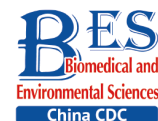## Original Article

Immunogenicity of Whole *Mycobacterium intracellulare* Proteins and Fingding on the Cross-Reactive Proteins between *M. intracellulare* and *M. tuberculosis*[*]

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Abstract

Objectives   To evaluate the immunogenicity of *Mycobacterium intracellulare* proteins and determine the cross-reactive proteins between *M. intracellulare* and *M. tuberculosis.*

Methods   Protein extracts from *M. intracellulare* were used to immunize BALB/c mice. The antigens were evaluated using cellular and humoral immunoassays. The common genes between *M. intracellular* and *M. tuberculosis* were identified using genome-wide comparative analysis, and cross-reactive proteins were screened using immunoproteome microarrays.

Results   Immunization with *M. intracellulare* proteins induced significantly higher levels of the cytokines interferon-γ (IFN-γ), interleukin-2 (IL-2), interleukin-12 (IL-12), interleukin-6 (IL-6) and immunoglobulins IgG, IgG1, IgM, and IgG2a in mouse serum. Bone marrow-derived macrophages isolated from mice immunized with *M. intracellulare* antigens displayed significantly lower bacillary loads than those isolated from mice immunized with adjuvants. Whole-genome sequence analysis revealed 396 common genes between *M. intracellulare* and *M. tuberculosis*. Microchip hybridization with *M. tuberculosis* proteins revealed the presence of 478 proteins in the serum of mice immunized with *M. intracellulare* protein extracts. Sixty common antigens were found using both microchip and genomic comparative analyses.

Conclusion   This is the advanced study to investigate the immunogenicity of *M. intracellulare* proteins and the cross-reactive proteins between *M. intracellulare* and *M. tuberculosis*. The results revealed the presence of a number of cross-reactive proteins between *M. intracellulare* and *M. tuberculosis*. Therefore, this study provides a new way of identifying immunogenic proteins for use in tuberculosis vaccines against both *M. intracellulare* and *M. tuberculosis* in future.

Key words: *Mycobacterium intracellulare*; *Mycobacterium tuberculosis*; Cross-reactive immune response; Vaccine; Antigen

INTRODUCTION

Tuberculosis (TB) is a serious infectious disease and the ninth leading cause of death worldwide. Therefore, it remains one of the most important public health problems in the world. According to the Global TB Report 2019, there were approximately 10 million active cases of TB worldwide in 2018, of which approximately 1.24 million died of the disease. In the meantime, a quarter of the world's population has been latently infected with *Mycobacterium tuberculosis*[1]. Furthermore, co-infection of *M. tuberculosis* and human immunodeficiency virus (HIV), prevalence of multidrug-resistant TB (MDR-TB) and emergence of extensively drug-resistant TB (XDR-TB), controlling the current TB epidemic remains a severe challenge[2]. Vaccination is the most efficient method for controlling communicable diseases; the Bacillus Calmette-Guérin (BCG) vaccine has been the first and only licensed vaccine used for TB prevention since 1921 globally. It was developed after long-term subculture of *M. bovis*, whereby the cells almost completely lost all pathogenicity to humans, but maintained strong immunogenicity, making it an ideal vaccine at the time[3]. According to current statistics, more than 3 billion people have been vaccinated with the BCG vaccine worldwide; however, its immune protection effect has always been a controversial topic. It is generally considered that the BCG vaccine is effective for the prevention and control of TB, especially tuberculous meningitis in infants, while its immune protection against adult TB is inferior or even ineffective[4]. Therefore, there is an urgent need to develop new and effective TB vaccines to end the epidemic, and researchers worldwide are working towards this goal using different approaches[5-7]. The key to these different approaches depends on finding new and effective antigens[8-11]. The effectiveness of the BCG vaccine against TB depends on the cross-reactivity of immune responses induced by both *M. bovis* and *M. tuberculosis* in humans[4]. Therefore, scientists have hypothesized that identifying new antigens from non-tuberculous mycobacteria (NTM) such as *M. vaccae* is a promising approach for determining suitable antigens for use in vaccines[12,13]. *M. intracellulare*, named by Runyon in the 1960s, is one of the most common pathogenic NTM and is widely distributed worldwide. *M. intracellulare* infection usually causes lung damage in the host. *M. intracellulare, M. avium, and M. tuberculosis* have been shown to induce greater, albeit similar, levels of chemokines compared to those induced by *M. smegmatis* and *M. abscessus*, whereas *M. intracellulare* showed higher levels of phagosome-lysosome fusion and apoptosis than *M. tuberculosis* in macrophages *in vitro*[14]. However, studies on the immune mechanisms induced by *M. intracellulare* infection are required, especially to characterize the cross-immunity with *M. tuberculosis*.

In this study, we aimed to identify antigens that induce cross-immunity between *M. intracellulare* and *M. tuberculosis.* First, we used whole bacterial protein extracts from *M. intracellulare* to immunize mice and evaluated the antigen performance using cellular and humoral immunoassays and the capability of macrophages to control intracellular *M. tuberculosis* growth. We then determined cross-reactive antigens between *M. intracellulare and M. tuberculosis* by comparative genomic analysis and immunoproteome microarray hybridization. The results of this study will help identify effective antigens among *M. intracellulare* proteins and may provide potential candidates for the development of new types of TB vaccines and immunotherapies against *M. intracellulare* and *M. tuberculosis* infection.

MATERIALS AND METHODS

Ethics Statement

All animal experiments conducted in the study were approved by the Ethics Committee and the Animal Experimental Ethical Committee of the National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention.

Bacterial Strains and Protein Preparation

M. intracellulare (ATCC13950) was cultured in Löwenstein–Jensen medium (ENCODE, China) at 37 °C. The bacterial colonies were washed in PBS buffer three times before being harvested into lysis buffer (10 mmol/L Tris-HCl + 100 mmol/L NaH_2PO_4 + 8 mol/L urea + 50 mmol/L IAA + 1× protease inhibitor cocktail). The harvested bacteria were then shock-crushed with magnetic beads four times (6.5 m/s, 2 min, ice for 1 min) to release the whole bacterial protein extract. Protein concentration was determined using a BCA kit (TransGen Biotech, China). All protein products were stored at −80 °C until use.

Immunological Evaluation of Whole M. intracellulare Protein Extracts in Mice

Mouse Immunization   Female BALB/c mice at 6–8

weeks were used in this study. Five mice were included in both experimental group and control group, and at least three mice were included to ensure reliability and statistical analysis. The experimental group was immunized with a 200 μL mixture containing 100 μg whole *M. intracellulare* protein extract, 50 μL 0.5 mg/mL PolyI:C, and 100 μL 2.5 mg/mL Dimethyl-dioctyldecylammonium bromide (DDA) as adjuvants. The control group was immunized with adjuvant mixture only (50 μL 0.5 mg/mL PolyI:C and 100 μL 2.5 mg/mL DDA). Each mouse was immunized 3 times, with subcutaneous injections at ten-day intervals.

Detection of Cytokines   On the 10th day after the last vaccination, spleens from immunized mice were surgically removed, crushed with a syringe plunger into Roswell Park Memorial Institute (RPMI) 1,640 medium (with 1% penicillin-streptomycin) (Gibco, USA), and filtered into centrifuge tubes with a cell strainer. Cell suspensions were centrifuged at 1,000 rpm (r = 8.5 cm) for 5 min, and erythrocytes were lysed with ACK lysis buffer (Solarbio, China). Finally, the splenocytes were collected and quantified using Millipore Scepter™ 2.0 (merckmillipore, Germany).

For each well, splenocytes (2×10^6 cells/mL, 500 μL) were co-cultured with 10 μg of corresponding bacterial antigens for 48 h. RPMI 1640 + ConA (5 μg/mL) or just RPMI 1640 were used as positive and negative controls, respectively. The BD OptEIA ELISA kits were used for detection of cytokines, including IFN-γ, IL-2, IL-4, IL-6, and IL-12, based on absorbance at 450 nm.

Detection of Immunoglobulins in the Serum   Ten days after the first, second, and third immunization, 120 μL mouse blood was collected from the orbital vein. Blood samples were stored at 4 °C overnight and then centrifuged at 2,000 rpm (r = 8.5 cm) for 10 min. The sera were collected and stored at −80 °C. ELISA was performed as follows: The 96-well ELISA plates were coated with antigens (the whole bacterial protein extract) at a final concentration of 10 μg/mL at 4 °C overnight. Blocking was achieved by adding 200 μL 3% BSA (Saibao, China) in each well, and extracts were incubated at 37 °C for 2 h. Sera (diluted 1:100, 1:1,000, 1:10,000, 2:10,000, 4:10,000, 8:10,000, and 1:100,000) were added and incubated for 1 h. Next, 100 μL 1:3,000 diluted secondary antibody (Southern Biotech, USA) was added and incubated at 37 °C for 50 min. TMB substrate (100 μL/well) (InnoReagents, China) was then added and the reaction was terminated by adding 2 mol/L H_2SO_4 (50 μL/well). Absorbance was read at 450 nm after 10 min.

Ability of Bone Marrow-derived Macrophages to Inhibit M. tuberculosis Growth ex vivo

1) Isolation of bone marrow-derived macrophage Bone marrow-derived macrophages (BMDMs) were isolated from mouse femurs at day 10 after the last immunization. First, the bone was cut at the hip joint, the entire leg was removed, and the skin and muscle were removed very carefully. Then, the bone was cut with scissors at the knee joint. Femurs were crushed using sterilized mortar pestle in 5 mL complete Dulbecco's Modified Eagle Media (DMEM) containing 10% FBS and 1% penicillin-streptomycin (Gibco, USA) and the cells were cultured in complete DMEM (with 10 μg/mL M-CSF) to differentiate BMDMs at 37 °C in 5% CO_2. Mature macrophages could be observed under the microscope by checking the morphology of cells after approximately 10 days.

2) H37Rv infection model BMDMs (2×10^6 cells) were plated in 24-well plates (Nunc, Denmark) in complete DMEM (10% FBS, 1% penicillin-streptomycin) per well and left to adhere for 12 h. BMDMs were then infected with log phase *M. tuberculosis* H37Rv for 4 h (day 0) and 3 and 5 days in a BSL-3 laboratory. *M. tuberculosis* H37Rv infected BMDMs were lysed with 1 mL ddH$_2$O and serially diluted (1:10, 1:100, and 1:1,000). Then, 100 μL from each preparation were inoculated on 7H10 media containing 50 μg/mL cycloheximide, 25 μg/mL polymixin B, 50 μg/mL carbenicillin, and 20 μg/mL trimethoprim and incubated at 37 °C. BMDMs isolated from adjuvant-immunized mice were used as controls. The multiplicity of infection (MOI) was set as 3 and 5. The count of intracellular *M. tuberculosis* was determined after 3 weeks.

Comparative Genomic Analysis

The complete genome sequences and coding sequences (CDSs) of *M. intracellulare* (NC_016946) and *M. tuberculosis* (NC_000962) were downloaded from the National Center of Biotechnology Information (NCBI) genome website. BLASTN in the NCBI BLAST+ software package (Version 2.6.0+) was used to determine the sequence similarity (parameters were set as follows: -perc_identity 85.00, -qcov_hsp_perc 90.00, -outfmt 5, -num_threads 32, and -evalue 1e-5) between the two species. The genes that were common between the two species were classified into functional categories according to S. T. Cole's classification[15].

Immune Proteomics

Antigens common between the species were

determined using protein microarrays (CapitalBio, China) spotted with 3791 H37Rv proteins and 428 CCDC1551 proteins. First, 3 mL serum sample diluted 1:200 with PBST was overlaid on the arrays and incubated at room temperature (RT) for 1 h. After washing three times with PBST, goat anti-mouse IgG (H+L) antibodies with Alexa-Fluor 532 or IgM with Alexa-Fluor 635 (diluted 1:1,000 with PBST) were added and incubated at RT for 1 h. Finally, arrays were washed with PBST, dried in a SlideWasher (CapitalBio, China), and scanned with GenePix 4200A (Molecular Devices, USA). Data were analyzed using GenePix 6.0 (Molecular Devices, USA). The signal-to-noise ratio (SNR) of each spot was defined as the ratio of the foreground to the background median intensity. To eliminate the systematic error between the protein arrays and different serum samples, quantile normalization was employed between the arrays. Spots with IgG-SNR > 3 or IgM-SNR > 5 were determined as positive. Gene ontology (GO) enrichment analysis and KEGG pathway analysis were performed at http://geneontology.org/ and https://www.genome.jp/kegg/tool/map_pathway2 .html, respectively. Protein-protein interaction analysis was performed using STRING 11.0 online (http://string-db.org/). Venn diagrams were generated using the R VennDiagram package https://rdrr.io/bioc/limma/man/venn.html.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism8 (GraphPad software, USA). The immunological data were compared using a two-tailed *t*-test. A two-sided *P* value ≤ 0.05 was used to determine statistical significance in all analyses.

RESULTS

Mycobacteria Intracellulare Proteins Predominantly Induce Th1-type Immunity in the Host

After immunizing mice three times with *M. intracellulare* protein extracts or adjuvants only, splenic lymphocytes were isolated and stimulated with *M. intracellulare* proteins. *M. intracellulare* protein extracts induced significantly higher levels of IFN-γ, IL-2, IL-12, and IL-6 (*P* < 0.0001, *P* < 0.001, *P* < 0.05, and *P* < 0.01, respectively), compared with control mice; however, IL-4 levels were similar in these mice (*P* > 0.05). Further, IL-4 concentrations obtained using both the immunization methods were low (< 30 pg/mL) (Figure 1).

Mycobacterium Intracellulare Protein Extracts Markedly Enhanced Antibody Responses

As shown in Figure 2, the prime and two prime-boost immunizations with *M. intracellulare* protein extracts induced strong and significant increase in IgG, IgG1, IgM, and IgG2a titers compared with pre-immunization and the adjuvant alone group at each detection point (*P* values were all < 0.001).

By day 30, after the second boost immunization, the IgG titer reached 1:800,000 and the titer of IgG1 also reached a high level of 1:400,000, while the titer of IgM was 1:32,000 and that of IgG2a was only 1:4,000 (Figure 2). All IgG, IgG1, IgM, and IgG2a titers were significantly higher than those acquired from the prime and the first boost immunization.

Immunization Significantly Improved the Ability of Bone Marrow-derived Macrophage to Inhibit M. tuberculosis Growth ex vivo

BMDMs isolated from mice immunized with *M. intracellulare* protein antigens were found to display significantly lower bacterial loads than those isolated from mice immunized only with adjuvants (control group) and inhibited the growth of intracellular *M. tuberculosis* in a dose- and time-dependent manner (Figure 3). Figure 3A shows that in MOI = 3 with 2.62 CFUs/mL (log10) H37Rv in day 0, the colony counts of the immunized group increased to 2.68 CFUs/mL (log10) when BMDMs were infected with H37Rv for 3 days, which was less than those of the control group [2.97 CFUs/mL (log10)] by 0.28 CFU/mL (log10) (*P* < 0.01); when BMDMs were infected with H37Rv for 5 days, the colony counts of the immunized group increased to 2.76 CFUs/mL (log10), which was significantly less than those obtained for 3.20 CFUs/mL (log10) of the control group by 0.45 CFUs/mL (log10) (*P* < 0.01). Figure 3B shows that in MOI = 5 with 3.01 CFUs/mL (log10) on day 0, the colony counts of the immunized group increased to 3.13 CFUs/mL (log10) when BMDMs were infected with H37Rv for 3 days, which was less than those of the control group [3.54 CFUs/mL (log10)] by 0.40 CFUs/mL (log10) (*P* < 0.05). When BMDMs were infected with H37Rv for 5 days, the colony counts of the immunized group increased to 3.37 CFUs/mL (log10), significantly less than those of the control group, which was 3.74 CFUs/mL (log10) by 0.3 CFUs/mL (log10) (*P* < 0.01).

Comparative Genomic Analysis of Mycobacterium

Basic information regarding the two downloaded genomic sequences is shown in Table 1. In total, 369

common genes were found between these two genomes; the classification of these genes is shown in Table 2.

Identification of Common Antigens

In the serum of *M. intracellulare*-immunized mice, 478 proteins were recognized by IgG and IgM antibodies in the protein microarray. GO enrichment analysis based on the Gene Ontology database revealed the top 15 GO terms to be in three categories according to the GO classification, of which "binding" "catalytic activity" "metabolic process" "cellular process" "cell" "cell part" were dominant, as shown in Figure 4.

We also conducted KEGG pathway analysis on these microarray-screened proteins. The names, functions, and pathways of 478 proteins are listed in Supplementary Table S1, available in www. besjournal.com. The top 15 enrichment pathways classified based on the screened proteins are displayed in Figure 5. Of the enriched pathways, "metabolic pathways" was the predominant pathway with 30 proteins, followed by "biosynthesis of secondary metabolites" with 19 proteins and "microbial metabolism in diverse environments" with 12 proteins.

The results from the protein-protein interaction analysis are shown in Figure 6. Of the proteins recognized by the microchip, three main interaction network clusters were found. The cluster with the
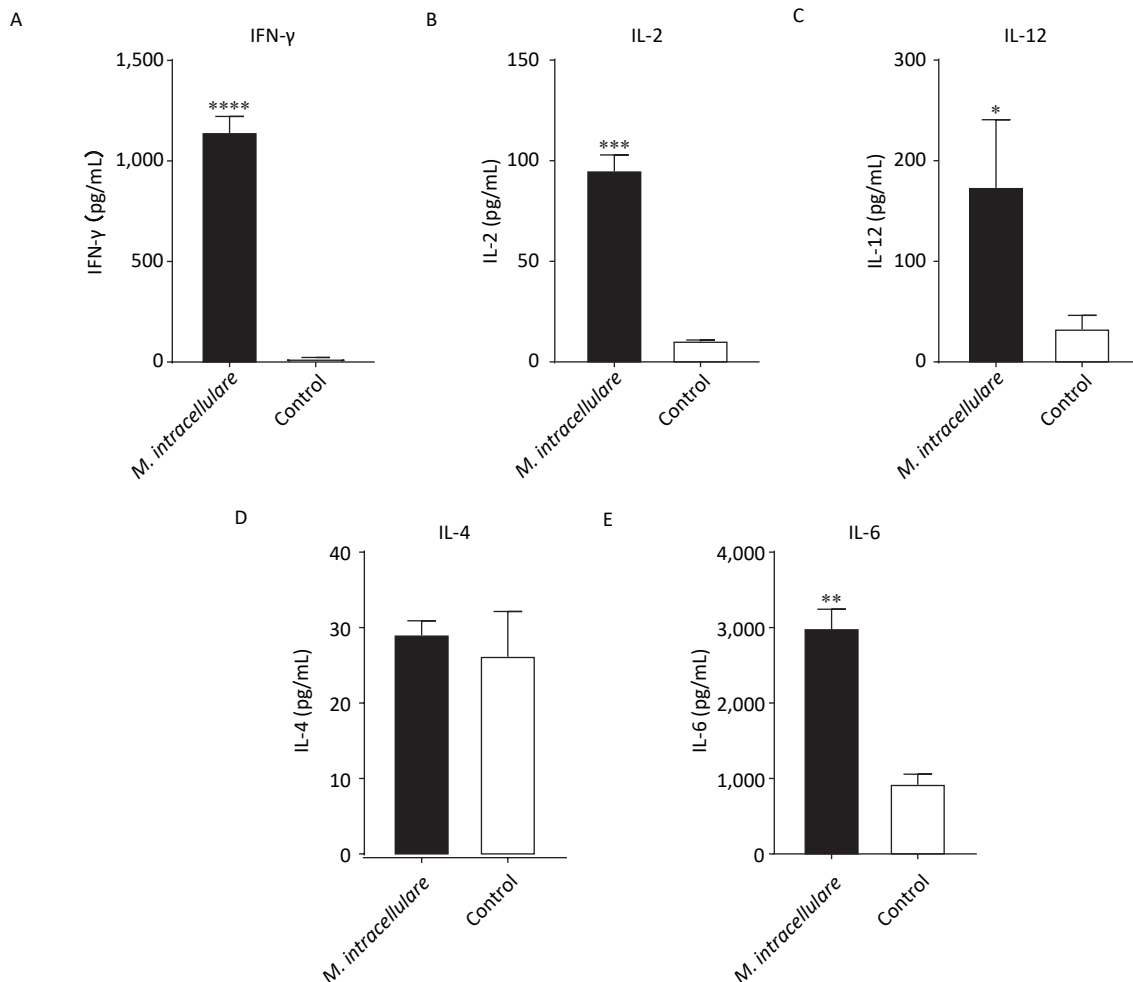
Figure 1. Cellular immune responses in immunized BALB/c mice. Lymphocytes isolated from BALB/c mice immunized with *M. intracellulare* protein extracts or adjuvants only were stimulated with *M. intracellulare* protein extracts, and the concentration of five different cytokines (A) IFN-γ, (B) IL-2, (C) IL-12, (D) IL-4, and (E) IL-6 were determined after 48 h. Capped line with asterisk * indicates significant difference between the two immunization methods, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. Columns indicate the mean from at least three samples, and error bars denote the standard deviation (SD).

highest score contained 13 proteins (Rv1308, Rv2925c, Rv2909c, Rv0041, Rv1306, Rv0732, Rv0006, Rv0440, Rv0053, Rv0684, Rv3417c, Rv3457c, Rv1650) and belonged to the cell composition cluster (GO:0044464). The second cluster contained seven proteins (Rv2460c, Rv2299c, Rv0350, Rv2703, Rv2534c, Rv3628, Rv0685) and belonged to the cytoplasmic composition modification (GO:0005737) related cluster. The third cluster contained four proteins (Rv2830c, Rv2546, Rv3321c, Rv0657c) and belonged to the toxin-antitoxin system (kw-1277) related cluster.

The results from the comparative analysis of antigens in protein microarray and comparative genomic analysis are shown in Figure 7. Of the 478 genes and 369 proteins found by microchip and comparative genomic analysis, 60 shared common antigens were found. In total, 418 exclusive antigens were revealed from protein microarray analysis and 309 from comparative genomic analysis. The names of the proteins in each group are listed in Supplementary Table S2, available in www.besjournal.com.

DISCUSSION

TB is an ancient infectious respiratory disease that seriously endangers human health. Almost a quarter of the world's population is estimated to have latent TB infection (LTBI). Numerous potential risk factors may promote the progression of LTBI to active TB once the efficiency of the immune system declines[16]. Therefore, there is an imperative need to develop new improved vaccines that protect against both active TB and LTBI[17]. To date, vaccination continues to be the most effective method to prevent and control TB[18]. However, the protection capacity of traditional BCG vaccines is limited. As a result, new strategies to improve the effectiveness of vaccination are required.

Bacterial proteins are important potential candidates for the development of new anti-TB
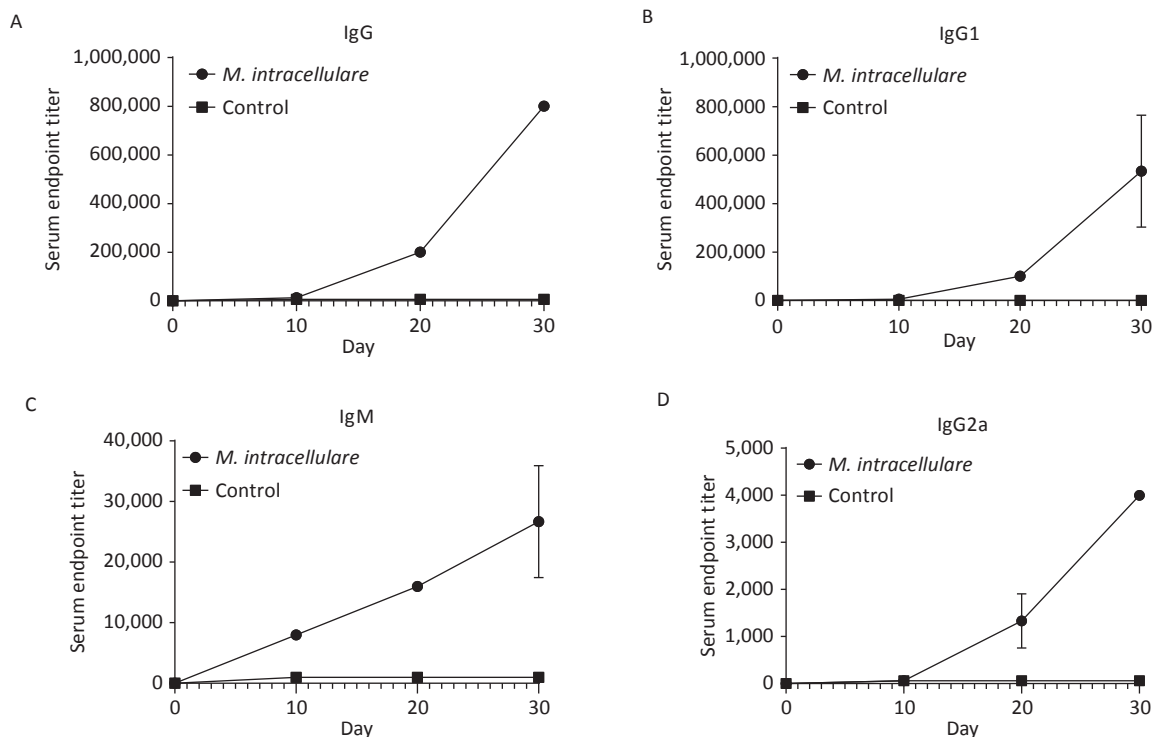
Figure 2. Antibody level monitoring in mice that had received three interval immunizations with *M. intracellulare* protein extracts or adjuvants. The change trends of titer levels of IgG, IgG1, IgM, and IgG2a are shown in (A), (B), (C), and (D), respectively. Dotted lines show the antibody titer levels in BALB/c mice immunized with *M. intracellulare* bacterial proteins + PolyI:C and DDA as an adjuvant; lines with squares show the antibody titer levels in BALB/c mice immunized with PolyI:C and DDA. The antibody titer levels were monitored at day 0, day 10 (10 days after the first immunization), day 20 (10 days after the second immunization), and day 30 (10 days after the third immunization). Points indicate the mean of at least three different serum samples in a group, and error bars denote standard deviation (SD).

vaccines because they can interact with host cells as key cell antigens[19]. Several *M. tuberculosis* secretory proteins have been used in newly designed anti-TB vaccines, and some of them showed excellent
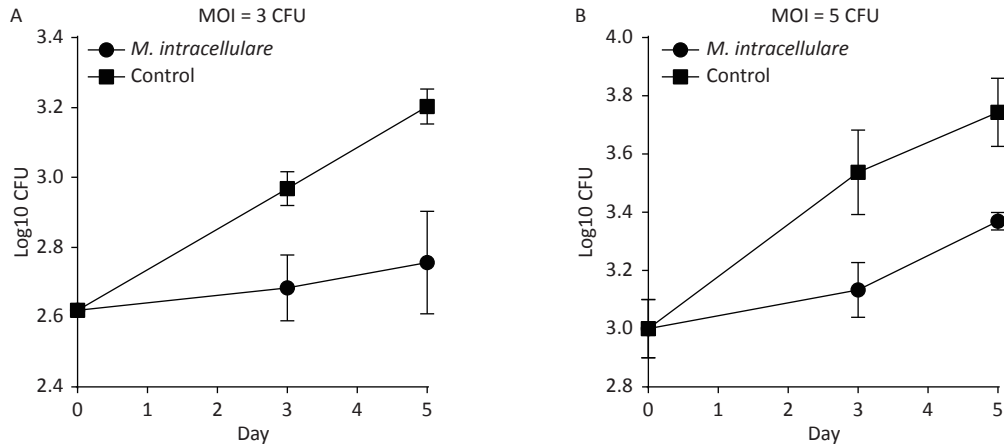
Figure 3. Immunization with *M. intracellulare* protein extracts enhanced the ability of bone marrow-derived macrophages to inhibit *M. tuberculosis* growth *ex vivo*. (A) BMDMs were infected with H37Rv at MOI = 3. (B) BMDMs were infected with H37Rv at MOI = 5. Colony counts were obtained for 0, 3, and 5 days of BMDM infection were compared with those obtained for H37RV. Points indicate the mean from triplicate cultures, and error bars denote standard deviation (SD). *P*-values were determined with two-tailed Student's *t* test.

Table 1. Annotation information for the two genomic sequences

Characters	NC_000962[*]	NC_016946[**]
Genome size (Mb)	4.41	5.4
GC (%)	65.60	68.10
No. of proteins	3,906	5,000
No. of rRNA	3	3
No. of tRNA	45	46
No. of other RNA	22	3
No. of genes	4,008	5,104
No. of pseudogenes	30	52

Note. [*]https://www.ncbi.nlm.nih.gov/genome/166?genome_assembly_id=159857. [**]https://www.ncbi.nlm.nih.gov/genome/1703?genome_assembly_id=171542.

Table 2. Functional classification of the 369 common genes found in the genomes of *M. tuberculosis* and *M. intracellulare*

No.	Function	Number
1	Virulence, detoxification, adaptation	10
2	Lipid metabolism	35
3	Information pathways	73
4	Cell wall and cell processes	39
5	Intermediary metabolism and respiration	143
6	Regulatory proteins	24
7	Conserved hypotheticals	41

immune responses and protective efficacy against *M. tuberculosis* infection[20-22]. For example, Ag85, EAST-6, CFP10, and Rv1886c are excellent antigens; one or more of these proteins have been used in subunit and viral-vector vaccines, some of which have successfully entered phases I–III clinical trials

and showed better protection ability than BCG[23-26].

A recent study showed that mycobacterial extracts from the rapidly growing NTM *M. vaccae* used in vaccines had promising protective effects against TB[12]. Several vaccines based on *M. vaccae* antigens, such as Vaccae™, DAR-901, and SRL172, have entered
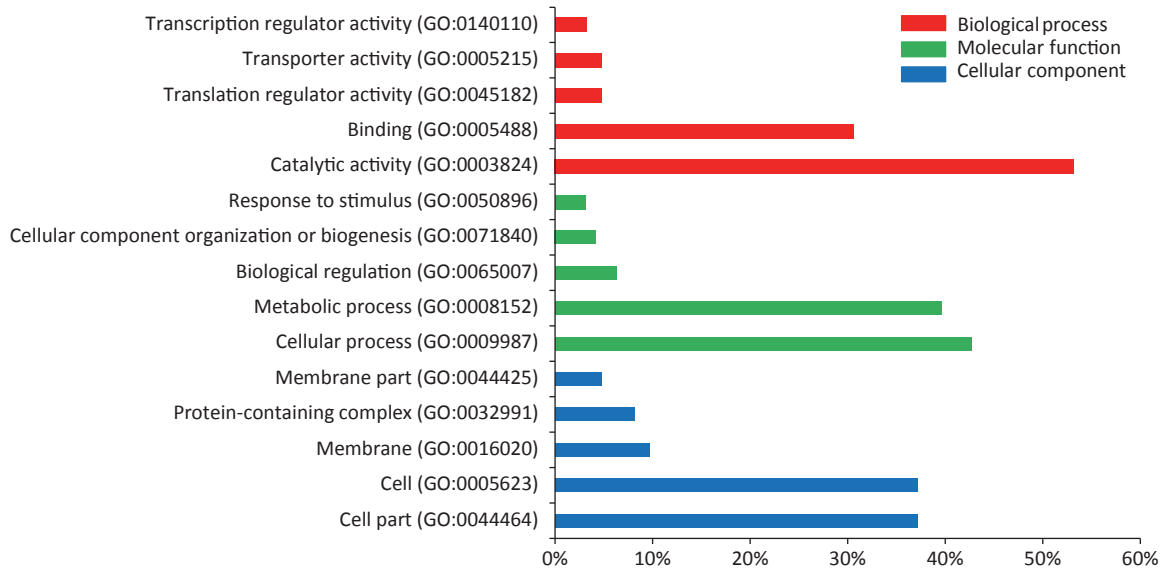
Figure 4. Top 15 GO terms identified based on the proteins found in the serum of *M. intracellulare* immunized mice. The results include three main categories: red bars represent biological processes, green bars represent molecular functions, and blue bars represent cellular components. The x-axis shows the percentage of the specific GO term in each category. GO, gene ontology.
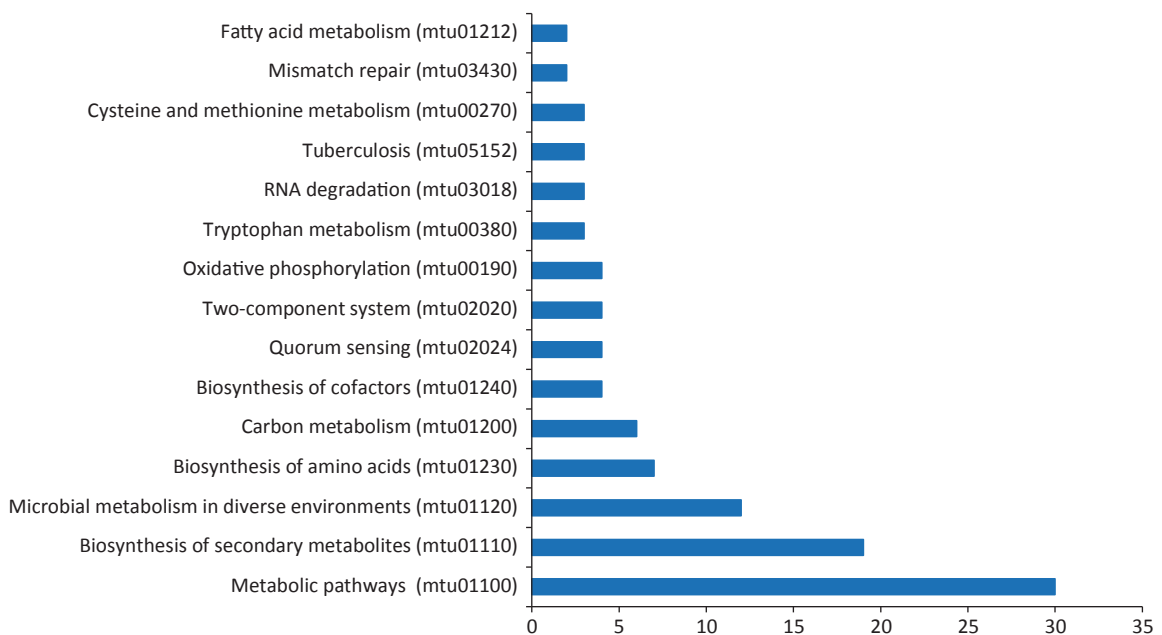
Figure 5. Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis for screened antigens. Blue bars represent each category with the name labeled in the left; x-axis shows the number of antigens in each specific KEGG term in each category.

different phases of clinical trials[27]. SRL172, an inactivated, whole-cell vaccine prepared from *M. vaccae*, was safe, well-tolerated, and immunogenic in humans[13,28]. A randomized controlled phase III trial in Tanzania demonstrated that boosting with SRL172 could protect against culture-confirmed TB in HIV-infected adults who had received BCG at birth[29]. The excellent immune effect of *M. vaccae* indicates that NTMs could also serve as a good source for designing new effective anti-TB vaccines. In the present study, we evaluated the immunogenicity and protective efficacy of *M. intracellulare* whole bacterial protein extracts in BALB/c mice to explore the possibility of using *M. intracellulare* as a source of immune effective antigens for developing anti-TB vaccines. Our results showed *that M. intracellulare* bacterial proteins promote high-level production of IFN-γ, IL-2, IL-6, and

IL-12 in mice, while IL-4 remained at a very low level, thus proving that immunization with *M. intracellulare* bacterial proteins predominantly elicited Th1-type cytokine production in BALB/c mice. Although the immune mechanism of vaccine protection from TB remains unclear, high expression levels of IFN-γ have been associated with enhanced protection against mycobacterial infection[30,31], and studies have shown that IFN-γ responses against multiple mycobacterial antigens could predict protection against TB[32,33]. IL-2 is also an important indicator in most clinical trials for evaluating TB-vaccine efficacy; high IL-2 levels indicate high concentrations of vaccine-induced activated CD4 and/or CD8 cells. These cells are necessary, although not sufficient, for protective immunity against *M. tuberculosis* in both animal models and humans[34,35]. IL-6 is a well-known inflammatory marker cytokine.
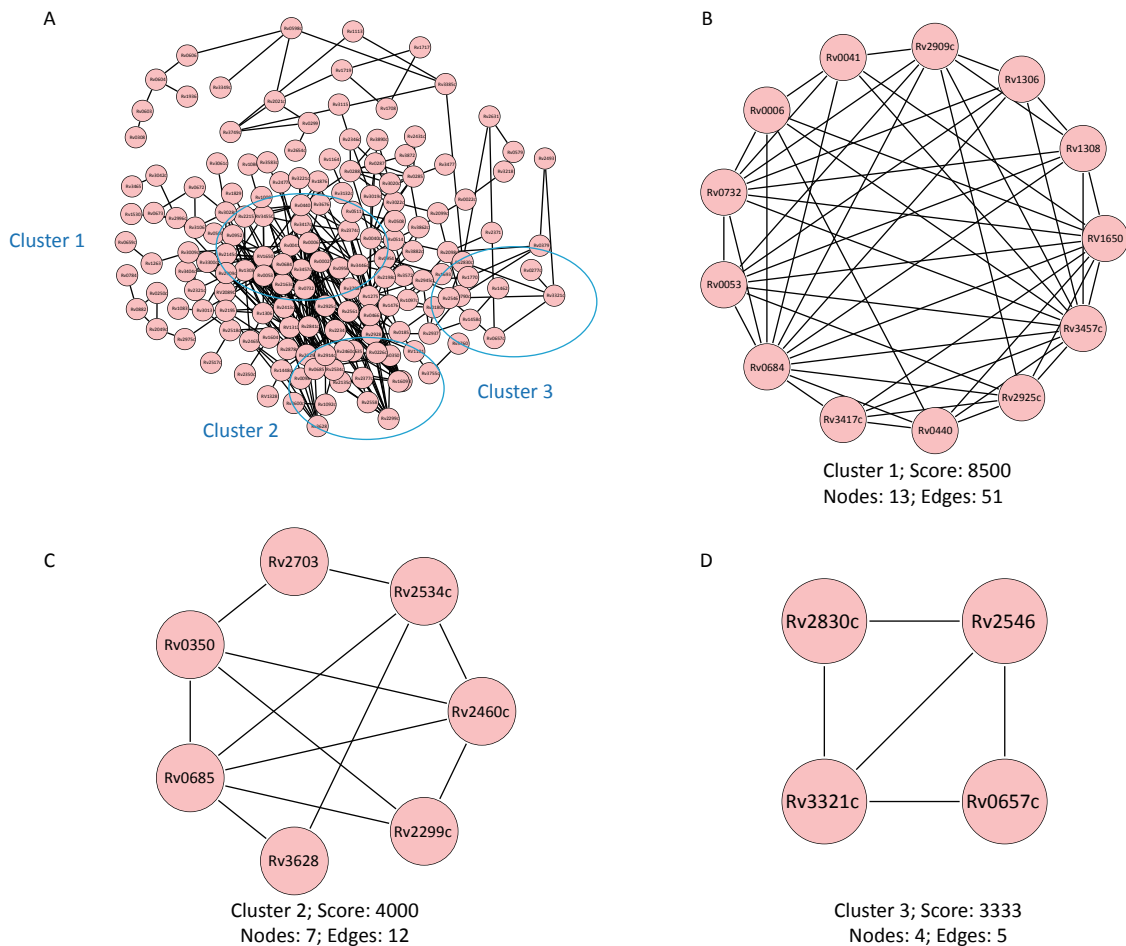
Figure 6. Protein-protein interaction network. The protein interaction network was constructed based on the String database. (A) Map of the interaction network of all screened antigenic proteins. (B) Cell composition (GO:0044464) correlated cluster. (C) Cytoplasmic composition modification (GO:0005737) correlated cluster. (D) Toxin-antitoxin system (kw-1277) correlated cluster. Lines represent specific protein-protein associations. GO, gene ontology.

Although several studies have confirmed that the level of IL-6 could be used to monitor the progress of infection and infer the risk of progression to active TB, its role in vaccine-mediated immune protection against TB is still unclear[36]. A previous study showed that IL-6 plays a major role in priming but not in late Th1 response to a TB vaccine, thus regulating the phenotype of the immune response[37]. The high concentration of IL-6 detected in this study was possibly induced by the antigenic proteins in *M. intracellulare*, which are also critical for immunity[38]. IL-12 is a key cytokine that mediates the immune response of Th1 cells. Its main immunomodulatory effect is to induce differentiation of early T helper cells into Th1 cells and promote the development and proliferation of Th1 cells. High levels of IL-12 indicate cellular immunity rather than humoral immunity[39]. A recent study reported that IL-12 production could also inhibit intracellular mycobacterial growth by enhancing autophagy[40]. Another cytokine we detected is IL-4, which is a central cytokine produced by Th2 cells and promotes B cell proliferation[41]. We found that IL-4 was at a very low concentration in both groups, and there was no significant difference between the two groups.

Data from previous studies suggested that both Th1 and Th2 immune responses play important roles in host protection against *M. tuberculosis* infection, and Th1 is essential against this intracellular pathogen. Current attempts to generate a vaccine against TB are generally based on the assumption that it must drive a Th1 response[42]. Our results showed that *M. intracellulare* proteins could induce a Th1/Th2 balance shift toward Th1 in BALB/c mice,
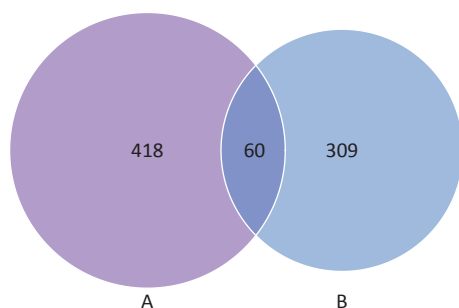

Figure 7. Comparative analysis of antigens in protein microarray and comparative genomic analysis. Venn diagram showing the distribution of common antigens and exclusive antigens between protein microarrays (A) and comparative genomic analysis (B). The numbers shown in the diagram represent the number of proteins in each group.

which favors the control of *M. tuberculosis in vivo*.

While the role of cell-mediated immunity in the protective immune response against TB has been well established, the role of B cells in this process is not clearly understood[43]. Emerging evidence suggests that B cell-dominated humoral immunity can modulate the host immune response to various intracellular pathogens, including *M. tuberculosis*, by regulating the level of granulomatous reaction, cytokine production, and T cell response[44,45]. Therefore, we also monitored antibody production in our animal model. The levels of the four antibodies (IgG, IgG1, IgM, and IgG2a) were measured in a time- and dose-dependent manner; results showed an increase in antibody titer levels. After three immunizations, they all reached a significantly higher quantity than that in the negative control group mice, suggesting that *M. intracellulare* bacterial proteins potentially improve the humoral immune response in mice. The results also indicated that PolyI:C and DDA function well as subcutaneous adjuvants to promote the production of antibodies without eliciting immune responses of their own.

Macrophages are the first line of defense of the host immune system against *M. tuberculosis* infection[46,47]. During its long-term interaction with macrophages, *M. tuberculosis* develops many effective strategies to avoid elimination, while surviving and proliferating inside macrophages[48]. Although a series of complex interactions between the host and pathogen ultimately determine the outcome of infection, the mechanisms of macrophage-bacillus interactions are complicated and still under investigation. The improved clearance ability of macrophages is essential for the host to fight TB. Our results showed that BMDMs from *M. intracellulare*-immunized mice had a significant ability to inhibit the growth of *M. tuberculosis* compared to those from control mice *in vitro*, indicating that *M. intracellulare* bacterial proteins can effectively change the host immune system environment to impair *M. tuberculosis* survival. One explanation for this result may be that bacterial protein immunization upregulates the expression of specific genes in BMDMs and thus helps to impede *M. tuberculosis* growth. Therefore, more specific experiments, including transcriptome analysis or other methods, are required to reveal the relevant mechanisms. Our results also suggest the possibility of using *M. intracellulare* antigenic proteins for the development of TB vaccines.

To gain insights into the antigens expressed among the *M. intracellulare* bacterial proteins that changed

the immune properties of mice to prevent *M. tuberculosis* infection, a protein microarray coated with 4,219 proteins of *M. tuberculosis* was used to identify the antigen repertoire by cross-reacting with IgG and IgM in the sera of mice immunized with whole *M. intracellulare* proteins. A total of 478 *M. tuberculosis* proteins were recognized by the serum antibodies IgG and IgM. Through GO enrichment analysis and KEGG pathway analysis of the 478 proteins as well as the comparative genomic analysis between *M. intracellulare* and *M. tuberculosis*, we primarily obtained an insight into the main biological processes and pathways involved in shared antigens between *M. tuberculosis* and *M. intracellulare*. In the protein-protein interaction analysis, we selected specific antigens that formed three particular clusters: cell composition, cytoplasmic composition modification, and toxin-antitoxin system-related cluster. The strong interactions between these proteins suggest that they may work together to regulate the host immune profile. These protein combinations may therefore be potential targets for the development of anti-TB vaccines. In addition, we compared the antigens detected by combinational genomics and proteomics; among 478 genes and 369 proteins identified in protein microarray and comparative genomic analysis, 60 common antigens were found. These antigens may possibly be potential cross-reactive antigens that may be used for the subsequent development of cross-immune vaccines for both *M. intracellulare* and *M. tuberculosis* in the future.

In summary, the present study demonstrates the possibility of whole bacterial protein extracts of *M. intracellulare* to serve as antigens against TB vaccines and reveals a number of proteins with cross-reactivity between *M. intracellulare* and *M. tuberculosis*. One limitation of the present study is that we still cannot screen for more specific antigens with clear immunogenicity to provide candidates for TB vaccines that can effectively protect against *M. tuberculosis* infection. Further studies are required to cautiously choose immunodominant antigens, which are crucial for developing novel vaccines for the control of TB.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

FENG Wen Hai, LIU Hai Can, and LI Gui Lian

designed this study, revised the manuscript, and edited the manuscript. XIAO Shi Qi prepared the first draft of the manuscript. XIAO Shi Qi, XU Da, DUAN Hong Yang, FAN Xue Ting, LI Xin Yao, LI Na, LI Ma Chao, and ZHAO Li Lan performed experiments. ZHAO Xiu Qin cultured the strains. ZHANG Wen and HAN Na performed genome sequencing of the *M. intracellulare* strain. WAN Kang Lin provided analysis support. All authors provided suggestions on the preparation of the manuscript and read and approved the final draft of the manuscript.

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Supplementary Table S1. Names, functions, and pathways of 478 proteins identified in the sera
of *M. intracellulare*-immunized mice by protein microarrays

ID	KEGG pathway	No. of genes	Genes mapped
mtu01100	Metabolic pathways	30	mtu:Rv0091 mtn; 5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase mtu:Rv0500 proC; pyrroline-5-carboxylate reductase mtu:Rv0511 hemD; uroporphyrin-III C-methyltransferase mtu:Rv0673 echA4; enoyl-CoA hydratase EchA4 mtu:Rv0769 oxidoreductase mtu:Rv0848 cysK2; cysteine synthase CysK mtu:Rv0952 sucD; succinyl-CoA ligase subunit alpha mtu:Rv0956 purN; phosphoribosylglycinamide formyltransferase PurN mtu:Rv1092c coaA; pantothenate kinase mtu:Rv1164 narI; nitrate reductase subunit gamma mtu:Rv1257c oxidoreductase mtu:Rv1263 amiB2; amidase AmiB mtu:Rv1306 atpF; ATP synthase subunit B mtu:Rv1308 atpA; ATP synthase subunit alpha mtu:Rv1448c tal; transaldolase mtu:Rv1530 adh; alcohol dehydrogenase mtu:Rv1604 impA; inositol-monophosphatase ImpA mtu:Rv1609 trpE; anthranilate synthase component I mtu:Rv2195 qcrA; ubiquinol-cytochrome C reductase rieske iron-sulfur subunit mtu:Rv2215 dlaT; pyruvate dehydrogenase E2 component dihydrolipoamide acyltransferase mtu:Rv2350c plcB; membrane-associated phospholipase B mtu:Rv2377c hypothetical protein mtu:Rv2465c rpiB; ribose-5-phosphate isomerase B mtu:Rv2996c serA1; D-3-phosphoglycerate dehydrogenase mtu:Rv3009c gatB; aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit B mtu:Rv3042c serB2; phosphoserine phosphatase SerB mtu:Rv3106 fprA; NADPH-ferredoxin reductase FprA mtu:Rv3465 rmlC; dTDP-4-dehydrorhamnose 3,5-epimerase mtu:Rv3600c type III pantothenate kinase mtu:Rv3703c etgB; iron(II)-dependent oxidoreductase EgtB
mtu01110	Biosynthesis of secondary metabolites	19	mtu:Rv0500 proC; pyrroline-5-carboxylate reductase mtu:Rv0511 hemD; uroporphyrin-III C-methyltransferase mtu:Rv0673 echA4; enoyl-CoA hydratase EchA4 mtu:Rv0769 oxidoreductase mtu:Rv0952 sucD; succinyl-CoA ligase subunit alpha mtu:Rv0956 purN; phosphoribosylglycinamide formyltransferase PurN mtu:Rv1086 (2Z,6E)-farnesyl diphosphate synthase mtu:Rv1257c oxidoreductase mtu:Rv1448c tal; transaldolase mtu:Rv1530 adh; alcohol dehydrogenase mtu:Rv1604 impA; inositol-monophosphatase ImpA mtu:Rv1609 trpE; anthranilate synthase component I mtu:Rv2215 dlaT; pyruvate dehydrogenase E2 component dihydrolipoamide acyltransferase mtu:Rv2350c plcB; membrane-associated phospholipase B mtu:Rv2377c hypothetical protein mtu:Rv2465c rpiB; ribose-5-phosphate isomerase B mtu:Rv2996c serA1; D-3-phosphoglycerate dehydrogenase mtu:Rv3042c serB2; phosphoserine phosphatase SerB mtu:Rv3465 rmlC; dTDP-4-dehydrorhamnose 3,5-epimerase
mtu01120	Microbial metabolism in diverse environments	12	mtu:Rv0511 hemD; uroporphyrin-III C-methyltransferase mtu:Rv0673 echA4; enoyl-CoA hydratase EchA4 mtu:Rv0952 sucD; succinyl-CoA ligase subunit alpha mtu:Rv1164 narI; nitrate reductase subunit gamma mtu:Rv1257c oxidoreductase mtu:Rv1263 amiB2; amidase AmiB mtu:Rv1448c tal; transaldolase mtu:Rv1530 adh; alcohol dehydrogenase mtu:Rv2215 dlaT; pyruvate dehydrogenase E2 component dihydrolipoamide acyltransferase mtu:Rv2465c rpiB; ribose-5-phosphate isomerase B mtu:Rv2996c serA1; D-3-phosphoglycerate dehydrogenase mtu:Rv3042c serB2; phosphoserine phosphatase SerB
mtu01230	Biosynthesis of amino acids	7	mtu:Rv0091 mtn; 5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase mtu:Rv0500 proC; pyrroline-5-carboxylate reductase mtu:Rv1448c tal; transaldolase mtu:Rv1609 trpE; anthranilate synthase component I mtu:Rv2465c rpiB; ribose-5-phosphate isomerase B mtu:Rv2996c serA1; D-3-phosphoglycerate dehydrogenase mtu:Rv3042c serB2; phosphoserine phosphatase SerB
mtu01200	Carbon metabolism	6	mtu:Rv0952 sucD; succinyl-CoA ligase subunit alpha mtu:Rv1448c tal; transaldolase mtu:Rv2215 dlaT; pyruvate dehydrogenase E2 component dihydrolipoamide acyltransferase mtu:Rv2465c rpiB; ribose-5-phosphate isomerase B mtu:Rv2996c serA1; D-3-phosphoglycerate dehydrogenase mtu:Rv3042c serB2; phosphoserine phosphatase SerB
mtu01240	Biosynthesis of cofactors	4	mtu:Rv0511 hemD; uroporphyrin-III C-methyltransferase mtu:Rv0769 oxidoreductase mtu:Rv1092c coaA; pantothenate kinase mtu:Rv3600c type III pantothenate kinase
mtu02024	Quorum sensing	4	mtu:Rv0732 secY; preprotein translocase SecY mtu:Rv1609 trpE; anthranilate synthase component I mtu:Rv2350c plcB; membrane-associated phospholipase B mtu:Rv3676 crp; cAMP receptor protein

Continued

ID	KEGG pathway	No. of genes	Genes mapped
mtu00190	Oxidative phosphorylation	4	mtu:Rv1306 atpF; ATP synthase subunit B mtu:Rv1308 atpA; ATP synthase subunit alpha mtu:Rv2195 qcrA; ubiquinol-cytochrome C reductase rieske iron-sulfur subunit mtu:Rv3628 ppa; inorganic pyrophosphatase
mtu02020	Two-component system	4	mtu:Rv1164 narI; nitrate reductase subunit gamma mtu:Rv2234 ptpA; protein-tyrosine-phosphatase mtu:Rv3132c devS; two component sensor histidine kinase DevS mtu:Rv3676 crp; cAMP receptor protein
mtu00270	Cysteine and methionine metabolism	3	mtu:Rv0091 mtn; 5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase mtu:Rv0848 cysK2; cysteine synthase CysK mtu:Rv2996c serA1; D-3-phosphoglycerate dehydrogenase
mtu00380	Tryptophan metabolism	3	mtu:Rv0673 echA4; enoyl-CoA hydratase EchA4 mtu:Rv1263 amiB2; amidase AmiB mtu:Rv2215 dlaT; pyruvate dehydrogenase E2 component dihydrolipoamide acyltransferase
mtu03018	RNA degradation	3	mtu:Rv0350 dnaK; chaperone protein DnaK mtu:Rv0440 groEL2; molecular chaperone GroEL mtu:Rv3417c groEL1; chaperonin GroEL
mtu05152	Tuberculosis	3	mtu:Rv0350 dnaK; chaperone protein DnaK mtu:Rv0440 groEL2; molecular chaperone GroEL mtu:Rv3417c groEL1; chaperonin GroEL
mtu03430	Mismatch repair	2	mtu:Rv0002 dnaN; DNA polymerase III subunit beta mtu:Rv2413c hypothetical protein
mtu01212	Fatty acid metabolism	2	mtu:Rv0673 echA4; enoyl-CoA hydratase EchA4 mtu:Rv0769 oxidoreductase

Supplementary Table S2. Names of common antigens identified by microchip and comparative genomic analysis

No.	Genome	BioChip	Common
1	Rv0009	MT0066.1	Rv0009
2	Rv0011c	MT0066.2	Rv0046c
3	Rv0019c	MT0116.1	Rv0053
4	Rv0046c	MT0250	Rv0337c
5	Rv0053	MT0270.1	Rv0350
6	Rv0055	MT0383	Rv0384c
7	Rv0126	MT0392	Rv0423c
8	Rv0130	MT0407	Rv0440
9	Rv0137c	MT0470	Rv0491
10	Rv0156	MT0487	Rv0551c
11	Rv0157	MT0543	Rv0667
12	Rv0158	MT0555	Rv0672
13	Rv0189c	MT0610	Rv0673
14	Rv0211	MT0946	Rv0684
15	Rv0230c	MT0968.1	Rv0685
16	Rv0236A	MT1029	Rv0732
17	Rv0238	MT1040.1	Rv0861c
18	Rv0242c	MT1055	Rv0984
19	Rv0244c	MT1083.1	Rv1013
20	Rv0267	MT1172.1	Rv1070c
21	Rv0321	MT1264.1	Rv1080c
22	Rv0337c	MT1305.1	Rv1086
23	Rv0350	MT1329	Rv1092c

Continued

No.	Genome	BioChip	Common
24	Rv0352	MT1479.1	Rv1257c
25	Rv0357c	MT1534	Rv1292
26	Rv0363c	MT1555.1	Rv1308
27	Rv0380c	MT1775	Rv1458c
28	Rv0384c	MT1849.1	Rv1654
29	Rv0391	MT2068	Rv1829
30	Rv0407	MT2113	Rv2115c
31	Rv0411c	MT2138.2	Rv2241
32	Rv0423c	MT2142	Rv2346c
33	Rv0430	MT2283	Rv2374c
34	Rv0440	MT2291	Rv2457c
35	Rv0458	MT2316	Rv2460c
36	Rv0465c	MT2330.1	Rv2465c
37	Rv0467	MT2334.1	Rv2477c
38	Rv0491	MT2361.1	Rv2534c
39	Rv0498	MT2405	Rv2558
40	Rv0500A	MT2455	Rv2697c
41	Rv0510	MT2488.1	Rv2711
42	Rv0527	MT2501	Rv2754c
43	Rv0548c	MT2502	Rv2788
44	Rv0551c	MT2520.1	Rv2795c
45	Rv0566c	MT2554.1	Rv2909c
46	Rv0634B	MT2625	Rv3009c
47	Rv0636	MT2626	Rv3028c
48	Rv0639	MT2637.1	Rv3118
49	Rv0640	MT2721	Rv3221c
50	Rv0641	MT2779	Rv3248c
51	Rv0642c	MT2871	Rv3412
52	Rv0647c	MT2958.1	Rv3442c
53	Rv0651	MT3139.1	Rv3457c
54	Rv0652	MT3270.1	Rv3551
55	Rv0655	MT3279	Rv3583c
56	Rv0667	MT3284	Rv3609c
57	Rv0668	MT3289	Rv3628
58	Rv0672	MT3290.2	Rv3676
59	Rv0673	MT3532.1	Rv3710
60	Rv0682	MT3573.12	Rv3791
61	Rv0683	MT3631	
62	Rv0684	MT3770	
63	Rv0685	MT3858	

Continued

No.	Genome	BioChip	Common
64	Rv0691A	MT3876	
65	Rv0693	MT3878	
66	Rv0700	MT4026.1	
67	Rv0701	Rv0002	
68	Rv0702	Rv0006	
69	Rv0703	Rv0009	
70	Rv0704	Rv0022c	
71	Rv0705	Rv0025	
72	Rv0707	Rv0028	
73	Rv0708	rv0036	
74	Rv0709	Rv0040c	
75	Rv0714	Rv0041	
76	Rv0716	Rv0043c	
77	Rv0717	Rv0045c	
78	Rv0718	Rv0046c	
79	Rv0719	Rv0053	
80	Rv0721	Rv0063	
81	Rv0723	Rv0076c	
82	Rv0732	Rv0089	
83	Rv0733	Rv0091	
84	Rv0737	Rv0095c	
85	Rv0753c	Rv0098	
86	Rv0803	Rv0100	
87	Rv0808	Rv0110	
88	Rv0814c	Rv0119	
89	Rv0815c	Rv0145	
90	Rv0820	Rv0150c	
91	Rv0821c	Rv0155	
92	Rv0859	Rv0180c	
93	Rv0861c	Rv0185	
94	Rv0889c	Rv0187	
95	Rv0896	Rv0226c	
96	Rv0903c	Rv0232	
97	Rv0946c	Rv0248c	
98	Rv0958	Rv0250c	
99	Rv0974c	Rv0264c	
100	Rv0975c	Rv0277c	
101	Rv0981	Rv0281	
102	Rv0984	Rv0285	
103	Rv1013	Rv0287	

Continued

No.	Genome	BioChip	Common
104	Rv1017c	Rv0288	
105	Rv1019	Rv0290	
106	Rv1023	Rv0295c	
107	Rv1038c	Rv0299	
108	Rv1070c	Rv0301	
109	Rv1074c	Rv0308	
110	Rv1077	Rv0333	
111	Rv1080c	Rv0337c	
112	Rv1086	Rv0350	
113	Rv1092c	Rv0369c	
114	Rv1095	Rv0379	
115	Rv1098c	Rv0384c	
116	Rv1099c	Rv0385	
117	Rv1151c	Rv0387c	
118	Rv1177	Rv0398c	
119	Rv1187	Rv0423c	
120	Rv1197	Rv0429c	
121	Rv1198	Rv0437c	
122	Rv1211	Rv0440	
123	Rv1213	Rv0446c	
124	Rv1240	Rv0459	
125	Rv1248c	Rv0466	
126	Rv1257c	Rv0489	
127	Rv1262c	Rv0491	
128	Rv1292	Rv0500	
129	Rv1298	Rv0508	
130	Rv1305	Rv0511	
131	Rv1308	Rv0514	
132	Rv1310	Rv0518	
133	Rv1311	Rv0521c	
134	Rv1315	Rv0546c	
135	Rv1321	Rv0551c	
136	Rv1331	Rv0561c	
137	Rv1380	Rv0571c	
138	Rv1381	Rv0577	
139	Rv1383	Rv0579	
140	Rv1384	Rv0580c	
141	Rv1388	Rv0598c	
142	Rv1392	Rv0600c	
143	Rv1415	Rv0603	

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No.	Genome	BioChip	Common
144	Rv1423	Rv0604	
145	Rv1436	Rv0606	
146	Rv1447c	Rv0612	
147	Rv1458c	Rv0635	
148	Rv1474c	Rv0657c	
149	Rv1475c	Rv0659c	
150	Rv1479	Rv0666	
151	Rv1481	Rv0667	
152	Rv1483	Rv0672	
153	Rv1484	Rv0673	
154	Rv1488	Rv0678	
155	Rv1493	Rv0684	
156	Rv1547	Rv0685	
157	Rv1589	Rv0699	
158	Rv1601	Rv0730	
159	Rv1611	Rv0731c	
160	Rv1617	Rv0732	
161	Rv1627c	Rv0750	
162	Rv1630	Rv0764c	
163	Rv1633	Rv0766c	
164	Rv1638	Rv0769	
165	Rv1641	Rv0772	
166	Rv1642	Rv0784	
167	Rv1643	Rv0790c	
168	Rv1654	Rv0793	
169	Rv1655	Rv0801	
170	Rv1657	Rv0810c	
171	Rv1658	Rv0819	
172	Rv1659	Rv0828c	
173	Rv1729c	Rv0837c	
174	Rv1730c	Rv0848	
175	Rv1731	Rv0857	
176	Rv1783	Rv0861c	
177	Rv1793	Rv0865	
178	Rv1821	Rv0869c	
179	Rv1827	Rv0882	
180	Rv1829	Rv0887c	
181	Rv2050	Rv0937c	
182	Rv2062c	Rv0950c	
183	Rv2097c	Rv0952	

Continued

No.	Genome	BioChip	Common
184	Rv2111c	Rv0954	
185	Rv2112c	Rv0956	
186	Rv2115c	Rv0970	
187	Rv2122c	Rv0984	
188	Rv2134c	Rv1008	
189	Rv2146c	Rv1012	
190	Rv2150c	RV1013	
191	Rv2156c	Rv1046c	
192	Rv2166c	Rv1056	
193	Rv2178c	Rv1070c	
194	Rv2193	Rv1080c	
195	Rv2204c	Rv1083	
196	Rv2218	Rv1086	
197	Rv2220	Rv1092c	
198	Rv2222c	Rv1097c	
199	Rv2225	Rv1109c	
200	Rv2241	Rv1112	
201	Rv2244	Rv1113	
202	Rv2245	Rv1124	
203	Rv2247	Rv1132	
204	Rv2256c	Rv1153c	
205	Rv2259	Rv1164	
206	Rv2346c	RV1181	
207	Rv2347c	Rv1202	
208	Rv2362c	Rv1208	
209	Rv2367c	Rv1239c	
210	Rv2374c	Rv1257c	
211	Rv2375	RV1259	
212	Rv2402	Rv1263	
213	Rv2404c	RV1264	
214	Rv2406c	Rv1275	
215	Rv2412	RV1282C	
216	Rv2420c	Rv1284	
217	Rv2421c	Rv1292	
218	Rv2426c	Rv1306	
219	Rv2428	Rv1308	
220	Rv2441c	Rv1309	
221	Rv2442c	RV1312	
222	Rv2448c	RV1328	
223	Rv2457c	Rv1371	

Continued

No.	Genome	BioChip	Common
224	Rv2460c	Rv1373	
225	Rv2461c	Rv1377c	
226	Rv2465c	RV1395	
227	Rv2466c	RV1404	
228	Rv2477c	Rv1414	
229	Rv2502c	Rv1427c	
230	Rv2511	Rv1428c	
231	Rv2534c	Rv1448c	
232	Rv2539c	Rv1451	
233	Rv2540c	Rv1458c	
234	Rv2558	Rv1462	
235	Rv2572c	Rv1463	
236	Rv2583c	Rv1476	
237	Rv2592c	Rv1501	
238	Rv2603c	Rv1530	
239	Rv2605c	Rv1531	
240	Rv2606c	Rv1536	
241	Rv2674	Rv1544	
242	Rv2676c	Rv1584c	
243	Rv2692	Rv1604	
244	Rv2697c	Rv1609	
245	Rv2699c	RV1650	
246	Rv2708c	Rv1654	
247	Rv2710	Rv1677	
248	Rv2711	Rv1685c	
249	Rv2713	Rv1692	
250	Rv2720	Rv1693	
251	Rv2725c	Rv1695	
252	Rv2733c	Rv1708	
253	Rv2744c	Rv1710	
254	Rv2754c	Rv1717	
255	Rv2764c	RV1718	
256	Rv2783c	Rv1719	
257	Rv2788	Rv1724c	
258	Rv2795c	Rv1742	
259	Rv2831	Rv1770	
260	Rv2840c	Rv1791	
261	Rv2861c	Rv1806	
262	Rv2868c	Rv1828	
263	Rv2882c	Rv1829	

Continued

No.	Genome	BioChip	Common
264	Rv2890c	RV1837c	
265	Rv2901c	Rv1848	
266	Rv2904c	Rv1875	
267	Rv2909c	Rv1876	
268	Rv2911	Rv1893	
269	Rv2919c	Rv1894c	
270	Rv2927c	Rv1896c	
271	Rv2965c	Rv1898	
272	Rv2975a	RV1912C	
273	Rv2987c	Rv1936	
274	Rv3003c	Rv1959c	
275	Rv3009c	Rv1968	
276	Rv3011c	Rv1988	
277	Rv3012c	Rv1992c	
278	Rv3028c	Rv2021c	
279	Rv3029c	Rv2042c	
280	Rv3043c	Rv2043c	
281	Rv3048c	Rv2049c	
282	Rv3051c	RV2089C	
283	Rv3053c	Rv2098c	
284	Rv3102c	Rv2099c	
285	Rv3105c	Rv2102	
286	Rv3117	Rv2104c	
287	Rv3118	Rv2107	
288	Rv3146	Rv2115c	
289	Rv3148	Rv2130c	
290	Rv3150	Rv2135c	
291	Rv3155	Rv2140c	
292	Rv3219	Rv2145c	
293	Rv3221c	Rv2158c	
294	Rv3240c	Rv2163c	
295	Rv3246c	Rv2195	
296	Rv3248c	Rv2198c	
297	Rv3270	Rv2215	
298	Rv3280	Rv2226	
299	Rv3303c	Rv2229c	
300	Rv3318	Rv2233	
301	Rv3319	Rv2234	
302	Rv3339c	Rv2239c	
303	Rv3340	Rv2241	

Continued

No.	Genome	BioChip	Common
304	Rv3356c	Rv2258c	
305	Rv3362c	Rv2293c	
306	Rv3368c	Rv2299c	
307	Rv3370c	Rv2321c	
308	Rv3396c	Rv2324	
309	Rv3409c	Rv2346c	
310	Rv3410c	Rv2350c	
311	Rv3411c	Rv2351c	
312	Rv3412	Rv2360c	
313	Rv3418c	Rv2368c	
314	Rv3432c	Rv2371	
315	Rv3436c	Rv2374c	
316	Rv3442c	Rv2376c	
317	Rv3443c	Rv2377c	
318	Rv3457c	Rv2413c	
319	Rv3458c	Rv2431c	
320	Rv3459c	Rv2436	
321	Rv3460c	RV2438C	
322	Rv3461c	Rv2445c	
323	Rv3462c	Rv2452c	
324	Rv3464	Rv2457c	
325	Rv3489	Rv2460c	
326	Rv3501c	Rv2465c	
327	Rv3515c	RV2467	
328	Rv3516	Rv2471	
329	Rv3526	Rv2473	
330	Rv3534c	Rv2477c	
331	Rv3535c	Rv2493	
332	Rv3543c	Rv2499c	
333	Rv3550	Rv2505c	
334	Rv3551	Rv2517c	
335	Rv3553	Rv2518c	
336	Rv3556c	Rv2528c	
337	Rv3557c	Rv2534c	
338	Rv3559c	Rv2546	
339	Rv3560c	Rv2555c	
340	Rv3562	Rv2558	
341	Rv3567c	Rv2561	
342	Rv3568c	Rv2564	
343	Rv3570c	Rv2576c	

Continued

No.	Genome	BioChip	Common
344	Rv3574	Rv2579	
345	Rv3583c	Rv2595	
346	Rv3586	Rv2614A	
347	Rv3592	Rv2631	
348	Rv3596c	Rv2638	
349	Rv3597c	Rv2641	
350	Rv3609c	Rv2654c	
351	Rv3610c	Rv2666	
352	Rv3620c	Rv2680	
353	Rv3628	Rv2684	
354	Rv3648c	Rv2685	
355	Rv3676	Rv2697c	
356	Rv3678A	Rv2703	
357	Rv3692	Rv2711	
358	Rv3708c	Rv2731	
359	Rv3709c	Rv2754c	
360	Rv3710	Rv2775	
361	Rv3715c	Rv2788	
362	Rv3753c	Rv2795c	
363	Rv3783	Rv2806	
364	Rv3789	Rv2830c	
365	Rv3791	Rv2835c	
366	Rv3809c	Rv2837c	
367	Rv3842c	Rv2841c	
368	Rv3856c	Rv2863	
369	Rv3859c	Rv2878c	
		Rv2885c	
		Rv2889c	
		Rv2903c	
		Rv2907c	
		Rv2909c	
		Rv2914c	
		Rv2925c	
		Rv2928	
		Rv2937	
		Rv2944	
		Rv2945c	
		RV2951C	
		Rv2975c	
		Rv2984	

Continued

No.	Genome	BioChip	Common
		Rv2996c	
		Rv3002c	
		Rv3007c	
		Rv3009c	
		Rv3013	
		Rv3019c	
		Rv3020c	
		Rv3022c	
		Rv3024c	
		Rv3028c	
		Rv3042c	
		Rv3050c	
		Rv3061c	
		Rv3071	
		Rv3072c	
		Rv3076	
		Rv3089	
		Rv3106	
		Rv3115	
		Rv3118	
		Rv3132c	
		Rv3160c	
		Rv3169	
		Rv3179	
		Rv3196	
		Rv3198c	
		RV3213C	
		Rv3218	
		Rv3221c	
		Rv3232c	
		Rv3237c	
		Rv3248c	
		Rv3257c	
		Rv3283	
		RV3284	
		Rv3285	
		Rv3300c	
		Rv3309c	
		Rv3311	
		Rv3315c	

Continued

No.	Genome	BioChip	Common
		Rv3321c	
		Rv3322c	
		Rv3341	
		Rv3349c	
		Rv3385c	
		Rv3395c	
		Rv3404c	
		Rv3406	
		Rv3412	
		Rv3416	
		Rv3417c	
		Rv3437	
		Rv3442c	
		Rv3446c	
		RV3455C	
		Rv3457c	
		Rv3465	
		Rv3477	
		RV3503C	
		Rv3519	
		Rv3551	
		Rv3555c	
		Rv3572	
		Rv3575c	
		Rv3583c	
		Rv3600c	
		Rv3609c	
		Rv3614c	
		Rv3628	
		Rv3653	
		Rv3672c	
		Rv3676	
		Rv3688c	
		Rv3703c	
		Rv3710	
		Rv3717	
		Rv3733c	
		Rv3735	
		Rv3749c	
		Rv3755c	

Continued

No.	Genome	BioChip	Common
		Rv3756c	
		Rv3760	
		Rv3768	
		Rv3788	
		Rv3791	
		Rv3799c	
		Rv3836	
		Rv3841	
		Rv3855	
		Rv3862c	
		Rv3872	
		Rv3882c	
		Rv3890c	
		Rv3908	
		Rv3918c	