Identification of *M. tuberculosis* Rv3441c and *M. smegmatis* MSMEG_1556 and Essentiality of *M. smegmatis* MSMEG_1556

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**Abstract**

The normal growth of mycobacteria attributes to the integrity of cell wall core which consists of peptidoglycan (PG), arabinogalactan (AG) and mycolic acids. N-acetyl glucosamine (GlcNAc) is an essential component in both PG and AG of mycobacterial cell wall. The biosynthetic pathway for UDP-N-acetylglicosamine (UDP-GlcNAc), as a sugar donor of GlcNAc, is different in prokaryotes and eukaryotes. The conversion of glucosamine-6-phosphate to glucosamine-1-phosphate, which is catalyzed by phosphoglucosamine mutase (GlmM), is unique to prokaryotes. Bioinformatic analysis showed that Msm MSMEG_1556 and Mtb Rv3441c are homologous to Ec GlmM. In this study, soluble Msm MSMEG_1556 protein and Mtb Rv3441c protein were expressed in *E. coli* BL21(DE3) and their phosphoglucosamine mutase activity were detected. In order to further investigate the essentiality of MSMEG_1556 for the growth of *M. smegmatis*, we generated a conditional MSMEG_1556 knockout mutant, which harbored thermo-sensitive rescue plasmid carrying Mtb Rv3441c. As the rescue plasmid was unable to complement MSMEG_1556 deficiency at 42°C, MSMEG_1556 knockout mutant did not grow. The dramatic morphological changes of MSMEG_1556 knockout mutant after temperature shift from 30°C to 42°C have been observed by scanning electron microscope. These results demonstrated that MSMEG_1556 is essential for growth of *M. smegmatis*. This study provided evidence that GlmM enzyme could be as a potential target for developing anti-tuberculosis drugs.

**Introduction**

Nowadays, two millions deaths each year (2% increased incidence) caused by *Mycobacterium tuberculosis* has been considered to be a major public health threat [1]. However, vaccine failed to provide protective immunity and any efforts to control tuberculosis were compromised as they evolved into stronger, more drug-resistant forms [2,3,4,5]. As we know, the cell wall of all Mycobacterium species is very waxy, hydrophobic, and thicker than other bacteria, the low permeability and resistance of cell wall partially contributes to the defense of adverse factors [6,7]. Thereby, the enzymes involved in the metabolic pathways of the cell wall are potential excellent targets for new anti-tuberculosis drugs [8,9].

The mycobacterial cell wall consists of the mycolate and peptidoglycan (PG) layer held together by arabinogalactan (AG) layer [10,11]. AG is attached to the muramic acid residue of the PG through a disaccharide linker (α-L-rhamnosyl-α-D-N-acetyl-glucosaminosyl-1-phosphate), and the glycan of PG is a disaccharide repeat unit (N-acetylmuramic acid-N-acetyl glucosamine). UDP-N-acetylglucosamine (UDP-GlcNAc) is an important pre-cursor for the synthesis of PG layer, and also a direct glycosyl donor for disaccharide linker, therefore, it plays a critical role in mycobacterial growth [12,13,14]. Three enzymes glutamine fructose-6-phosphate transferase (GlmS), phosphoglucosamine mutase (GlmM), glucosamine-1-phosphate acetyl transferase/N-Acetylglicosamine-1-phosphate uridil transferase (GlmU) involve in the metabolic pathway of UDP-GlcNAc in *E. coli* [13,15,16,17,18]. It is noteworthy that reactions catalyzed by GlmM and GlmU are unique to prokaryotes (Fig. 1). The function of Msm GlmU and Mtb GlmU had been identified and the GlmU had been confirmed being essential for growth of *M. smegmatis* and *M. tuberculosis* [19,20]. Therefore, GlmM may also be essential for mycobacteria and could be used as a potential target of anti-tuberculosis drug.

The bioinformatic analysis data showed that Msm MSMEG_1556 and Mtb Rv3441c are homologous to Ec GlmM. We proposed Msm MSMEG_1556 protein and Mtb Rv3441c protein catalyzing the same reaction in biosynthetic pathway of UDP-GlcNAc as in *E. coli*. In this study, we identified that both Msm MSMEG_1556 and Mtb Rv3441c protein had the phosphoglucosamine mutase activity. The essentiality of

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MSMEG_1556 for mycobacteria was determined by observing growth curve, colony forming unit (CFU) and morphology of MSMEG_1556 knockout mutant. In addition, dinitrothiocyanobenzene (DNTB) chromogenic method [20,21] in microtiter plate for the measurement of phosphoglucosamine mutases activity was described.

Materials and Methods

Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this study were listed in Table S1. E.coli DH5α, NovaBlue and BL21(DE3) cells were grown in Luria-Bertani (LB) medium at 37°C routinely. M. smegmatis mc2155 strain was grown in LB broth containing 0.05% Tween 80 or on LB agar at 37°C routinely. M. smegmatis mc2155 was used for cloning MSMEG_1556 gene with its upstream region and constructing a conditional M. smegmatis MSMEG_1556 gene knockout strain by allelic exchange recombinant experiments. Sucrose was added to the LB agar at final concentration of 10% when required. Antibiotics were added at the following final concentrations: ampicillin (Amp), 100 μg/ml; gentamicin (Gen), 5 μg/ml for E. coli; kanamycin (Kan), 50 μg/ml for E. coli and 25 μg/ml for M. smegmatis; streptomycin (Str), 25 μg/ml for E. coli and 12.5 μg/ml for M. smegmatis.

Sequence alignment of Ec GlmM, Msm MSMEG_1556 and Mtb Rv3441c

The amino acid sequences of Ec GlmM protein, Msm MSMEG_1556 protein and Mtb Rv3441c protein were aligned by using Multalin online tool [http://multalin.toulouse.inra.fr/multalin/] [22].

Expression and purification of Ec GlmM protein, Msm MSMEG_1556 protein, Mtb Rv3441c protein, and Mtb GlmU protein

The genomic DNA of E. coli BL21(DE3) was prepared as described previously [23], with modification. Ec glmM gene was amplified from E. coli BL21(DE3) genomic DNA using the Ec glmM1 and Ec glmM2 primers (Table 1) and was cloned into pET1.2/blunt vector to generate a plasmid pKJ3 (pET-Ec glmM) (Table S1). The genomic DNA of M. smegmatis was amplified from M. smegmatis mc2155 genomic DNA by PCR using Msm glmM1 and Msm glmM2 primers (Table 1) and was cloned into pMD18-T vector to generate pLS1 (pMD18-Mtb Rv3441c) plasmid (Table S1). After confirmation by DNA sequencing, the Ec glmM was ligated into the NcoI and BamHI sites of pCold II and the Msm glmM was ligated into the NdeI and EcoRI sites of pCold II for overexpression of Ec GlmM protein, Msm MSMEG_1556 protein and Mtb Rv3441c protein, respectively.

Table 1. Primers used in this study.

| Primers | Primer sequences (5'→3') |
|---------|--------------------------|
| Ec glmM1 | GCCCATGGTATAGTGAATCTGGAATATTTTCG (underlined is Ncol site) |
| Ec glmM2 | TGAGATCTTAAATGGTATGGTATGATGAAAGGGCTTTTTACTGATCGCGG (underlined is BamHI site) |
| Msm glmM1 | TCTATGCGAGTCTGCATGCCTGCCAC (underlined is Ndel site) |
| Msm glmM2 | CGGAATTCATCTCGAGACGACTCAC (underlined is EcoRI site) |
| Mtb glmM1 | GGCGCATTGATGGCTGCACTCGTGGGCAC (underlined is Ndel site) |
| Mtb glmM2 | TAATGGAATGTCATTGCACGGCCGCTGCAAG (underlined is BamHI site) |
| Msm glmM3 | GACTAGTGTTGCTCTCGAAGACGTTGGCCATCGG (underlined is SpeI site) |
| Msm glmM4 | TGCCCGCCGCCGCTTATCCCTGACGATCAGAAATT (underlined is NotI site) |
| Msm glmM5 | ACTGGGGACCGAGGAGTGC |
| Msm glmM6 | GTCGGCGACATCTCCCGC |

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**Figure 2. Assay of phosphoglucomutase.** Phosphoglucomutase catalyzes the conversion of GlcNH$_2$-6-P to GlcNH$_2$-1-P. GlcNH$_2$-1-P and acetyl CoA were catalyzed by glucosamine-1-phosphate acetyltransferase activity of GlmU to produce GlcNAC-1-P and CoA-SH which was detected by DTNB. TNB, the product generated from the reaction of CoA-SH and DTNB, could be monitored at 405 nm. doi:10.1371/journal.pone.0042769.g002

_E. coli_ BL21(DE3) strains carrying different expression plasmids were grown at 37°C in 300 ml LB broth containing Amp. When OD$_{600}$ of the culture reached to 0.5, BL21(DE3)/pKJ4 culture was induced by 0.4 mM isopropyl-D-thiogalactopyranoside (IPTG) at 37°C for 3 hours; BL21(DE3)/pKJ2 and BL21(DE3)/pLS2 was transferred to a 15°C-incubator and induced by 1 mM IPTG for 24 hours. The cells were then harvested and resuspended in 8 ml lysis buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl and 20% glycerol) with 1 mM phenylmethyl-sulphonyl fluoride (PMSF) followed by sonication. After centrifugation at 20,000 g for 30 minutes, the resulting supernatant was loaded onto 1 ml Ni-NTA column (Qiagen) previously equilibrated with equilibrium buffer. The column was washed with 20 ml wash buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 20% glycerol and 45 mM imidazole). The purified protein was eluted with 15 ml elute buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 20% glycerol and 200 mM imidazole) with 1 mM PMSF, and the first 7 ml was collected for analyses of SDS-PAGE and Western blot as well as detection of phosphoglucomutase mutase activity.

The purified Es GlmM protein, Msm MSMEG_1556 protein and Mtb Rv3441c protein were run on 12% SDS-PAGE and transferred to a nitrocellulose membrane (Pall Corp) in blotting buffer (20 mM Tris-base, 150 mM glycine and 20% methanol). The membrane was blocked with 1% BSA in TBS buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) and incubated with (anti)-polyhistidine clone His-1 antibody (Sigma) at 4°C for 30 minutes, the resulting supernatant was loaded onto 1 ml Ni-NTA column (Qiagen) previously equilibrated with equilibrium buffer. The column was washed with 20 ml wash buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 20% glycerol and 45 mM imidazole). The purified protein was eluted with 15 ml elute buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 20% glycerol and 200 mM imidazole) with 1 mM PMSF, and the first 7 ml was collected for analyses of SDS-PAGE and Western blot as well as detection of phosphoglucomutase mutase activity.

The purified Ec GlmM protein, Msm MSMEG_1556 protein and Mtb Rv3441c protein were run on 12% SDS-PAGE and transferred to a nitrocellulose membrane (Pall Corp) in blotting buffer (20 mM Tris-base, 150 mM glycine and 20% methanol). The membrane was blocked with 1% BSA in TBS buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) and incubated with (anti)-polyhistidine clone His-1 antibody (Sigma) at 4°C for 30 minutes, the resulting supernatant was loaded onto 1 ml Ni-NTA column (Qiagen) previously equilibrated with equilibrium buffer. The column was washed with 20 ml wash buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 20% glycerol and 45 mM imidazole). The purified protein was eluted with 15 ml elute buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 20% glycerol and 200 mM imidazole) with 1 mM PMSF, and the first 7 ml was collected for analyses of SDS-PAGE and Western blot as well as detection of phosphoglucomutase mutase activity.

The purified Ec GlmM protein, Msm MSMEG_1556 protein and Mtb Rv3441c protein were run on 12% SDS-PAGE and transferred to a nitrocellulose membrane (Pall Corp) in blotting buffer (20 mM Tris-base, 150 mM glycine and 20% methanol). The membrane was blocked with 1% BSA in TBS buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) and incubated with (anti)-polyhistidine clone His-1 antibody (Sigma) at 4°C for 30 minutes, the resulting supernatant was loaded onto 1 ml Ni-NTA column (Qiagen) previously equilibrated with equilibrium buffer. The column was washed with 20 ml wash buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 20% glycerol and 45 mM imidazole). The purified protein was eluted with 15 ml elute buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 20% glycerol and 200 mM imidazole) with 1 mM PMSF, and the first 7 ml was collected for analyses of SDS-PAGE and Western blot as well as detection of phosphoglucomutase mutase activity.
Figure 3. Sequence alignment of Ec GlmM, Msm MSMEG_1556 and Mtb Rv3441c. Msm MSMEG_1556 protein was 38% identical to Ec GlmM and Mtb Rv3441c protein was 41% identical to Ec GlmM. Mtb Rv3441c protein and Msm MSMEG_1556 protein had 79% identities. * indicates the homology between all three organisms, # indicates the homology between *M. tuberculosis* and *M. smegmatis*, and ! indicates the homology between *E. coli* and *M. smegmatis* or *E. coli* and *M. tuberculosis*.

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Mycobacterial GlmM

gene knockout strain (Table S1) where Msm MSMEG_1556 gene was knocked out through the second homologues recombination was selected by using Southern blot. To confirm M. smegmatis LS2, the DNA fragment containing Msm MSMEG_1556::kanR and its flanking sequences at upstream and downstream of Msm MSMEG_1556 was amplified from M. smegmatis LS2 genome by using Msm glmM5 and Msm glmM6 primers (Table 1). The amplified PCR products of Msm MSMEG_1556::kanR from M. smegmatis LS2 and Msm MSMEG_1556 from wild type M. smegmatis mc²155 were distinguished by 1% agarose gel.

Southern blot analysis

The MSMEG_1556 DNA probe was prepared by using DIG High Prime Labeling and Detection Starter Kit I (Roche). The pLS3 was digested by SpeI and NotI and Msm MSMEG_1556 was purified. One microliter of the purified Msm MSMEG_1556 was used as a template to generate probe. The genomic DNA isolated from different colonies was digested overnight by SmaI was used as a template to generate probe. The genomic DNA was purified. One microliter of the purified Msm MSMEG_1556 DNA fragment containing Msm MSMEG_1556::kanR from M. smegmatis LS2, M. smegmatis mc²155 carrying the rescue plasmid pLS7 alld wild type M. smegmatis mc²155 were used as controls. The OD₆₀₀ of the cultures was monitored at interval of 24 h and the growth curves at both 30°C and 42°C were obtained.

To measure CFU, M. smegmatis LS2 was grown in LB medium containing 0.05% Tween 80 and appropriate antibiotics, and the cells were incubated at 30°C and 42°C respectively. M. smegmatis mc²155 was amplified from plasmid pLS7 and wild type M. smegmatis mc²155 were used as controls. The OD₆₀₀ of the cultures was determined at the interval of 24 h for CFU determinations (taken at the same time points), dilutions were spread on LB agar plates containing Str and Kan. The plates were incubated at 30°C before CFU were counted.

Expression of MSMEG_1556 protein in M. smegmatis LS2 strain

Western blot was used to analyze the expression levels of GlmM protein in M. smegmatis LS2. M. smegmatis LS2 was grown in 500 ml LB broth at 30°C, and then switched to 42°C when OD₆₀₀ of the culture was 0.01. M. smegmatis LS2 kept growing at 30°C was as control. The OD₆₀₀ was determined at the interval of 24 hours. For CFU determinations (taken at the same time points), dilutions were spread on LB agar plates containing Str and Kan. The plates were incubated at 30°C before CFU were counted.

Morphology of M. smegmatis LS2 strain after shifting temperature from 30°C to 42°C

M. smegmatis LS2 was grown in 20 ml LB broth at 30°C, and then switched to 42°C when OD₆₀₀ of the culture was 0.01. M. smegmatis LS2 kept growing at 30°C was as control. The cells were harvested after shifting temperature for 72 h, 120 h and 192 h, respectively. Scanning electron microscopy (SEM) samples were prepared as previously described [19]. Briefly, the cell pellet was washed three times in the 0.1 M Phosphate buffer (pH 7.4) and fixed with 2.5% glutaraldehyde followed by fixation with 1% OsO₄. The cells were then dehydrated in a graded series of ethanol (20, 40, 60, 70, 80, 90, and 100%). After critical point dry the cells were applied to a silicon wafer slide and coated by gold.

| Table 2. Specific phosphoglucomamase mutase activity of GlmM proteins. |
|-----------------|---------------------|
| Proteins        | Specific enzyme activity of (nmol·min⁻¹·mg⁻¹) |
| Purified Ec GlmM| 156                 |
| Purified Msm MSMEG_1556| 145               |
| Purified Mtb Rv3441c| 151               |

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The cells were then observed by using a JSM-6360 at 15 kV of accelerating voltage.

Results

Msm MSMEG_1556 and Mtb Rv3441c were homologous to Ec GlmM

The bioinformatics analysis data showed that Msm MSMEG_1556 protein was 38% identical to Ec GlmM and Mtb Rv3441c protein was 41% identical to Ec GlmM. Msm MSMEG_1556 protein and Mtb Rv3441c protein had 79% identities (Fig. 3). The results demonstrated that Rv3441c is the ortholog of GlmM in M. tuberculosis and MSMEG_1556 is the ortholog of GlmM in M. smegmatis.

Soluble Ec GlmM protein, Msm MSMEG_1556 protein and Mtb Rv3441c protein were overexpressed

Soluble Ec GlmM protein, Msm MSMEG_1556 protein and Mtb Rv3441c protein were overexpressed in BL21(DE3) respectively and purified by Ni-NTA affinity chromatography. The purified proteins were analyzed by SDS-PAGE and Western blot. The results showed that purified Ec GlmM protein, Msm MSMEG_1556 protein and Mtb Rv3441c protein had the expected molecular weight of 48.99 kD, 47.99 kD, and 47.31 kD, respectively (Fig. 4).

Msm MSMEG_1556 protein and Mtb Rv3441c protein had phosphoglucomamine mutase activity

The specific enzyme activity of Ec GlmM, which could be as a positive control, was 156 mmol·min−1·mg−1. The specific enzyme activity of Msm MSMEG_1556 protein and Mtb Rv3441c protein were 145 and 151 mmol·min−1·mg−1, respectively. The results demonstrated that both Msm MSMEG_1556 protein and Mtb Rv3441c protein had phosphoglucomamine mutase activity same as Ec GlmM protein (Table 2). In the reaction catalyzed by Mtb Rv3441c protein, the product CoA-SH was also detected by Nova-Pak C18 and its retention time was 9.1 min, which is consistent with CoA-SH standard (data not shown).

Conditional M. smegmatis MSMEG_1556 gene knockout strain LS2 was constructed

Once conditional replication plasmid pLS5 was electroporated to M. smegmatis mc2_155, the MSMEG_1556::kanR in pLS5 made single crossover at upstream or downstream of the MSMEG_1556 locus in M. smegmatis genome of colonies grown at 42°C, resulting in M. smegmatis LS1 mutants which integrated the MSMEG_1556::kanR at the MSMEG_1556 locus through the first homologous recombination. M. smegmatis LS1 mutants were selected by Southern blot (data not shown). The results suggested that MSMEG_1556 was an essential gene. When rescue plasmid pLS7 was electroporated to M. smegmatis LS1 mutant, the colonies grown on LB agar plate containing Kan, Str and Sucrose at 30°C had undergone the second homologous recombination, resulting in M. smegmatis gene knockout. Five M. smegmatis LS2 (MSMEG_1556 gene knockout) strains were selected by Southern blot. All five M. smegmatis LS2 strains showed the expected DNA fragments of 2.12 kb and 2.55 kb (Fig. 5A, 5C). The PCR result indicated that all five M. smegmatis LS2 strains had the expected PCR product of 1.88 kb (Fig. 5D).

MSMEG_1556 gene was essential for mycobacterial growth

Growth curves of M. smegmatis LS2 and M. smegmatis mc2_155 carrying pCG76 at 30°C and 42°C were obtained to determine the essentiality of MSMEG_1556 for the growth of mycobacteria. The results showed that M. smegmatis LS2 was only able to grow at 30°C but not at 42°C, whereas M. smegmatis mc2_155 carrying pCG76 was able to grow at both 30°C and 42°C (Fig. 6A), which was consistent to the growth curve of wild type M. smegmatis mc2_155 (data not shown). To measure CFU, M. smegmatis LS2 was
shifting temperature from 30°C to 0.10 OD, and then the culture was switched to an incubator at 42°C. The OD and CFU over time were obtained as shown (Fig. 6B, 6C). Since Rs3441c protein was produced in M. smegmatis LS2 cells at 30°C, the cells multiplied for the first 24 hours after the temperature shift. Then dramatically, with increased incubation time at 42°C, the CFUs dropped as the amount of Rs3441c protein decreased. These results demonstrated that MSMEG_1556 gene is essential in M. smegmatis.

Rv3441c protein was not detected in M. smegmatis LS2 strain

Western blot was used to analyze the expression levels of Rv3441c protein. (Anti)-MSMEG_1556 antibody performed a cross reaction with Rv3441c protein (Fig. 7A), demonstrated that (anti)-MSMEG_1556 antibody could be used for detect the Rv3441c protein in M. smegmatis LS2 strain. Western blot results showed that when M. smegmatis LS2 was grown at 42°C, the expression of Rs3441c protein was not detected compared to the Rv3441c protein in M. smegmatis LS2 grown at 30°C (Fig. 7B). The MSMEG_1556 in wild type M. smegmatis was also detected. The data indicated that lacking Rs3441c protein in M. smegmatis LS2 had effect on bacterial growth at 42°C.

M. smegmatis LS2 grown at 42°C had a morphological change

The morphology of M. smegmatis mc²155, M. smegmatis LS2 grown at 30°C and M. smegmatis LS2 grown at 42°C were observed by using SEM. The results showed that the cellular shape and size of M. smegmatis LS2 grown at 42°C (switched from 30°C) changed compared to those kept growing at 30°C and wild type M. smegmatis mc²155. As the incubation time extended, M. smegmatis LS2 strain at 42°C became longer shapes, “bulb” heads, rougher cell surface and even lysis (Fig. 8D, 8E, 8F). Whereas, the shape and size of M. smegmatis LS2 strain kept growing at 30°C (Fig. 8A, 8B, 8C) were similar to those of wild type M. smegmatis mc²155 (Fig. 8G).

Discussion

UDP-GlcNAc is an essential common precursor of bacterial cell wall PG and outer membrane lipopolysaccharide, as well as important for the synthesis of the enterobacterial common antigen [13,14]. The pathway for UDP-GlcNAc synthesis in prokaryotes is somewhat different from that in eukaryotes [17]. Three enzymes, glutamine fructose-6-phosphate transferase (GlmS), phosphoglucosamine mutase (GlmM) and glucosamine-1-phosphate acetyl transferase/N-Acetylglicosamine-1-phosphate uridyl transferase (GlmU), catalyzed the formation of UDP-GlcNAc in E. coli have been identified [13,15,16,17,18]. GlmM catalyzes the conversion of GlcNH₂-6-P to GlcNH₂-1-P. The homologs of GlmM were also found in other Gram-negative bacteria e.g. H. influenzae, H. pylori, P. aeruginosa [27,28,29] and Gram-positive bacteria e.g. S. gordonii and S. aureus [30,31]. The glmM gene was essential for cell growth in Gram-negative bacteria (e.g. E. coli). The inactivation of glmM gene in E. coli was followed by various alterations of cell shape and finally cells were lysed [13]. This was most probably due to the progressive depletion of precursors for PG and lipopolysaccharide synthesis. However, the mutation of glmM gene only reduced the growth rate and increase cell autolysis in Gram-positive bacteria [30,32]. Furthermore, the glmM mutation also reduced the...
formation of bacterial biofilm and increased sensitivity to penicillins [30,32].

In mycobacteria, UDP-GlcNAc is an important sugar donor for both formation of disaccharide linker and biosynthesis of peptidoglycan in mycobacterial cell wall, therefore, lacking UDP-GlcNAc could have effect on the structural integration of mycobacterial cell wall and further on their cell morphology. Ec GlmM has been well characterized as phosphoglucosamine mutase to catalyze the second step in the synthesis of E. coli UDP-GlcNAc [13], and Msm MSMEG_1556 protein and Mtb Rv3441c protein have significant homology to Ec GlmM.

To detect the phosphoglucosamine mutase activity of Msm MSMEG_1556 protein and Mtb Rv3441c protein, it is required to acquire soluble protein. Mtb Rv3441c protein was produced by using pET16b vector, unfortunately, Mtb Rv3441c protein was insoluble (data not shown). To avoid the formation of inclusion bodies, a cold-shock expression vector pCold II with the Rv3441c gene was constructed. This vector was designed to perform efficient protein expression utilizing promoter derived from cspA gene, which was one of the cold-shock gene. When the incubation temperature of E. coli host cells was reduced sufficiently, the growth is temporarily halted and almost of protein expression decrease, while expression of a group of proteins called “cold-shock proteins” was specifically induced. A significant overproduction of soluble Mtb Rv3441c protein in E. coli BL21(DE3) was observed. The Msm MSMEG_1556 protein was also produced by using the same protocol.

In our study, we also set up an enzyme assay for detection of phosphoglucosamine mutase activity. DNTB chromogenic method was more convenient and time saving, which facilitates high-throughput inhibitor screening, compared with previous methods by autoradiography and HPLC [13,17].

The activity of both Msm MSMEG_1556 protein and Mtb Rv3441c protein was dependent on the presence of Mg2+. Glc-1,6-diP was also required for activity. The purified proteins exhibited little activity without Glc-1,6-diP, however, phosphoglucosamine mutase activity was remarkably enhanced in the presence of this compound. The phosphorylated phosphoglucosamine mutase was assumed to be active [13]. It is unclear that Glc-1,6-diP is a

![Figure 7. Western blot analysis of proteins from M. smegmatis mc^{-155}, M. smegmatis LS2 at 30°C and M. smegmatis LS2 at 42°C. A. The purified Mtb Rv3441c protein was detected by using (anti)-MSMEG_1556 antibody. The result showed (anti)-MSMEG_1556 antibody had a cross reaction with Rv3441c. M. PageRuler prestained protein ladder; lane 1. the purified Mtb Rv3441c protein. B. Rv3441c protein in M. smegmatis LS2 grown at 42°C and 30°C and MSMEG_1556 protein in M. smegmatis mc^{-155} strain were analyzed by using (anti)-MSMEG_1556 antibody. M. PageRuler prestained protein ladder; lane 1. the Rv3441c protein in M. smegmatis LS2 grown at 42°C was not detectable; lane 2. the Rv3441c protein in M. smegmatis LS2 grown at 30°C was detectable; lane 3. the MSMEG_1556 protein in M. smegmatis mc^{-155} strain was detectable. doi:10.1371/journal.pone.0042769.g007](#)

![Figure 8. Scanning electron micrographs of M. smegmatis LS2 strain after shifting temperature from 30°C to 42°C. M. smegmatis LS2 grown at 30°C was switched to 42°C when OD600 reached to 0.01, and M. smegmatis LS2 strain kept growing at 30°C was as control. The cells were harvested after temperature switched for 72 h, 120 h and 192 h, respectively. The micrographs showed that shape and size of M. smegmatis LS2 strain kept growing at 30°C (A, B, C) were similar to those of wild type strains, whereas the M. smegmatis LS2 strain at 42°C became longer shapes, “bulb” heads, rougher cell surface and even lysis (D, E, F). A, B, C. M. smegmatis LS2 grown at 30°C for 72, 120 and 192 h (10000×); D, E, F. M. smegmatis LS2 grown at 42°C for 72, 120 and 192 h (10000×); H, I. M. smegmatis LS2 grown at 42°C for 120 and 192 h (20000×); G. Wild type M. smegmatis mc^{-155} (10000×). doi:10.1371/journal.pone.0042769.g008](#)
phosphorylating agent or activator. We attempt to co-crystallize Glc-1,6-diP and Mtb Rx3441c protein so as to reveal this mechanism in follow-up research.

Mtb Rx3441c gene (annotated as msc4 in Tuberculist Server) has been proved to be essential for the growth of cells by using Herpesvirus pseudorabies virus transposon site hybridization (TraSH) methodology [33]. To assess the effect of mutated glmM gene on cell growth, morphology, cell wall structure, etc., we used a model mycobacterial strain, M. smegmatis, to construct conditional MSMEG_1556 gene knockout strain LS2 by inserting kan^R cassette. M. smegmatis LS2 was unable to grow at 42°C (non-permissive temperature) since the rescue plasmid carrying Mtb Rx3441c gene could not replicate. It demonstrated that the MSMEG_1556 gene is essential for the growth of M. smegmatis. Furthermore, we found that GlmM protein was not expressed in M. smegmatis LS2 strain. To observe morphological change of LS2 strain, the LS2 cells were harvested after shifting temperature from 30°C (permissive temperature) to 42°C. The morphological analysis of LS2 by SEM revealed that the LS2 cells were longer and had rougher surface compared to the wild type cells. With the increased incubation time at 42°C, many cells fused and lysed eventually. These results suggested that lacking GlmM could block the PG synthesis and make the structure of cell wall changed, resulting in cell death. Therefore, GlmM is a potential target for development of anti-tuberculosis drugs.

Supporting Information

Table S1  Bacterial strains and plasmin used in this study, (DOC)

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Author Contributions

Conceived and designed the experiments: YM SL JK. Performed the experiments: SL JK WY WZ YX. Analyzed the data: SL JK YZ YM. Contributed reagents/materials/analysis tools: YX YM. Wrote the paper: YM SL JK.

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