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GarA is an essential regulator of metabolism in Mycobacterium tuberculosis

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Summary

Alpha-ketoglutarate is a key metabolic intermediate at the crossroads of carbon and nitrogen metabolism, whose fate is tightly regulated. In mycobacteria the protein GarA regulates the tricarboxylic acid cycle and glutamate synthesis by direct binding and regulation of three enzymes that use α-ketoglutarate. GarA, in turn, is thought to be regulated via phosphorylation by protein kinase G and other kinases. We have investigated the requirement for GarA for metabolic regulation during growth in vitro and in macrophages. GarA was found to be essential to Mycobacterium tuberculosis, but dispensable in non-pathogenic Mycobacterium smegmatis. Disruption of garA caused a distinctive, nutrient-dependent phenotype, fitting with its proposed role in regulating glutamate metabolism. The data underline the importance of the TCA cycle and the balance with glutamate synthesis in M. tuberculosis and reveal vulnerability to disruption of these pathways.

Introduction

Mycobacterium tuberculosis is an obligate pathogen that multiplies inside macrophages and granulomas, possibly using host fatty acids and cholesterol as carbon sources (Lee et al., 2013). In vitro M. tuberculosis is able to utilize diverse carbon sources, since it possesses complete pathways for glycolysis, the tricarboxylic acid (TCA) cycle, the pentose phosphate pathway, glyoxylate cycle and methylcitrate cycle (Beste et al., 2007). Efficient carbon metabolism is required for growth and persistence in vivo, since disruption of gluconeogenesis, the glyoxylate cycle and the methylcitrate cycle have each been found to reduce the virulence of M. tuberculosis in mice (McKinney et al., 2000; Munoz-Elias et al., 2006; Marrero et al., 2010).

The TCA cycle is the major energy-generating pathway in aerobic organisms, with the α-ketoglutarate dehydrogenase complex (KDH) being a major point of control of flux through the cycle (Bunik and Fernie, 2009). In most aerobic organisms KDH is regulated at the level of gene expression and also by key metabolites that are allosteric activators or inhibitors (Bunik and Fernie, 2009). The KDH of M. tuberculosis is encoded by Rv1248c (α-ketoglutarate decarboxylase, Kgd), Rv2215 [dihydrolipoamide acyltransferase, DlaT (Tian et al., 2005)] and Rv0462 [dihydrolipoamide dehydrogenase, Lpd (Argyrou and Blanchard, 2001)] (Wagner et al., 2011). The Kgd subunit of M. tuberculosis, like that of Corynebacterium glutamicum and the majority of other Actinobacteria, has an additional acyltransferase domain, enabling the same DlaT subunit to function in both KDH and the pyruvate dehydrogenase complex (Niebisch et al., 2006; Wagner et al., 2011). The KDH complex appears to be a key point of control in M. tuberculosis. In addition to allosteric activation by acetyl-coenzyme A, M. tuberculosis KDH is also regulated by binding to an inhibitor protein called GarA (O’Hare et al., 2008; Wagner et al., 2011). This unconventional regulator was first identified in C. glutamicum (Niebisch et al., 2006) and may operate in many other organisms since GarA homologues are widespread in the Actinobacteria.

GarA is a small protein consisting of a forkhead-associated (FHA) domain with N- and C-terminal extensions. The typical function of an FHA domain is protein-protein interaction mediated by specific recognition of phosphorylated threonine residues (Durocher et al., 1999), and indeed when GarA is phosphorylated at its N-terminus the FHA domain is able to bind to phosphothreonine within the N-terminus in an auto-recognition event (Barthe et al., 2009; England et al., 2009; Nott et al., 2009) that blocks binding to KDH, relieving inhibition. Thus the GarA-KDH complex is the endpoint of a kinase signalling pathway to control metabolism.

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The substrate of KDH, α-ketoglutarate, lies at the crossroads of carbon and nitrogen metabolism, as it is also a substrate for glutamate synthesis either by transamination or using ammonia. In addition to regulation of the TCA cycle, GarA regulates the balance between the TCA cycle and glutamate metabolism by inhibiting glutamate dehydrogenase (GDH), involved in glutamate breakdown, and activating glutamate synthase (GltS), involved in glutamate synthesis (Nott et al., 2009). The net result is that unphosphorylated GarA is predicted to promote glutamate synthesis (Fig. 1).

Glutamate is the major amino group donor in anabolism and one of the most abundant cellular metabolites. As such, glutamate biosynthesis and degradation are subject to complex regulation. In bacteria glutamate is synthesized by glutamate dehydrogenase or, during nitrogen limitation, by the co-ordinated activity of glutamate synthase and ATP-dependent glutamine synthetase. This latter pathway is predicted to be the main route of glutamate synthesis in M. tuberculosis, since the only GDH encoded by the genome is predicted to be NADH-dependent and catabolic. The genome of M. smegmatis, by contrast, encodes two additional predicted NADPH-dependent anabolic GDH enzymes.

Regulation of nitrogen metabolism has been reviewed for Gram-positive Bacillus subtilis (Gunka and Commichau, 2012) as well as the Actinomycetes C. glutamicum (Burkovski, 2007) and Streptomyces coelicolor (Reuther and Wohlleben, 2007). In these organisms important control mechanisms include global regulators of transcription as well as post-translational control of enzyme activities, with α-ketoglutarate, glutamine and ATP serving as markers for cellular nitrogen and carbon limitation or sufficiency. However, the mechanisms of control are distinctive in the different organisms. In M. smegmatis GlnR is thought to be the global nitrogen response regulator (Jenkins et al., 2013). The finding that GarA binds in vitro to GDH and GltS suggests that it could be an important player in nitrogen regulation in the mycobacteria and potentially in other Actinobacteria (O’Hare et al., 2008; Nott et al., 2009).

To date GarA has been studied using recombinant proteins but there was only indirect evidence for the impact of this regulation on mycobacterial cells, coming from over-expression of GarA (Belanger and Hatfull, 1999; O’Hare et al., 2008) and disruption of the kinase that phosphorylates GarA, protein kinase G (PknG) (Cowley et al., 2004). More recently, genome-wide transposon mutagenesis predicted that garA could be essential in M. tuberculosis (Griffin et al., 2011). The position of garA immediately upstream of a predicted cotranscribed essential gene might cloud interpretation of the results, although the transposon in question is not known to cause polar effects (Sassetti et al., 2003). Here we used targeted gene disruption, phenotypic profiling and site-directed mutagenesis to address the physiological function of GarA in M. smegmatis and M. tuberculosis.

Results

Deletion of garA in M. smegmatis leads to a nutrient-dependent growth defect

To assess whether metabolic regulation by GarA is required for growth we attempted to construct in-frame,
Disruption of substrates that enter glycolysis (glucose and glycerol), loss and or no growth (Fig. 3). Deletion of the nitrogen source and testing single variable sources of modified Sauton’s medium using ammonium chloride as mentioned by the introduction of plasmid-borne individually (Fig. 3) and these defects were partially complemented by the introduction of plasmid-borne transcriptional regulator and a conserved hypothetical protein a putative operon containing a conserved putative transcriptional regulator and a conserved hypothetical protein (Fig. 2A). The garA deletion in ΔgarAΔMS was confirmed by PCR and Western blotting (Fig. 2B and C). Colonies grew slightly slower than those of the parent strain, and had a smoother appearance (Fig. 2D).

The optimal carbon and nitrogen sources utilized by M. tuberculosis are glycerol and asparagine, as used in Sauton’s medium (Lyon et al., 1974), or glucose, glycerol, glutamate and ammonia as used in Middlebrook 7H9 medium (Middlebrook et al., 1954). However, M. tuberculosis and M. smegmatis are metabolically versatile and able to utilize a large variety of carbon and nitrogen sources. Since GarA is thought to be a metabolic regulator, we hypothesized that deletion of garA would affect the ability of M. smegmatis to utilize different carbon and nitrogen sources. We measured the growth of ΔgarAΔMS in modified Sauton’s medium using ammonium chloride as the nitrogen source and testing single variable sources of carbon. Deletion of garA reduced the growth rate and maximal optical density for all carbon sources tested individually (Fig. 3) and these defects were partially complemented by the introduction of plasmid-borne garA. Using substrates that enter glycolysis (glucose and glycerol), ΔgarAΔMS grew at almost the same rate as the wild type strain, but using substrates that enter the TCA cycle (acetate, propionate and succinate) ΔgarAΔMS showed little or no growth (Fig. 3).

Growth of ΔgarAΔMS was tested using a variety of nitrogen sources, and the growth defect was most pronounced when ammonium chloride was supplied (Fig. 4A and B). To investigate whether the phenotype of ΔgarAΔMS was simply due to a defect in the ability to assimilate inorganic ammonia/ammonium salts, we also tested the ability of ΔgarAΔMS to grow using asparagine plus acetate or propionate (Fig. 4C and D). In these conditions ΔgarAΔMS was able to grow but had a pronounced growth defect compared with the parent strain, indicating that deletion of garA affects the use of both carbon and nitrogen sources.

Since GarA may regulate ammonia metabolism by inhibiting glutamate dehydrogenase, we wanted to test whether the poor growth of ΔgarAΔMS on ammonia could be due to toxicity of ammonia, however this strain was not inhibited by the addition of ammonium chloride (30 mM) to standard Sauton’s or Middlebrook 7H9 medium (not shown).

Similarly, growth on propionate is known to intoxicate mutants of M. smegmatis and M. tuberculosis deficient in the methyl citrate cycle as these strains accumulate toxic levels of propionyl-CoA (Gould et al., 2006; Upton and McKinney, 2007). To test whether the growth defects of ΔgarAΔMS are due to toxic accumulation of metabolites, we measured growth in media supplemented sequentially with TCA cycle intermediates or amino acids. None of the tested supplements reduced growth, so there was no evidence of intoxication, but glutamate, glutamine and asparagine preferentially stimulated the growth of ΔgarAΔMS to wild type-like levels, suggesting that ΔgarAΔMS may be deficient in these metabolites. In contrast supplementation with succinate and other TCA cycle intermediates led to no stimulation of ΔgarAΔMS (Figs 5 and S1).
Fig. 3. Deletion of garA prevents utilization of some carbon sources. Growth curves of M. smegmatis mc²155 (□), ΔgarA<sub>MS</sub> (■), and a complemented strain (ΔgarA<sub>MS</sub> carrying pRBexint-garA: ●) in Middlebrook 7H9 medium with ADN and Tween-80 (A) or modified Sauton’s medium containing 10 mM ammonium chloride as the sole nitrogen source and 0.05% tyloxapol to disperse growth (B–F). The following sole carbon sources were used:

- B. glycerol 1%;
- C. glucose 1%;
- D. sodium propionate 10 mM;
- E. sodium acetate 10 mM;
- F. sodium succinate 10 mM.

Error bars represent standard deviation of five replicates and each graph is representative of at least three independent experiments.

Fig. 4. The growth defect of ΔgarA<sub>MS</sub> is dependent on the nitrogen and carbon sources. Growth curves of M. smegmatis mc²155 (□), ΔgarA<sub>MS</sub> (■), and a complemented strain (ΔgarA<sub>MS</sub> carrying pRBexint-GarA: ●) in modified Sauton’s medium.

- A. 1% glycerol and 10 mM ammonium chloride are the sole carbon and nitrogen sources, Tween-80 prevents clumping.
- B. 1% glycerol and 10 mM glutamate are the sole carbon and nitrogen sources, Tween-80 prevents clumping.
- C. 10 mM sodium acetate and 3 mM asparagine are the sole carbon and nitrogen sources, tyloxapol prevents clumping.
- D. 10 mM sodium propionate and 3 mM asparagine are the sole carbon and nitrogen sources, tyloxapol prevents clumping.

Error bars represent standard deviation of five replicates and each graph is representative of at least three independent experiments.
GarA is essential in M. tuberculosis

As mentioned above, attempts to delete garA in M. tuberculosis were unsuccessful, suggesting that the gene may be essential. Therefore, the conditional knockout strain \( c\Delta garA_{Mtb} \) was constructed. In this strain garA was subjected to an in-frame deletion allowing the expression of the downstream genes, while a copy of garA was integrated at the L5 \( att \) site under transcriptional control of a promoter repressible by anhydrotetracycline (ATc) (Fig. 6). This strain showed rapid loss of growth and viability when garA transcription was repressed (Fig. 7A and B) indicating that GarA is essential in \( M. tuberculosis \).

We hypothesize that the biochemical effect of GarA depletion is that GDH and KDH cannot be inactivated resulting in the continued transformation of glutamate and glutamine into succinate. To confirm this hypothesis, \( c\Delta garA_{Mtb} \) was grown on Middlebrook 7H10 agar plates supplemented with ATc and glutamine or glutamate. Supplementation with glutamate or glutamine (Fig. 7C), but not succinate (Fig. S2), allowed the conditional knockdown strain to grow even when garA transcription was repressed, confirming our hypothesis.

Since several amino acids can be converted into glutamate, one possible explanation for this phenotype is that the drainage of glutamate from the cytoplasm leads to a sink effect reducing the concentration of these amino acids to a level not compatible with growth. An alternative explanation is that the phenotype is due to depletion of glutamate and glutamine, which act as nitrogen donors. To discriminate between these two hypotheses \( c\Delta garA_{Mtb} \) was grown on Middlebrook 7H10 agar plates supplemented with several amino acids at the concentration of 10 mM. The results show that only asparagine, glutamate or glutamine were able to restore the phenotype (Figs 7C and S2). The fact that the addition of asparagine, but not other amino acids which can be converted into glutamate, allowed the mutant to grow suggests that the growth inhibition does not result from amino acid drainage due to the attempt of the cell to replenish the glutamate pool. However, since the only amino acids able to restore the growth are those that can act as nitrogen donors (glutamine and glutamate directly and asparagine following release of ammonia by the asparaginase Rv1538c), the effect may be due to the deficiency of nitrogen donors.

GarA is essential for intracellular growth and survival of \( M. tuberculosis \)

In order to determine if GarA is essential also during intracellular growth, we infected THP-1-derived human macrophages with \( c\Delta garA_{Mtb} \). When ATc was added to the cell culture medium, bacteria grew during the first two days, and then quickly lost viability (Fig. 8). These findings clearly show that GarA is essential for intracellular growth of \( M. tuberculosis \).

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Variants of GarA disrupted for binding to KDH cannot complement the growth of garA knockout M. smegmatis. GarA binds three enzymes involved in central metabolism: KDH, GDH and GltS, via its FHA domain. Mutations in the FHA domain have been identified that preferentially disrupt binding to some or all three of the enzymes (Nott et al., 2009). The mutation S94A disrupts binding to GDH and GltS, R142A to KDH and GDH, and K140E disrupts binding to all three enzymes. These variant garA genes were tested for their ability to complement the phenotype of ΔgarA(MS) and cΔgarA(Mtb) (Table 1 and Fig. 9). Variant K140E, which is unable to bind any of the three enzymes in vitro, is poorly able to complement the growth of ΔgarA(MS).

**Fig. 6.** The strategy used to create a conditional mutant for garA in *M. tuberculosis.*
A. In *M. tuberculosis,* garA is the first gene in an operon.
B. In the conditional mutant cΔgarA(Mtb) there is an unmarked deletion of garA, which leaves the garA promoter (PgarA) and two downstream genes intact. The att site contains the TetR/Pip OFF repressible promoter system: tetR is transcribed constitutively and TetR represses pip transcription. When anhydrotetracycline is added it binds to TetR, allowing transcription of pip. Pip then prevents transcription of garA.

**Variants of GarA disrupted for binding to KDH cannot complement the growth of garA knockout M. smegmatis**

GarA binds three enzymes involved in central metabolism: KDH, GDH and GltS, via its FHA domain. Mutations in the FHA domain have been identified that preferentially disrupt binding to some or all three of the enzymes (Nott et al., 2009). The mutation S94A disrupts binding to GDH and GltS, R142A to KDH and GDH, and K140E disrupts binding to all three enzymes. These variant garA genes were tested for their ability to complement the phenotype of ΔgarA(MS) and cΔgarA(Mtb) (Table 1 and Fig. 9). Variant K140E, which is unable to bind any of the three enzymes in vitro, is poorly able to complement the growth of ΔgarA(MS).

**Fig. 7.** garA is an essential gene in *M. tuberculosis.* Repression of garA transcription in the conditional mutant cΔgarA(Mtb) leads to loss of growth and viability.
A. Graphs show the optical density of cΔgarA(Mtb) cultured in the presence (circles) or absence (squares) of anhydrotetracycline.
B. cΔgarA(Mtb) cultured in the presence of anhydrotetracycline was tested for viability by counting cfu on 7H10 agar. The culture on day 14 gave no colonies, meaning that there were fewer than 200 cfu ml⁻¹. Error bars show the standard deviation for duplicate measurements. Results shown are representative of two independent experiments.
C. Supplementation with glutamate, glutamine or asparagine restores the growth defect of cΔgarA(Mtb). Serial dilutions were spotted onto 7H10 plates containing zero or 500 ng ml⁻¹ anhydrotetracycline, ATc, plus specific supplements added at 10 mM. Gln, glutamine; Glu, glutamate; Asn, asparagine.
By contrast, the S94A variant, which shows reduced binding to GDH or GltS but retains binding to KDH, was able to restore growth almost as well as the wild type gene (Fig. 9). This complementation suggests that regulation of KDH, GDH and GltS is the main function of GarA and that inhibition of KDH in particular is crucial for normal growth of M. smegmatis.

**Discussion**

We have previously proposed that GarA acts as a regulator of metabolism in mycobacteria, since recombinant GarA acts on the activities of KDH, GDH and GltS, whereas overexpression of GarA inhibits the growth of M. smegmatis (O’Hare et al., 2008). Supporting this hypothesis we present data showing that gene disruption of garA leads to a specific nutrient-dependent growth defect.

Based on the phenotype of pknG disruption [glutamate accumulation (Cowley et al., 2004)], and the enzyme-modulatory effects of recombinant GarA (O’Hare et al., 2008; Nott et al., 2009), we have established a model in which GarA influences the distribution of α-ketoglutarate between the TCA cycle and glutamate synthesis by inhibiting KDH, activating glutamate synthesis and inhibiting glutamate degradation (Fig. 1). This model is supported by the fact that supplementation with glutamate restores normal growth of garA deficient M. smegmatis and M. tuberculosis (Figs 5 and 7C). Based on these results we propose that unphosphorylated GarA promotes glutamate synthesis and PknG reduces glutamate synthesis via phosphorylation of GarA.

In contrast to the nutrient-dependent growth defect of ΔgarA MS, garA knockdown in M. tuberculosis caused rapid growth defect (Fig. 8).

**Table 1.** Complementation of ΔgarA MS with plasmid borne garA variants.

| GarA variant | Variant binds to: | Complementation of ΔgarA MS | Conclusion |
|-------------|------------------|-----------------------------|------------|
| K140E       | N                | Poor                        | Enzyme regulation is important for GarA function. |
| R142A       | N                | Intermediate                | GltS activation is needed but is not sufficient for GarA function. |
| S94A        | Y                | Full                        | KDH inhibition is crucial for GarA function. |

**Fig. 8.** garA is essential for growth and survival of M. tuberculosis in macrophages. Differentiated THP-1 cells infected with cΔgarA MS were incubated in the presence (circles) or absence (squares) of anhydrotetracycline then lysed and bacterial viability measured. In the presence of anhydrotetracycline macrophages yielded no colonies on days 6 and 7, indicating fewer than 200 bacilli per well. Error bars show the standard deviation for duplicate measurements. Results shown are representative of two independent experiments.

**Fig. 9.** Variants of GarA deficient in KDH binding are unable to complement the phenotype of ΔgarA MS. ΔgarA MS carrying control plasmid (■) or plasmids bearing wild type garA (wt, ●) or site-directed mutants of garA were grown in modified Sauton’s medium containing 10 mM NH₄Cl as the sole nitrogen source and either 20 mM propionate (A) or 20 mM acetate (B) as the sole carbon source. Variants of garA carried the following single mutations: S94A (♦), K140E (▼) or R142A (○). Error bars represent standard deviation of five replicates and each graph is representative of at least three independent experiments.
loss of growth and viability, indicating that GarA regulation of glutamate synthesis plays a more important role in this organism. GarA, PknG and the enzymes they control are conserved in all members of the *M. tuberculosis* complex as well as other sequenced mycobacteria (Table S1). However, the genome of *M. smegmatis* encodes two additional GDHs not present in *M. tuberculosis*. Indeed, the predominant GDH activity in cell extracts is NADP*-dependent and is not regulated by GarA (29 nmol mg⁻¹ min⁻¹, data not shown). The additional capability of *M. smegmatis* to accumulate and degrade glutamate by alternative enzymes is the most likely reason for the non-essentiality of GarA in this organism.

Although GarA is essential for growth of *M. tuberculosis* in standard conditions, specific supplementation with glutamate, glutamine or asparagine could restore the ability of cΔgarAΔms to grow *in vitro* (Fig. 7). The fact that cΔgarAΔms could not replicate but was rapidly killed in macrophages (Fig. 8) could report on the nutritional environment inside the phagosome, seemingly to indicate that intracellular *M. tuberculosis* does not experience nutritionally permissive, amino acid rich conditions, consistent with an earlier study (Tullius et al., 2003). The cell culture conditions mimes the concentration of amino acids in normal human plasma, with the exception of glutamine, which is 2 mM compared with approximately 0.6 mM in plasma, and so it is tempting to speculate that GarA would also be essential for *M. tuberculosis* to cause disease in humans.

In mycobacteria GarA can bind to three different enzyme targets, whereas the homologous protein in *C. glutamicum* is only thought to regulate KDH. We have previously used site-directed mutagenesis to define the overlapping binding sites for each enzyme on the FHA domain of GarA and to produce mutant versions of GarA that are deficient in binding to one or all enzyme partners. Here we used these mutant versions of GarA to complement the growth defect of ΔgarAMS. The data confirm that enzyme binding is necessary for GarA function and could also suggest the relative importance of regulating each individual enzyme activity. These data must be interpreted with caution since the FHA domain of GarA is also involved in binding to protein kinases and therefore these mutations may reduce the ability of the kinase to phosphorylate GarA. Nevertheless, Table 1 clearly indicates the ability to bind KDH is crucial for the function of GarA.

The essentiality of GarA in *M. tuberculosis* points to vulnerability in this pathway that could potentially be exploited for anti-tuberculosis drug development. Indeed, inhibition of another enzyme involved in nitrogen acquisition and glutamine synthesis, glutamine synthetase, prevents growth *in vitro*, in macrophages and in animals (Harth and Horwitz, 1999; 2003). Unfortunately the high frequency of generation of resistant mutants by upregulation of glutamine synthetase (Carroll et al., 2011) makes this a problematic target for drug development but alternative steps on this pathway may prove to be more tractable targets.

The mechanism of action of GarA, namely direct binding to activate or inhibit multiple enzyme targets with PknG providing an ‘off’ switch, is unusual and unprecedented. Furthermore the multi-specificity of the binding site on the FHA domain of GarA is also unusual as it binds at least five different proteins: PknG and other kinases via phosphorylated threonine (Villarino et al., 2005) plus three enzyme targets via phosphorylation-independent interaction. Despite the lack of precedent, the physiological relevance of this regulatory pathway is clearly demonstrated here. This particular system is specific to the Actinobacteria, but other FHA domain proteins and serine threonine protein kinases are widespread in prokaryotes and the functions of most are still unknown.

**Experimental procedures**

**Bacterial strains, media, and culture**

*Mycobacterium smegmatis* mc²155 and *M. tuberculosis* H37Rv were routinely cultured in Middlebrook 7H9 medium (Oxoid) supplemented with 10% ADN (0.5% bovine serum albumin, 0.2% dextrose, 0.085% NaCl), 0.05% w/v Tween-80 or Middlebrook 7H10 agar with 10% ADN. A list of strains used in this study is provided in Table S2. To analyse nutrient utilization, a minimal version of Sauton’s medium was prepared [3.7 mM KH₂PO₄, 2 mM MgSO₄, 9.5 mM sodium citrate, 0.17 μM ferric ammonium citrate, pH 7.0 (Lyon et al., 1974)], to which carbon and nitrogen sources were added: glycerol or glucose at 1% v/v or sodium acetate, sodium propionate or sodium succinate at 10 mM, ammonium chloride at 10 mM or asparagine, sodium aspartate, sodium glutamate or glutamine at 30 mM. To disperse the culture surfactants were added at 0.05% w/v: either Tween-80, which can be utilized as a carbon source, or tyloxapol, which cannot. When required antibiotics were used at the following concentrations: kanamycin (50 μg ml⁻¹) and hygromycin (100 μg ml⁻¹). Bacterial viability was estimated by measuring colony-forming units (cfu) per ml by plating aliquots of bacterial suspension on Middlebrook 7H10 agar plates containing 10% ADN.

**Construction and characterization of the *M. smegmatis* garA mutant**

An unmarked garA deletion mutant was constructed according to a published method (Parish and Stoker, 2000). Briefly, two ~1.5 kb fragments containing the downstream and the upstream regions of garA were amplified from genomic DNA of *M. smegmatis* mc²155 with primers DF-GarAMS-F/DF-GarAMS-R and UF-GarAMS-F/UF-GarAMS-R (Table S3). Each fragment was cloned into pGEM-T Easy (Promega) and confirmed by sequencing. Fragments were cut out through introduced restriction sites HindIII and Scal and jointly cloned into the HindIII site in p2NIL. The marker cassette from
The initial OD540 was 0.06 and cultures were passaged by to attempt to delete mutants as white colonies. The same method was employed sucrose (2%) to identify putative unmarked gene deletion on Middlebrook 7H10 without antibiotics but with Xgal and A single cross-over mutant (verified by PCR) was used to parallel cultures were cultured with zero or 500 ng ml−1 of warm phosphate buffered saline to remove extracellular bacteria. Finally, 100 µl of warm RPMI with or without ATc (500 ng ml−1), was added to each well and the plate was incubated at 37°C. RPMI with or without ATc was replaced every 48 h. For 8 days, every 24 h, starting from 90 min after the initial washes, the medium was removed from three wells, and then intracellular bacteria were released by lysing the macrophages with 100 µl of 0.05% SDS. The suspensions obtained from the lysed macrophages were immediately diluted in 7H9 and plated to determine viable counts. About 95% of macrophages remained viable during the entire experiment, as determined by Trypan blue exclusion.

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