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ORIGINAL PAPER

Proteomic analysis of drug-resistant *Mycobacterium tuberculosis* by one-dimensional gel electrophoresis and charge chromatography

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Abstract Multidrug-resistant tuberculosis (MDR-TB) is a form of TB caused by *Mycobacterium tuberculosis* (*M. tuberculosis*) that do not respond to, at least, isoniazid and rifampicin, the two most powerful, first-line (or standard) anti-TB drugs. Novel intervention strategies for eliminating this disease were based on finding proteins that can be used for designing new drugs or new and reliable kits for diagnosis. The aim of this study was to compare the protein profiles of MDR-TB with sensitive isolates. Proteomic analysis of *M. tuberculosis* MDR-TB and sensitive isolates was obtained with ion exchange chromatography coupled with MALDI-TOF–TOF (matrix-assisted laser desorption/ionization) in order to identify individual proteins that have different expression in MDR-TB to be used as a drug target or diagnostic marker for designing valuable TB vaccines or TB rapid tests. We identified eight proteins in MDR-TB isolates, and analyses showed that these proteins are absent in *M. tuberculosis*-sensitive isolates: (Rv2140c, Rv0009, Rv1932, Rv0251c, Rv2558, Rv1284, Rv3699 and MMP major membrane proteins). These data will provide valuable clues in further investigation for suitable TB rapid tests or drug targets against drug-resistant and sensitive *M. tuberculosis* isolates.

Keywords MDR-TB · Sensitive TB · TB proteomics · Ion exchange chromatography (IEC) · Mass spectrometry

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Introduction

Tuberculosis (TB) remains a major global health problem. In 2012, an estimated 8.6 million people developed TB, and 1.3 million died from the disease (World Health Organization 2013). To reach the TB eradication to less than 1 case/1,000,000 population in 2050, the incidence needs to decline by 20 % per year (World Health Organization 2012). A major recent problem is the emergence of drug-resistant strains, mainly because people do not complete their treatment plans or have been incorrectly treated and so have remained infectious. Standard anti-TB drugs have been used for decades, and resistance to the medicines is widespread. Pathogenic strains that are resistant to a single anti-TB drug have been documented in every country surveyed. Multidrug-resistant tuberculosis (MDR-TB) is a form of TB caused by bacteria that do not respond to, at least, isoniazid and rifampicin, the two most powerful, first-line (or standard) anti-TB drugs. Inappropriate or incorrect use of antimicrobial drugs, or use of ineffective formulations of drugs (e.g. use of single drugs, or poor quality medicines), and premature treatment interruption can cause drug resistance, which can then be transmitted, especially in crowded settings such as prisons and hospitals. Disease

caused by resistant bacteria fails to respond to conventional, first-line treatments. MDR-TB is treatable and curable by using second-line drugs.

However, second-line treatment options are limited, and recommended medicines are not always available. The extensive chemotherapy required (up to 2 years of treatment) is more costly and can produce severe adverse drug reactions in patients. In some cases, more severe drug resistance can develop. Extensively drug-resistant TB (XDR-TB) is a form of multidrug-resistant tuberculosis that responds to even fewer available medicines, including the most effective second-line anti-TB drugs. In 2013, about 480,000 people developed MDR-TB in the world. It is estimated that about 9.0 % of MDR-TB cases had XDR-TB. At least one-third of people living with HIV worldwide in 2013 were infected with TB bacteria though they did not become ill with active TB. People living with HIV are 26–31 times more likely to develop active TB than healthy people. HIV and TB form a lethal combination, each speeding the other's progress. In 2013, about 360,000 people died of HIV-associated TB. Approximately 25 % of deaths among HIV-positive people are due to TB. There were an estimated 1.1 million new cases of TB among the HIV-positive people in 2013. The complexity and prohibitive cost of MDR-TB treatment mean that few of the world's MDR-TB patients receive proper treatment. Without a significantly simpler, faster, cheaper and oral treatment for MDR-TB, countries cannot scale up treatment to serve their populations (Luetkemeyer et al. 2011; Gandhi et al. 2010).

The interface between the host cells and *M. tuberculosis* includes surface proteins or membrane-associated proteins that are required for the pathogenicity of bacteria and involved in intracellular multiplication, lipid metabolism and transport across the cell envelope and bacterial response to host microbicidal processes. These proteins are exposed to the external environment (Malen et al. 2010; Camacho et al. 1999). As a result, they are ideal for drug targets, diagnostic probes and components of vaccines (Daffé and Etienne 1999; Hopkins and Groom 2002).

The present study analyzes and compares the protein expression profile of *M. tuberculosis* MDR and sensitive isolates to identify proteins, which could be used as a drug target or diagnostic marker for future designing of valuable TB vaccine or TB rapid test.

Ion exchange chromatography (IEC) coupled with MALDI-TOF–TOF (matrix-assisted laser desorption/ionization) was very efficient in the analysis of complex protein/peptide mixtures. In contrast to conventional protein sequencing procedures, mass spectrometry allows the rapid and automated analysis of many proteins in a short time frame (Rabilloud 2003; Shevchenko et al (1996); Shevchenko et al. 2006).

IEC is the most commonly practiced chromatographic method of protein purification and separates proteins, at first, on the basis of their charge type (cationic or anionic) and, secondly, on the basis of relative charge strength. Separation in this method depends on the reversible adsorption of charged solute molecules to the immobilized ion exchange groups of opposite charge. Most *M. tuberculosis* proteins have isoelectric point (pI) in the range of 3–7. Accordingly, we used anion exchanger, and the functional group was DEAE (Sakash and Kantrowitz 2000; Himmelhoch 1971).

In this study, due to complexity of the protein mixture of *M. tuberculosis*, the proteins were eluted by increasing the ionic strength of the buffer, which compete with the salt ions existing in the buffer. Each component has a distinct net charge and requires a specific ionic strength to elute it from the column. When a salt gradient of increasing concentration is used, proteins are eluted at specific salt concentrations, effectively fractionating the mixture.

Materials and methods

Source of culture and extraction of *M. tuberculosis* proteins

M. tuberculosis (MDR and sensitive clinical isolates) were obtained from Iranian Mycobacterial Collection, Pasteur Institute-Tehran, Iran. In this study, we used five MDR-TB isolates and five sensitive isolates. They were cultured in 7H9 medium for 4 weeks at 37 °C. Bacteria were extracted from the culture medium by centrifugation at 5000 rpm for 45 min and washed with PBS, pH 7.4. Protein extraction was carried out by sequential extraction method (Moloy et al. 1998; Moloy 2000). The collected bacterial cells were suspended in reagent 1 (Tris 40 mM) consisting of DNase (1 µg/ml) and protease inhibitor, PMSF (1 Mm), sonicated for 1 h at 50 HZ (Bandelin, GM-220, Germany); the unbroken cells and cell wall debris were removed by centrifugation at 5000 rpm for 45 min to yield a firm pellet. The supernatant was collected and labeled as extract 1. Then, its protein concentration was determined. Reagent 2 was added to the pellet (8 M urea, 4 % CHAPS, 2 mM TBP, 40 mM Tris). Then, the mixture was vortexed and sonicated. The mixture was centrifuged to yield a firm pellet and clear supernatant. The supernatant was labeled as extract 2, and its protein concentration was determined. Reagent 3 was added to the pellet (5 M urea, 2 M thiourea, 2 % CHAPS, 2 mM TBP, 40 mM Tris) and mixed for 5 min. Sonication and centrifugation yielded a firm pellet and clear supernatant, and we named it extract 3. Then, its protein concentration was determined by using Bradford method (Bradford 1976; Dunn 1999). Extracts 1, 2 and 3

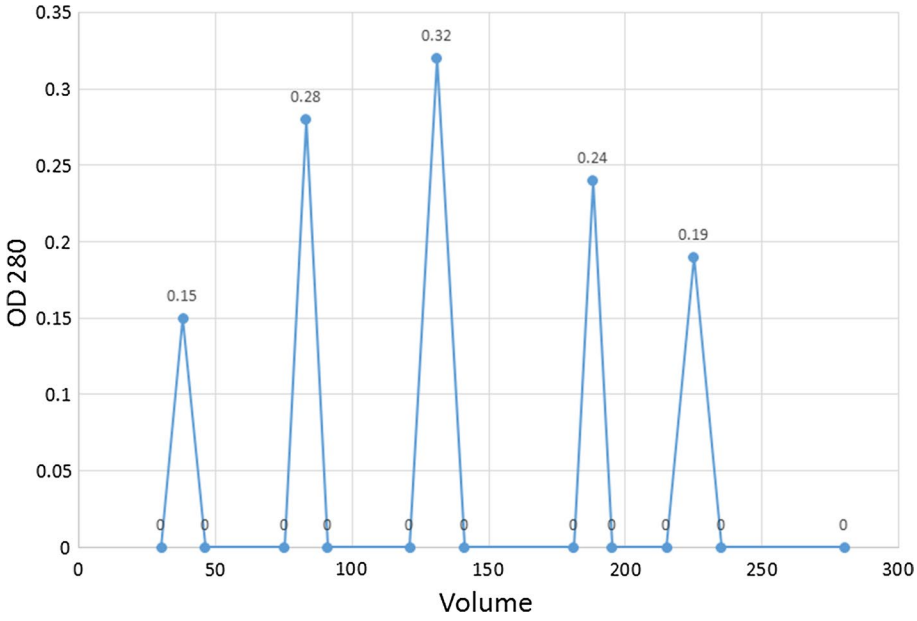

Fig. 1 Ion exchange chromatography of MDR-TB proteins on a column of DEAE-cellulose. Optical density (OD) at 280 nm, indicating the protein content in eluted fractions

were mixed and dialyzed against ammonium bicarbonate 0.1 M, pH 8.0. After dialysis, the proteins were lyophilized.

Ion exchange chromatography (IEC)

DEAE-cellulose resin (Sigma) was suspended in 300 ml 0.1 M NaOH for 30 min at pH 13.0. The sodium hydroxide solution was discarded, and resin was poured in slurry into a Buchner funnel while applying gentle suction. We poured the resin in slurry until all of it was added to the funnel and washed with distilled water until pH reached 8.0. Then, the resin was suspended in 0.1 M HCl with gentle shaking for 30 min (pH reached to 1.0). In this step, the resin was resuspended in distilled water. Washing was continued with distilled water until the effluent pH was 5 or greater, which was replaced with 10× buffer (500 mM Tris, pH 8.5) for 30 min. The buffer was discarded, and resin was equilibrated with 1 × buffer (50 mM Tris, pH 8.5). The resin was packed into the column and equilibrated with the buffer. The samples (1 mg of protein from each of the MDR and sensitive isolates) were adjusted to the Tris 50 mM, pH 8.5 and then were loaded to the column (Sakash and Kantrowitz 2000). We chose step-by-step ionic strength elution, and in every step, ionic strength of the elution buffer was increased by adding 0.05, 0.07, 0.1, 0.15, 0.2, 0.33, 0.5, 0.67, 0.8 and 1 M NaCl. All fractions were assayed for the protein concentration in 280 nm (Zavaran et al. 1997).

Mass spectrometry and data analysis

Protein bands of polyacrylamide gel electrophoresis were excised in 1 × 1 mm pieces and remitted to the University of York (Department of Biology). Purified peptides were applied to an AnchorChip (Bruker) and analyzed by MALDI-TOF (Bruker Daltronic Mass Analyzer Reflex III). Aliquot of each peptide mixture was applied to a ground steel MALDI target plate. Fragmentation was performed in LIFT mode without the introduction of a collision gas. The default calibration was used for MS/MS spectra, which were baseline-subtracted and smoothed (Savitsky-Golay, width 0.15 m/z, cycles 4); monoisotopic peak detection used a SNAP averaging algorithm (C 4.9384, N 1.3577, O 1.4773, S 0.0417, H 7.7583) with a minimum S/N of 6. Bruker Flex Analysis software (version 3.3) was used to perform spectral processing and peak list generation. Tandem mass spectral data were submitted to database searching using a locally running copy of the MASCOT program (Matrix Science Ltd., version 2.4) through the Bruker ProteinScape interface (version 2.1). The results were filtered to accept only peptides with an expect score of 0.05 or lower, allowing to obtain higher confidence identifications by including tandem spectral data in MASCOT searches, and the data were compared with the MASCOT database for sequence matches (Shevchenko et al. 2006).

Results and discussion

Proteins were eluted with 250-ml buffer at chosen ionic strength and pH 8.0. IEC results revealed five peaks for each of the MDR-TB and sensitive isolates (Fig. 1). The first peak was eluted with Tris 50 mM, and the other peaks were eluted by increasing the concentration of NaCl (0.2, 0.4, 0.6 and 0.8 M). Protein concentration was measured by using Bradford method. Fifty micrograms from each peak was loaded into the gradient (5–15 %) polyacrylamide

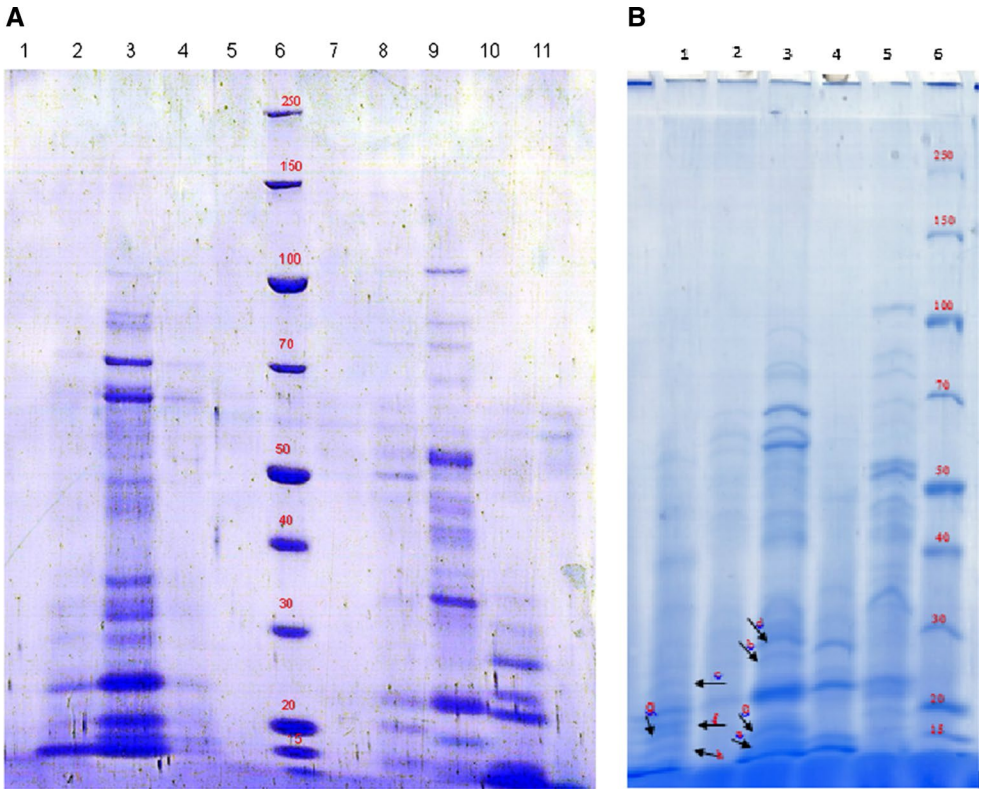

Fig. 2 **a** Protein fraction peaks, from sensitive and MDR-TB isolates in gradient SDS-PAGE (5–15 %). *Lanes* 1, 2, 3, 4 and 5 belong to peaks one, two, three, four and five from MDR isolates, respectively. *Lane* 6 has shown molecular mass marker (250.0, 150.0, 100.0, 70.0, 50.0, 40.0, 30.0, 20.0 and 15.0 KD). *Lanes* 7, 8, 9, 10 and 11 belong to peaks one, two, three, four and five from sensitive isolates. **b** Protein fraction peaks, from sensitive and MDR-TB isolates in gradient SDS-PAGE (5–15 %). *Lane* 6 indicates molecular mass marker; *lanes* 1 and 3 belong to two and three peaks from MDR isolates. And lanes 4 and 5 belong to two and three peaks from sensitive isolates. Four bands are indicated in peak three (a, b, c and d), and four bands are indicated in peak two (e, f, g and h)

gel. After electrophoresis, the proteins could be visualized with Coomassie Brilliant Blue R-250 stain for detection in polyacrylamide gels (Fig. 2a). Analysis and comparison of the protein bands in five peaks of *M. tuberculosis* (MDR and sensitive isolates) led us to identify eight proteins in the MDR-TB isolates, which were not observed in the sensitive isolates. Most of the proteins and most of the differences belonged to the second and third peaks. There were four bands in the peak 3 (a, b, c and d) and four bands in the peak 2 (e, f, g and h) ranging from 15 to 30 and 15 to 20 kDa, respectively (Fig. 2b). Eight protein bands (a, b, c, d, e, f, g and h) were excised from the gel in 1 × 1 mm pieces and analyzed by mass spectrometry; the results are given in Table 1.

For eight identified proteins, GRAVY value was calculated with GRAVY calculator (http://www.GRAVY-CALCULATOR.de). Seven proteins (MMP, Rv2140c, Rv0009, Rv0251c, Rv2558, Rv1284 and Rv3699) had negative GRAVY values, and one protein (Rv1932) had positive GRAVY value. Proteins with negative GRAVY values are hydrophilic. TMHMM (new membrane topology prediction method) correctly predicts 97–98 % of transmembrane helices. Additionally, it can discriminate between soluble and membrane proteins. TMHMM is available at http://www.cbs.dtv.dk/services/TMHMM. This method was used to identify transmembrane proteins in our data profile. It further predicts protein topology based on the sequences in FASTA format (Malen et al. 2011; Xiong et al. 2005). In this study, we identified proteins with negative GRAVY values and lack of predicted transmembrane helices; however, it seems they are membrane or cell wall proteins. Lipoproteins do not have TMHS and exhibit negative GRAVY values but still are associated with the plasma membrane (Rv0251c, MMP) (Zhiguo and Jeroen 2010).

In the present study, IEC was used and coupled with proteomic analysis in demonstration of proteins that are differentially expressed in MDR-TB isolates compared with sensitive isolates. Surface-exposed proteins are potential targets of drugs. Here, identifying the surface-exposed proteins of MDR-TB is a good strategy for designing new drugs or new and reliable kits for diagnosis of TB (Eichacker et al. 2004).

It is concluded that MDR-TB isolates have different characteristic banding patterns in polyacrylamide gel

Table 1 Details of identified proteins of *M. tuberculosis* MDR isolates

Protein description RefSeq ID/Sanger ID	Pfam	GRAVY score	Mass (M_r)	Band	pI	Score	aa	RV GENE	Functional category
gil248681 MMP = major membrane protein	–	−0.5370	16,086	a	5.0	44	143	–	Virulence, detoxification and adaptation
gil15610835 conserved protein	TPMT	−0.0957	25,040	b	4.69	367	233	Rv3699 Rv3699	Conserved hypotheticals
gil15608424 beta-carbonic anhydrase	Pro_CA	−0.1619	18,348	c	5.48	87	163	Rv1284 canA	Intermediary metabolism and respiration
gil15609695 conserved protein	–	−0.0292	26,044	d	6.18	133	236	Rv2558 Rv2558	Conserved hypotheticals
gil15607392 heat-shock protein Hsp (heat-stress-induced ribosome-binding protein A)	HSP20	−0.4729	17,775	e	5.21	98	159	Rv0251c Hsp	Virulence, detoxification and adaptation
gil15609277 conserved protein TB18.6	PBP	−0.1039	18,622	f	5.41	132	176	Rv2140c TB18.6	Conserved hypotheticals
gil15607151 iron-regulated peptidyl-prolyl *cis*–trans isomerase A PpiA (PPIase A) (rotamase A)	Pro isomerase	−0.3027	19,285	g	5.8	366	182	Rv0009 ppiA	Information pathways
gil15609069 probable thiol peroxidase Tpx	Redoxin	0.2860	17,057	h	4.36	272	165	Rv1932 tpx	Virulence, detoxification and adaptation

electrophoresis, which can be used in immunoblotting and antigen detection (Fig. 2b). Our results are in accordance with the findings of Bai Yu Lee et al. (1992) who showed that major membrane protein (MMP) is highly expressed in the virulent strains of *M. tuberculosis*, and its detection in sera from patients with pulmonary tuberculosis might be a serodiagnostic tool. MMP is present only in membrane preparations from *M. tuberculosis*. This protein was also identified as Hsp16.3, which is maximally synthesized at the *M. tuberculosis* stationary phase and is strongly associated with the cell envelope and intracellular clusters (Yang et al. 1999). Our results further showed that MMP is 16 kDa and is similar to the findings of Bai Yu Lee et al. (1992). They showed that MMP of *M. tuberculosis* is not synonymous with other heat-shock proteins (e.g., 19-kDa) lipoprotein and that it is apparently different from 14 to 15 kDa proteins. Braibant et al. (2000) described that many of those exporting systems are potentially implicated in the export of drugs and probably contribute to the intrinsic resistance of *M. tuberculosis* to many antibiotics, a characteristic usually attributed to the relative impermeability of its cell wall. Our analysis revealed that Rv3699 could be active Rv2686c, which is an antibiotic ABC transporter transmembrane protein. Probably it is responsible for the translocation of the substrate across the membrane (Cole et al. 1998).

In this manner, Hsp20 has been located in the cell wall and plasma membrane and could be used as potential immunodiagnosis of MDR-TB antigen. Also, there exist obvious reasons for activation of Rv0667 by Hsp20. Rv0667 or rpoB is expressed in RIF-resistant TB (Wolfe et al. 2010; Miller et al 1994). These data could be used in planning for diagnosis or drug design.

By our analysis, a member of the β-carbonic anhydrase family (BCA) was expressed in MDR-TB. This is in agreement with the findings of Suarez Covarrubias et al. (2005) who showed that Rv1284 was particular in *M. tuberculosis*. The new sulfonamides were evaluated as inhibitors of two β-carbonic anhydrases from *M. tuberculosis* (Rv1284 and Rv3273) (Nienaber et al. 2015).

Peptidyl-prolyl *cis*–trans isomerase (RV0009) is necessary for protein folding and is thought to participate in processes such as signaling, cell surface recognition and chaperoning. Probably, iron-regulated RV0009, also known as cyclophilins, shares a domain consisting of 109 amino acids and is a major cellular target for the immunosuppressive drug cyclosporin (Fleischmann et al. 2002).

Previous comparison of the protein patterns of INH-monoresistant *M. tuberculosis* strains and H37RV revealed that five protein spots are upregulated in INH-resistant consisting of Rv1446, Rv3028c, Rv0491, Rv2971 and Rv2145 (Jiang et al. 2006). In another study, four proteins of Rv0635, Rv1827, Rv0036c and Rv2032 were identified as intracellular MDR, and two proteins of Rv2896c and Rv2558c were revealed to belong to sensitive isolates (Singhal et al. 2012). One other study found nine proteins

as overexpressed in SM-resistant isolates consisting of Rv0350, Rv0440, Rv1240, Rv3057c, Rv3028c, Rv2145c, Rv2031c and Rv0569 (Neelja et al. 2010).

Zhang et al. (2012) suggest that Rv2031c, Rv3692 and Rv0444c are possible candidate biomarkers for effective use in the serodiagnosis of drug-resistant tuberculosis infection.

Surface proteins are ideally positioned to protect the bacterium or to modify the host immune response to the bacillus. So research on the cell wall or membrane proteome can promise candidates for vaccine and drug development against pathogenic *M. tuberculosis*.

A major problem is the isolation and separation of these proteins prior to mass spectrometric analysis. The insolubility of the membrane proteins at the isoelectric focusing step has been shown to be difficult with 2D electrophoresis procedures. In order to alleviate this problem, we replaced the one-dimensional SDS-PAGE because the membrane proteins are soluble in SDS buffers. In previous studies, 2DE methods have been used for comparison of the protein patterns of MDR-TB and sensitive *M. tuberculosis* strains. Our result is important for detecting these proteins because we used IEC-coupled SDS-PAGE, in practice. In other words, we considered pI and molecular weight simultaneously.

In conclusion, we have separated membrane and membrane-associated proteins directly from sonicated MDR-TB and susceptible *M. tuberculosis* bacilli. The protein profile data of the two strains (susceptible and MDR-TB) were further analyzed with the aim of finding relative quantitative differences of the observed proteins. Majority of commonly upregulated or expressed proteins belonged to the respiration category and cellular metabolism. Identification of these proteins is crucial in order to fully understand the pathogenicity of the bacteria. Thus, this study leads to the identification of different proteins between MDR and sensitive *M. tuberculosis* strains, which can be possible candidates for use in immunodiagnostic kits or drug development.

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