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## Mycobacterium Lysine $\epsilon$ -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  $\epsilon$ -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.

Bacterial persistence, a ubiquitous bacterial physiological state phenotypically tolerant to antibiotics, is increasingly recognized as the culprit of the intractability of chronic and relapsing infections. Bacterial persisters are specialized survivors genetically identical to nontolerant kins but under a non-growing or extremely slow-growing, non-replicating dormant state<sup>1</sup>. Sequestration of the targets instead of targets mutation contribute to the antibiotics tolerance of persister<sup>2</sup>. Persister cells are stochastically developed during mid-exponential phase<sup>3</sup> under favorable conditions<sup>4</sup>. Despite more than 70 years after the first report of persisters<sup>5</sup>, the molecular mechanisms underlying bacterial persistence remain largely elusive. Several pathogens, such as *Mycobacteria*, *Pseudomonas* and *Staphylococcus*<sup>6–8</sup>, are remarkable persisters.

*Mycobacteria* have two notorious pathogens, *M. tuberculosis* (*Mtb*) for tuberculosis and *M. leprae* for leprosy, and nontuberculous *Mycobacterium* (NTM)<sup>9</sup>. Many *M. tuberculosis* genes have been reported to be associated with persister formation and essential for persistence in mouse model, such as those involved in energy metabolism<sup>10–14</sup>, *RelA* (ppGpp synthase)<sup>15</sup>, *M. tuberculosis* *PhoY2*, a homolog of *E.coli* *PhoU*<sup>16,17</sup>, RpsA (S1 protein), a protein involved intrans-translation, proteasome *PrcBA*<sup>18,19</sup>, Toxin-antitoxin (TA) modules<sup>20,21</sup>, TA locus *vapBC*<sup>22</sup>, *RelE*<sup>20</sup>, other 88 putative TA system candidates in *M. tuberculosis* genome and 30 TA modules<sup>23–27</sup>. The differential expression of *Rv3290c* in persisters<sup>27,28</sup> implicates an important role in persistence.

Lysine  $\epsilon$ -aminotransferase (LAT) is a pyridoxal 5'-phosphate (PLP)-dependent enzyme that converts l-lysine to  $\alpha$ -aminoadipate- $\delta$ -semialdehyde and glutamate, which is subsequently converted to A<sup>1</sup>-piperidine-6-carboxylic

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Description	Genotype or relevant phenotype	Source or reference
Strains		
<i>E. Coli</i> DH5 $\alpha$	supE44 $\Delta$ lacU169( $\phi$ 80lacZ $\Delta$ M15)hsdR17recA1 endA1 gyrA96thi-1 relA1 $\lambda$ pir	
<i>M. smegmatis</i> mc <sup>2</sup> 155	High-frequency transformation mutant of <i>M. smegmatis</i> ATCC 607	
Plasmids		
PET-28	Kanamycin resistance plasmid	
pALACE	<i>E. coli</i> - <i>Mycobacteria</i> shuttle vector, hygromycin resistance plasmid	38
PJV53	Expression of Che9c gp61, Kanamycin resistance plasmid	34
PAL-75	Providing hygromycin resistance cassette	34
Primers		
LAT-F	5'-CGCGGATCCGCGTCCTGCTATCATAGCGTCATG-3'	This study
LAT-R	5'-GGAATTCATATGGAATTCGCGCTGCCTTACGTACCAC-3'	
KO-F1	5'-GCACTAGTGGGGTTGTGCGTGGTGAT-3'	This study
KO-R1	5'-CGAAGATCTCCAGGAAACGCTAGATCTCCGCGCTCGACCAGATGCG-3'	
KO-F2	5'-ACAAGATCTAGCGTTTCTCGAGATCTGTCGCGCTGCCCGTGA-3'	This study
KO-R2	5'-GCAGAAGCTTATCACATCTCCCGCTCG-3'	
lat-F1	5'-TGCTTCATCGCCGAACCCATCCA-3'	This study
lat-R1	5'-CGAACGAGACCACATCGGCATCACA-3'	
lrpA-F	5'-ATTCTGGTCCGCGAACTCGTGCCA-3'	This study
lrpA-R	5'-CGACGAACGCCGAAAGCATGTTGC-3'	
rel-F	5'-AAGTCATTGCCCACTTGCCCATC-3'	This study
rel-R	5'-CGGCCCTTGACGATCATCTTCTGGT-3'	
lysA-F	5'-CAGAAGTTCGGTTTGTGCTGGCCA-3'	This study
lysA-R	5'-GCTTGTGCGCCAGTTCCTTCATG-3'	
sigA-F	5'-AAGACACCACCTGGAATC-3'	38
sigA-R	5'-AGCTTCTTCTCCTCGTCCTC-3'	

**Table 1. Bacterial strains, plasmids, and primers used in this study.**

acid<sup>29,30</sup>. In the  $\beta$ -lactam-producing *Actinomycetes*, LAT has been shown to catalyze the first steps of  $\beta$ -lactam antibiotic biosynthesis pathway<sup>31</sup>. *Rv3290c*, encoding a Lysine  $\epsilon$ -aminotransferase (LAT) in *M. tuberculosis*, was upregulated over 40-fold in nutrient-starved persistence models<sup>32</sup>. In this study, we initially reported the involvement of LAT in mycobacterium persister formation.

## Materials and Methods

**Antibiotics.** Ampicillin, kanamycin, hygromycin, norfloxacin were bought from Sangon Biotech Co., and their stock solutions were freshly prepared, filter-sterilized, and used at indicated concentrations.

**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* mc<sup>2</sup>155 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<sup>32,33</sup> with minor modifications. In brief, exponential phase cultures were pelleted and washed twice with 1  $\times$  PBS before being resuspended in 1  $\times$  PBS, transferred to standing flasks or microwell and incubated at 37  $^{\circ}$ 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  $\mu$ g/ml; kanamycin, 500  $\mu$ g/ml for *E. coli* or 200  $\mu$ g/ml for *M. smegmatis*; hygromycin, 50  $\mu$ g/ml.

**Knockout mutant construction and complementation.** The *lat* gene of *M. smegmatis* mc<sup>2</sup>155 was disrupted using specialized transduction previously described<sup>34</sup>. PCR and sequencing of *lat*<sub>Msm</sub> 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'-CGCGGATCCGCGTCCTGCTATCATAGCGTCATG-3', bearing a *Bam*H I restriction site followed by the start codon of *Rv3290c*; and 5'-GGAATTCATATGGAATTCGCGCTGCCTTACGTACCAC-3', consisting of the final three C-terminal amino acids of *Rv3290c* and a TAA termination codon followed by a *Nde* I restriction site. The gel-purified polymerase chain reaction product was digested with *Bam*H I and *Nde* I, yielding a 1395-base pair *Bam*H I/*Nde* I fragment. The fragments were ligated into the plasmid pALACE digested by *Bam*H I and *Nde*I, to produce the plasmid pALACE-*lat*<sub>Mtb</sub>. Sequencing of pALACE-*lat*<sub>Mtb</sub> confirmed the correctness of the constructed fragment. Competent cells of *lat*<sub>Msm</sub> mutant were prepared as described<sup>34</sup>, and pALACE containing *lat*<sub>Mtb</sub> gene was used to transform *lat*<sub>Msm</sub> mutant competent cells. This was followed by electroporation<sup>35</sup>

into the mutant as previously described<sup>34</sup>. 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<sub>Mtb</sub>, bacterial pellets were harvested and disrupted by ultrasonication. Samples were then subjected to SDS-PAGE and the His-tagged LAT<sub>Mtb</sub> protein was detected by mouse anti-His antibodies (TIANGEN, China).

**Amino acids determination.** Overnight cultures of *M. smegmatis* mc<sup>2</sup>155 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 ddH<sub>2</sub>O and resuspended in 5 ml of ddH<sub>2</sub>O. Then the bacteria were pipetted into the dialysis tube and dialyzed in ddH<sub>2</sub>O 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<sup>36</sup> 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 µm 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<sup>37</sup>. The initial cell densities were 10<sup>8</sup> 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 after 24 h and 72 h of starvation. Control samples were prepared by washing log-phase cultures twice with PBS then resuspended in PBS, as described for the preparation of starved bacteria, and harvesting 50 ml culture 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<sup>38</sup>.

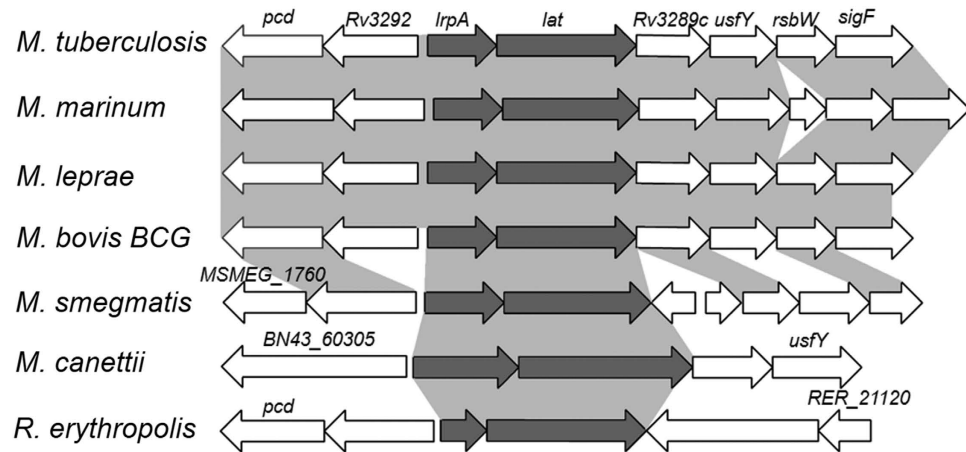
**Real-Time PCR.** 1 µg of total RNA was reversely transcribed to cDNA using a first strand cDNA synthesis kit (Roche) according to the manufacturer's instructions. Resultant cDNA was used for real-time PCR. Advanced SYBRGreen Supermix (BIO-RAD) were used to quantify cDNA in a 20 µL reaction containing 10 µM each primer, 10 µl supermix (2X), 4 µL 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

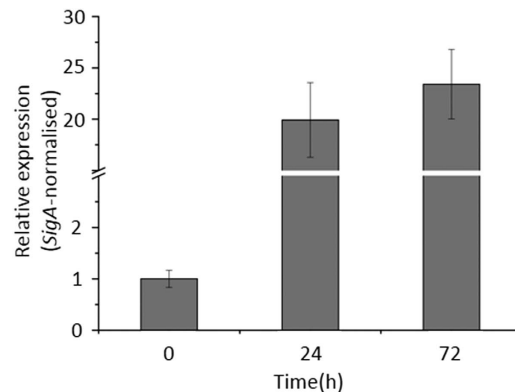
***lat* is conserved among Mycobacteria.** *lat* is conserved among *M. tuberculosis*, *M. marinum*, *M. leprae*, *M. bovis* BCG, *M. smegmatis*, *M. canettii*, and *Rhodococcus erythropolis* PR4 by BLAST analysis. The amino acid identity between *M. tuberculosis* LAT and its homologs is greater than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

***lat*<sub>Msm</sub> 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.*<sup>39</sup> 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 *M. smegmatis lat* 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<sup>40</sup>, in particular to study the persistence under nutrient starvation<sup>41</sup>, *M. smegmatis* was adopted as a model in our study.

***lat* (*MSMEG\_1764*) is nonessential and can be deleted in *M. smegmatis* mc<sup>2</sup>155.** 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 1565 bp (1365 bp of the wild type *lat*<sub>MS</sub>



**Figure 1. Genomic context of *lat* among *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.

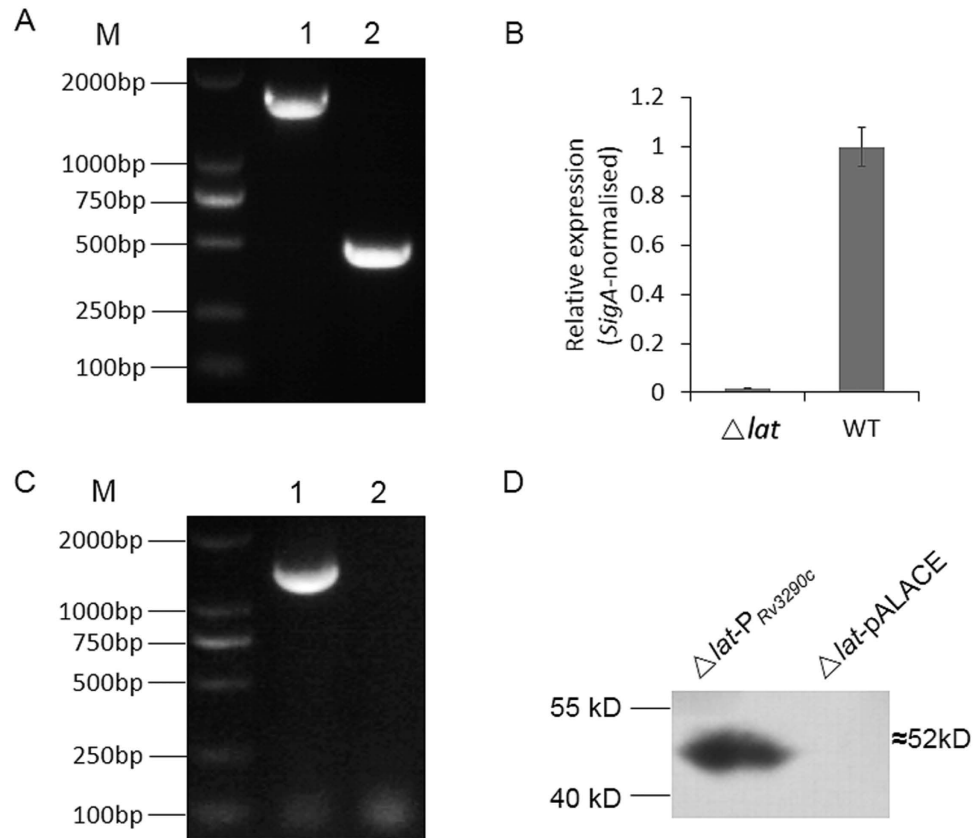


**Figure 2. The expression of *M. smegmatis lat* under nutrient starvation.** Cells of *M. smegmatis* were initially grown in 7H9 medium to log-phase and then washed by  $1 \times$  PBS and resuspended in  $1 \times$  PBS, incubate at  $37^\circ\text{C}$ , 110 rpm. RNAs were extracted from bacteria harvested at indicated time points. RT-PCR was performed as described in Materials and Methods. Data are means  $\pm$  s.d. of triplicates in one of at least three experiments.

and extra 200 bp from the genome) fragment can be amplified from the wild-type, while there was only 449 bp (249 bp of  $\Delta lat_{MS}$  and extra 200 bp from the genome) amplicon from the  $lat_{Msm}$  knockout mutant (Fig. 3A). The  $lat_{Msm}$  knockout mutant was further confirmed by the absence of transcription product (Fig. 3B), indicating that the  $\Delta lat$  mutant of *M. smegmatis* was successfully constructed. Blastp shows that MSMEG\_1764 is the homolog of Rv3290c in *M. smegmatis* and shares 77% identity with Rv3290c. Hence, we use *M. 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  $\Delta lat$  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  $\sim 52$  kDa LAT<sup>Mtb</sup>-His fusion protein in the cell lysates of the complemented strain, while absent in the parental strain (Fig. 3D).

***lat* deleted *M. smegmatis* is hypersensitive to norfloxacin and shows declined persistence under nutrient starvation.** LAT was profoundly upregulated during *M. tuberculosis* starvation persistence model. The effect of *lat* deletion on *M. 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 *lat* has no effect upon the antibiotic sensitivity for the *M. smegmatis* wild-type and complement strain ( $2 \mu\text{g}\cdot\text{ml}^{-1}$  for norfloxacin,  $8 \mu\text{g}\cdot\text{ml}^{-1}$  for rifampicin and  $4 \mu\text{g}\cdot\text{ml}^{-1}$  for isoniazid). The deletion of genes involved in persistence usually has no effect upon MIC<sup>42,43</sup>. To test the effect of *lat* deletion on the MIC, the strains were exposed to high concentration antibiotics. The survival rate of  $\Delta lat_{Msm}$  strain is lower than wild-type and complement strain under  $20 \mu\text{g}\cdot\text{ml}^{-1}$  norfloxacin exposure (Fig. 4A).

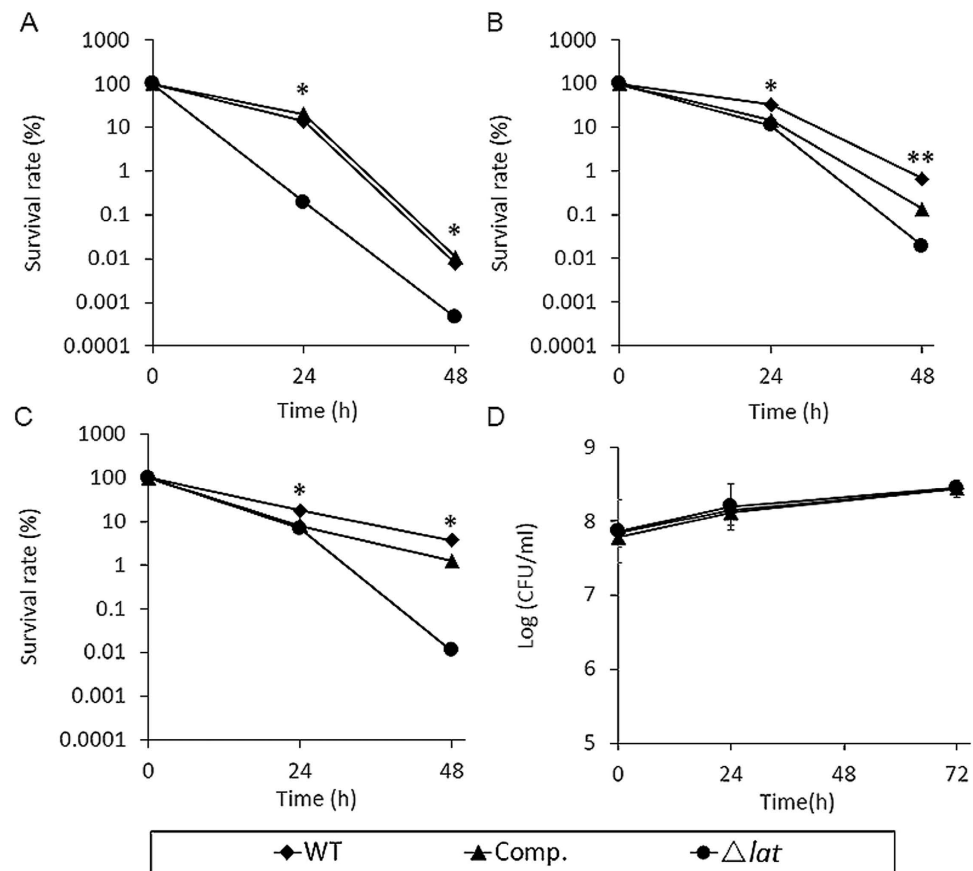
Since LAT was significantly upregulated under nutrient starvation in *M. tuberculosis*. It is interesting to know whether the deletion of *lat* will compromise the survival of *M. smegmatis* under starvation. Therefore, we tested the viability of mutant strain in  $1 \times$  PBS buffer as described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



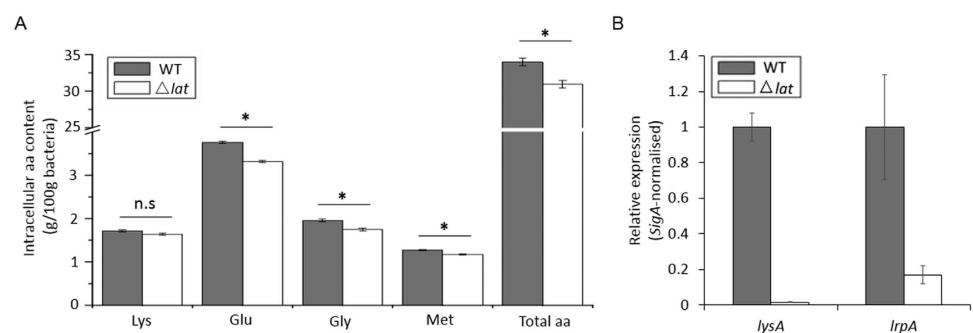
**Figure 3. Construction of *MSMEG\_1764* knockout mutant and complement strains.** (A) PCR verification of the construction *MSMEG\_1764* knockout strain. Lanes: 1. Wild-type MS; 2. *MSMEG\_1764* knockout strain. (B) Verify the transcription of *MSMEG\_1764* knockout strain by RT-PCR. Wild type and  $\Delta lat_{Msm}$  strains were grown at 37C 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 *lat\_{Msm}* gene. (C) Construction of  $\Delta lat$ -*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.

can be seen between wild-type and  $\Delta lat_{Msm}$  strain after 72 hours starvation. To explore whether the deletion of *lat\_{Msm}* 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  $\Delta lat_{Msm}$  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 *lat\_{Msm}* failed to affect the viability under nutrient starvation, but can impair the persistence of the mutant strain under norfloxacin treatment.

**Inactivation of *lat\_{Msm}* decreased the intracellular amino acids content.**  $^{13}\text{C}$ -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<sup>44</sup>. 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<sup>45</sup>. 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  $\Delta MSMEG_1764$  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<sup>46</sup>. To test whether *lysA* control the lysine level in *M. smegmatis*, we measured the expression level of *lysA* in these strains. As expected, the transcription of *lysA* in  $\Delta lat_{Msm}$  strain was decreased (Fig. 5B). Genomic context analysis showed that there is a transcriptional

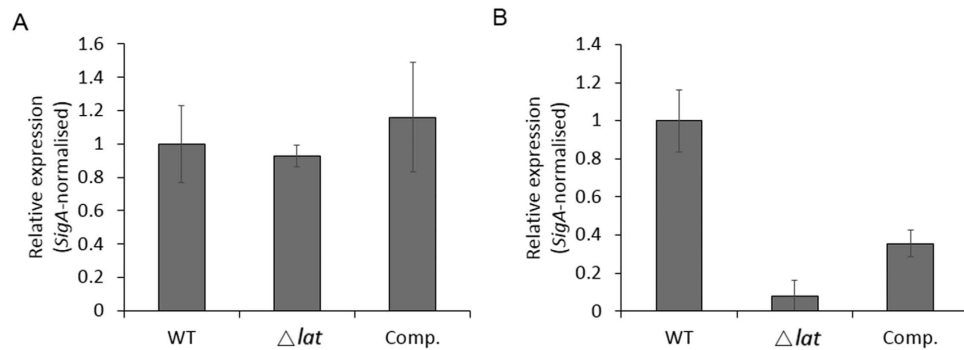


**Figure 4.** Survival of strains under norfloxacin exposure in 1 x PBS buffer. Wild type, complement strain and  $\Delta lat_{Msm}$  strain treated with norfloxacin after 0 h (A), 24 h (B) and 72 h (C) starvation. Data are shown as means  $\pm$  SD of triplicate wells. Similar results were obtained in three independent experiments. (D) Starvation time and persister formation. Log phase cultures of wild type, complement and  $lat_{Msm}$  knockout strain were washed by 1 x PBS and then cultured under starvation condition. Data points are averages of 3 independent experiments. \*\* $P < 0.01$ , \* $P < 0.05$ , Student's  $t$  test, significant difference from the  $\Delta lat_{MS}$  and the wild type strain.



**Figure 5.** Deletion of  $lat_{Msm}$  resulted in the decrease of intracellular amino acid content. (A) Determination of intracellular amino acid content in WT and  $\Delta lat_{Msm}$  strain. (B) The transcription level of *lysA* and *lrpA* in wild type and  $\Delta lat_{Msm}$  strains. Data are shown as means  $\pm$  SD of triplicate wells. Similar results were obtained in three independent experiments. n.s indicates no significant difference ( $P \geq 0.1$ ), \* $P < 0.05$ , Student's  $t$  test, significant difference from the  $\Delta lat_{MS}$  and the wild type strain.

regulator leucine-responsive regulatory protein (LrpA) upstream of *lat* (Fig. 2), a regulator capable of directly binding to the upstream region of *lat*<sup>A7</sup>. To test whether the transcription of *lrpA* was also decreased, RT-PCR was applied. The result showed that *lrpA* transcription was also lowered in  $\Delta lat$  strain (Fig. 5B). The results suggested



**Figure 6. Response of the *rel<sub>Msm</sub>* to starvation.** Profile of the relative expression levels of *rel<sub>Msm</sub>* across 0 h and 24 h time course. Data are means  $\pm$  s.d. of triplicates in one of at least three experiments.

that the deletion of *lat* in *M. smegmatis* decreased the intracellular amino acids amount and downregulated the transcription of *lrpA*.

***relA<sub>Msm</sub>* was downregulated in *lat<sub>Msm</sub>* deletion strain under nutrient starvation.** The stress alarmone guanosine 3',5'-bispyrophosphate ((p)ppGpp), a key molecule in antibiotic resistance<sup>48</sup> and bacterial persistence<sup>49</sup>, was found to accumulate rapidly under starvation. The relationship between *relA* and *lrpA* has previously been noted<sup>50,51</sup>. The expression of *lrp* is positively controlled by ppGpp, namely the lower ppGpp level means fewer Lrp<sup>50</sup>. Moreover, the expression of *lrp* was significantly reduced in the strains failed to produce ppGpp<sup>50</sup>. Rel<sub>Mtb</sub>, the *M. tuberculosis* bifunctional enzyme responsible for both (p)ppGpp synthesis and hydrolysis<sup>52</sup>, and has been shown to play an important role in the survival of bacteria during nutrient starvation condition<sup>53</sup>. To test whether the downregulation of *lrpA* resulted from the decreased expression of *Rel* in *M. smegmatis*, the transcription level of *rel<sub>Msm</sub>*, the *rel<sub>Mtb</sub>* homolog of *M. smegmatis*<sup>54</sup>, 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 *rel<sub>Msm</sub>* was markedly downregulated in  $\Delta lat_{Msm}$  strain after 24 h starvation (Fig. 6B). Complementation of *M. smegmatis*  $\Delta lat_{Msm}$  with the *M. tuberculosis lat Rv3290c* can partially restore the expression of *rel<sub>Msm</sub>*. This result suggested that knockout *lat<sub>Msm</sub>* has no effect upon the intracellular (p)ppGpp content under normal conditions, but can influence the synthesis and hydrolysis of (p)ppGpp under nutrient starvation.

## Discussion

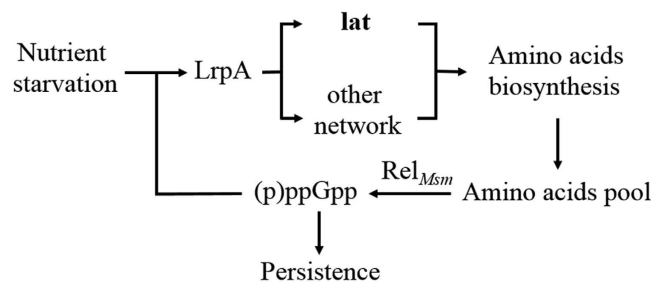
Bactericidal antibiotics usually can sterilize most bacteria rapidly. However, a sub-population will survive and be reactivated upon the withdrawal of the antibiotics<sup>55</sup>. Genes involving in the persisters formation are intensively studied<sup>56</sup> both for fundamental insights and translational 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 persistence<sup>42,43</sup>. LAT was previously showed to be involved in the L-lysine metabolism<sup>57</sup>. The data here showed that the knockout of *lat<sub>Msm</sub>* 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 metabolism<sup>58</sup>, 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 anabolism<sup>59,60</sup>. The knockout of *lrp* in *E. coli* downregulated the expression of the amino acid metabolism-related genes<sup>61</sup>. In our study, we found the expression of *lrpA* was downregulated in *lat<sub>Msm</sub>* 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. tuberculosis*<sup>39</sup>, as well as in *M. smegmatis* (Fig. 5B). The persistence of *lrpA* inactivated *Mycobacterium fortuitum* was attenuated in a murine infection model<sup>62</sup>. The expression of *lrp* is stimulated by (p)ppGpp and LRP can regulate the biosynthesis of amino acid<sup>50</sup>. Here we have shown that the deletion of *lat<sub>Msm</sub>* downregulated the expression of *rel<sub>Msm</sub>* (Fig. 6). *M. tuberculosis* failed to produce (p)ppGpp under starvation was defective in long-term survival both *in vitro*<sup>53</sup> and *in vivo*<sup>15</sup>. In our study, the downregulation of *rel<sub>Msm</sub>* 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)ppGpp is produced by Rel<sub>Msm</sub> 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)ppGpp, and (p)ppGpp controls the dormant persister formation (Fig. 7). The *lat* deleted mutant strain failed to replenish the amino acid pool, then downregulated



**Figure 7. A model of feed-forward loop controlling *M. smegmatis* persistence in response to nutrition starvation .**

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<sup>63</sup> will promisingly facilitate the discovery of novel LAT inhibitors<sup>64</sup>. 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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### Author Contributions

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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