The Nitrogen Regulator GlnR Directly Controls Transcription of the prpDBC Operon Involved in Methylcitrate Cycle in Mycobacterium smegmatis

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ABSTRACT  Mycobacterium tuberculosis utilizes fatty acids of the host as the carbon source. Metabolism of odd-chain fatty acids by Mycobacterium tuberculosis produces propionyl coenzyme A (propionyl-CoA). The methylcitrate cycle is essential for mycobacteria to utilize the propionyl-CoA to persist and grow on these fatty acids. In M. smegmatis, methylcitrate synthase, methylcitrate dehydratase, and methylisocitrate lyase involved in the methylcitrate cycle are encoded by prpC, prpD, and prpB, respectively, in operon prpDBC. In this study, we found that the nitrogen regulator GlnR directly binds to the promoter region of the prpDBC operon and inhibits its transcription. The binding motif of GlnR was identified by bioinformatic analysis and validated using DNase I footprinting and electrophoretic mobility shift assays. The GlnR-binding motif is separated by a 164-bp sequence from the binding site of PrpR, a pathway-specific transcriptional activator of methylcitrate cycle, but the binding affinity of GlnR to prpDBC is much stronger than that of PrpR. Deletion of glnR resulted in faster growth in propionate or cholesterol medium compared with the wild-type strain. The ΔglnR mutant strain also showed a higher survival rate in macrophages. These results illustrated that the nitrogen regulator GlnR regulates the methylcitrate cycle through direct repression of the transcription of the prpDBC operon. This finding not only suggests an unprecedented link between nitrogen metabolism and the methylcitrate pathway but also reveals a potential target for controlling the growth of pathogenic mycobacteria.

IMPORTANCE The success of mycobacteria survival in macrophage depends on its ability to assimilate fatty acids and cholesterol from the host. The cholesterol and fatty acids are catabolized via β-oxidation to generate propionyl coenzyme A (propionyl-CoA), which is then primarily metabolized via the methylcitrate cycle. Here, we found a typical GlnR binding box in the prp operon, and the affinity of GlnR to prpDBC is much stronger than that of PrpR. Deletion of glnR resulted in faster growth in propionate or cholesterol medium compared with the wild-type strain. The ΔglnR mutant strain also showed a higher survival rate in macrophages. These results illustrated that the nitrogen regulator GlnR regulates the methylcitrate cycle through direct repression of the transcription of the prpDBC operon. This finding not only suggests an unprecedented link between nitrogen metabolism and the methylcitrate pathway but also reveals a potential target for controlling the growth of pathogenic mycobacteria.

KEYWORDS Mycobacterium smegmatis, nitrogen regulator GlnR, methylcitrate cycle, prpDBC operon

Tuberculosis is a chronic bacterial infectious disease, and Mycobacterium tuberculosis is the leading cause from a single infectious agent, ranking above HIV/AIDS. In 2017, there were an estimated 1.3 million tuberculosis (TB) deaths among HIV-negative
Recent research indicated that fatty acids and cholesterol are preferred carbon sources for *Mycobacterium tuberculosis* when growing within animal macrophages (2–5). β-Oxidation of even-chain fatty acids can yield acetyl coenzyme A (acetyl-CoA), while odd-chain fatty acids yield propionyl-CoA as an additional product (6). Degradation of cholesterol also produces acetyl-CoA and propionyl-CoA (7). Further metabolism of acetyl-CoA and propionyl-CoA needs the glyoxylate and methylcitrate cycles, respectively, in mycobacteria. The methylcitrate cycle converts propionyl-CoA to pyruvate at a molar ratio of 1:1 (8–14). In *M. tuberculosis*, the methylcitrate cycle is not only essential for them to metabolize odd-chain fatty acids but also essential for them to grow in murine bone marrow-derived macrophages. Thus, the methylcitrate cycle plays an important role for *M. tuberculosis* to grow, persist, and keep virulence.

Methylcitrate dehydratase (MCD; encoded by *prpD*), methylisocitrate lyase (MCL; encoded by *prpB*), and methylcitrate synthase (MCS; encoded by *prpC*) are specific to the methylcitrate cycle and essential for mycobacteria to grow on propionate as the sole carbon source (4, 8, 9, 11–13, 15). *M. tuberculosis* contains only MCD and MCS (encoded by operon *prpDC*) (16–18), and the activity of MCL is provided by isocitrate lyase 1 (ICL1) and 2 (ICL2) which are two isoforms involved in the glyoxylate pathway. *Mycobacterium smegmatis* contains a *prpDBC* operon encoding the three enzymes involved in the methylcitrate cycle (*msmeg_6645*, *msmeg_6646*, and *msmeg_6647* products). The *prpDIBC* operon plays a key role in assimilation of propionyl-CoA for both *M. tuberculosis* and *M. smegmatis* to obtain carbon and energy and to prevent the accumulation of toxic metabolite (6, 19). However, so far, the regulation mechanism underlying the methylcitrate cycle and the *prpDIBC* operon is not well known. PrpR, a transcriptional regulator, was reported to regulate the methylcitrate pathway by activating the *prp* operon in *M. tuberculosis*, while the *prpR* knockout strain exhibited an inhibited growth (4). In *Salmonella enterica* serovar Typhimurium, PrpR activates the *prp* operon in response to 2-methylcitrate (20).

**RESULTS**

Nitrogen response regulator GlnR binds the promoter region of the *prpDBC* operon. In previous work, we found that the nitrogen regulator GlnR in actinobacteria directly regulated carbon metabolisms, including the uptake and utilization of non-phosphotransferase-system carbon sources (22), degradation of starch (23), synthesis of citrate (24), and synthesis of acetyl-CoA/propionyl-CoA (25, 26). In this study, we found a typical GlnR-binding motif (GGACCGGCACC-GTAAC) was identified at the upstream region of the *prpDBC* operon in *M. smegmatis*. We found that GlnR directly binds to this region and represses *prpDBC* operon transcription. The finding suggests an unprecedented link between nitrogen metabolism and propionyl-CoA assimilation involved in the utilization of fatty acids or cholesterol and provides new insights into the sensing and metabolism of nutrients of mycobacteria in host cells.
GlnR repressed the transcription of prpDBC operon in M. smegmatis. To investigate the regulatory effect of GlnR on prpDBC in vivo, we constructed a glnR-deleted strain (ΔglnR) and the complementary strain (attB::pMV361-glnR) of M. smegmatis. Three strains (wild-type, ΔglnR, and attB::pMV361-glnR strains) were cultured in nitrogen-limited M9 medium. The transcriptional levels of prpD, prpB, and prpC in the three strains were compared. As shown in Fig. 2, the transcriptional level of prpDBC increased significantly in the glnR-deleted strain compared with the wild-type strain. The transcripts were increased 8-fold for prpD, 4-fold for prpB, and 6-fold for prpC in the ΔglnR strain. The complementation of the glnR gene into the ΔglnR strain resulted in normal expression of prpDBC transcripts (Fig. 2). The data showed that GlnR repressed the transcription of prpD, prpB, and prpC in vivo in M. smegmatis.

The transcription of prpDBC is responsive to nitrogen availability. GlnR is a global nitrogen regulator that regulates the transcription of the genes involved in nitrogen metabolism and is responsive to nitrogen availability (26, 27). Therefore, we investigated the transcriptional response of the prpDBC operon under nitrogen-limited (N\(^{-}\)) and nitrogen-rich (N\(^{+}\)) conditions. As shown in Fig. 3, the limitation of nitrogen resulted in a 5-fold increase for glnR but a 73% decrease for prpD, a 65% decrease for prpB, and a 61% decrease for prpC. This result further demonstrated that GlnR directly controls transcription of the prpDBC operon involved in methylcitrate cycle in response to nitrogen availability in M. smegmatis.

GlnR shows higher affinity to prpDBC promoter than PrpR in vitro. A typical GlnR binding motif (GGACC-GGACC-GTAAAC) was found in the upstream region of the prpDBC operon and confirmed by DNase I footprinting assay (Fig. 4A). To verify whether the motif sequence is key for GlnR binding, two biotin-labeled synthetic probes (128 bp) containing the predicted binding motif (P1) and mutant motif (P2) were used for EMSAs. As shown in Fig. 4B and C, no band shift was observed for probe P2, indicating that the predicted
GlnR-binding sequence in the promoter region of prpDBC was directly bound by GlnR. Transcriptional regulator PrpR was reported to directly regulate the prpD(B)C operon in *M. tuberculosis*, *M. smegmatis*, and *S. enterica*. An 8-bp PrpR-binding motif sequence (TTTGCAAA) was identified in *M. tuberculosis* H37Rv. In this work, a PrpR-binding motif (TTTGCAAA)
in the upstream region of prpDBC was found 164 bp downstream of the GlnR binding sequence (Fig. 1A). To determine the binding affinities of these two regulators to the upstream region of prpDBC, Octet assays were performed using proteins with different dilutions. The dissociation constant (K_d) values of GlnR and PrpR for prpDBC are 38.5 and 404 nM, respectively (Fig. 4D and E). These results revealed that GlnR has a 10-fold higher affinity for the promoter region of prpDBC than PrpR.

GlnR affects the growth of M. smegmatis on propanoate or cholesterol. GlnR repressed the transcription of the prpDBC operon, which was involved in the methylcitrate cycle and played an important role in metabolisms of fatty acid and cholesterol for mycobacteria in host cells. It is, thus, reasonable to expect that GlnR can have an effect on the growth of M. smegmatis on fatty acid or cholesterol as carbon source. To investigate the regulatory effect of GlnR on utilization of fatty acid or cholesterol, three M. smegmatis strains (wild type [WT], ΔglnR, and attB::pMV361-glnR) were cultivated respectively on 10 mM propanoate or cholesterol. As shown in Fig. 5A and B, ΔglnR strains grew much better than then wild-type strain both on 10 mM propanoate or cholesterol under nitrogen-limited conditions. The deletion of glnR alleviated GlnR-mediated repression of the prpDBC operon and increased activity of the methylcitrate pathway (assimilation of propanoate and propionyl-CoA).

The resazurin reduction assay was also employed to examine the growth of M. smegmatis strains on propionate or cholesterol (28). Blue compound resazurin, which can be reduced to pink fluorescent product by the metabolically active cells, usually was used to report the cell metabolism activity and cell number. Three M. smegmatis strains (WT, ΔglnR, and attB::pMV361-glnR) were grown in nitrogen starvation M9 medium with propionate or cholesterol as a carbon source. After 36 h, 100 µl of the cultures was transferred to wells on a 96-well plate and resazurin was added. As shown in Fig. 5C, the ΔglnR strain showed a more obvious pink color (reduced resazurin) than the wild type and complementary strain. No reduction of resazurin was observed in control wells (no cells). These observations were consistent with the previous conclusion that GlnR repressed the transcription of prpDBC and inhibited the methylcitrate pathway. The upregulation of prpDBC transcription in the ΔglnR strain resulted in improvement of propional-CoA assimilation for accelerating utilization of propionate or cholesterol.
GlnR affects the survival of *M. smegmatis* in macrophages. Last, we investigated the effect of GlnR on the survival of *M. smegmatis* in macrophages after infection. Equal amounts of *M. smegmatis* WT, Δ*glnR*, and attB::pMV361-glnR strains were used to infect the macrophage, and their viability was determined at different time points. As shown in Fig. 6, the Δ*glnR* strain revealed a similar survival in macrophages with the wild-type strain in the initial 2 h after infection. After 12 h or 24 h, the Δ*glnR* strain had a 2× survival compared with the wild-type and attB::pMV361-glnR strains. These results indicated that GlnR exerted a negative effect on the survival of *M. smegmatis* in macrophages. Taken together, GlnR repressed the methylcitrate pathway and propional-CoA assimilation in response to nutrient signals in macrophages, which affected the viability and infection of *M. smegmatis*.

**DISCUSSION**

In the present study, we identified a new regulator, GlnR (the nitrogen transcriptional regulator), that repressed the transcription of the *prp* operon involved in the methylcitrate cycle in *M. smegmatis*. Our finding reveals an unprecedented link between nitrogen metabolism and propional-CoA assimilation in the utilization of fatty acids and cholesterol and provides new insights into the sensing and metabolism of nutrients of mycobacteria in host cells (Fig. 7).

Fatty acids and cholesterol metabolism are essential for mycobacteria to grow in macrophages and infect human and animals (29). However, the degradation of fatty acids and cholesterol results in accumulation of propionyl-CoA (30–32). Propionyl-CoA is an inhibitor of several key metabolic enzymes, such as pyruvate dehydrogenase, succinyl-CoA synthetase, and ATP citrate lyase, and, thus, is considered as a toxic metabolite. Mycobacteria are highly sensitive to increases in the propionyl-CoA pool, and it has evolved several different mechanisms for the detoxification of propionyl-CoA or assimilation of propionyl-CoA. So far, two pathways have been found for propionyl-CoA assimilation in mycobacteria, namely, the methylcitrate cycle (MCC) that converts propionyl-CoA to pyruvate/succinyl-CoA, and the propionyl-CoA carboxylase (PCC) pathway, which is responsible for the metabolism of propionyl-CoA to methylmalonyl-CoA (32, 33). These carbon intermediates can be used as precursors for the synthesis of pathogenic cell wall lipids. The methylcitrate cycle is the major pathway for propional-CoA utilization for both bacteria and fungi (34). The assimilation of propionyl-CoA needs to be tightly regulated to prevent its accumulation and alleviate toxicity in cell. The propionyl-CoA assimilation has been investigated in *M. tuberculosis*. It was found that the pathway-specific regulator PrpR regulated methylcitrate cycle by activating the *prp* operon in mycobacteria (4, 33). Previous works have demonstrated that the transcripts of the *prp* operon increased substantially after infection of the mouse lung (19, 35) and that the Δ*prp* strain could not grow or reproduce in murine macrophages (4).

Activated under conditions of nitrogen starvation, the nitrogen regulator GlnR, inhibits the assimilation of propional-CoA through direct repression of the transcription of *prpDBC* in the methylcitrate cycle. The repression of the propional-CoA assimilation results in poor growth of mycobacteria in propionate...
or cholesterol. By integrating environmental nitrogen signals to modulate the propional-CoA assimilation involved in the utilization of fatty acids or cholesterol, GlnR mediates the interplay between nitrogen and carbon metabolism of mycobacteria during infection. *M. tuberculosis* uses the fatty acids and cholesterol from the host as a carbon source, generating propional-CoA. However, there are few reports about the nitrogen metabolism of *M. tuberculosis* living in the host cell. Various nitrogen sources can be used by *M. tuberculosis*, but organic sources (such as amino acids) are more efficient (36), and host-acquired Asp and Asn have been experimentally confirmed as a nitrogen source. This study explains GlnR sensing the nitrogen signal and then regulating propional-CoA assimilation, which may affect virulence lipids synthesis (37).

The findings not only provide new insights into the regulatory mechanism underlying cross talk of nitrogen and carbon metabolism but also reveal a potential target for controlling the growth of pathogenic mycobacteria.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** All bacterial strains and plasmids used in this experiment are listed in Table 1. *Mycobacterium smegmatis* strains were cultured in LB broth (supplemented with 0.05% Tween 80) at 37°C, 200 rpm or onto LB agar plates at 37°C. For growth on different carbon sources, strains were grown in M9 medium containing 12.6 mM Na2HPO4, 2 mM KH2PO4, 8 mM NaCl, 19 mM NH4Cl, 2 mM MgSO4, 0.1 mM CaCl2, and 0.1% tyloxapol. Provided carbon sources included 10 mM propionate or 10 mM cholesterol or other odd-chain fatty acids. All *Escherichia coli* strains used in this experiment were grown in LB broth at 37°C, 200 rpm.

**Cloning, overexpression, and purification of GlnR and PrpR.** The genes *glnR* and *prpR* were amplified by PCR from the genome of *M. smegmatis* mc2 155. A seamless cloning and assembly kit was used. After purification, the PCR product was introduced into pET-28a to generate recombinant vector, pET-28a-glnR or pET-28a-prpR. The clones were confirmed by PCR and sequencing. The proteins were expressed by *E. coli* BL21(DE3). A single clone was cultured in 5 ml LB (1% kanamycin) overnight, and

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**FIG 7** The regulatory mechanism of GlnR on prpDBC involved in methylcitrate cycle. MCC, methylcitrate cycle; MC, methylcitrate; MIC, methylisocitrate; SUC, succinate; MAL, malate; OAA, oxaloacetate.
then transferred to 50 ml LB (1‰ kanamycin). A total of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added when the optical density at 600 nm (OD600) of the cells was about 0.7. Then, the cells were grown at 20°C overnight.

Cells were collected by centrifugation at 8,000 \(g\) for 10 min, and then resuspended in 25 ml phosphate-buffered saline (PBS) buffer. The cells were disrupted using sonication, and the cell debris was removed by centrifugation. The supernatant was purified using an Ni-nitrilotriacetic acid (Ni-NTA)–agarose column (Merck) that was preequilibrated in 10 mM imidazole in 50 mM NaH2PO4 and 300 mM NaCl (pH 8.0). The desired protein was eluted with 20 to 250 mM imidazole in 50 mM NaH2PO4 and 300 mM NaCl (pH 8.0). The fractions were analyzed by SDS-PAGE electrophoresis. The protein concentration was determined by the bicinchoninic acid (BCA) method.

**Electrophoretic mobility shift assay.** The upstream region (from –300 to 50) of prpDBC containing GlnR-binding and PrpR-binding sites were amplified by PCR with biotin-labeled primer (5=–biotin-AGCC AGTGGCGATAAG-3=). The PCR product was analyzed by agarose gel electrophoresis and purified using a PCR purification kit (Shanghai Generay Biotech). The concentrations of biotin-labeled DNA probes were determined with a microplate reader (Biotek, USA). EMSAs using N-terminally His-tagged GlnR or PrpR were carried out following the chemiluminescent EMSA kit (Beyotime Biotechnology, China) manual. The binding mixture (total volume, 10 μl) containing 1 μl DNA probes, various amounts of purified GlnR or PrpR and 1 μl gel-shift binding buffer were incubated at room temperature for 20 min. After binding, the mixture was separated on a nondenaturing PAGE gel in ice-bathed 0.5 Tris-borate-EDTA at 100 V, and bands were detected by BeyoECL Plus.

**Quantitative real-time PCR.** Cells at exponential stage in nitrogen-limited medium were collected by centrifugation. Total RNA was prepared using an RNeasy mini kit (Qiagen, Valencia, CA). The RNA quality was analyzed by 1% agarose gel electrophoresis, and the concentration was determined by microplate reader (BioTek, USA). The RNA was reverse transcribed to cDNA using a PrimeScript reverse transcription (RT) reagent kit with gDNA Eraser (TaKaRa, Shiga, Japan), and DNase digestion was performed to remove genomic DNA before reverse transcription for 5 min at 42°C. PCRs were performed with primers listed in Table 2.

Real-time PCRs were performed using 2X RealStar green fast mixture (GeneStar, Beijing, China) in a 20-μl final volume containing 50 ng cDNA on a CFX96 real-time system (Bio-Rad, USA). The PCR conditions were as follows: 95°C for 5 min, 40 cycles of 95°C for 5 s and 60°C for 30 s, and then extended at 72°C for 10 min. For all the real-time (RT-PCR) assays, 16S RNA was used as an internal control. The transcriptional fold changes of target genes were calculated using the \(2^{-ΔΔCT}\) method.

**Kinetic binding analysis using Octet system.** The binding affinities of GlnR and PrpR proteins to the upstream region of the prpDBC operon were determined by Bio-Layer interferometry using an Octet System (Octet QX4; ForteBio, USA). Streptavidin biosensors were loaded with biotinylated DNA fragment (upstream region of prpDBC) by incubation for 5 min in 7-μg/ml DNA solution, and then washed in loading buffer for 5 min. After that, the biosensors were moved to protein solutions to allow association for 10 min, and then transferred into PBS buffer to detect dissociation. All samples were performed

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**TABLE 1** Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Bacterial strain or plasmid</th>
<th>Source or reference</th>
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<td><strong>Strains</strong></td>
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<tr>
<td>ΔglnR</td>
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</tr>
<tr>
<td>attB::pMV361-glnR</td>
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<td>Novagen</td>
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<td>E. coli BL21(DE3)</td>
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<tr>
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<tr>
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<td>43</td>
</tr>
<tr>
<td>pET-28a-prpR</td>
<td>This study</td>
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**TABLE 2** Primers used for real-time PCR^a

<table>
<thead>
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<th>Primer</th>
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<tr>
<td>GlnR (5784)-RT-F</td>
<td>AGACGATCAGAGCACCCATCAAGA</td>
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<tr>
<td>GlnR (5784)-RT-R</td>
<td>CACCCAGGAGCTGTCTGGACACTA</td>
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<tr>
<td>16S-RT-F</td>
<td>TGGGTGCTAGCCGTTGGAATG</td>
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<tr>
<td>16S-RT-R</td>
<td>CGGGTCGTTGCTGGCTGTT</td>
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<tr>
<td>PrpD (6645)-RT-F</td>
<td>GGACGGAGGTATCGCCTTGAAG</td>
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<td>PrpD (6645)-RT-R</td>
<td>GGGTCTGGTGCTGGCTGTT</td>
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<tr>
<td>PrpB (6646)-RT-F</td>
<td>GCACCGCCGACTTACCCCTC</td>
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<td>PrpB (6646)-RT-R</td>
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<td>PrpC (6647)-RT-F</td>
<td>CGGCAAATCTTCCCGCAAGG</td>
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<tr>
<td>PrpC (6647)-RT-R</td>
<td>GATGTCGAGCCAACGGCTTC</td>
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</tbody>
</table>

^aRT, real time; F, forward; R, reverse.
in a 96-well plate at 37°C and at 1,000 rpm in a volume of 100 µl. The proteins were diluted in the PBS buffer containing 10% glycerol, 10 µg/ml bovine serum albumin (BSA), and 0.02% Tween 20. The kinetic parameters \( k_{\text{on}}, k_{\text{off}}, k_{\text{cat}} \) and \( K_{M} \) were calculated by 1:1 binding model using the Octet Data Analysis version 7.0.

**DNase I footprinting assay for binding site identification.** DNase I footprinting assays were carried out according to the method of Wang et al. (38) and Zianni et al. (39). Briefly, the probes of p5900 and p6645 were amplified with the primers SCamtBFP (M13F) and SCamtBFP (M13R), and then the 400-ng probes were used to bind to GlnR protein at 30°C for 30 min. Following, the product was used to digest with 0.015 units DNase I (Promega) at 37°C for 1 min. The reaction was stopped by adding 140 µl DNase I stop solution (200 mM unbuffered sodium acetate, 30 mM EDTA, and 0.15% SDS). Samples were firstly extracted with phenol-chloroform and then precipitated with ethanol, and the pellets were dissolved in 30 µl MniIQ water. The preparation of DNA ladder, electrophoresis, and DNA analysis were done as the same as described before (38), except that the GeneScan-LIZ500 size standard (Applied Biosystems) was used.

**Resazurin assays.** To monitor the cell metabolism activity of three *M. smegmatis* strains (wild type, glnR-deleted mutant ΔglnR, and complementary strain attB::pMV361-glnR) on different carbon sources, the resazurin assay was performed according to previous descriptions (28, 40). Briefly, strains were activated by culturing in LB containing 0.05% Tween 80, and then transferred to M9 medium to be cultured to OD\(_{600}\) of 1.0. Strains were harvested and diluted to 10\(^5\) CFU/ml in M9, and 10\(^4\) CFU was added into 96-well plates with M9 containing different carbon resources. After 36 h of growth, the resazurin solution (12.5-mg/ml final concentration, Sigma) was added into the plate. The change in color was observed every ten minutes. Medium without cells and cells without a carbon source were used as negative controls.

**Survival of *M. smegmatis* in macrophages.** The human mononuclear macrophage THP-1 cell was from the Cell Bank of Typical Culture Preservation Committee of Chinese Academy of Science (Shanghai, China) and was cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS) at 37°C in the presence of 5% CO\(_2\). They were divided into 12-well plates (2.0 × 10\(^5\) cells per well) and differentiated using phorbol myristate acetate (PMA) when the cells were at the best state (41). After 12 h, the cells were washed and then cultured in fresh RPMI 1640 medium for 12 h. The *M. smegmatis* bacteria were added into the plates (10 times more than the THP-1 cells). After incubating for 2 h, the cells were washed three times using fresh medium to remove the uninfected bacteria. The infected cells were cultured in fresh medium with gentamicin. At the different infection times, the cells were washed three times with fresh medium, and then LB medium containing 0.05% SDS was added to lyse the cells for 10 min. The lysates were collected and diluted at different gradients to the inoculate plate. The *M. smegmatis* colony-forming units were counted after 3 days of culturing (26).

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We have no conflict of interest to declare.

B.-C.Y. conceived the concept and supervised the project; W.-B.L. and X.-X.L. designed the experiments and completed them with the help of M.-J.S. and G.-L.S.; W.-B.L. and X.-X.L. wrote the paper and B.-C.Y. revised it. All authors discussed the results and commented on the manuscript.

**REFERENCES**


