



# Rv2629 Overexpression Delays *Mycobacterium smegmatis* and *Mycobacteria tuberculosis* Entry into Log-Phase and Increases Pathogenicity of *Mycobacterium smegmatis* in Mice

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**Objective:** The aim of the present study was to explore the potential biological role of Rv2629 in *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*.

**Methods:** Recombinant wild type and mutant Rv2629 strains were constructed. Rv2629 expression was evaluated by real-time PCR and western blot. Microarray and interaction network analyses were used to identify the gene interactions associated with wild type and mutant Rv2629. Bacterial growth was assessed in Balb/c mice infected with wild type and mutant Rv2629 strains using CFU assay and histological analysis of the organs.

**Results:** Overexpression of Rv2629 could delay the entry of the *Mycobacterium tuberculosis* cells into the log-phase, while Rv2629 decreased the number of ribosomes and the expression of uridylylate kinase in *Mycobacterium smegmatis*. The Gene Ontology (GO) and pathway analysis indicated that 122 genes correlated with wild type Rv2629, whereas the Rv2629 mutation led to decrease in the ribosome production, oxidative phosphorylation, and virulence in *Mycobacterium tuberculosis*. Overexpression of Rv2629 slightly enhanced the drug resistance of *Mycobacterium smegmatis* to antibiotics, and increased its survival and pathogenicity in Balb/c mice.

**Conclusion:** It is suggested that Rv2629 is involved in the survival of the clinical drug-resistant strain via bacterial growth repression and bacterial persistence induction.

**Keywords:** Rv2629, *Mycobacterium smegmatis*, *Mycobacterium tuberculosis*, bacterial physiology, dormancy

**Abbreviations:** AA, amino-acid acetyltransferase; ACP, acr-coregulated promoter; ADC, albumin-dextrose-catalase; BCIP/NBT, 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium; CHP, conserved hypothetical protein; FFP, ferritin family protein; FtsZ, filamentous temperature-sensitive protein Z; GO, Gene Ontology; MDR, multidrug resistance; MS, mass spectrometry; SD, standard deviation; TB, tuberculosis; TF, trigger factor; UK, uridylylate kinase.

## INTRODUCTION

*Mycobacterium tuberculosis* (*M. tuberculosis*) infection is the major cause of TB. In 2016, 9287 new TB cases were reported in the United States and the successful elimination of TB in the United States is not expected to be achieved during this century (Doosti-Irani et al., 2016). The successful survival and evolution of *M. tuberculosis* is attributed to a large extent to its ability to persist for long periods within the human body in a latent and/or dormant state (Peddireddy et al., 2017). During latent infection, *M. tuberculosis* bacilli are retained within granulomas, which offers a niche with increased concentration of nitric oxide, low oxygen, and absence of nutrients (Bartek et al., 2009; Trauner et al., 2012). The non-replicating persistent form of *M. tuberculosis* aids the escape of the pathogen from the host defense mechanisms and provides additional advantage to the bacilli as regards the effect of standard anti-TB drugs. This process results in the long term survival of the bacteria for several decades (Voskuil et al., 2003; Keshari et al., 2017).

Genes such as *dosR*, *icl*, *acr* (*hspX*), and *lsr2*, which were demonstrated to be stimulated by hypoxia (Boon and Dick, 2002; Florczyk et al., 2003; Park et al., 2003; Starck et al., 2004; Fu and Fu-Liu, 2007), are expressed at low levels and/or are absent in the log-phase of bacterial growth. These genes were shown to be associated with dormancy of *Mycobacterium* (Boon and Dick, 2002; Park et al., 2003). Among the aforementioned genes, the dormancy survival regulon, regulated by the response regulator DosR, appears to be essential for hypoxic survival in a vast number of mycobacterial species, including *M. tuberculosis* (Park et al., 2003; Domenech et al., 2017), *Mycobacterium bovis* BCG (Alonso et al., 2011), and *Mycobacterium smegmatis* (*M. smegmatis*) (Homolka et al., 2009). DosR was shown to initiate transcription of an array of genes known as the *dosR* regulon, which comprises 48 genes containing the DosR-binding site and an ACP family sequence motif (Florczyk et al., 2003). The expression of these genes allows long-term survival of *M. tuberculosis* under anaerobic conditions in the host (Errey and Blanchard, 2005; Homolka et al., 2009; Alonso et al., 2011).

*Rv2629* is a member of the *dosR* dormancy regulon (Park et al., 2003) due to the presence of an ACP motif predicted by the Gibbs Recursive Sampler and SCAN (Florczyk et al., 2003). The *Rv2629* gene is conserved with regard to its expression in several environmental mycobacteria and related strains of *M. tuberculosis* (Wang et al., 2007). *Rv2629* has been reported to be induced by hypoxia and nitric oxide, while it is upregulated under dormancy conditions (Boon and Dick, 2002; Park et al., 2003; Voskuil et al., 2003; Starck et al., 2004), suggesting an association with the dormant state of *M. tuberculosis* (Starck et al., 2004). *Rv2629* was further identified as a drug-resistant protein by 2D-gel electrophoresis and MS analysis (Wang et al., 2007). A previous study determined the *Rv2629* sequence variations in 58 MDR strains and in 55 strains from a reference collection that represented the major *M. tuberculosis* complex pathogens namely, *M. tuberculosis* Beijing, *M. africanum* West African 1, *M. tuberculosis* (H37Rv;

ATCC 27294), *M. bovis* (ATCC 19210), and *M. africanum* (ATCC 25420) (Homolka et al., 2009). Among these 58 MDR strains, 36 (62%) had the 191A/C mutation (codon 64, Asp to Ala) in *Rv2629* that supported an association with rifampicin resistance (Chakravorty et al., 2008; Homolka et al., 2009). The 191C allele was present in the Beijing strains when the MDR strains were stratified according to different phylogenetic lineages that indicated a classical Beijing IS6110 restriction fragment length polymorphism pattern (Chakravorty et al., 2008; Homolka et al., 2009). The IS6110-restriction fragment length polymorphism (IS6110-RFLP) has been applied for the identification of the Beijing strains based on their hybridization patterns (Alonso et al., 2011). The Beijing strains evade the innate immune response and exhibit high capacity to cause and spread TB in humans compared with other *M. tuberculosis* lineages. Nevertheless, the exact mechanism of this action is yet to be determined (Alonso et al., 2011). Although the biological function of *Rv2629* remains unclear, it is speculated that its association with drug resistance involves maintenance of the persistent state of *M. tuberculosis*.

The present study aimed to investigate the function of the *Rv2629* protein, notably the relationship between *Rv2629* over-expression and bacterial physiological changes.

## MATERIALS AND METHODS

### Strains and Growth Media

*Mycobacterium smegmatis* (MC2 155) and *M. tuberculosis* H37Ra were grown in Middlebrook 7H9 broth or Middlebrook 7H10 agar (DIFCO, United States) supplemented with 10% ADC (BD, United States) and kept in a biosafety P1 laboratory. The media for recombinant *M. smegmatis* and *M. tuberculosis* H37Ra that over-expressed *Rv2629* were supplemented with 50 µg/mL kanamycin, while for recombinant *M. smegmatis* that expressed low levels of *MSMEG\_1130*, the medium was supplemented with 50 µg/mL kanamycin and 0.1% acetamide (w/v). Transformed *Escherichia coli* DH5α was cultured in Luria-Bertani broth and/or agar with the appropriate antibiotic (50 µg/mL kanamycin).

Strains isolated from clinical drug-resistant patients (strains 1570, 934, 945, 1573, 1176, 522, 1219, and 1221, all of the Beijing type) were obtained and cultured in a P2 laboratory at the Shanghai Pulmonary Hospital. All clinical strains included were rifampicin-resistant. Strain 934 was rifampicin (RIF) resistant with a minimum inhibitory concentrations (MIC) of 32 µg/mL. Strains 1570, 945, 1573, 1176, 1219, 522, and 1221 were MDR. All strains were identified and drug-susceptibility was evaluated as previously described (Ramaswamy et al., 2003; Wang et al., 2007). Clinical isolates and *M. tuberculosis* H37Rv were cultured under the same conditions as *M. tuberculosis* H37Ra (Kwon et al., 2017).

### Cell Culture

THP-1 cells were grown in house and were cultured in RPMI-1640 (Gibco, Foster City, CA, United States) supplemented with 10% (w/v) fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin (Gibco). THP-1 cells were maintained at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>.

## Cloning and Transformation

Recombinant plasmids encoding wild type *Rv2629* (*Rv2629<sup>W</sup>*) and the 191C mutant (*Rv2629<sup>M</sup>*) were constructed. The full length *Rv2629* genes were amplified from genomic DNA extracted from H37Rv (for *Rv2629<sup>W</sup>*) and from clinical strains (for *Rv2629<sup>M</sup>* genotype). Recombinant plasmids encoding the antisense strand of *MSMEG\_1130*, an ortholog of *Rv2629* in *M. smegmatis*, was constructed. The amplification was conducted by PCR using the appropriate primers (listed in Supplementary Table S1). The PCR products were digested with the corresponding enzymes (NEB, United States) (Supplementary Table S1) for 3 h at 37°C. Plasmid pMV261 (for over-expression) and/or pACT (an acetamide-inducible expression vector, for reduced expression) (Invitrogen, Waltham, MA, United States) were digested with the same restriction enzymes. The two fragments were ligated, transformed into *E. coli* DH5 $\alpha$  and plated on LB agar containing kanamycin (50  $\mu$ g/mL). Following overnight incubation at 37°C, single colonies were randomly selected and grown in LB broth. The plasmids were isolated from *E. coli* DH5 $\alpha$  culture using an Axygen Miniprep Kit (Axygen, Union City, CA, United States) and confirmed by DNA sequencing.

The recombinant pMV261 and/or pACT plasmids were transformed into the *M. smegmatis* strain MC2 155 (*MS*) and/or *M. tuberculosis* H37Ra, respectively by electroporation (Reed et al., 2007). The study of *M. tuberculosis* genes in recombinant *M. smegmatis* is a recognized approach (Falcone et al., 1995; Daugelat et al., 2003; Andreu et al., 2004; Zimhony et al., 2004; Altaf et al., 2010; Sweeney et al., 2011; Bae et al., 2017; Sun et al., 2017; Yu et al., 2017; Wang et al., 2017). Recombinant *M. smegmatis* over-expressing *Rv2629* was identified as *MSW* (*Rv2629* wild type) and *MSM* (*Rv2629* mutant). Recombinant *M. tuberculosis* H37Ra over-expressing *Rv2629* was identified as *RaW* (*Rv2629* wild type) and *RaM* (*Rv2629* mutant). Recombinant *M. smegmatis* expressing low levels of *MSMEG\_1130* was identified as *MSL*. Recombinant strains with the plasmid pMV261 and/or pACT were constructed (*MSP*, *RaP*, or *MS pACT*) and used as controls. The types of strains with the corresponding genes and plasmids are presented in Supplementary Table S2.

## RNA Extraction and Real Time PCR (RT-qPCR)

Total RNA was isolated from *M. smegmatis* and *M. tuberculosis* using an RNA extraction kit (Omega, United States), according to the manufacturer's instructions. Contaminating DNA was digested with RNase-free DNase I (Omega, United States) for 15 min at 37°C. Prior to further analysis the quality check of the RNA was determined by the estimation of the A260/A280 ratio. For RT-qPCR, cDNA was synthesized according to the manufacturer's instructions (ReverTraAce qPCR RT kit, Toyobo, Japan).

qPCR analysis was conducted using *Rv2629*-specific primers (see Supplementary Table S1) with SYBR Green Real-Time PCR Master Mix (QPK-201) (Toyobo, Japan). 16S rRNA and *Rv2703* (*SigA*) were used as reference sequences. The reaction conditions

were: 94°C for 10 min, followed by 40 cycles of 94°C for 30 s, 56 to 65°C for 45 s, and 72°C for 30 s. A standard curve of copy number (range: 10<sup>1</sup>–10<sup>8</sup>) was constructed. Data were collected using a BIO-RAD iCycler (BIO-RAD, United States). RT-negative (without reverse transcriptase) reactions were used to account for residual DNA, and the copy numbers were normalized to those of 16S rRNA. The normalized values were used to determine the relative fold change in expression. The estimation of the relative expression of each gene was carried out using the ddCT method. The experiments were carried out in triplicate, and the results are expressed as mean  $\pm$  SD.

## Western Blot Analysis

Whole cells were harvested from 20 mL cultures of *M. smegmatis* strains and *M. tuberculosis* isolates. The bacterial pellets were washed twice with 0.02 M PBS, resuspended, and sonicated in 200  $\mu$ L of 0.02M PBS (supplemented with 1 mM PMSF and 10 mM EDTA) and centrifuged. The supernatant was treated with lysis buffer (8 M urea, 2 M thiourea, 140 mM DTT, 0.5% Biolyte pH 4–7, and 4% CHAPs). The protein concentration was estimated using the Bradford assay. Equal amounts of protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12% gel), transferred to a PVDF membrane and detected with anti-*Rv2629* rabbit antiserum (in house preparation) and/or the FtsZ monoclonal antibody (4°C, overnight) followed by a goat anti-rabbit IgG antibody conjugated with alkaline phosphatase (Sigma, United States) (37°C, 1 h). The blots were visualized with BCIP/NBT solution (room temperature, 20 min). The reaction was terminated by addition of deionized water. FtsZ was used as a loading control.

## Minimum Inhibitory Concentration Assay

Minimum inhibitory concentrations assay of recombinant *M. smegmatis* was carried out to detect the relationship between over-expressing *Rv2629* proteins and the susceptibility to anti-TB drugs (streptomycin, isoniazid, rifampicin, ethambutol, ofloxacin, levofloxacin, moxifloxacin, amikacin, kanamycin, and capreomycin). Antibiotic dilutions and 96-well plate preparations (Falcon 3072; Becton Dickinson, Lincoln Park, NJ, United States) were carried out as described by Caviedes et al. (2002). Briefly, *Rv2629* overexpressing strains were grown in 7H9/ADC at 37°C until an OD<sub>600</sub> value of 0.5 was obtained. The bacterial suspensions were diluted 1000 times in 7H9 broth and the final diluted broth was dispensed into each well of a 96-well cell culture plate (100  $\mu$ L/well). The drug storage solutions were diluted to prepare two-fold serial diluted solutions in 7H9 broth and subsequently added to the wells containing the bacterial suspensions. The plate was incubated for 48 h at 37°C. The wells containing bacterial pellets were denoted as the positive wells, whereas the wells containing clear broth were denoted as the negative wells. The minimum drug concentrations of the negative wells were the MICs of the drugs. All the experiments were carried out in triplicate.

## Microarray Analysis

Total RNA from each sample was quantified using the NanoDrop 1000 (Thermo Fisher, United States). RNA integrity was assessed using standard denaturing agarose gel electrophoresis. For microarray analysis, the Agilent Array platform was used according to the manufacturer's standard protocols for sample preparation and microarray hybridization. Briefly, total RNA (1  $\mu$ g) was amplified and transcribed into fluorescent cRNA, following the manufacturer's Quick Amp Labeling protocol (Version 5.7, Agilent Technologies). The labeled samples were hybridized toward the *M. tuberculosis* H37Ra custom Oligo Array (8x15K, Agilent array) and were scanned using the Agilent Scanner G2505B (Agilent Technologies, United States). The Agilent Feature Extraction Software (version 10.5.1.1) (Agilent Technologies, United States) was used to analyze acquired array images. The median normalization and subsequent data processing was carried out using the GeneSpring GX v11.0 software package (Agilent Technologies, United States). Following Median normalization of the raw data, genes of at least one out of nine samples that exhibited flags in Present and/or Marginal ("All Targets Value") were selected for further data analysis. Differentially expressed genes were identified using Volcano Plot filtering. Pathway and GO analyses were conducted in order to reveal the biological functions of this subset of differentially expressed genes. Finally, hierarchical clustering was carried out in order to show distinguishable gene expression profiling among samples.

## Transmission Electron Microscopy

For ultrathin cryo sections, log-phase *M. Smegmatis* cultures were harvested (5 mL). The cells were washed twice with 0.1 M phosphate buffer (PB) and fixed with 2.5% glutaraldehyde in 0.1 M PB (2 h). The log phase was estimated as determined previously (Belanger and Hatfull, 1999). Notably, a culture of *M. smegmatis* mc<sup>2</sup>155 was grown at 37°C, until an OD<sub>600</sub> reading of approximately 0.8 was obtained. The cells were rinsed three times (15 min per wash) with 0.1 M PBS. After fixation in 1% osmium tetroxide (3 h), the cells were rinsed as previously described prior to dehydration with solutions containing increasing concentrations of ethanol and/or acetone at 4°C. The samples were subjected to gradual infiltration with Epon resin (3 days). Subsequently, the samples were solidified by incubation at increasing temperature for a total period of 2 days. Thin sections (70–80 nm) were prepared and stained with 3% uranyl acetate and lead citrate prior to examination under a Philips CM-120 transmission electron microscope (TEM) (Philips, United States).

## Growth Curve

To determine whether *Rv2629* expression levels among strains would lead to observable phenotypic growth and survival differences, recombinant *M. tuberculosis* H37Ra and *M. smegmatis* strains were subcultured thrice in Middlebrook 7H9 broth supplemented with 10% ADC and 0.05% Tween-80 (OD<sub>600</sub> = 0.4). The *M. smegmatis* cultures were diluted when

an OD<sub>600</sub> value of 0.05 was obtained. Aliquots of 50 mL were dispensed in conical flasks and grown under continuous shaking at 220 rpm and aerobic conditions. The growth and survival were assayed by optical density readings (OD<sub>600</sub>) every 3 h following inoculation. The experiments were performed in triplicate.

The growth curve of recombinant *M. tuberculosis* H37Ra was detected using the BACTEC MGIT 960 Mycobacterial Detection System (Becton, Dickinson and Company, United States), as determined by the manufacturer's instructions.

## Infection of THP-1 Cells with *M. tuberculosis* Strains and *M. smegmatis* Strains

THP-1 cells ( $5 \times 10^5$ ) were stimulated with 5 ng/mL PMA (Sigma Chemical Co, St. Louis, MO, United States) for 24 h prior to infection. The infection was conducted using H37Rv, clinical *M. tuberculosis* strain 934, and recombinant *M. smegmatis* in the log-phase at a multiplicity of infection (MOI) value of 1:10 (37°C, 5% CO<sub>2</sub>, 4 h). Extracellular bacilli were removed by washing cells three times with PBS and the cells were cultured for 1 h in complete medium in the presence of 100  $\mu$ g/mL gentamycin. The medium was replaced every 12 h during incubation, and the cells were lysed using 1% Triton X-100 solution at specific time points (0, 24, 48, 72, and 96 h). The precipitates were collected by centrifugation (2000 g, 20 min) for total RNA extraction, as described above. The number of CFU of intracellular mycobacteria and the viable bacteria present in the supernatant were determined on Middlebrook 7H10 agar with 10 % ADC.

## Mouse Infection

The culture of MSP and MSW was conducted in 5 mL of Middlebrook7H9 medium supplemented with 10% ADC and 500 mg sterile 2-mm glass beads at 37°C under continuous shaking. The cultures isolated during the log phase were centrifuged at 500 g in order to remove clumps, and the cells were washed twice with sterile saline. The cell suspension was adjusted to an OD<sub>600</sub> of 0.8, corresponding to a concentration of  $5 \times 10^7$  CFU/mL. Female Balb/c mice (6–8-week-old) were infected intravenously with 0.1 mL of the bacterial suspension. The size of inoculum was confirmed by CFU counts.

The ability of survival and dissemination of each strain *in vivo* was assessed in six mice per group that were sacrificed by cervical dislocation on 0, 3, 6, 9, 12, 15, 20, and 35 days after infection. Lung, spleen, and kidney tissue specimens were weighed and homogenized in saline. The specimens were cultured in Middlebrook 7H10 medium (Becton Dickinson, United States) supplemented with 10% OADC following appropriate dilutions. CFUs were enumerated following 3–4 days of incubation at 37°C. All of the animal procedures were approved by the animal care committee of the Fudan University.

## 2D Gel Electrophoresis and MS Analysis

Whole cell extracts were prepared from 100 mL of cultures of each *M. smegmatis* strain in the log-phase. Bacterial pellets were washed twice and centrifuged (15°C, 8000 rpm, 15 min) in 0.02

M PBS (pH 7.4). The cells were sonicated in 500  $\mu$ L of 0.02 PBS (0.02 M supplemented with 1 mM PMSF and 10 mM EDTA) and centrifuged at 12,000 rpm for 20 min at 4°C. The cells were treated with lysis buffer (8 M urea, 2 M thiourea, 140 mM DTT, 0.5% Biolyte pH 4-7, and 4% CHAPs) for 1 h and centrifuged (4°C, 12,000 rpm, 15 min). The supernatant was harvested and the protein concentration was estimated using the Bradford assay. Total protein (80  $\mu$ g) were loaded on linear pH 4-7 IPG strips (Bio-Rad, United States) that were allowed to rehydrate at 50 V for 13 h. Isoelectric focusing was carried out using a Protean IEF (Bio-Rad, United States). The proteins were separated using 2-dimensional SDS-PAGE (12% gels) and stained with silver nitrate, as reported previously (Scheler et al., 1998; Gharahdaghi et al., 1999). The gels were scanned using Molecular Image FX (Bio-Rad, United States) and analyzed with the PD Quest 6.0 software (Bio-Rad, United States). The proteins of interest were excised from the silver-stained 2D gels and identified by MALDI-TOF-MS (Fu and Fu-Liu, 2007).

## Statistical Analysis

Statistical analysis was conducted using SPSS 11.5 (SPSS Inc., Chicago, IL, United States). Statistical differences of the continuous variables were analyzed by one-way analysis of variance (ANOVA) and the Student–Newman–Keuls test and/or Student *t*-test. The statistical level of significance was set at  $P < 0.05$ .

## RESULTS

### Expression of *Rv2629* in Different Clinical Strains

Previous studies have demonstrated an increase in the expression of *Rv2629* at the transcriptional and/or translational levels induced by hypoxia and nitric oxide in clinical isolates of *M. tuberculosis* (Voskuil et al., 2003; Kassa et al., 2012; Zhang et al., 2014). Thus, we investigated the expression of the *Rv2629* protein and mRNA levels among clinical isolates of *M. tuberculosis*. Quantitative RT-PCR was conducted using cDNA synthesized from total RNA isolated from bacilli from seven clinical strains (1573, 1570, 945, 1176, 1219, 522, and 1221) and the *M. tuberculosis* H37Rv control strain in the log and/or stationary-phases respectively (Figure 1A). The stationary phase was observed notably following 10 days of growth and the bacterial cultures were harvested at 3 h intervals for further analysis. It was observed that the average transcriptional level of *Rv2629* in clinical isolates during the logarithmic phase was approximately two-fold lower than that noted in *M. tuberculosis* H37Rv, while during the stationary phase, this level was higher in the clinical isolates than in the control strain. The expression of *Rv2629* at the protein level was further measured by Western blot analysis (Figures 1B,C). The cytoplasmic fraction of the cell extracts obtained from the clinical isolates indicated a two-fold increase in the stationary phase compared to that noted in *M. tuberculosis* H37Rv. The over-expression of *Rv2629* at both the protein and the mRNA levels in clinical *M. tuberculosis* isolates was related to the

stationary phases of the strains. *Rv2629* exhibited reduced expression during the log-phase, when bacilli were actively duplicating.

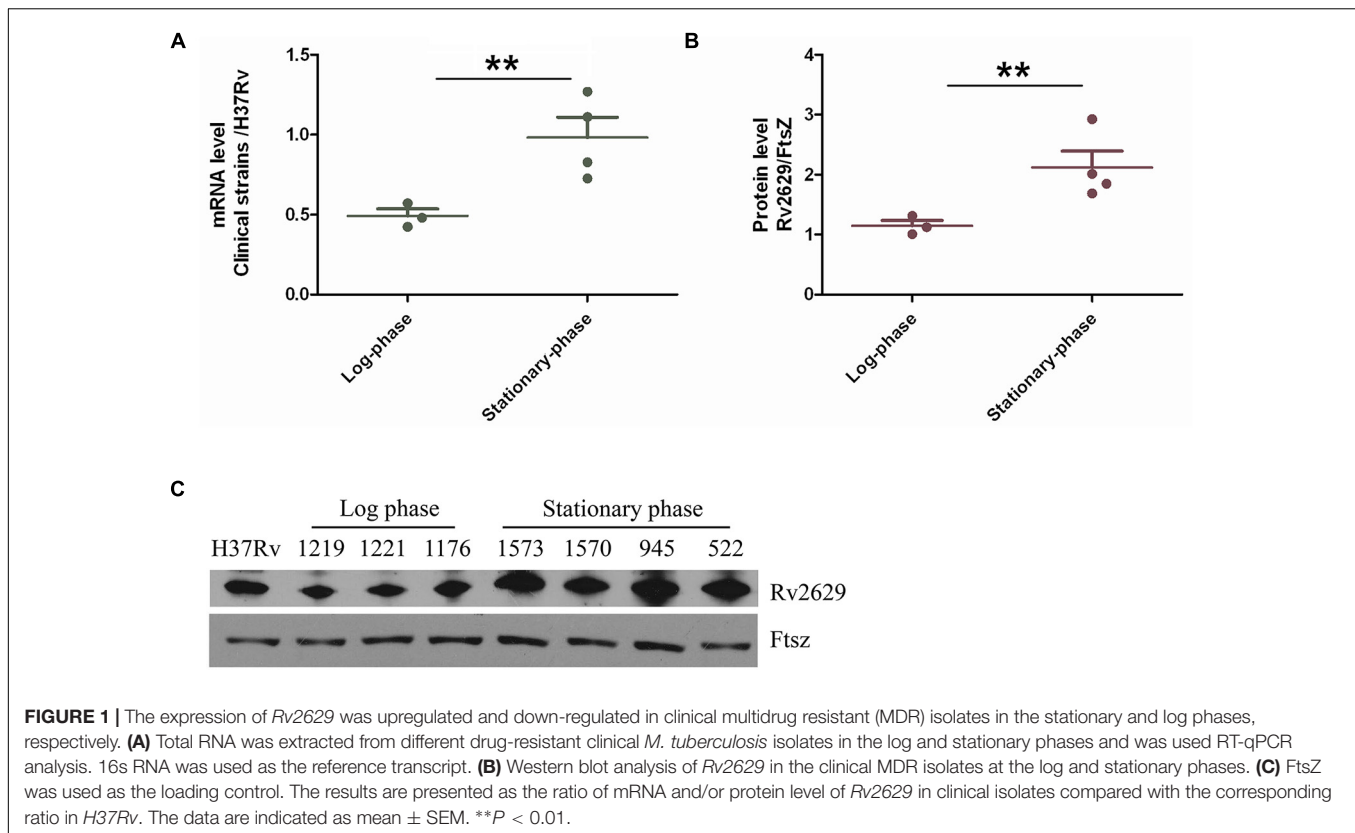
### Expression of *Rv2629* in Clinical *M. tuberculosis* Strain 934 in THP-1 Cells

In order to investigate the association of *Rv2629* with dormancy, the THP-1 human leukemic monocyte cell line was infected with H37Rv and/or a clinical RIF-resistant isolate 934 in the log-phase at MOI = 10, following stimulation with 5 ng/mL PMA for 24 h. The mRNA levels of *Rv2629* expressed in bacteria were measured at 0, 3, 24, and 72 h. Strain 934 was cultured in the absence of THP-1 cells and served as control. *Rv2629* gene intracellular expression was further determined during several time periods (Figure 2). The results indicated that the ratios of the *Rv2629* mRNA levels of strain 934 to H37Rv at 3 and 24 h were higher in the extracellular compared with the intracellular compartment ( $P < 0.01$ ), whereas the opposite was noted for the 72 h period ( $P < 0.001$ ) (Figure 2). Moreover, a high upregulation (45-fold) of intracellular *Rv2629* expression in the clinical strain was observed at 72 h post-infection compared with the extracellular control (Figure 2), supporting the hypothesis that intracellular bacilli can remain in a dormant phenotype up to day 3 by over-expressing *Rv2629*.

### Confirmation of Overexpression and Reduced-Expression of *Rv2629* in *Mycobacteria*

*M. smegmatis* over-expressing *Rv2629*<sup>W</sup> (MSW) and *Rv2629*<sup>M</sup> (MSM) were produced in order to simulate *Rv2629* expression levels in clinical *M. tuberculosis* strains and investigate the function of the *Rv2629* protein. Western blot and RT-qPCR analysis of four *M. smegmatis* strains during the log-phase confirmed the increased expression of *Rv2629* in the MSW and MSM compared with the control MS and MSP strains (Figures 3A,B).

The ortholog of *Rv2629* in H37Ra and *M. smegmatis* strains are *MRA\_2657* and *MSMEG\_1130*, respectively. The DNA and amino acid sequences of *MRA\_2657* are completely identical with *Rv2629*, while *MSMEG\_1130* and *Rv2629* are highly homologous, in which the overlap value between *MSMEG\_1130* and *Rv2629* was 366 based on the data of KEGG database. *M. smegmatis* MSL corresponded to a bacterial strain expressing low levels of *MSMEG\_1130*. The strain was constructed to investigate the function of *Rv2629*, whereas the plasmid pACT, an acetamide-inducible expression vector, served as control. For generating the MSL strain, the plasmid pACT was subjected to recombination with the DNA fragment, whose mRNA production could complement with the *MSMEG\_1130* mRNA sequence reversely and thus could inhibit the expression of *MSMEG\_1130* gene. Western blot analysis in the two *M. smegmatis* strains during log-phase confirmed low expression of *MSMEG\_1130* in MSL compared with control MSpACT (Figure 3C). The use of *M. smegmatis* as a parental strain for the overexpression of *Rv2629* has been reported in a previous study (Wang et al., 2007).

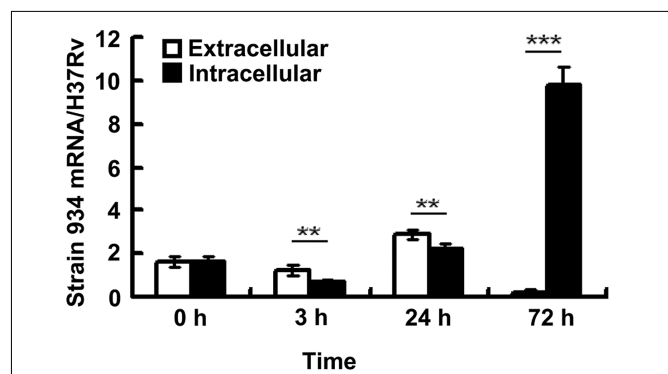


## Minimum Inhibitory Concentration Assay of Recombinant *M. smegmatis*

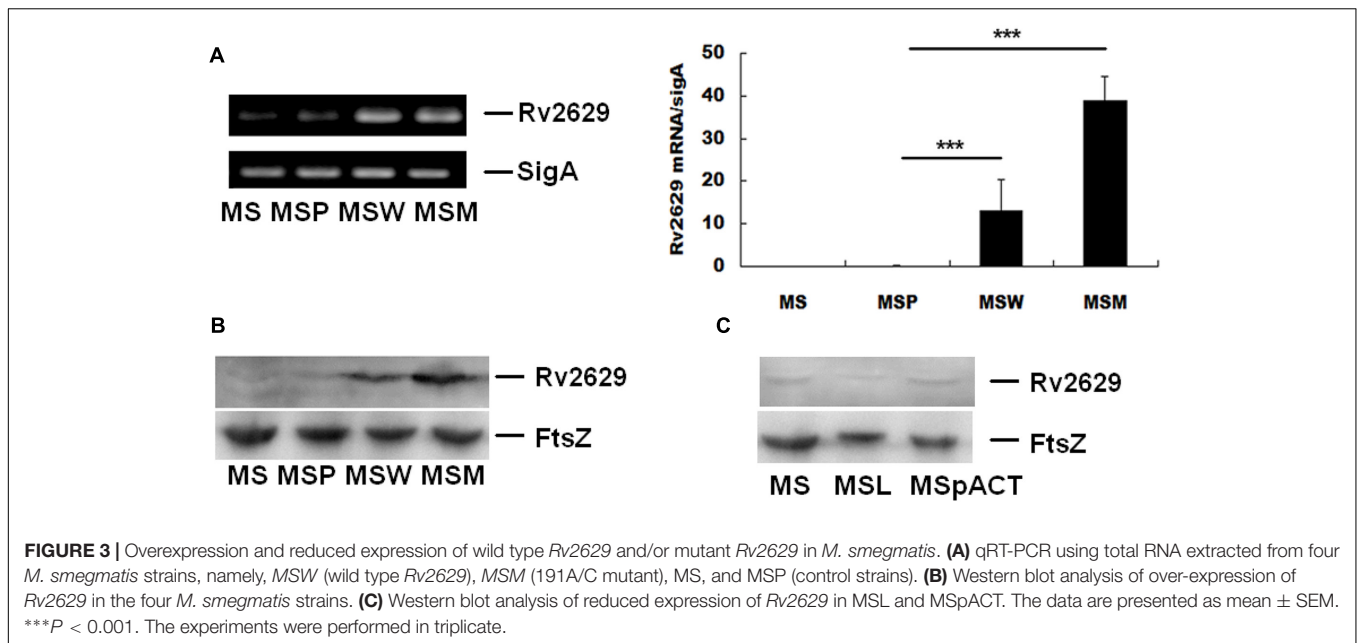
In order to investigate the association of *Rv2629* with drug-resistance, 10 anti-TB drugs were selected including four first-line drugs (streptomycin, isoniazid, rifampicin, and ethambutol) and six second-line drugs (ofloxacin, levofloxacin, moxifloxacin, amikacin, kanamycin, and capreomycin). The MIC of recombinant *M. Smegmatis* was determined. The results are shown in **Table 1**. The *Rv2629* over-expressing strains enhanced drug-resistance of recombinant *M. smegmatis* to ethambutol, moxifloxacin, and levofloxacin at least two-fold compared with the MSP strain.

## Microarray Analysis of *M. tuberculosis* H37Ra Overexpressing *Rv2629*

The present study aimed to investigate the differences in gene expression caused by *Rv2629* overexpression in *M. tuberculosis* strains and the potential interplay in the biological function of the *Rv2629* gene. Microarray analysis was conducted on recombinant *M. tuberculosis* H37Ra overexpressing *Rv2629* in the log phase. The results indicated that recombinant *M. tuberculosis* H37Ra overexpressing *Rv2629* (*RaW*) and H37Ra overexpressing 191 A/C mutated *Rv2629* (*RaM*) remained in the log phase of growth, respectively. In addition, 122 genes correlated with wild type *Rv2629* (Supplementary Table S3), whereas 92 genes correlated with 191A/C mutated *Rv2629*. The genes from the two strains were shown in different sizes (**Figure 4**). As shown in **Figure 4**,



two genes are connected if the correlation between them is greater than a selected threshold (0.9). The correlation is based on a correlation coefficient between each particular gene and the two



**TABLE 1** | Minimal inhibition concentrations (MICs) of recombinant *M. smegmatis* against first- and second-line drug treatment.

	MSW	MSM	MSP
Streptomycin	0.5	0.5	0.5
Isoniazid	4	4	4
Rifampicin	4	2–4	2–4
Ethambutol	0.5	0.5	$\leq 0.25$
Ofloxacin	0.25	0.25	0.25
Levofloxacin	0.25	0.25	$\leq 0.125$
Moxifloxacin	0.125	0.125	$\leq 0.06$
Amikacin	1	1	1
Kanamycin	>32	>32	>32
Capreomycin	4	4	8

modules. A total of 30 genes correlated with wild type *Rv2629* and to a lesser extent with 191 A/C mutated *Rv2629* (Table 2). The correlation coefficient was lower than the selected threshold in *RaM*. The remaining 92 genes correlated with *Rv2629* in the two bacterial strains.

The GO and pathway analysis indicated that 122 genes correlated with wild type *Rv2629*; they were involved in ribosome construction, toluene degradation, arginine and proline metabolism, oxidative phosphorylation, and homologous recombination. The genes were notably related to the ribosome construction and the Fisher  $P$ -value was  $4.28 \times 10^{-10}$  (Supplementary Table S4). The genes that solely correlated with wild type *Rv2629* were notably related to ribosome production, oxidative phosphorylation, and virulence (Table 2). The results indicated that the 191A/C mutation in *Rv2629* could influence the function of wild type *Rv2629* partially. The mutation may lead to the decreased effect of *Rv2629* on ribosome production, oxidative phosphorylation, and virulence.

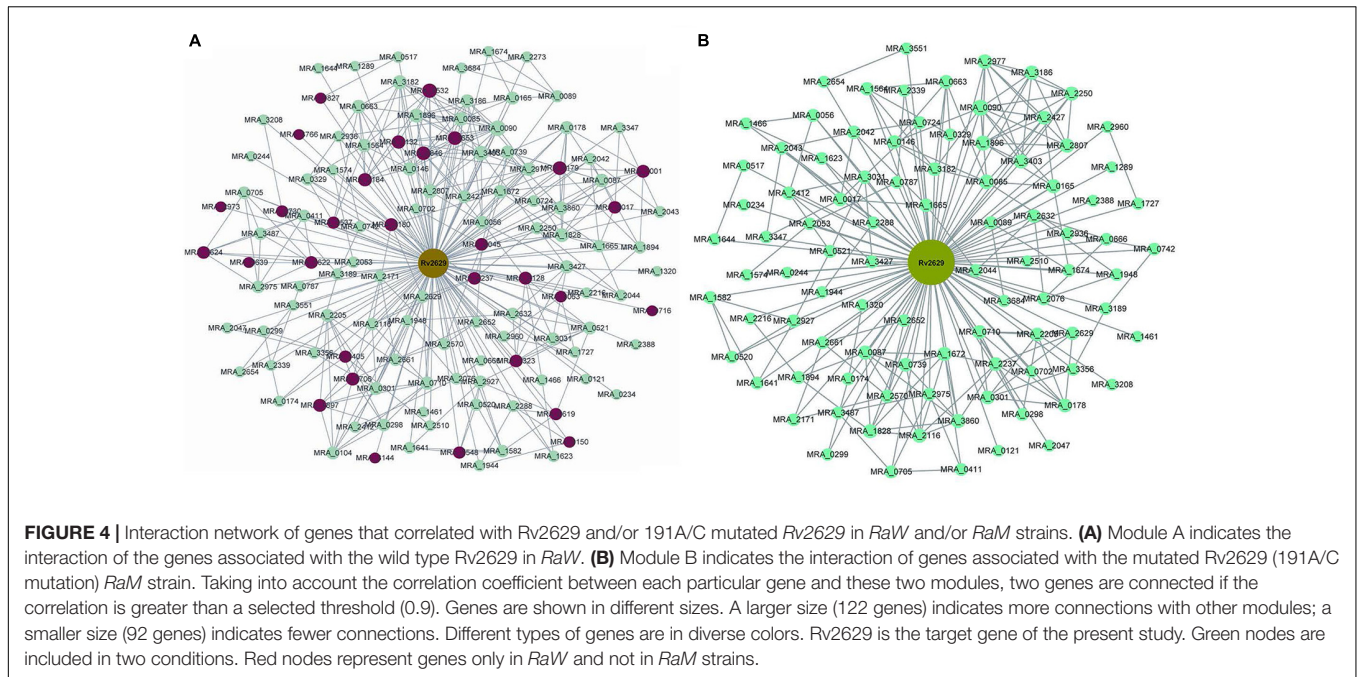
## Reduced Ribosome Content in *M. smegmatis* Overexpressing *Rv2629*

The effects of the *Rv2629* gene on the morphological features of *MSW* and *MSM* at the log-phase were investigated by TEM, as demonstrated by previous studies (Tumminia et al., 1991). No difference was noted in cell wall thickness and morphology, although recombinant *M. smegmatis* *MSW* revealed apparent differences in their cytoplasmic component compared to the *MSM*, *MSP*, and *MS* strains (Figure 5). The cytoplasm of *MSW* cells exhibited lower electron density and the cytoplasmic ribosomal content was reduced significantly compared to the *MS* and *MSP* strains, with the exception of the *MSM* strain (Figure 5).

## Growth Characteristic of Recombinant *M. smegmatis*

The microarray results indicated that *Rv2629* might be related to ribosome production. Consequently, the growth curves of the *MSW* and *MSM* strains were investigated by harvesting cultures at 3-h intervals and by measuring the  $OD_{600}$ . *MS* and *MSP* served as controls. The results of the two independent experiments demonstrated comparatively slow growth of *MSW* (Figure 6A) and indicated that *Rv2629* overexpression inhibits bacterial growth and delays entry in the log phase. The growth of *MSM* was more rapid compared with *MSW* and slower than *MSP* and *MS*, which suggested that the 191A/C mutation of the *Rv2629* gene weakens the inhibitory function of *Rv2629*. A similar effect was noted in the *RaW* and *RaM* strains (Supplementary Figure S1).

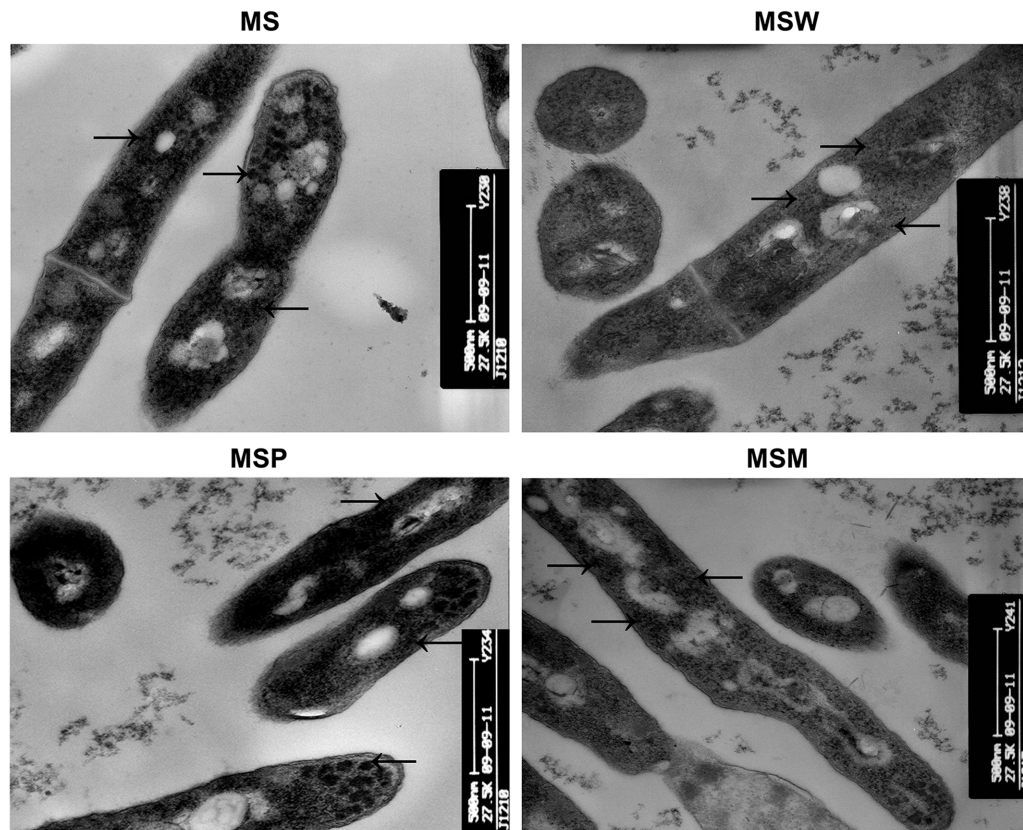
*MSL* strains that expressed low levels of *MSMEG\_1130* were investigated, as described above. *MSpACT* was used as the control. The results of two independent experiments indicated comparatively rapid growth of *MSL* (Figure 6B), while the low



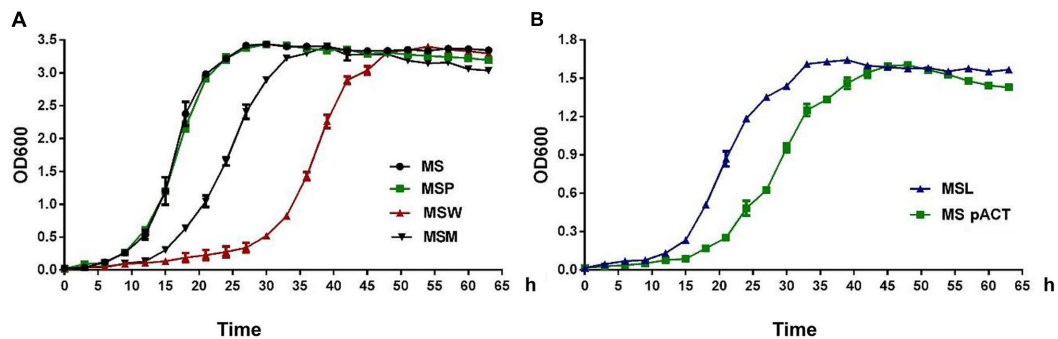
**TABLE 2 |** Thirty genes solely correlated with wild type *Rv2629*.

Probe Name	Protein ID	Annotation	RaW vs. Control	RaM vs. Control	RaW vs. RaM
MRA_3766	YP_001285117.1	Hypothetical protein	Up	Up	Up
MRA_3537	YP_001284884.1	MCE-family protein Mce4C	Up	Down	Up
MRA_3532	YP_001284879.1	Mce associated protein	Up	Up	Up
MRA_3405	YP_001284751.1	Hypothetical protein	Up	Up	Up
MRA_3184	YP_001284529.1	NADH dehydrogenase subunit G	Up	Up	Up
MRA_3180	YP_001284525.1	NADH dehydrogenase subunit C	Up	Up	Up
MRA_3179	YP_001284524.1	NADH dehydrogenase subunit B	Up	Up	Up
MRA_3144	YP_001284489.1	Molybdenum cofactor biosynthesis protein D1	Up	down	up
MRA_3063	YP_001284407.1	Glycosyltransferase	Up	Up	Up
MRA_2973	YP_001284316.1	Putative polyketide synthase Pks1	Up	Up	Up
MRA_2846	YP_001284187.1	Hypothetical protein	Up	Up	Down
MRA_2273	YP_001283597.1	Putative secreted protein	Up	Up	Up
MRA_2128	YP_001283451.1	Putative integral membrane protein	Up	Down	Up
MRA_2045	YP_001283367.1	Hypothetical protein	Up	Down	Up
MRA_1897	YP_001283215.1	Secreted antigen 85-B FbpB	Up	Down	Up
MRA_1706	YP_001283017.1	Hypothetical protein	Up	Up	Up
MRA_1653	YP_001282961.1	50S ribosomal protein L35	Up	Up	Up
MRA_1624	YP_001282932.1	Prolipoprotein diacylglyceryl transferase	Up	Up	Up
MRA_1622	YP_001282930.1	Tryptophan synthase subunit beta	Up	Up	Up
MRA_1323	YP_001282626.1	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	Up	Up	Up
MRA_1132	YP_001282432.1	Putative peroxidase BpoB	Up	Down	Up
MRA_1001	YP_001282298.1	Molybdopterin biosynthesis protein MoeA1	Up	Up	Up
MRA_0827	YP_001282118.1	Hypothetical protein	Up	Up	Up
MRA_0730	YP_001282020.1	50S ribosomal protein L30	Up	Up	Up
MRA_0716	YP_001282006.1	50S ribosomal protein L16	Up	Up	Up
MRA_0639	YP_001281925.1	Exodeoxyribonuclease V subunit beta	Up	Up	Up
MRA_0619	YP_001281905.1	Hypothetical protein	Up	Up	Up
MRA_0548	YP_001281833.1	Putative integral membrane protein	Up	Up	Up
MRA_0150	YP_001281430.1	Chloride channel	Up	Up	Up
MRA_0104	YP_001281385.1	Hypothetical protein	Up	Up	Up





**FIGURE 5 |** Transmission electron microscope analysis of ribosomal content. The ribosomal content (arrows) of *MS*, *MSW*, *MSP*, and *MSM* strains. The data are representative of two independent experiments with three samples in each experiment. Five random fields were observed.

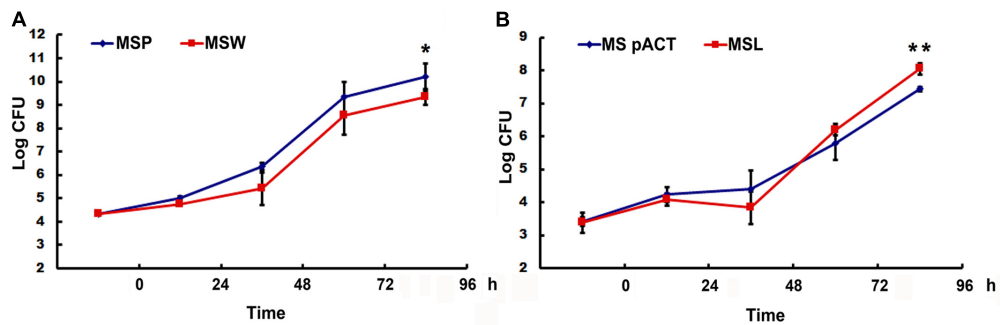


**FIGURE 6 |** The expression of *Rv2629* modulates the growth of *M. smegmatis* under aerobic conditions. The growth curve of recombinant *M. smegmatis* strains that exhibited reduced expression of *Rv2629* and overexpression of the *Rv2629<sup>W</sup>* and/or the *Rv2629<sup>M</sup>* genes, respectively. The growth of the strains was measured by harvesting cultures at 3-h intervals and subsequent measurement of the absorbance values at 600 nm (OD<sub>600</sub>). The abbreviations were: *MS* (wild type), *MSP* (control strain transformed with the pMV261 plasmid), and *MSpACT* (control strain transformed with the pACT plasmid). The results indicated **(A)** comparatively slow growth of *MSW*. **(B)** Comparatively rapid growth of *MSL*. The data are representative of two independent experiments with two samples in each experiment which shows similar results.

expression of *MSMEG\_1130* promoted bacterial growth and entry to the log phase.

The enhanced and/or attenuated *in vitro* growth phenotypes of recombinant *M. smegmatis* prompted the examination of the correlation between *Rv2629* expression and dormant phenotype.

THP-1 cells were infected with the bacteria. No major differences in the intracellular number of viable bacteria were observed at 24 h, suggesting that recombinant *M. smegmatis* strains that expressed high and low levels of *Rv2629* exhibited similar rates of growth to that of control strains within the first day after



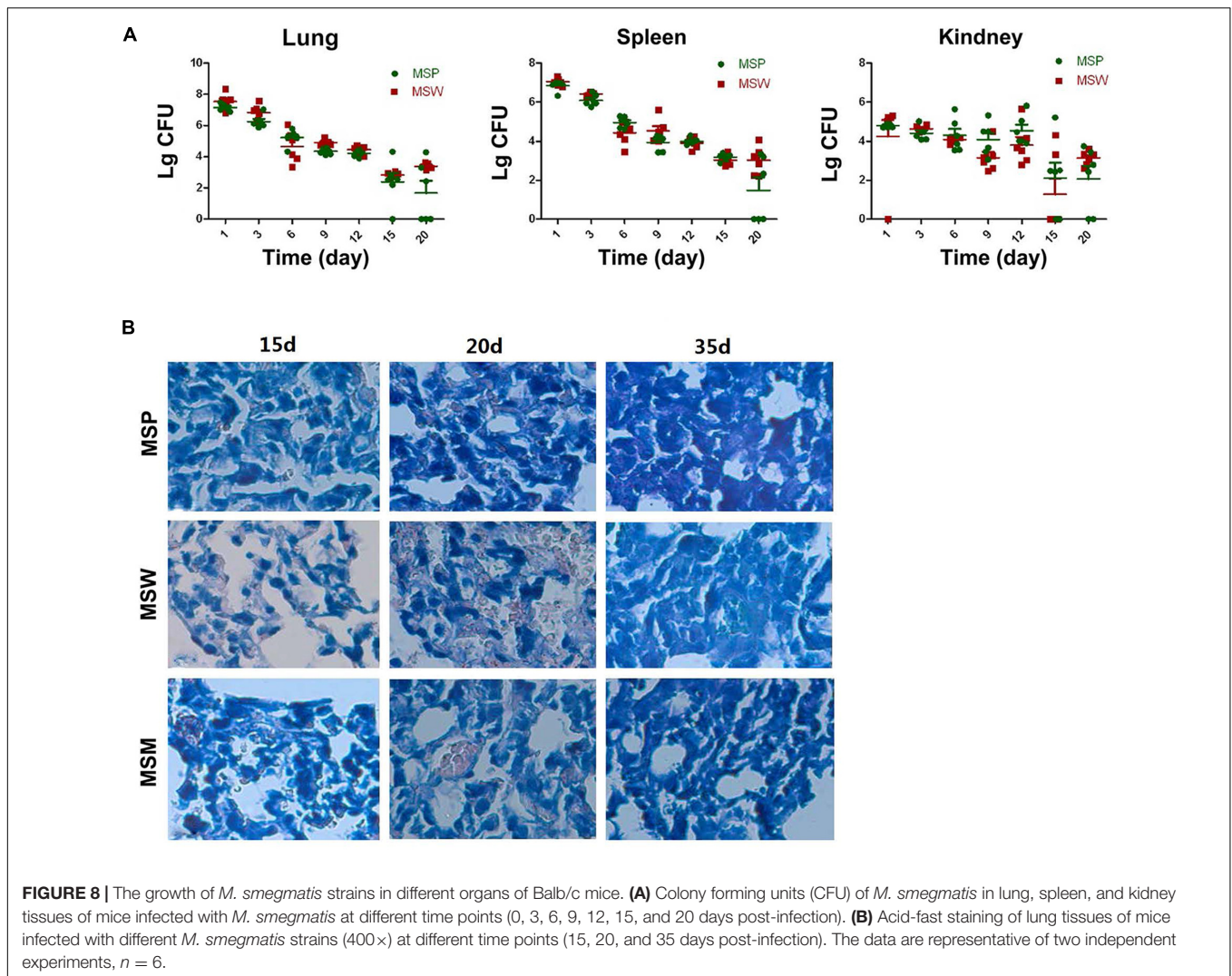
**FIGURE 7 |** The expression of *Rv2629* influences the growth of *M. smegmatis* in human THP-1 cells. **(A)** *M. smegmatis* over-expression of *Rv2629* (MSW) resulted in the slow growth of THP-1 following infection ( $P < 0.01$ , 96 h). **(B)** *M. smegmatis* low-expressing *MSMEG\_1130* (MSL) strain exhibited a rapid growth in THP-1 cells at 96 h post-infection,  $P < 0.01$  (96 h). The data of each time point was analyzed by the Student's *t*-test after homogeneity test of variance,  $n = 4$ . The experiments were performed in triplicate.

the infection (Figure 7A). Nevertheless, the bacterial growth of the over-expressing *Rv2629* strains was impaired at day 2 post-infection, whereas the viable bacilli number at the turn point of growth from lag to the log-phase was decreased. This result is consistent to that found in the *in vitro* experiments. Furthermore, over-expression of *Rv2629* decreased the growth rate of the bacilli and further prevented them from achieving higher cell density in the stationary phase. This conclusion was further confirmed by comparison of the growth capability observed in THP1 cells of low-expressing the *MSMEG\_1130* *M. smegmatis* strains. The bacilli showed increased growth rate compared with the control samples from 48 to 96 h post-infection (Figure 7B). The growth of MSL in THP-1 cells was evaluated at MOI values of 1:5 and 1:20. The results were consistent with the results observed at MOI of 1:10 (Supplementary Figure S2).

## Growth and Pathogenicity of *Rv2629* Overexpressing *M. smegmatis* Strains in Mice

The attenuated reproductive capacity of MSW in macrophage infection prompted us to investigate whether MSW could further decrease the virulence and pathogenicity of host tissues. Balb/c mice were infected with MSP, MSW, and MSM in order to identify the different pathogenicity and survival ability among these strains *in vivo*. The CFU values of bacteria in the lung, spleen, and kidney tissues were counted. The results indicated that the MSW strain exhibited a longer survival time, notably in the lung tissues compared with MSP, while with regard to the MSW strain the detection of the bacterial growth was evident in the lungs of the mice following 20 days of infection (Figure 8A). This observation occurred despite the absence of CFU counts between MSP and MSW at each time point. The acid-fast staining of lung tissue indicated that overexpression of wild type *Rv2629* could promote the survival of *M. smegmatis* in granulomas of the lung. No significant differences among the three strains were noted at the 15th day after infection. The number of MSW strains that overexpressed the wild type *Rv2629* increased significantly compared to the other two strains

in the granuloma of the lung at the 20th day after infection. No visible MSP and/or MSM could be found in the diseased location at the 35th day post-infection, although a high number of MSW strains formed granulomas in the lung (Figure 8B). Additional pathological tests indicated that the pathogenicity of MSW increased and additional damage was caused in the lung tissues compared to the MSP and/or MSM strains. The pulmonary alveolus of mice infected with MSP was damaged slightly and it was the sole cause of bleeding in the bronchial lumen in the absence of granuloma formation during the 15th day following infection. Sporadic granuloma could be found in the small part of the lungs at the 20th day after infection, whereas the inflammatory exudate was absorbed and the main part of the lung recovered to the normal structure 35 days post-infection (Figure 9A). Apparent changes could be observed in the lung of MSW-infected mice. The normal structure of the major part of the pulmonary alveolus disappeared, which was permeated with inflammatory exudate and associated with the infiltration of a great number of inflammatory cells. The alveolar wall indicated congestion and incrustation, whereas apparent granuloma could be found in the lungs 15 days post-infection. The area and degree of damage increased further 20 days post-infection, and the normal structure of the complete part of the pulmonary alveolus disappeared. The structure of the majority of the pulmonary alveolus recovered, although a certain part of granuloma could be found in the lung (Figure 9B). The pathogenicity that was caused by the *Rv2629* expressing strains decreased when a missense mutation (Asp64 to Ala64, A/C) was present at position 191 of the genetic sequence of *Rv2629*. The pulmonary alveolus of mice infected with MSM was damaged partly and sporadic granuloma could be found in certain parts of the lungs 15 days post-infection. The damaged structure of pulmonary alveolus recovered gradually and the majority of the lung tissue recovered to the normal structure at the 20th and the 35th day week post-infection, respectively, as noted for the MSP strain (Figure 9C). Consequently, the overexpression of the wild type *Rv2629* could increase the survival and pathogenicity of *M. smegmatis*, whereas the 191 A/C mutation in *Rv2629* could weaken the pathogenicity of the *Rv2629*-expressing strains.



## Identification of Protein Expression by MALDI-TOF-MS in *M. smegmatis* Overexpressing *Rv2629*

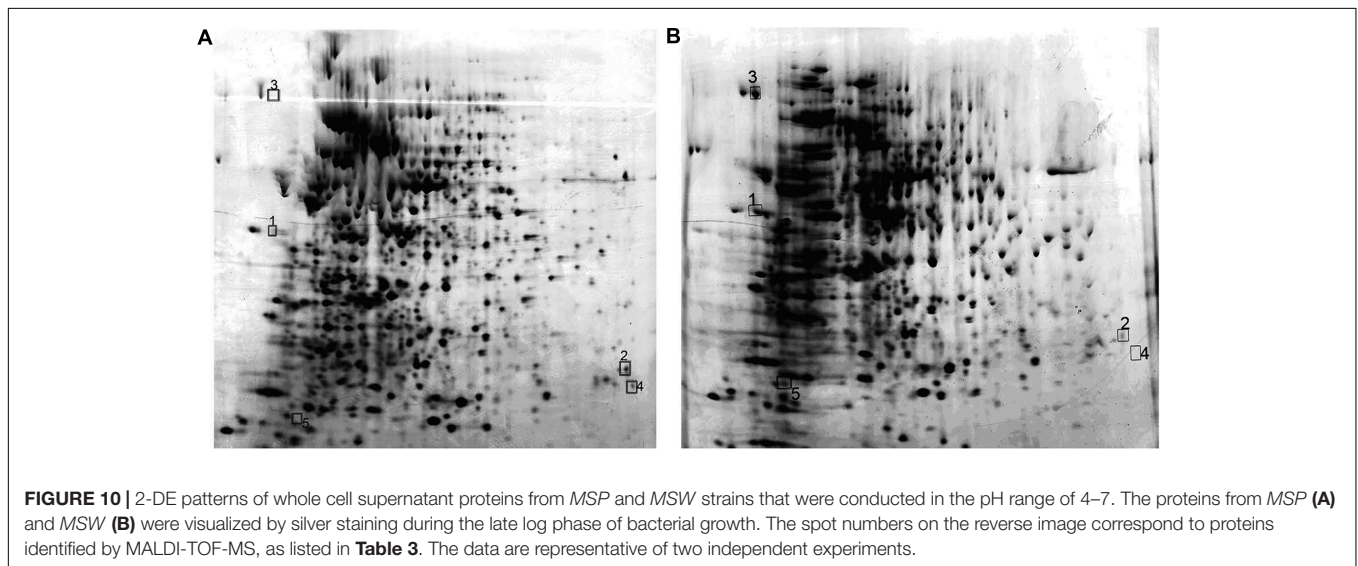
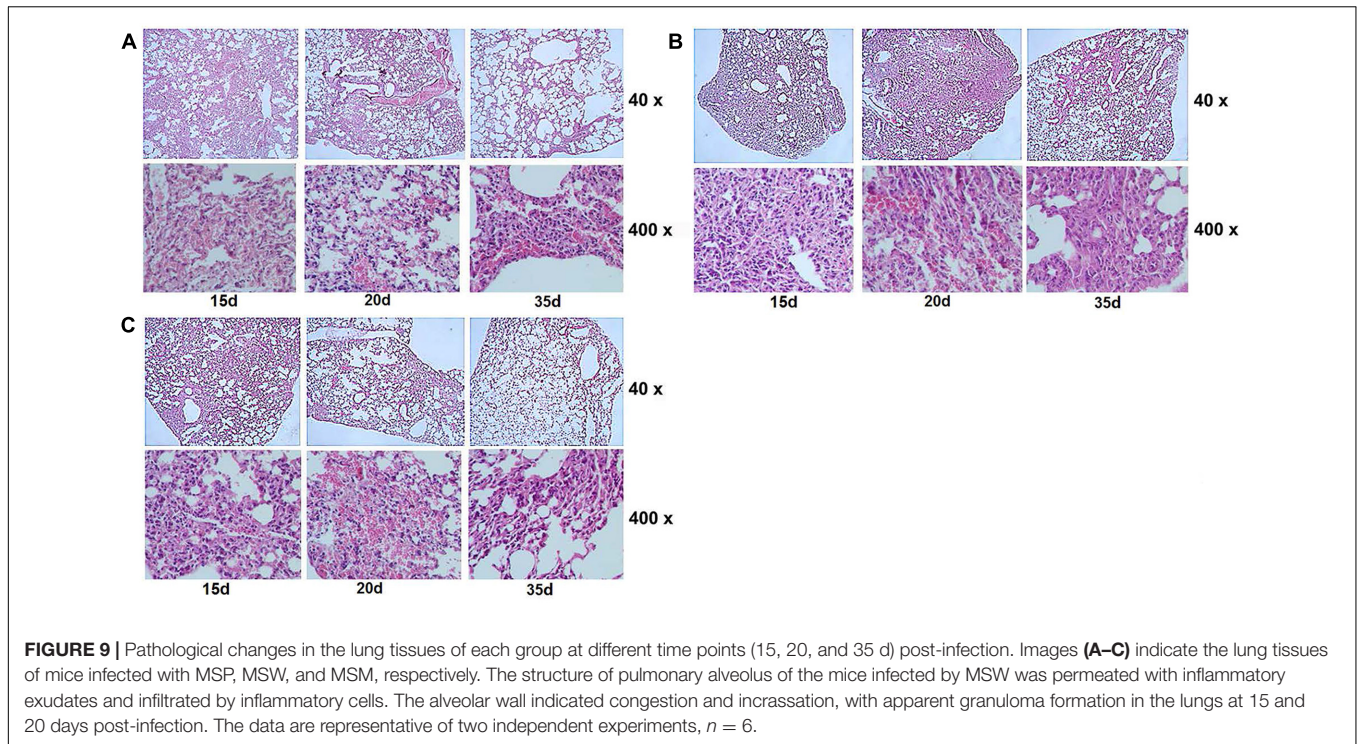
Protein expression maps of *M. smegmatis* overexpressing *Rv2629* were obtained by 2-DE (Figure 10). A total of eight differential proteins were found by comparing the protein differential expressing map on 2-DE gels. A total of five proteins, namely, CHP, UK, TF, AA, and FFP, were identified by MALDI-TOF-MS (Table 3). Nevertheless, the expression pattern of these proteins differed among the strains. TF and FFP were present only on the MSW 2-DE gel, as opposed to the MSP gel. AA was not detected on the MSW 2-DE gel. The expression of UK decreased, while the expression of CHP increased in the MSW compared with the MSP strains.

## DISCUSSION

In the present study, an expression analysis of the *Rv2629* native protein was conducted in recombinant *M. smegmatis* and

*M. tuberculosis* strains and in clinical isolates. In addition, the growth characteristics of recombinant *M. smegmatis*, expressing *Rv2629* wild type and mutant genes were evaluated *in vitro* and *in vivo*. It is suggested that *Rv2629* is correlated with the survival of the clinical drug-resistant *M. tuberculosis* strains via the repression of bacterial growth and the induction of bacterial persistence.

It has long been speculated that a latent and/or dormant state may play a role in drug tolerance of *M. tuberculosis* (Florczyk et al., 2003; Starck et al., 2004; Errey and Blanchard, 2005; Bartek et al., 2009; Peddireddy et al., 2017). The first-line antibiotics used to treat TB are all active against replicating bacteria (Hoffmann et al., 2010) but not against anaerobic dormant bacilli (Ramaswamy et al., 2003; Fu and Fu-Liu, 2007). The fact that the *DosR* regulon is required for long-term survival under anaerobic conditions has led to the notion that *DosR*-regulated genes may contribute to the higher level of *M. tuberculosis* drug tolerance during infection (Boon and Dick, 2002; Florczyk et al., 2003; Ramaswamy et al., 2003; Starck et al., 2004; Errey and Blanchard, 2005; Bartek et al., 2009; Alonso et al., 2011;



Peddireddy et al., 2017). The present study indicated that *Rv2629* expression was decreased in clinical drug-resistant isolates during the log-phase compared with H37Rv, although the expression of the protein was increased during the stationary-phase. It can be speculated that upregulation of *Rv2629* in the stationary-phase is beneficial to the survival of drug-resistant strains *in vivo*, and therefore the protein may be indirectly associated with drug resistance. The drug tolerance observed in the present study may be due to alternative mechanisms, such as the survival of the bacilli in a non-replicating and/or low metabolic state environment.

The present study indicated that overexpression of *Rv2629* may inhibit the biosynthesis of *M. smegmatis* in the lag-phase, during which the synthesis of the enzymes and proteins required for metabolism and rapid reproduction occurs. TEM indicated that the ribosome content was decreased in *MSW* in the log phase compared with the *MSP* strain. Furthermore, 2D-gel electrophoresis indicated a decrease of UK and a deletion of AA proteins in *MSW* compared with the *MSP* strain. The enzymes are known to be involved in pyridine synthesis and arginine metabolism in *M. tuberculosis* (Errey and Blanchard, 2005; Rostirolla et al., 2011). UKs, also known as UMP kinases,

**TABLE 3** | Proteins expressed differentially between *MSW* and *MSP*.

Spot no.	Gene	GI in NCBI <sup>a</sup>	Locus tag <sup>a</sup>	Peptide sequence matched by MS/MS	Protein score	Expression level <sup>b</sup>
1	Conserved hypothetical protein	gi  118174177	MSMEG_2381	AGTLIAAVPLDPALSVAGAER	74	Increased
2	Uridylate kinase	gi  118170082	MSMEG_2540	TNPDAELITAISHR	203	Decreased
3	Trigger factor	gi  118173001	MSMEG_2540	GAVLEQVNDALPSR	318	New
4	Amino-acid acetyltransferase	gi  118172835	MSMEG_5612	AADDGLSFVVR	100	Deletion
5	Ferritin family protein	gi  118170246	MSMEG_6422	DALALALDQER	134	New

<sup>a</sup>GI in NCBI and locus tag according to the complete genome of *M. smegmatis* strain MC2 155 (<http://www.ncbi.nlm.nih.gov>). <sup>b</sup>Expression of identified proteins in *MSW* compared with *MSP*.

are key enzymes in the synthesis of nucleoside triphosphates. The members of the UMP kinase family catalyze the conversion of UMP to UDP, an essential step in the pyrimidine metabolic pathway in a variety of bacteria (Yoshida et al., 2012). In many bacterial genomes, the gene tends to be located immediately downstream of elongation factor T and upstream of ribosome recycling factor. This enzyme has been shown to be essential in bacteria. The product of *pyrH* (*Rv2883*) gene, a member of the UMP kinase family, has been shown to be essential for *M. tuberculosis* growth by the rapid screening method (Lee et al., 2007; Rostirolla et al., 2011). Genetic studies have provided evidence that UMP kinases are essential for the growth of both Gram-negative (e.g., *E. coli*) and Gram-positive bacteria (e.g., *Streptococcus pneumoniae*) (Yamanaka et al., 1992; Fassy et al., 2004). Therefore, it can be speculated that *Rv2629* delays entry in the log-phase by inhibiting protein biosynthesis. Delayed growth under conditions of dormancy is likely to be beneficial for the survival of bacteria in the host, and therefore may contribute to the increased pathogenicity of the strain. Although *Rv2629* has been characterized as a dormancy protein that is induced under anaerobic conditions of *M. tuberculosis*, the main focus of research has been toward the ability of this protein to confer resistance to bacterial strains (Florczyk et al., 2003; Wang et al., 2007). The current study investigates the novel hypothesis that the reduced growth and dormancy-type characteristics of *M. smegmatis* and *M. tuberculosis* are not entirely dependent on antibiotic resistance. The drug tolerance observed in the present study may be due to alternative mechanisms, such as the survival of the bacilli in a non-replicating and/or low metabolic state. Similarly, it has been reported that the phenotypic tolerance acquired in *M. tuberculosis* to isoniazid treatment is via the activation of mycobacterial DNA activating protein (MDP1) and the regulation of *kat G* transcription (Niki et al., 2012).

The aforementioned results supported the functional overlap of *Rv2629* between *M. smegmatis* and *M. tuberculosis*. The overlap between the two strains has been proposed by a previous study (Homolka et al., 2009). The *dosR* gene exhibited a severe survival defect in *M. smegmatis* under hypoxic conditions and this phenotype could be complemented by the *dosR* gene from *M. tuberculosis*, which supported the functional overlap between *DosR* from *M. smegmatis* and *M. tuberculosis* (Boon and Dick, 2002; Park et al., 2003; Homolka et al., 2009).

The pathogenicity of the *Rv2629* overexpressing *M. smegmatis* strain in the tissues of BalB/c mice was investigated and it was

found that the overexpression of *Rv2629* increased the bacterial survival in the lung and spleen organs. The exact cause of this effect remains unknown. Microarray analysis indicated that the mRNA levels of several proteins, namely, MCE-family protein *Mce4C* (MRA\_3537), secreted antigen 85-B *FbpB* (MRA\_1897), and *Mce* associated protein (MRA\_3532), were upregulated in the recombinant strain wild type *Rv2629* compared with the *Rv2629* mutant strain, which might be associated with the increased pathogenicity and dormancy resistance. *Mce4* encodes a cholesterol transporter that is associated with cholesterol metabolism and is essential for mycobacterial persistence. The disruption of *mce4* results in failure of *M. tuberculosis* to maintain chronic infection in mice, while retaining full virulence during the acute phase (Mohn et al., 2008; Pandey and Sasseti, 2008). In contrast to these findings, the MCE family protein and the *Mce*-associated protein are generally considered as virulence factors, and are associated with lipid transport and host cell invasion (Rodriguez et al., 2015; Perkowski et al., 2016). Ag85B may also contribute to the adherence, invasion, and dissemination of organisms in the host tissue, which can bind to Fn and play a critical role in mycobacterial adherence to the host cells (Kassa et al., 2012). Furthermore, Ag85B serves as an important colonization factor potentially contributing to mycobacterial virulence (Kuo et al., 2012). To date, no proteomic studies have been conducted with regard to the *Rv2629* mutant strain of *M. smegmatis* and/or *M. tuberculosis*. The 2D-gel electrophoresis indicated that the proteins TF and FFP were expressed in *MSW*, but not in *MSP* strains. TF is a FK506 binding protein (FKBP)-type peptidyl-prolyl cis-trans isomerase (PPIase) that is considered a highly conserved ribosome-associated chaperone found in a vast number of bacteria (Hoffmann et al., 2010). This protein has been reported to be essential for protein synthesis and survival under conditions of stress (Wu et al., 2011). The present study indicated that overexpression of *Rv2629* induced the expression of TF and FFP and might improve bacterial survival in the persistence state.

The success of the pathogenicity of *M. tuberculosis* is largely attributed to its ability to manipulate the host immune responses. The genome of *M. tuberculosis* encodes multiple immune-modulatory proteins that have been implicated in the pathogenicity of this strain. In agreement with the main concept of the current study, it has been shown that the immune-modulatory protein PE\_PGRS41 boosted the survival of *M. smegmatis* within macrophages and was accompanied by

enhanced cytotoxic cell death via inhibition of apoptosis and autophagy. This indicated that specific bacterial proteins can act as virulence factors that enhance the pathogenic properties of *M. smegmatis* (Deng et al., 2017). To the best of our knowledge the implication of the antigenic protein *Rv2629* in the virulence of *M. smegmatis* has not been reported to date. The number of studies that have examined the contribution of this antigen in the immune response elicited against *M. tuberculosis* is very limited (eight in total). The majority of the studies on *Rv2629* have focused on the contribution of this protein to the immune response elicited against *M. tuberculosis* as well as in the resistance developed against agents such as Rifampicin (Chakravorty et al., 2008; Louw et al., 2009; Niki et al., 2012). In addition, polymorphisms of *Rv2629* have been used as potential markers for the identification process of certain *M. tuberculosis* genotypes (Alonso et al., 2011; Zhang et al., 2014). Consequently, the current study offers novel evidence on the potential interplay of the *Rv2629* protein regarding the survival of *M. tuberculosis*.

Based on KEGG database, the overlap value between MSMEG\_1130 and *Rv2629* was 366. Moreover, Blastn data showed that there are 54% identical nucleotide sequences and Blastp data showed that there are 59% similar or identical amino acid sequences between them. The nucleotide and amino acid sequences of two genes are similar, there was certain homology of nucleotide and amino acid sequences between the two genes. Therefore, MSMEG\_1130 was the most homologous protein with *Rv2629* in *M. smegmatis* strains, with more than 50% amino acids identical or similar with *Rv2629*. To further clarify the problem, other low-expression models such as the MRA\_2657 low-expression H37Ra strain should be further investigated. Furthermore, there was no experimental data supporting the different function of MSMEG\_1130 from *Rv2629*. On the contrary, MSMEG\_1130 might have similar function with *Rv2629* related with the growth of *M. smegmatis*, for our data show MSMEG\_1130 low-expression strain exhibited a rapid growth compared with the control. MSMEG\_1130 was also considered as a protein related with the growth of *M. smegmatis* in other study (Lauten et al., 2010).

## CONCLUSION

The present study strongly suggests that the overexpression of the wild type *Rv2629* protein could delay the growth of the *M. smegmatis* by modifying the expression of specific proteins involved in bacterial metabolism. The overexpression of both WT and mutant *Rv2629* did not affect the susceptibility of *M. smegmatis* to antibiotics, although it increased its survival

and pathogenicity *in vivo*. The findings propose a potential explanation regarding the association of the protein *Rv2629* with the survival of the clinical drug resistant *M. tuberculosis* strain via the repression of bacterial growth and the induction of bacterial persistence *in vivo*. Nevertheless, the biological function and signaling pathway of *Rv2629* warrants further investigation.

## AUTHOR CONTRIBUTIONS

QW and LZ conceived and supervised the study; DL, KH, WW, CP, and YD performed experiments. RJ and WX provided new tools and reagents; HyW and HhW analyzed data and helped to design experiments; DL, QW and LZ wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2017.02231/full#supplementary-material>

**FIGURE S1** | Expression levels of *Rv2629* modulate the growth of *H37Ra* strains under aerobic conditions. The growth curve of recombinant *H37Ra* overexpressing the *Rv2629<sup>W</sup>* (*RaW*) and/or *Rv2629<sup>M</sup>* (*RaM*) genes were measured using the BD BACTEC MGIT 960 *Mycobacterium* growth and detection system. The results are shown as the fluorescence intensity acquired automatically. *RaP* (transformed with the pMV261 plasmid) was used as a control. The results indicated comparatively slow growth of *RaW*. Data are representative of two experiments.

**FIGURE S2** | The growth of MSL in THP-1 cells infected at different multiplicity of infections (MOI). **(A)** The *M. smegmatis* low-expressing MSMEG\_1130 (MSL) strain exhibited a rapid growth in THP-1 cells at 72 and 96 h post-infection, when infected at a MOI value of 1:5,  $P < 0.001$  (72 and 96 h). **(B)** *M. smegmatis* low-expressing MSMEG\_1130 (MSL) strain exhibited a rapid growth in THP-1 cells at 72 and 96 h post-infection, when infected at a MOI value of 1:20,  $P < 0.05$  (72 and 96 h). The data of each time point was analyzed by the Student's *t*-test after homogeneity test of variance,  $n = 4$ .

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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