Mycobacterium Lysine ε-aminotransferase is a novel alarmone metabolism related persister gene via dysregulating the intracellular amino acid level

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Bacterial persisters, usually slow-growing, non-replicating cells highly tolerant to antibiotics, play a crucial role contributing to the recalcitrance of chronic infections and treatment failure. Understanding the molecular mechanism of persister cells formation and maintenance would obviously inspire the discovery of new antibiotics. The significant upregulation of Mycobacterium tuberculosis Rv3290c, a highly conserved mycobacterial lysine ε-aminotransferase (LAT) during hypoxia persistent model, suggested a role of LAT in persistence. To test this, a lat deleted Mycobacterium smegmatis was constructed. The expression of transcriptional regulator leucine-responsive regulatory protein (LrpA) and the amino acids abundance in M. smegmatis lat deletion mutants were lowered. Thus, the persistence capacity of the deletion mutant was impaired upon norfloxacin exposure under nutrient starvation. In summary, our study firstly reported the involvement of mycobacterium LAT in persister formation, and possibly through altering the intracellular amino acid metabolism balance.

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Bacterial culture and starvation conditions. The bacterial strains and plasmid used in this study are shown in Table 1. E. coli strains were grown on LB broth agar or in LB broth, Mycobacterium smegmatis mc²155 was grown in 7H9 liquid medium (Difco) supplemented with 0.05% w/v Tween 80, 0.5% glycerol and 0.5% glucose or were grown on 7H10 agar supplemented with 1% glycerol and 0.5% glucose. The starvation culture condition as described with minor modifications. In brief, exponential phase cultures were pelleted and washed twice with 1× PBS before being resuspended in 1× PBS, transferred to standing flasks or microwell and incubated at 37°C, 110 rpm. For viability determination during starvation, bacteria were cultured in 50 ml volumes in 250 ml bottles (Shuniu), and the number of cfu/ml was determined by plating serial dilutions onto 7H10 agar from triplicate cultures at several time points (0 h, 24 h, and 72 h).

When required, the following antibiotics were used at the final concentration: ampicillin, 100 μg/ml; kanamycin, 500 μg/ml for E. coli or 200 μg/ml for M. smegmatis; hygromycin, 50 μg/ml.

Knockout mutant construction and complementation. The lat gene of M. smegmatis mc²155 was disrupted using specialized transduction previously described. PCR and sequencing of latΔmut was used to confirm the deletion. For knockout mutant complementation, the M. tuberculosis H37Rv Rv3290c coding region was amplified by polymerase chain reaction using the primers: 5′-CGCGGATCCGGCTCGCTATCATGATTGGAC-3′, consisting of the final three C-terminal amino acids of Rv3290c and a TAA termination codon followed by a NdeI restriction site. The gel-purified polymerase chain reaction product was ligated into the pALACE digested by NdeI fragment. The fragments were ligated into the plasmid pALACE digested by BamH I and NdeI, to produce the plasmid pALACE-latΔmut. Sequencing of pALACE-latΔmut confirmed the correctness of the constructed fragment. Competent cells of latΔmut mutant were prepared as described, and pALACE containing latΔmut gene was used to transform latΔmut mutant competent cells. This was followed by electroporation.

Table 1. Bacterial strains, plasmids, and primers used in this study.
into the mutant as previously described. Transformed cells were streaked on 7H10 plates containing 100 μg/ml ampicillin and 50 μg/ml hygromycin. The desired complemented strain was identified by bacterial PCR and Western Blotting. To detect His-tagged LAT_{sm}, bacterial pellets were harvested and disrupted by ultrasonication. Samples were then subjected to SDS-PAGE and the His-tagged LAT_{sm} protein was detected by mouse anti-His antibodies (TIANGEN, China).

**Amino acids determination.** Overnight cultures of *M. smegmatis* mc²155 were diluted 1:100 in M9 medium and incubated at 37°C on shaker (PEIYING DHZ.CA, TAI CANG SHI YAN SHE BEICHANG) at 110 rpm. Exponential phase cultures were harvested by centrifugation at 8000 rpm, 4°C for 15 min. Harvested cells were washed three times with ddH₂O and resuspended in 5 ml of ddH₂O. Then the bacteria were pipetted into the dialysis tube and dialyzed in ddH₂O at 4°C for 24 h. Cells were collected and transferred to freeze-dried reagent bottle for freeze dehydration by vacuum pump. The procedure was from reference with slight modification. Briefly, 50 mg dried sample was put into a 15 × 150 mm testtube, and then 6 ml of 6 M HCl were added into the testtube containing bacterial cells. The upper part in the testtube was removed and the testtube was sealed after 10 min vacuumization. The treated testtube was hydrolyzed for 22 hours in a 110 °C ± 1 °C oven. The testtube was taken out and cooled to room temperature, mixed and filtered. 1 ml of filtrate was put into a 50 ml beaker, and waterbathing evaporated at 60 °C, 2-fold diluted by adding 0.02 M HCl, the sample was filtered by 0.22 um membrane, and loaded into a Hitachi L-8800 amino acid analyzer. The analysis cycle is 53 min, using two columns during the analysis process: (1) Separation column: (4.6 mm × 60 mm) Eluent flow rate is 0.4 ml/min, the column temperature was 70 °C, column pressure was 11.627 MPa. (2) Reaction column: Ninhydrin and ninhydrin buffer flow was 0.35 ml/min, the column temperature was 135 °C, column pressure was 1.078 MPa.

**MIC assay and drug treatment of cultures.** The MICs of antibiotics were determined by using serial two-fold dilution of the antibiotics in 7H9 medium as previously described in reference. The initial cell densities were 10^6 cfu/ml of exponential culture, and the samples were incubated for 3 days at 37°C. The MIC was recorded as the minimum drug concentration abolishing visible growth. For drug treatment assay, 50 ml *M. smegmatis* was cultured under starvation conditions in 250 ml bottles. At 0, 24 h and 72 h time point, one milliliter of a starvation culture of *M. smegmatis* was diluted 10 times in 1 × PBS, and 1 ml was aliquoted per well of a 48-well plate, every sample with 3 repeats. Norfloxacin was added to duplicate wells of cultures at final concentration of 20 μg/ml. Control wells for cultures received no drug or no cells. Cultures were incubated with or without drug at 37 °C for 24 h and 48 h, followed by serial diluting and plating on 7H10 agar to determine bacterial viability.

**RNA Isolation and reverse transcription-PCR (RT-PCR).** *M. smegmatis* cultured under starvation conditions in 50 ml volumes in 250 ml bottles (Shuniu). Three 50 ml cultures were harvested by centrifugation at time zero (t = 0). Pellets were pulverized in liquid nitrogen and homogenized in Trizol solution (Invitrogen) and RNA was isolated according to the manufacturer’s instructions. The subsequent steps were performed according to the reference.

**Real-Time PCR.** 1 μg of total RNA was reversely transcribed to cDNA using a first strand cDNA synthesis kit (Roche) according to the manufacturer’s instructions. Resultant cDNA was used for real-time PCR. Advanced SYBRGreen Supermix (BIO-RAD) were used to quantify cDNA in a 20 μl L reaction containing 10 μM each primer, 10 μl supermix (2X), 4 μl L cDNA. Primers used are listed in Table 1. Copy numbers of MSMEG _1764_ mRNA were normalized with copy numbers of sigA mRNA. Each reaction was run in triplicate in Bio-Rad CFX-96 Real-Time Detection System with the following parameters: 95°C for 2 Min, 40 cycles of 95°C for 10 Sec, and 64°C for 40 Sec.

**Results**

**lat is conserved among Mycobacteria.** *lat* is conserved among *M. tuberculosis*, *M. marinum*, *M. leprae*, *M. bovis* BCG, *M. smegmatis*, *M. canettii*, and Rhodococcus erythropolis PR4 by BLAST analysis. The amino acid identity between *M. tuberculosis* LAT and its homologs is greater than 59% in all cases, its neighboring gene *brpA* is conserved too. Most neighboring genes of *M. tuberculosis*, *M. marinum*, *M. leprae*, and *M. bovis* BCG are highly conserved (Fig. 1).

**lat_{sm} is upregulated under nutrient starvation in *M. smegmatis*.** MSMEG _1764_ is the homolog of *Rv3290c* in *M. smegmatis* and shares 77% identity with *Rv3290c*. Since *lat* was highly upregulated in *M. tuberculosis* under nutrient starvation, it is interesting to know whether this is the case in *M. smegmatis*. To this end, a nutrient starvation model according to Betts et al. was established. The transcription of *lat* in *M. smegmatis* at 0 h, 24 h and 72 h was measured. *lat* was upregulated 20 and 23-fold in *M. smegmatis* undergoing starvation after 24 and 72 hours, respectively (Fig. 2). The results showed that the expression pattern of *lat* in *M. smegmatis* under nutrient starvation is the same as that in *M. tuberculosis*. Given *M. smegmatis* is a well-recognized facile surrogate to address *M. tuberculosis* biology, in particular to study the persistence under nutrient starvation, *M. smegmatis* was adopted as a model in our study.

**lat (MSMEG _1764_) is nonessential and can be deleted in *M. smegmatis* mc²155.** To determine the effect of *lat* mutation on the persistence of mycobacterium, a *lat* knockout mutant was constructed by *Xer* site-specific recombination as described in the Materials and Methods section. *M. smegmatis* hygromycin-resistant colonies were selected and cultured consecutively for five generations. Primer lat-F1 and lat-R1 were then used to confirm the mutant genotype, with the wild-type strain as a control. The 1565bp (1365 bp of the wild type lat_{sm})
and extra 200 bp from the genome) fragment can be amplified from the wild-type, while there was only 449 bp (249 bp of \( \Delta \text{lat}_{\text{MS}} \) and extra 200 bp from the genome) amplicon from the \( \Delta \text{lat}_{\text{MS}} \) knockout mutant (Fig. 3A). The \( \Delta \text{lat}_{\text{MS}} \) knockout mutant was further confirmed by the absence of transcription product (Fig. 3B), indicating that the \( \Delta \text{lat} \) mutant of \( M. \text{smegmatis} \) was successfully constructed. Blastp shows that MSMEG_1764 is the homolog of Rv3290c in \( M. \text{smegmatis} \) and shares 77% identity with Rv3290c. Hence, we use \( M. \text{tuberculosis} \) Rv3290c gene for complementation assays. The recombination vector pALACE-Rv3290c in which Rv3290c is under the control of an ACE promoter was transformed into the \( \Delta \text{lat} \) mutant strain to get the complementary strain. Bacteria PCR result shows that a 1350 bp band was amplified in the complementary strain and no band for the parental strain (Fig. 3C). Western Blotting analysis using the anti-His antibody further confirmed the presence of the expressed ~52 kDa LAT\(_{\text{MS}}\)-His fusion protein in the cell lysates of the complemented strain, while absent in the parental strain (Fig. 3D).

\( \text{lat} \) deleted \( M. \text{smegmatis} \) is hypersensitive to norfloxacin and shows declined persistence under nutrient starvation. LAT was profoundly upregulated during \( M. \text{tuberculosis} \) starvation persistence model. The effect of \( \text{lat} \) deletion on \( M. \text{smegmatis} \) persistence remains elusive. To test this, we first measured the MIC of norfloxacin, rifampicin and isoniazid, the MIC value showed that the deletion of \( \text{lat} \) has no effect on the antibiotic sensitivity for the \( M. \text{smegmatis} \) wild-type and complement strain (2 \( \mu \text{g·ml}^{-1} \) for norfloxacin, 8 \( \mu \text{g·ml}^{-1} \) for rifampicin and 4 \( \mu \text{g·ml}^{-1} \) for isoniazid). The deletion of genes involved in persistence usually has no effect upon MIC\(^{42,43}\). To test the effect of \( \text{lat} \) deletion on the MIC, the strains were exposed to high concentration antibiotics. The survival rate of \( \Delta \text{lat}_{\text{MS}} \) strain is lower than wild-type and complement strain under 20 \( \mu \text{g·ml}^{-1} \) norfloxacin exposure (Fig. 4A).

Since LAT was significantly upregulated under nutrient starvation in \( M. \text{tuberculosis} \). It is interesting to know whether the deletion of \( \text{lat} \) will compromise the survival of \( M. \text{smegmatis} \) under starvation. Therefore, we tested the viability of mutant strain in 1 \( \times \) PBS buffer as described by Betts et al.\(^{39}\). As shown in Fig. 5, no difference

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**Figure 1.** Genomic context of \( \text{lat} \) among \textit{Mycobacteria} and close relatives. Arrows represented with a dark grey background correspond to genes conserved among all species, whereas genes without ortholog in at least one species are shown with white arrows.

**Figure 2.** The expression of \textit{M. smegmatis} \( \text{lat} \) under nutrient starvation. Cells of \textit{M. smegmatis} were initially grown in 7H9 medium to log-phase and then washed by 1 \( \times \) PBS and resuspended in 1 \( \times \) PBS, incubate at 37 \( ^\circ \text{C} \), 110 rpm. RNAs were extracted from bacteria harvested at indicated time points. RT-PCR was performed as described in Materials and Methods. Data are means ± s.d. of triplicates in one of at least three experiments.
can be seen between wild-type and ΔlatMsm strain after 72 hours starvation. To explore whether the deletion of latMsm only affect the persistence of mutant strain in high antibiotic concentrations, strains were subjected to antibiotic exposure and the ratio of persisters was determined. The 24 hours and 72 hours-starved cultures were exposed to norfloxacin for 48 h and the number of survived strains was assessed. We can see that 24 h and 72 h (Fig. 4B, C) starvation cultures of three strains exhibit higher level of norfloxacin persistence than 0 h time point. With the increase of starvation, wild type and complement strains persisted, but not ΔlatMsm strain (Fig. 4D).

Complementation of the mutant with Rv3290c restored the phenotype of wild type strain. After 24 h and 72 h starvation, all strains showed a higher tolerance to norfloxacin than non-starvation cultures. This result indicated that deletion of latMsm failed to affect the viability under nutrient starvation, but can impair the persistence of the mutant strain under norfloxacin treatment.

Inactivation of latMsm decreased the intracellular amino acids content. 13C-isotope profiling of the persisters of Staphylococcus aureus revealed an active amino acid anabolism in this subpopulation, including Ala, Asp, Glu, Ser, Gly and His. Dysregulation of intracellular amino acids level has been shown to lower the survival capability within macrophage exemplified by M. tuberculosis pknG mutants, which encodes protein serine/threonine kinase involved in the regulation of amino acids level. To examine whether the inactivation of MSMEG_1764 has an effect on the amount of intracellular amino acids, the intracellular levels of the amino acids were determined for the wild-type, mutant, and complemented strains. As shown in Fig. 5A, the concentration of glutamic acid, glycine, methionine and total amino acids content in the ΔMSMEG_1764 mutant strain was decreased, but without significant differences for the lysine content between the mutant strain and the wild-type strains. This might be due to the feedback repression of lysA (Diaminopimelate (DAP) - decarboxylase, the enzyme involved in the last step of lysine biosynthesis) by the excess of lysine. This shows that the rate of lysine biosynthesis was regulated by the intracellular lysine amount.

![Graph showing relative expression](image)

![Graph showing norfloxacin persistence](image)

**Figure 3.** Construction of MSMEG_1764 knockout mutant and complement strains. (A) PCR verification of the construction MSMEG_1764 knockout strain. Lanes: 1. Wild-type MS; 2. MSMEG_1764 knockout strain. (B) Verify the transcription of MSMEG_1764 knockout strain by RT-PCR. Wild type and ΔlatMsm strains were grown at 37°C in MB 7H9 liquid medium to an OD600 of 0.8–1.0. Total bacterial RNA was isolated and subjected to RT-PCR to detect the expression of the latMsm gene. (C) Construction of Δlat-Rv3290c strain; Lanes: 1. Knockout strain complement with pALACE-Rv3290c; 2. Knockout strain complement with pALACE plasmid. (D) Western-blotting to confirm the expression of Rv3290c. Lysates were prepared from bacterial cells cultured as in (B), after 16 h induction and subjected to Western blotting to detect His-tagged Rv3290c protein using mouse anti-His antibody.
regulator leucine-responsive regulatory protein (LrpA) upstream of *lat* (Fig. 2), a regulator capable of directly binding to the upstream region of *lat*. To test whether the transcription of *lrpA* was also decreased, RT-PCR was applied. The result showed that *lrpA* transcription was also lowered in Δ*lat* strain (Fig. 5B). The results suggested...
that the deletion of lat in M. smegmatis decreased the intracellular amino acids amount and downregulated the transcription of lrpA.

**relA** was downregulated in lat deletion strain under nutrient starvation. The stress alarmones guanosine 3',5'-bispyrophosphate ((p)pGpp), a key molecule in antibiotic resistance48 and bacterial persistence49, is found to accumulate rapidly under starvation. The relationship between relA and lrpA has previously been noted50,51. The expression of lrp is positively controlled by ppGpp, namely the lower ppGpp level means fewer Lrp50. Moreover, the expression of lrp was significantly reduced in the strains failed to produce ppGpp50. RelMtb, the M. tuberculosis bifunctional enzyme responsible for both (p)pGpp synthesis and hydrolysis52, and has been shown to play an important role in the survival of bacteria during nutrient starvation condition53. To test whether the downregulation of lrpA resulted from the decreased expression of Rel in M. smegmatis, the transcription level of relMsm, the relMtb homolog of M. smegmatis53, was measured at different intervals under starvation. No significant difference can be spotted between wild type and knockout strain at 0 h starvation (Fig. 6A). But relMsm was markedly downregulated in ΔlatMsm strain after 24 h starvation (Fig. 6B). Complementation of M. smegmatis ΔlatMsm with the M. tuberculosis lat Rv3290c can partially restore the expression of relMsm. This result suggested that knockout latMsm has no effect upon the intracellular (p)pGpp content under normal conditions, but can influence the synthesis and hydrolysis of (p)pGpp under nutrient starvation.

**Discussion**

Bactericidal antibiotics usually can sterilize most bacteria rapidly. However, a sub-population will survive and be reactivated upon the withdrawal of the antibiotics55. Genes involving in the persister formation are intensively studied56 both for fundamental insights and translational medicine ends.

Here we showed that lat gene MSMEG_1764, the homologue of Mtb lat Rv3290c is involved in the persister formation (specifically tolerance to norfloxacin) via mediating the intracellular amino acid contents and altering the expression of ppGpp synthase expression in M. smegmatis.

Inactivation of the homologue of Rv3290c in M. smegmatis leads to more sensitive to norfloxacin than the wild type strain (Fig. 4), but without change to the MIC (Data not shown). Generally, mutation of genes involved in persister formation should not alter the MIC, but only affect the persistence42,43. LAT was previously showed to be involved in the L-lysine metabolism57. The data here showed that the knockout of latMsm rendered a decrease of intracellular total amino acid, but without discernable effect on the accumulation of L-lysine (Fig. 5A). LAT is just one of the several genes involved in M. tuberculosis glutamate metabolism58, given the diversity and redundancy of genes to control the robustness of this important amino acid homeostasis, it is not quite unexpected that the disruption of LAT only slightly decreased the glutamate content in M. smegmatis.

Leucine-responsive regulatory protein (Lrp) is a global transcriptional regulator widespread among prokaryotes, and modulates the expression of a variety of genes involved in metabolism during starvation, especially in the amino acid catabolism and anabolism59,60. The knockout of lrp in E.coli downregulated the expression of the amino acid metabolism-related genes51. In our study, we found the expression of lrpA was downregulated in latMsm knockout strain (Fig. 5B), which can explain why the intracellular amino acid content was decreased in knockout mutants. Previous study showed that lrpA is profoundly up-regulated during nutrient starvation conditions characteristic of persistent/latent phase in M. tuberculosis50, as well as in M. smegmatis (Fig. 5B). The persistence of lrpA inactivated Mycobacterium fortuitum was attenuated in a murine infection model62. The expression of lrp is stimulated by (p)pGpp and LRP can regulate the biosynthesis of amino acid60. Here we have shown that the deletion of latMsm downregulated the expression of relMsm (Fig. 6). M. tuberculosis failed to produce (p)pGpp under starvation was defective in long-term survival both in vitro53 and in vivo15. In our study, the downregulation of relMsm is coincident with the diminished bacterial tolerance to antibiotics.

In conclusion, we have shown that LAT is involved in persister formation in mycobacteria. Under nutrition starvation, (p)pGpp is produced by RelMtb and accumulates within bacteria, then upregulates the transcription of LrpA gene, which in turn upregulates the protein level of LAT and response for amino acid metabolism. Intracellular amino acid level regulates the accumulation of (p)pGpp, and (p)pGpp controls the dormant persister formation (Fig. 7). The lat deleted mutant strain failed to replenish the amino acid pool, then downregulated

![Figure 6. Response of the relMsm to starvation. Profile of the relative expression levels of relMsm across 0 h and 24 h time course. Data are means ± s.d. of triplicates in one of at least three experiments.](image-url)
the synthesis of (p)ppGpp. This is largely due to the positive regulation of the transcription of LrpA by (p)ppGpp. The determination of the M. tuberculosis H37Rv LAT crystal structure and the identification of its active sites\(^6\) will promisingly facilitate the discovery of novel LAT inhibitors\(^6\). Our finding has shown that LAT is a new player in mycobacterial persistence provided a potential drug target for inhibitors against persisters.

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X.D., Y.L., Q.D., Q.H., M.X., Y.L. and S.G. performed the experiments. X.D. and J.X. analyzed the data. X.D., Z.L. and J.X. designed the study and wrote the paper. All authors have read and approved the manuscript.

Additional Information
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