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Comparative Proteomic Analysis of Virulent Korean *Mycobacterium tuberculosis* K-strain with Other Mycobacteria Strain Following Infection of U-937 Macrophage

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In Korea, the *Mycobacterium tuberculosis* K-strain is the most prevalent clinical isolates and belongs to the Beijing family. In this study, we conducted comparative porteomics of expressed proteins of clinical isolates of the K-strain with H37Rv, H37Ra as well as the vaccine strain of *Mycobacterium bovis* BCG following phagocytosis by the human monocytic cell line U-937. Proteins were analyzed by 2-D PAGE and MALDI-TOF-MS. Two proteins, Mb1363 (probable glycogen phosphorylase GlgP) and MT2656 (Haloalkane dehalogenase LinB) were most abundant after phagocytosis of *M. tuberculosis* K-strain. This approach provides a method to determine specific proteins that may have critical roles in tuberculosis pathogenesis.

Keywords: proteomic analysis, *Mycobacterium tuberculosis* K-strain, human monocytic cell line U-937, 2-D PAGE, MALDI-TOF-MS, Mb1363, MT2656

Almost one-third of the world population is infected with *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB). Per year, there are approximately 8 million tuberculosis cases and 1.87 million tuberculosis-related deaths (Corbett *et al.*, 2003). Despite recent economic prosperity, Korea still faces significant public health challenges in tuberculosis control (Hong *et al.,* 1995). There is evidence that specific genotypic traits may associate with Korean ethnicity prevalence of increased transmissibility and pathologic tuberculosis strains, particularly the so-called, K-strain, may be TB-contributing factors in the Korean population (Kim *et al.,* 2001). Proteomic characterization of *M. tuberculosis* K-strains could provide an initial map of the distinct proteins in various strains before and after infection of host cells. To date, comparative proteomic experiments have been performed to identify the culture filtrate protein (CFP) antigens which *M. tuberculosis* strains secrete in the medium (Wong *et al.,* 1999; Bahk *et al.,* 2004), but not in viable bacilli recovered from macrophages. In our study we compared qualitative and quantitative expression of cytosolic proteins in *M. tuberculosis* strains before and after phagocytosis by the human macrophage-like cell line U-937.

M. tuberculosis H37Rv (ATCC 27294) and H37Ra (ATCCC 35835) were obtained from ATCC (Koram Biotech, Korea). K-strain and 5 clinically isolated *M. tuberculosis* strains from tuberculosis patients were collected by the Korean Institute of Tuberculosis. K-strains were identified using RFLP by The Korea Center for Mycobacterial DNA Data Base, Korean Institute of Tuberculosis (Park *et al.,* 2000). Five clinically isolated *M. tuberculosis* strains showed different RFLP patterns with K-strain even though they were Beijing family. The vaccine strain *Mycobacterium bovis* BCG employed was Pasteur 1173 P_2. The human histocytic macrophage-like cell line U-937 was obtained from the Korean cell line bank (Korea). The U-937 cell line was thawed from a stock solution, maintained a cell density of 5×10^6 U-937 cells per 50 ml of RPMI-1640 supplemented with 10% Fetal Calf Serum (GIBCO, USA) in a 225 cm^2 flask. The cells were differentiated using of 5 ng/ml of phorbol myristic acid (PMA). The macrophages were infected using late-log phase culture of mycobacteria grown in Middlebrook 7H10 plus ADC and Tween 80. Late log phase cultures (OD_{550} of 0.6-0.8) were added at 10^7 CFU/culture flask, at a MOI of 1:5. Optical densities at 550 nm were measured on a plate reader using 1 ml culture of mycobacteria and adjusted for 1 cm path length. The macrophages were incubated overnight at 37°C, 5% CO_2, to allow phagocytosis to occur. U-937 cell pellets were washed twice in PBS with 1% (v/v) Tween 80. U-937 cell pellets and mycobacteria-infected cell pellets were resuspended in lysis buffer containing 0.3% (w/v) SDS, 200 mM DTT, 28 mM Tris/HCl, 22 mM Tris base, 1 mM PMSF. Resuspended pellets were sonicated by a

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sonic dismembrator (Fisher, USA) for further cell disruption. Lysates were then centrifuged and the supernatant was filtered by 0.22 μm syringe filter (Millipore, France). Each protein sample was precipitated for 12 h at -20°C. The precipitated proteins were then pelleted by centrifugation (12,000×g, 20 min) and washed with cold acetone containing 20 mM DTT (Bollag *et al.*, 1996). The protein concentration of each sample was estimated using the Bradford assay kit (BioRad protein assay, USA).

Two-dimensional gel electrophoresis was performed as described in detail (Rabilloud *et al.*, 1998). Total protein extract was separated by IPG at first dimension (Immobilize pH Gradient 4-10 NL, Purchased from Genomine Inc. Korea) and the large (26×20 cm) 2-D format was used for second dimensional separation of focused proteins. Separated spots were detected by alkaline silver staining as described in Rabilloud *et al.* (1998). Quantitative analysis of 2-D gel spots was carried out using the PDQuest^{TM} software (version 7.0, BioRad) according to the protocols provided by the manufacturer. The quantity of each spot was normalized by total valid spot intensity. The expression differences for each different mycobacterial strain before and after infection were compared using the same software. Protein identification was performed as described previously in detail (Bollag *et al.*, 1996) using Ettan MALDI-TOF (Amersham Biosciences). Protein spots was excised and digested with trypsin (Promega, USA) and digested peptides were mixed with α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% TFA and applied to MALDI-TOF analysis. The search program ProFound (Zhang *et al.*, 2000), developed by The Rockefeller University (http://129.85.19.192/profound_bin/Web ProFound.exe), was used for protein identification by peptide mass fingerprinting.

For subsequent identification of protein spots, 2-D gels were independently repeated five times for U-937 cell lines and mycobacterial strains before and after infection to ensure the reproducibility of experiments. Approximately 1000 protein spots (pI range 4-10) were detected in each intact U-937 cell line, PMA treated U-937 cells and mycobacteria infected U-937 cell lines. Also approximately the same number of spots was detected in each mycobacterial strains, *M. tuberculosis* K-strain, H37Rv, H37Ra, 5 clinical isolates and *M. bovis* BCG. After 16 h infection the PMA differentiated U-937 cells with the different mycobacterial strains there were average 300 differently expressed spots in 2-D profiles (Fig. 1). Thus, the 2-D comparisons indicate that infection induced quantitative changes in U-937 cells. Further analysis of the 300 differently expressed independent spots after phagocytosis using PDQuest 2-D analysis software revealed 50 spots that were more abundant in the K-strain infected U-937 cells when compared to other mycobacteria infected U-937 cells. Among these 50 spots, the 20 most highly expressed proteins after K-strain infection were identified by matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS). Eleven of these identified spots were differently expressed U-937 host pro-

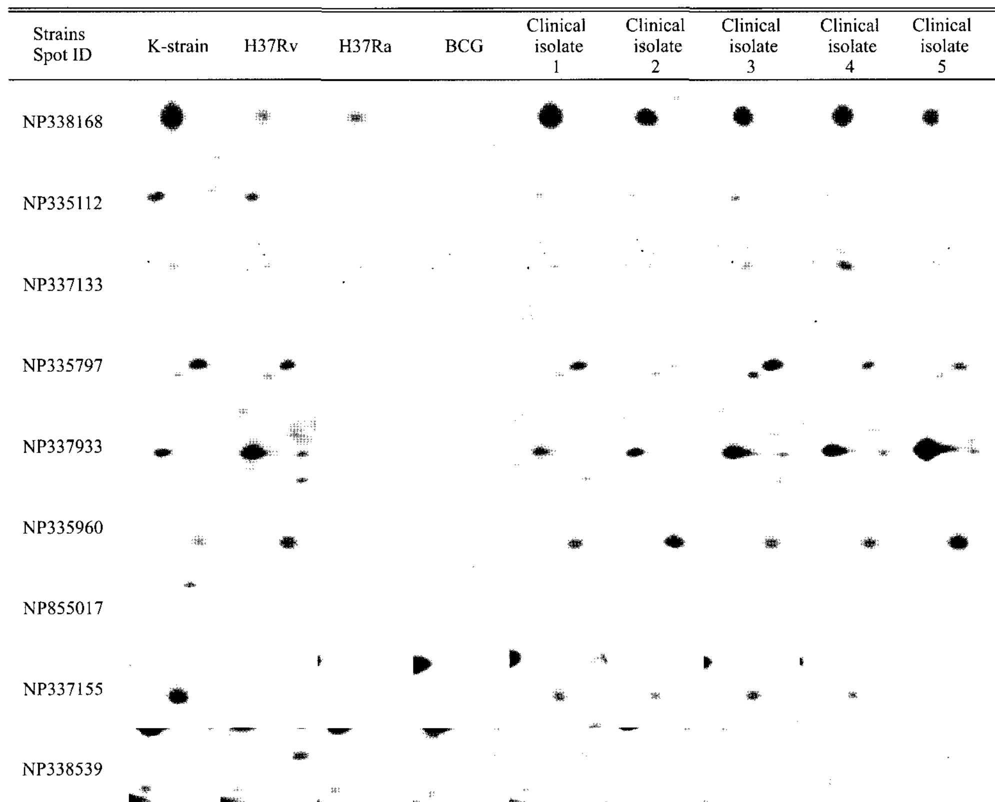

Fig. 1. 2-D spots of the cytosolic proteins from the K, H37Rv, H37Ra, BCG and clinical isolates in the pH rage 4-10 after infection of U-937 cells.

Table 1. List of proteins more abundantly expressed after *M. tuberculosis* K-strain infection to U-937 cells identified on the 2-D gels followed by analysis with MALDI-TOF and their amino acid sequences

NCBI reference no.	Sanger I.D.	Protein identification	Z Score	Cove-rage %	pI	kDa	Abundantly expressed strains after infection
NP338168	MT3622	Hypothetical protein	0.46	9%	5.7	36.19	K-strain, H37Rv, clinical isolates
NP335112	MT0701	Acyl-CoA dehydrogenase, putative	0.53	12%	5.7	58.52	
NP337133	MT2635	Hypothetical protein	0.67	23%	6.2	25.70	
NP335797	MT1349	ATP synthase F1, gamma subunit	2.33	48%	5.4	33.87	
NP337933	MT3404	Ama/HipO/HyuC family	2.34	32%	5.6	41.21	
NP335960	MT1510	ABC transporter, ATP-binding protein	2.34	52%	5.7	28.80	
NP855017	Mb1363	Probable glycogen phosphorylase GlgP	2.20	17%	5.2	95.50	K-strain
NP337155	MT2656	Haloalkane dehalogenase LinB	2.30	34%	5.1	33.71	
NP338539	MT3985	FtsK/SpoIIIE family protein	2.34	30%	6.2	64.54	H37Rv

teins (data not shown).

Seven mycobacteria-specific spots, MT3622, MT0701 (acyl-CoA dehydrogenase), MT2635, MT1349 (ATP synthase F1, gamma subunit), MT3404 (Ama/HipO/HyuC family), MT1510 (ABC transporter, ATP-binding protein) showed greater expression post-phagocytosis of the K-strain but also showed increased expression patterns in the H37Rv and clinically isolated strains after infection. In the K-strain, the two most relatively abundant expressed spots after K-strain phagocytosis were Mb1363 (probable glycogen phosphorylase GlgP) and MT2656 (Haloalkane dehalogenase LinB). One spot MT3985 (FtsK/SpoIIIE family protein) was highly expressed by the K-strain after phagocytosis but slightly more increased in the H37Rv strain (Table 1).

Mb1363, GlgP, one of the most abundantly expressed protein after K-strain phagocytosis is homologous to the *M. tuberculosis* H37Rv protein Rv1328. The two proteins share 99.7% identity for 863 overlap in amino acids. The glycogen phosphorylase functions in chaperone-like activity for the macrophage migration inhibitory factor (MIF). At heat stress temperatures, the large oligomers dissociate into monomers and bind and stabilize thermally denatured malate dehydrogenase and glycogen phosphorylase b thus preventing aggregation of the MIF (Cherepkova *et al.*, 2005). MIF is a ubiquitous multifunctional cytokine having diverse immunological and neuroendocrinal properties (Cherepkova *et al.*, 2004). It can be hypothesized that highly expressed glgP protein in K-strians induces MIF activation and may be advantageous for the bacteria to inhibit macrophage migration.

MT2656 (Haloalkane dehalogenase LinB), also highly expressed in the K-strian after phagocytosis. Haloalkane dehalogenases catalyze hydrolytic cleavage of carbon-halogen bonds in halogenated aliphatic compounds, leading to the formation of primary alcohols, halide ions, and protons. These enzymes are potentially useful for cleaning up contaminated subsurfaces (Stucki *et al.*, 1995), but the function of dehalogenating enzymes in mycobacteria is currently unknown. To test the role of mycobacterial dehalogenases in pathogenesis, the presence of the dehalogenase genes was screened in both virulent *M. bovis* strains and attenuated *M. bovis* BCG strains (Jesenska *et al.*, 2000). Dehalogenase genes, *dmbA* and *dmbB,* are thought to be widely distributed among species of *M. tuberculosis.* To invesgate whether these *dmbA* and *dmbB* genes are located in genomic regions of *M. bovis*, these regions were excised during serial passages of attenuated BCG (Mahairas *et al.*, 1996). Both genes were found in all of the tested isolates of *M. bovis* and *M. bovis* BCG, suggesting that they are not located in excised pathogenicity regions. However, the recent study of Mattow *et al.* (2001) suggests that the gene *dmbA* may be expressed in *M. tuberculosis* but not *M. bovis* BCG. Mattow *et al.* (2001) compared the cellular protein composition of two virulent strains of *M. tuberculosis* and two attenuated vaccine strains of *M. bovis* BCG by using 2-D electrophoresis and mass spectrometry (Mattow *et al.*, 2001). The haloalkane dehalogenase DmbA was missing in two attenuated strains of *M. bovis* BCG (Copenhagen and Chicago) but was present in two virulent strains of *M. tuberculosis* (Rv 2579 and Erdman). The lack of the DmbA protein in attenuated strains could be due to too low expression, repression, or lack of function. Another recent study revealed that *M. bovis* carries genes coding for enzymes with dehalogenating activities in their genome and that similar genes are widely distributed among other mycobacteria irrespective of their geographical and host type origin (Jesenska *et al.*, 2005).

The differential expression of proteins does not fully explain why K-strains are dominant in the Korean population. In addition, our culture conditions may have some critical points to study induced differential protein expressions compared to the actual mycobacterial environments in the human lung. We used Middlebrook 7H10 plus ADC and Tween-80 for mycobacterial growth but changed to RPMI-1640 media for infection and to allow phagocytosis by the U-937 cells to occur. The change in medium and not sufficient phagocytosis or the intracellular environment may have induced differential mycobacterial protein expression and the expressed patterns after phagocytosis may mingle cytosolic and secreted mycobacterial proteins. So the differential protein expression may have been due to the change in culture conditions and not in response to the phagocytic or intracellular environment. Additionally, the expressed patterns after phagocytosis may mingle cytosolic and secreted mycobacterial

proteins. Thus, the differential protein expression may have been the consequence of the change in culture conditions, and not in response to the phagocytic or intracellular environment. Additional experiments will be required in order to further characterize the GlgP and LinB proteins, such as recombinant proteins *in E. coli* or mycobacteria, and compare the expressions in the phagocytic environment, enzyme-linked immunoassays for each of the overexpressed proteins for the evaluation of immunological functions. Nevertheless, the principal shortcoming of our study, namely that we focused our interest on the characterization of differently expressed proteins after infection with the *M. tuberculosis* clinical K-strain, is an important shortcoming. The proteomic approach will give us an opportunity to further understand and characterize virulent *M. tuberculosis* strains and to assist in developing new effective tuberculosis vaccines.

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