in both induction conditions, as expected. The results from this study indicate that the induction parameter at 37 °C (for 5 h) was better for expressing proteins in native soluble form. However, the induction at 25 °C (for 12 h) increased the overall protein production by 1.5 fold (Table 1). The induction at lower temperature produced 1.4 fold higher soluble native protein than the induction at high temperature (Table 1). Although a large portion of protein aggregated in the undesirable pellet induced fraction, the induction at lower temperature still expressed higher protein quantity.

Lower temperature cultivation is associated with a slow growth rate and slow protein synthesis rate [15]. Slow expression conditions allow the forming proteins to adopt a well-folded conformation [14]. This corresponds to the temperature dependent property of hydrophobic interactions. At a higher temperature (37 °C), toxic phenotypes are associated with the expression system. Proteases are more active at a higher temperature, leading to possible protein degradation [17]. Although high-temperature induction at 37 °C for 5 h enables a robust process in which the proteins translation is maximal, the expressed proteins are frequently in the undesirable insoluble aggregate form [19].
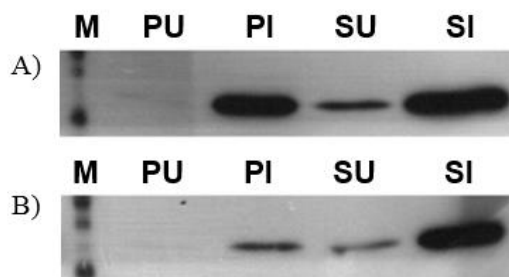
Fig. 2. Level of Ag85B protein expression (32 kDa) from A) low-temperature induction conditions and B) high-temperature induction conditions: M (protein marker, Bio-Rad), PU (pellet un-induced), PI (pellet induced), SU (supernatant un-induced), SI (supernatant induced).
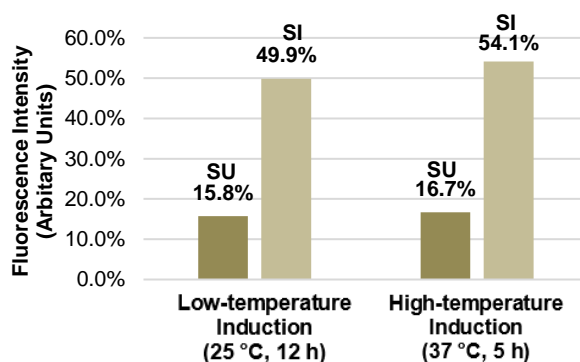
Fig. 3. The percentage of fluorescence intensity of the SU (supernatant un-induced, ■) and the SI (supernatant induced, ■) protein fractions from the low-temperature induction conditions at 25 °C for 12 h and high-temperature induction condition at 37 °C for 5 h.
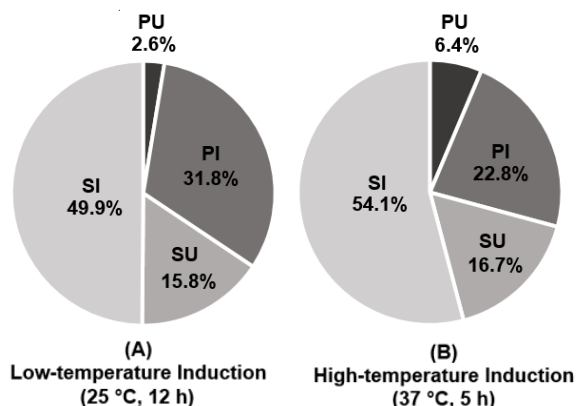
Fig. 4. The percentage of fluorescence intensity of each protein fraction from (A) the low-temperature induction conditions at 25 °C for 12 h, and (B) the high-temperature induction conditions at 37 °C for 5 h: PU (pellet un-induced, ■), PI (pellet induced, ■), SU (supernatant un-induced, ■), SI (supernatant induced, ■).

Table 1. The fluorescence intensity of each protein fraction from low and high-temperature induction conditions: PU (pellet un-induced), PI (pellet induced), SU (supernatant un-induced), SI (supernatant induced).

Protein Fraction	Induction Parameters	
	25 °C (12 h)	37 °C (5 h)
	Fluorescence Intensity	
Pellet Un-induced (PU)	4496	7334
Pellet Induced (PI)	55137	26222
Supernatant Un-induced (SU)	27364	19207
Supernatant Induced (SI)	86527	62207
Total	173524	114970

Conclusion

Two induction conditions, including low-temperature induction at 25 °C (for 12 h) and high-temperature induction at 37 °C (for 5 h), were employed for overexpressing native soluble *M. tuberculosis* Ag85B protein in *E. coli* in rich LB media. The soluble protein expression was more pronounced in the slow induction protocol performed at low temperature. Slow inducible expression at low temperature enhances protein correct folding and suppresses protease activity. The findings can be applied to achieve a highly functional soluble recombinant protein yield of the gene *Rv1886c*. Further study on the appropriate OD_{600} for induction and the IPTG concentration can be carried out to optimize the induction process. The expressed soluble Ag85B recombinant proteins will be used in the development of a conjugated vaccine against tuberculosis.

References

[1] World Health Organization [Internet]. Tuberculosis [cited 2016 Nov 1] Available from: http://www.who.int/mediacentre/factsheets/fs104/en/

[2] Kaufmann SHE. Is the development of a new tuberculosis vaccine possible? Nature Medicine 2000;6:955-60.

[3] Bentley-Hibbert SI, Quan X, Newman T, Huygen K, Godfrey HP. Pathophysiology of antigen 85 in patients with active tuberculosis: antigen 85 circulates as complexes with fibronectin and immunoglobulin G, Infect Immun 1999; 67(2):581-8.

[4] Floss DM, Mockey M, Zanello G, Brosson D, Diogon M, Frutos R, Bruel T, Rodrigues V, Garzon E, Chevaleyre C, Berri M, Salmon H, Conrad U, Dedieu L. Expression and immunogenicity of the mycobacterial Ag85B/ESAT-6 antigens produced in transgenic plants by elastin-like peptide fusion strategy, J. Biomed Biotech 2010;2010:1-1.

[5] Piubelli L, Campa M, Temporini C, Binda E, Mangione F, Amicosante M, Terreni M, Marinelli F, Pollegioni L.

Optimizing *Escherichia coli* as a protein expression platform to produce *Mycobacterium tuberculosis* immunogenic proteins. Microbial Cell Factories 2013;12:1-14.

[6] Geluk A, Lin MY, Meijgaarden KE van, Leyten EMS, Franken KLMC, Ottenhoff THM, Klein MR, T-cell recognition of the HspX protein of *Mycobacterium tuberculosis* correlates with latent *M. tuberculosis* Infection but not with *M. bovis* BCG vaccination. Infect Immun 2007;75(6):2914-21.

[7] Taylor JL, Wieczorek A, Keyser AR, Grover A, Flinkstrom R, Karls RK, Bielefeldt-Ohmann H, Dobos KM, Izzo AA. HspX-mediated protection against tuberculosis depends on its chaperoning of a mycobacterial molecule, Immun Cell Biol 2012;90:945–54.

[8] Orme IM, McMurray DN, Belisle JT. Tuberculosis vaccine development: recent progress. Trends in Microbiology 2011;9(3):115-8.

[9] Husain AA, Warke SR, Kalorey DR, Daginawala HF, Taori GM, Kashyap RS. Comparative evaluation of booster efficacies of BCG. Ag85B, and Ag85B peptides based vaccines to boost BCG induced immunity in BALB/c Mice: A pilot study clinical and experimental vaccine research 2015;4:83-7.

[10] Aghababa H, Mobarez AM, Behmanesh M, Khoramabadi N, Mobarhan M. Production and purification of mycolyl transferase B of *Mycobacterium tuberculosis*. Tanaffos 2011; Vol. 10, No.4, pp.23-30.

[11] Zarif R, Sankian M, Gholubi A, Farshadzadeh Z, Soleimanpour S, Youssefi F, Karamoddini MK, Ghazvini K, Varasteh AR. Cloning and expression of *Mycobacterium tuberculosis* major secreted protein antigen 85B (*Ag85B*) in *Escherichia coli,* Jundishapur J Microbiol 2013;6(2):112-6.

[12] Tao C, Zhao D, Dong H, Shan F, Lian K, Pan Z, Chen X, Yin Y, Jiao X. Prokaryotic expression and immunological characteristics of *Mycobacterium tuberculosis Rv1886c*. Acta Microbiologica Sinica 2014;54(3):330-7.

[13] Fan X, Gao Q, Fu R. Differential immunogenicity and protective efficacy of DNA vaccines expressing proteins of *Mycobacterium tuberculosis* in a mouse model. Microbiol Res 2009;164:374-82.

[14] Rosano GL, Ceccarelli EA. Recombinant protein expression in *Escherichia coli*: advances and challenges. Frontiers in Microbiol 2014;5(5):1-17.

[15] Rai M, Padh H. Expression systems for production of heterologous proteins. Current Science 2001;80(9):1121-8.

[16] The University of Sydney, Dale's Lecture 5 [Internet]. The Differences Between Eukaryotes and Prokaryotes). Available from: http://sydney.edu.au/science/molecular_bioscience/PHAR2811/PHARlectures/PHARlecture5/PHARlecture5notes.pdf

[17] Sunil S, Khattar SK, Saini KS. Production of active eukaryotic proteins through bacterial expression systems: A review of the existing biotechnology strategies. Mol Cell Biochem 2008;307:249-64.

[18] Yildir (Tamerler) C, Onsan ZI, Kirdar B. Optimization of starting time and period of induction and inducer concentration in the production of the restriction enzyme EcoRI from recombinant *Escherichia coli* 294. Turk J Chem 1998;22:221-6.

[19] San-Miguel T, Pérez-Bermúdez P, Gavidia I. Production of soluble

eukaryotic recombinant proteins in *E. coli* is favoured in early log-phase cultures induced at low temperature. Springerplus 2013;2(89):1-4.

[20] Kelley KD, Olive LQ, Hadziselimovic A, Sanders CR. Look and see if it is time to induce protein expression in *Eschericia coli* cultures. Biochem 2010;49(26):5405-7.

[21] Sezonov G, Joseleau-Petit D, D'Ari R. *Escherichia coli* physiology in Luria-Bertani broth, J Bacteriol 2007;189(23):8746-9.

[22] Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. Molecular biology of the cell. 4th ed. New York: Garland Science; 2002.