Proteomics and Transcriptomics Studies in Nonreplicating Persistence of *Mycobacterium tuberculosis* Beijing and Non-Beijing Strains

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ABSTRACT

Latent tuberculosis infection (LTBI) is an infectious disease which the pathogen can behave as non-replicating persistent (NRP) or dormancy cells, and it caused by *Mycobacterium tuberculosis* (*Mtb*) strains. Beijing and non-Beijing strains have been reported and suggested to facilitate rise of the re-infection rate in regions with a high incidence of TB disease. To better understanding of molecular mechanisms and explore biomarker of the pathogen via NRP stage, mimicking condition is thought to reflect of *Mtb* inside the host granulomas (intracellular environment). The experiments have been designed by hypoxia, low pH, iron deprivation and nutrient starvation. Proteins prepared from multiple stress cultures, were then performed in-gel digestion with Trypsin and characterized by Liquid chromatography tandem mass spectrometry and then validated by quantitative real-time PCR. The results showed that strains Beijing found to be up-regulation of cyp123, Rv1496, ppgK and folP1 and non-Beijing found to be up-regulation of mce1R and Rv1501. The unique feature of proteins presents in this study that allowed the investigation and identification of *Mtb* strains during multiple stresses. Which further benefit for identification of potential biomarkers lead to discovery new drug targets and vaccine development.

Keywords: *Mycobacterium tuberculosis*, Multiple stresses, Beijing

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Introduction

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (*Mtb*). Beijing (BJ) is a virulence strain, and globally outbreak. The infection of BJ-*Mtb* strain is associated with the emergence of multidrug-resistant (MDR), which is resisted to the first-line anti-TB drug. MDR-TB becomes more pathogenicity and virulent, a major problem of TB treatment [1]. WHO reported that one-third of the world population, are infected with *Mtb*. In addition, 2-3 billion of people who are infected with *Mtb* have become latent TB infection (LTBI). LTBI is characterize by a non-replicating persistent (NRP) and relatively associated with the resistant to anti-TB drug. Worldwide, approximately 5-10% of LTBI are estimated to be an active TB disease [2-3]. The NRP stage of *Mtb* is associated with the reduction of metabolic processes, activation of stress responses and the emergence of drug resistant. However, the metabolic processes during the NRP stage of *Mtb*, and the association of metabolic processes with the development of MDR-TB under survival the NRP stage, and reactivation of NRP are largely unclear.

A major global health concern of *Mtb* is due to the spread and association with the numerous outbreaks of *Mtb* BJ strain [4]. Previous studies demonstrated that the dissemination of the BJ strain might have a higher pathogenicity and virulent, as defined by the capacity to cause disease and severe disease compared to other strains of *Mtb* [5]. Therefore, the predominant serotype that has been emerging in various a of the world and the association with disease outbreaks including antibiotic resistance [6].

Previously, the *Mtb* strain that was used to investigate the metabolic and transcriptomic profiles under stress condition was non-BJ (*Mtb* H37Rv) [2, 7]. Therefore, we aimed to identify and characterize a possible biomarker of NRP stage of *Mtb*. In this study, we performed *in vitro* multiple stress conditions to mimic the condition of *Mtb* infection in granulomas of macrophage that *Mtb* usually become NRP stage using both BJ and non-BJ strains.

Methodology

Bacterial strains, media and growth condition

M. tuberculosis H37Rv laboratory strain (non-BJ) and clinical strains (BJ). The *Mtb* strains cultured in Middlebrook 7H9 (M7H9) medium (Sigma) and supplemented with 0.2% of glycerol and 10% of BBL™ Middlebrook OADC Enrichment (BD, US). Bacterial cells were grown at 37 °C for 7 days with shaking condition. The number of culturable cells units was determined by plate count technique and log phase *Mtb* were selected for further experiments.

The multiple stresses media were prepared as follows: M7H9 medium and either supplemented with iron chelators, methylene blue, 36% HCl and diluted M7H9 medium to 10% to mimic the nutrient starvation and media ware sterilized by filtration. Log phase of *Mtb* were then inoculated and cultured for 4 weeks at 37 °C without shaking. Methylene blue decolonization was used as an indicator for oxygen depletion to represent of dormancy cells. This project

was approved by the Khon Kaen University Ethics Committee (No. HE621448) to declare that the isolated microorganisms and laboratory cultured used in this study did not linked to the patient information.

Cell lysis and protein extraction

The *Mtb* cells were collected after 4 weeks of inoculation by centrifugation. Protein was extracted by lysis buffer (0.5 M Na_2HPO_4, 5 M NaCl, 1 M imidazole, 100 mg/ml lysozyme, 1X protease inhibitor cocktail (Amresco, USA), 1 M dithiothreitol) with 0.1 mm zirconia/silica beads (BioSpec Products, Inc.). Protein concentrations were determined by Bradford protein assay, Bradford reagent (Bio-Rad Laboratories, Inc.).

Preparation of protein for LC-MS/MS

Five micrograms proteins of each sample were separated by in-gel digestion using12.5% acrylamide gel, and centrifuged at high speed for 1 min and kept at room temperature for 20 min. Then gels were cut into small pieces and 200 µl of 100% acetonitrile (ACN) [8] was added and shake for 5 min. the supernatant was discarded and resuspended with 200 µl of 10 mM NH_4HCO_3 and shake for 5 min. After that, 200 µl of 10 mM DTT/10 mM NH_4HCO_3 was added and incubated for 1 h at 56 °C and supernatant was then discarded. The addition of 200 µl of 100 mM IAA/10 mM NH_4HCO_3 was performed and incubated at room temperature in the dark condition for 1 hr. The gels were grinded and 100 µl of 100% ACN can was added, then, samples were then centrifuged at 10,000 rpm for 5 min and allowed to dry at 56 °C for 5 min. Proteins were then digested with 10 ng of trypsin and incubated for overnight at 37 °C. After incubation, the supernatant was collected, evaporated, and re-suspended with 50 µl of 0.1% FA/LC. The samples were stored at −20 °C until the liquid chromatography with tandem mass spectrometry (LC−MS/MS) analysis.

LC−MS/MS, Bioinformatics and data analyses

LC-MS/MS analysis, bioinformatics and data analyses were performed as previously described [9]. The analyzed data from DeCyderMS were submitted to the database for searching against the reference *Mtb* H37Rv strain. Which was used for identification of the proteins and it offers a database for exploring details of individual proteins. All the results including data analysis of genes/proteins are given from UniPort (https://www.uniprot.org/), PANTHER14.1 (http://www.pantherdb.org/), NCBI (https://www.ncbi.nlm.nih.gov/), Mycobrowser (https://mycobrowser.epfl.ch/), TB Database (http://genome.tbdb.org/tbdb_sysbio/MultiHome.html), jvenn (http://jvenn.toulouse.inra.fr/app/example.html) and STRING version 11.0 (https://string-db.org/).

Quantitative real time PCR (qRT-PCR)

The *Mtb* cells were harvested after culturing for 4 weeks by centrifugation. RNA was isolated by using Trizol RNA isolation buffer (Invitrogen, USA) with silica beads, according to the manufacturer's instructions. To remove genomic DNA, RNA was treated with Deoxyribonuclease I (DNase I, Invitrogen, USA), according to the manufacturer's instructions. The cDNA was then synthesized by using SuperScript III Reverse Transcriptase (Invitrogen, USA), according to the manufacturer's instructions. All protocols were performed according to our previous study [10].

Primer synthesis was obtained and ordered from Ward Medic, Thailand. The primer sequences and target genes used in this study, and 16S ribosomal RNA (16S rRNA) gene forward and reverse primer sequences as previously described by Haile et al. (2002) [11] are listed in Table 1.

The expression of *Mtb* genes was examined by qRT-PCR. The qRT-PCR reactions were performed on real-time PCR instrument (Applied Biosystems QuantStudio 6 Flex Real-Time PCR System) using SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratory, Inc., USA). The relative expression of *Mtb* genes was examined by $2^{-\Delta\Delta Ct}$. The 16S rRNA was used as an internal control.

Statistical analysis

GraphPad prism was used for all data analysis. All of experiments were done by duplicates and values are expressed as mean±SD. Student's t-test was used to analyze the different among two groups of study. The statistical significance of the differences between the groups are indicated at $*p<0.05$; $**p<0.01$; $***p<0.001$.

Table 1 Primer pairs used to amplify cDNA.

Pair no.	Gene	Direction/position of primer	Sequence
1	16S rRNA	Forward, 469-491	5'-TTGACGGTAGGTGGAGAAGAAGC-3'
		Reverse, 909-888	5'-CCTTTGAGTTTTAGCCTTGCGG-3'
2	mce1R	Forward, 326-345	5'-CCATTGCCCAAGAACTTGCC-3'
		Reverse, 456-437	5'-GATCGACGCGATGGTTTTGG-3'
3	Rv1501	Forward, 583-602	5'-CGGGCTCGTTGTTCGTTTTT-3'
		Reverse, 684-665	5'-ACGAGCGCGTAAACTGATGA-3'
4	ppgK	Forward, 375-394	5'-CAGCAGGTCACCATCCTCAA-3'
		Reverse, 528-509	5'-TGTTGGGTATCAACGTCCCG-3'
5	cyp123	Forward, 477-496	5'-TTCAGAGCTGATAGGCGTGC-3'
		Reverse, 671-652	5'-AGTGCCGACGTCAGATTGTT-3'
7	folP1	Forward, 109-128	5'-GATCTCGACGATGCGGTGAA-3'
		Reverse, 292-273	5'-TGGTATCGATGCTGACGGTG-3'
9	Rv1496	Forward, 550-569	5'-CGTTCGTGTTGCTGACCTTG-3'
		Reverse, 732-713	5'-ACAGTGCTTCGCGAGGATAG-3'

Results

Unique proteins during NRP stage

To investigate the protein expression profiles of *Mtb* under the multiple-stress conditions, *Mtb* strains were grown under the multiple-stress conditions and LC-MS/MS was performed. The differentially expressed proteins that were expressed only in NRP stage, but not active stage of BJ and non-BJ strains were selected for further analyzed. The result showed that four differentially expressed proteins (cyp123, Rv1496, ppgK and folP1) that were upregulated in the NRP stage of BJ strain compared with active stage (Table 2). In addition, two proteins (mce1R and Rv1501) that were upregulated in the NRP stage of non-BJ strain when they compared to active stage (Table 2). Therefore, this result suggests that the up-regulation of these proteins in both of BJ and non-BJ strains may play important roles in the adaptation of *Mtb* under the multiple-stress condition to become NRP stage.

Table 2 The relative intensity of Beijing and non-Beijing strains during multiple stresses induced NRP stage

GI no.	ORF no.	Gene symbol	Gene description	Average Log2 fold	P-value
Beijing strain					
gi|15607906	Rv0766c	cyp123	Probable cytochrome P450 123 Cyp123	9.810	0.092
gi|15608634	Rv1496	-	Possible transport system kinase	10.327	0.092
gi|490009148	Rv2702	ppgK	Polyphosphate glucokinase PpgK (polyphosphate-glucose phosphotransferase)	5.487	0.211
gi|57117134	Rv3608c	folP1	Dihydropteroate synthase 1 FolP (DHPS 1) (dihydropteroate pyrophosphorylase 1) (dihydropteroate diphosphorylase 1)	5.348	0.211
Non-Beijing strain					
gi|489997814	Rv0165c	mce1R	Probable transcriptional regulatory protein Mce1R (probably GntR-family)	9.356	0.091
gi|15608639	Rv1501	-	Conserved hypothetical protein	5.175	0.211

Validation of proteomic analysis by quantitative real-time PCR

The LC-MS/MS results showed an increasing of *Mtb* proteins under the multiple-stress condition that may play roles in the adaptation of *Mtb*. Therefore, we further validated the expression of these proteins at the mRNA level by qRT-PCR. Present study, the relative expression levels of cyp123, folP1, ppgK and Rv1496 were significantly up-regulated greater than 2-, 11-, 22- and 46-fold, respectively, in NRP stage of BJ strain compared with active stage ($p<0.05$) (Fig.

1A). In contrast to LC MS/MS result, the expression of mce1R was significantly downregulated in NRP stage of non-BJ strain compared with active stage ($p<0.001$) (Fig. 1B). However, the expression of Rv1501 was not significantly different in NRP stage of non-BJ strain compared with active stage (Fig. 1B). Therefore, the differentially expressed of these genes in NRP of *Mtb* strains may play important roles in the adaptation to multiple-stress conditions. The upregulation of genes under NRP stage could be useful as a target for the detection of dormant *Mtb*.

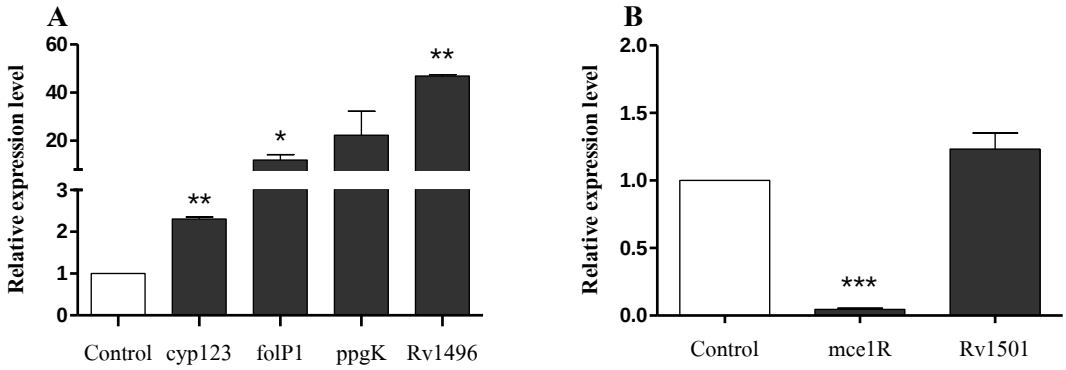

Figure 1 The relative expression levels of *Mtb* strains: Beijing (A) and non-Beijing (B). The bacterial cells were cultured for 4 weeks under the multiple stresses and the expression levels were determined by qRT-PCR. The active *Mtb* growth was used as control. The data was presented as a mean of expression fold changed which normalized by the expression level of 16S rRNA. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$ (T-test).

Discussion and conclusions

Tuberculosis (TB) is an infectious disease leading to the health problem and the causes of human deaths worldwide. Exposure to stress conditions of *M. tuberculosis* (*Mtb*), the pathogens are specifically adapted to host cells which can develop and enter a dormant stage also known as latent TB infection (LTBI). The emergence of LTBI is one of concerning problem of TB because this stage of *Mtb* can maintain a persistent infection, still alive within human body for long times (chronic infection) and can be reactivated in immunocompromised host. Therefore, in this study we aimed to investigate and identify the differentially expressed proteins of NRP stage induced by the multiple-stress conditions. Previously, the *Mtb* dormancy model has been proposed and the alteration protein and gene expression pattern could be used for the identification of a new therapeutic targets for of the dormancy stage of *Mtb*.

Beijing is one family of *Mtb* strains that has attracted special attention, hypervirulence family and this family are associated with multidrug resistance [12]. Therefore, Beijing genotype strains have been characterized by IS6110 restriction fragment length polymorphism (RFLP) and spoligotype patterns. Moreover, the genetic markers can

differentiate between Beijing and non-Beijing defined by DNA large sequence polymorphisms (LSP) to identified as deletion segments of DNA when compared with the reference strain of H37Rv also known as region of difference (RD) (RD105, RD149, RD207, RD181, RD150 and RD142) [5-6, 13-14].

In the present study, four proteins (cyp123, folP1, ppgK and Rv1496) were differentially expressed in BJ strain under the NRP stage. The cyp123 protein, probable cytochrome P450 123, is a group of heme-thiolate monooxygenases, which can oxidize a variety of structurally unrelated compounds, including steroids, fatty acids, and xenobiotics. The functional category of this protein is involved in an intermediary metabolism and respiration. Proteomics and transcriptomics analysis revealed that this protein was found in the cell membrane fraction. The expression of this protein was induced by a high temperature [15-16]. Gene array analysis of *Mtb* cells that were exposed to stress conditions has been suggested that the expression of P450 enzymes play important roles in the pathogen's response to environmental stimuli, immune and chemical [17].

The Rv1496, possible transport system kinase involved in transport (possibly arginine). The functional category of this protein is involved in cell wall and cell processes. In addition, ppgK, polyphosphate glucokinase PpgK (polyphosphate-glucose phosphotransferase), is an enzyme that catalyzes the phosphorylation of glucose using polyphosphate or ATP as the phosphoryl donor. The GTP, UTP and CTP can replace ATP as phosphoryl donor [18-19]. The functional category of this protein is involved in intermediary metabolism and respiration. The last protein, folP1, dihydropteroate synthase 1 FolP (DHPS 1), is involved in the sub-pathway (tetrahydrofolate biosynthesis) that synthesizes 7,8-dihydrofolate from 2-amino-4-hydroxy-6-hydroxymethyl-7, 8-dihydropteridine diphosphate and 4-aminobenzoate and also dihydropteroate synthase. This protein can also catalyze the formation of the immediate precursor of folic acid and it is implicated in the resistance to sulfonamide [20]. The functional category of this protein is involved in intermediary metabolism and respiration.

In addition to BJ strain, in non-BJ strain, two proteins (Rv1501 and mce1R) were differentially expressed in NRP stage. In contrast to protein level, mce1R was significantly downregulated in NRP stage of non-BJ strain at mRNA level. The mce1R, probable transcriptional regulatory protein Mce1R (probably GntR-family), is involved in the transcriptional mechanism and also involved in regulatory proteins. Mce1R protein facilitates the balanced of temporal expression of the mce1 products that is required for the formation granulomas. In addition, this protein is necessary for the *Mtb* dormancy stage [21]. The Rv1501, conserved hypothetical protein, plays an important role in mycobacterial virulence. The functional category of this protein is involved in the conserved hypotheticals [22].

In conclusion, the BJ lineage is globally outbreak and is associated with the emergence of MDR, leading to the high pathogenicity and virulence. Our proteomics analysis of BJ and non-BJ that were exposed to the multiple-stress condition demonstrate the unique protein expression profiles that differ from an active *Mtb* stage. The unique proteins of NRP stage could be used as a biomarker for the distinguishing LTBI from active TB infection or could be used a

therapeutic target for LTBI. In addition, these proteins may play a crucial role for the adaptation and survival of *Mtb* under the stress conditions in host.

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