Housecleaning of pyrimidine nucleotide pool coordinates metabolic adaptation of nongrowing *Mycobacterium tuberculosis*

Kun-Xiong Shi*, Yong-Kai Wu, Bi-Kui Tang, Guo-Ping Zhao and Liang-Dong Lyu

*Key Laboratory of Medical Molecular Virology of MOE/MOH, School of Basic Medical Sciences, Fudan University, Shanghai, People’s Republic of China; †Department of Microbiology, School of Life Sciences, Fudan University, Shanghai, People’s Republic of China; ‡Department of Life Science, Bengbu Medical College, Bengbu, People’s Republic of China; †Department of Microbiology and Li Ka Shing Institute of Health Sciences, The Chinese University of Hong Kong, Prince of Wales Hospital, New Territories, Hong Kong, People’s Republic of China; ‡CAS-Key Laboratory of Synthetic Biology, Shanghai Institute of Plant Physiology and Ecology, Chinese Academy of Sciences, Shanghai, People’s Republic of China

**ABSTRACT**

The ability of *Mycobacterium tuberculosis* (*Mtb*) to adopt a slowly growing or nongrowing state within the host plays a critical role for the bacilli to persist in the face of a prolonged multidrug therapy, establish latency and sustain chronic infection. In our previous study, we revealed that genome maintenance via MazG-mediated elimination of oxidized dCTP contributes to the antibiotic tolerance of nongrowing *Mtb*. Here, we provide evidence that housecleaning of pyrimidine nucleotide pool via MazG coordinates metabolic adaptation of *Mtb* to nongrowing state. We found that the Δ*mazG* mutant fails to maintain a nongrowing and metabolic quiescence state under dormancy models in vitro. To investigate bacterial metabolic changes during infection, we employed RNA-seq to compare the global transcriptional response of wild-type *Mtb* and the Δ*mazG* mutant after infection of macrophages. Pathway enrichment analyses of the differentially regulated genes indicate that the deletion of *mazG* in *Mtb* not only results in DNA instability, but also perturbs pyrimidine metabolism, iron and carbon source uptake, catabolism of propionate and TCA cycle. Moreover, these transcriptional signatures reflect anticipatory metabolism and regulatory activities observed during cell cycle re-entry in the Δ*mazG* mutant. Taken together, these results provide evidence that pyrimidine metabolism is a metabolic checkpoint during mycobacterial adaptation to nongrowing state.

**ARTICLE HISTORY** Received 21 October 2018; Revised 26 November 2018; Accepted 3 December 2018

**KEYWORDS** *Mycobacterium tuberculosis*; dormancy; nongrowing state; pyrimidine metabolism; *mazG*

Despite the availability of chemotherapy, tuberculosis remains a leading cause of death due to a single bacterium infection. Increasing evidence shows that the ability of *Mycobacterium tuberculosis* (*Mtb*), the causative agent of tuberculosis to adopt a nongrowing state within the host plays a critical role for the bacilli to persist in the face of a prolonged multidrug therapy, establish latency and sustain chronic infection. Therefore, understanding the molecular events underlying growth control and metabolic adaptation of nongrowing *Mtb* is believed to be particularly important for the development of new therapeutic strategies [1,2]. In our previous studies, we showed that elimination of oxidized dCTP via NTP pyrophosphohydrolase MazG is required for the persistence of *Mtb* during chronic infection of mouse and contributed to antibiotic tolerance of stationary-phase culture and intracellular *Mtb* [3,4]. These results established that housecleaning of the pyrimidine nucleotide pool plays a crucial role in *Mtb* genome maintenance under stress environments. Given that all of these phenotypes are akin to a nongrowing state, we speculate that *mazG* may implicate in mycobacterial adaptation to growth-limiting environments. In this preliminary data report, we provide evidence that housecleaning of pyrimidine nucleotide pool coordinates metabolic adaptation of nongrowing *Mtb*.

To test whether *mazG* is required for mycobacterial adaptation to nongrowing state, we measured the survival of wild-type *Mtb* and the Δ*mazG* mutant under hypoxic and nutrient-starvation conditions, i.e. the two in vitro models of mycobacterial dormancy. Under both conditions, while the wild-type *Mtb* and the complemented Δ*mazG* mutant maintained slightly decreased level (Figure 1(a)) or the same level (Figure 1(b)) of colony forming units (CFUs) during the course...
of treatment, the ΔmazG mutants exhibited a survival advantage over that of the wild type (Figure 1(a,b)). Most dramatically, the deletion of mazG results in a CFU counts 10 times higher than that of wild-type Mtb after 5 weeks hypoxic treatment (Figure 1(a)), consistent with the results of a previous transposon library-screening study showing that inactivation of Mtb mazG (Rv1021, which was annotated as a conserved hypothetical protein in the Supporting Information of Ref. [5]) results in a survival advantage phenotype under hypoxic condition [5]. These results demonstrate that the ΔmazG mutant is unable to arrest its growth in response to growth-limiting environments.

Our previous studies demonstrated that the deletion of mazG could result in the accumulation of an oxidized pyrimidine nucleotide in mycobacteria, which may affect nucleotide homeostasis and cellular metabolism [3,4]. To investigate whether the observed growth advantage phenotype is accompanied with altered cellular metabolism, we assessed the bacterial NADH/NAD\(^+\) redox balance, given that the regeneration of reducing equivalents is critical for the maintenance of cellular metabolism in nongrowing Mtb [2]. As shown in Figure 1(c), the ΔmazG mutant showed decreased NADH/NAD\(^+\) ratios compared with that of the wild-type Mtb, indicating that the cellular redox balance is shifted towards an oxidizing state [2]. These results indicate that MazG-mediated maintenance of pyrimidine nucleotide pool plays a crucial role in the metabolic adaptation of nongrowing Mtb.

A recent study demonstrated that the stress environment of macrophages could induce nongrowing or slow-growing forms of Mtb [6]. To investigate whether the deletion of mazG affects metabolic adaptation of Mtb during infection, we employed RNA-seq to profile the global transcriptional response of wild-type Mtb and the ΔmazG mutant at 1-day post-infection of human macrophage-like THP-1 cells (Supplementary Figure S1), which was when the ΔmazG mutant begins to show reduced survival compared with that of wild type [3]. Totally, 109 upregulated and 25 downregulated genes with fold change >2 were identified in the ΔmazG mutant compared with wild-type Mtb (Dataset S1). For a global view of key regulational changes, differentially regulated genes were grouped into functional categories according to TuberculList server classification (Figure 1(d)). Among the upregulated genes, the most significantly enriched categories are insertion sequences and phages (\(p = .0005\)). This transcriptional signature is an indication of DNA instability in the ΔmazG mutant, given that the induction of transposase was shown to be a common response to DNA damage in Mtb and Escherichia coli [7,8]. Moreover, the upregulation of genes involved in DNA maintenance (cas1/2, mrr, ung, ligA and mutY) also reflects increased DNA damage in the ΔmazG mutant (Dataset S1). Significantly upregulated category was also observed for detoxification and adaptation processes, such as mazEF, vapC and ephE (involves in detoxification of oxidatively damaged lipids). Together, these transcriptional signatures corroborate the genome safeguarding and antioxidative roles of MazG [3,4].

Among the differentially regulated genes, functional categories relative to metabolism were also significantly enriched, including cell wall and cell processes (\(p = .021\)), intermediary metabolism and respiration (\(p = .042\)) and lipid metabolism (\(p = .003\)) (Figure 1(d)). To further delineate the metabolic changes, we performed pathway enrichment analyses according to the KEGG pathway with manual inspection. Significantly upregulated metabolic pathways include biosynthesis of mycobactin (a molecule that scavenges iron from the environment), ABC transporter, citrate cycle and starch and sucrose metabolism, whereas degradation of cholesterol [9] and branch chain amino acids (BCAAs), propionate metabolism and glycerine, serine and threonine metabolism were significantly downregulated (Figure 1(e)). Overall, these results indicate that the deletion of mazG impacts cellular metabolism of Mtb during infection. The mechanism that underlies the observed metabolic changes due to mazG deletion remains to be determined. However, the upregulation of cdd (cytidine deaminase), dcd (dCTP deaminase) and pyrR (repressor of pyrimidine synthesis) in the ΔmazG mutant [10], taken together with the characterized role of MazG in eliminating oxidized dCTP, suggests that the observed metabolic changes may stem from dysfunction of pyrimidine metabolism.

The most intriguing metabolic change in the ΔmazG mutant is the simultaneous downregulation of catalytic pathways that could give rise to propionyl-CoA, a toxic metabolic intermediate generated during degradation of cholesterol, BCAA and odd chain fatty acids (Figure 1(e–f)). Although mycobacterial persistence requires the utilization of host cholesterol, Mtb is extraordinarily sensitive to increases in the propionyl-CoA pool unless further detoxified [9,11]. Intriguingly, genes that belong to the propionyl-CoA detoxification pathways, including the methylcitrate cycle (prpC and prpR) and synthesis of methyl-branched lipids (ppsB, papA5, pks1 and drrBC), were upregulated in the ΔmazG mutant (Figure 1(f)). Thereafter, these transcriptional changes reflect a unique metabolic state that could alleviate the metabolic poisoning by propionyl-CoA. In addition, accompanying with the downregulation of cholesterol and BCAA catalytic pathways, genes involved in the uptake of nutrients other than lipids, including sugar (uspB), glycerol-3-phosphate (ugpA) and peptides (drrBC and dppB), as well as catalabolism of diaccharide (malQ) were significantly
Figure 1. Deletion of mazG impairs mycobacterial metabolic adaptation in dormancy model in vitro and during infection of macrophages. Survival of Mtb stains under hypoxic (a) and nutrient-starvation (b) conditions in vitro. The surviving cells were quantified by counting colonies after plating. Compl, complemented mutant strain. Data shown are mean ± SE in triplicate. (c) Cellular NADH/NAD+ ratios in Mtb strains under hypoxic and nutrient-starvation conditions. Thirty-five days after hypoxic/starvation treatment intracellular NADH and NAD+ were extracted and measured as described in Supplementary Materials. Data shown are mean ± SE in duplicate. (d) Functional categories of the differentially expressed genes in the ΔmazG mutant (increase or decrease with fold change >2 vs wt) at 1-day post-infection of THP-1 macrophages. Enrichment analyses of the differentially expressed genes in the ΔmazG mutant according to KEGG pathway. (f) Graphic representation of global metabolic changes in the ΔmazG mutant deduced from enriched pathways according to TubercuList classification and KEGG pathway. Genes showed upregulation and downregulation (ΔmazG vs wt, fold change >2) are indicated in red type and blue type, respectively. Genes required for growth arrest of Mtb under hypoxic condition (Ref. [5]) are underlined. FC, fold change. *p < .05, **p < .01, ***p < .001.
upregulated in the ΔmazG mutant (Figure 1(e–f)). Together, these transcriptional signatures reflect a switch in the usage of carbon sources in the ΔmazG mutant during infection [1].

Compared with wild-type Mtb, several genes involved in cell cycle control were upregulated in the ΔmazG mutant. For instance, the expression of the resuscitation-promoting factor (rpfF), which is implicated in the resuscitation of mycobacteria from dormancy via a mechanism of cell wall remodeling [12], was increased 2.4-fold. Moreover, the upregulation of malQ reflects an increased usage of maltose derived from trehalose (Figure 1(f)), which was shown to be a critical carbon source required for the re-initiation of de novo synthesis of peptidoglycan for cell cycle re-entry in hypoxic nongrowing Mtb [13]. In line with this, we found that murB, the gene involved in synthesis UDP-N-acetylmuramic acid (a precursor of peptidoglycan), was also upregulated (1.7-fold) in the ΔmazG mutant (Dataset S1). Expression of whiB5, which encodes a transcriptional regulator involved in the resume of growth after reactivation from chronic infection and maintenance of metabolic activity following prolonged starvation [14], was increased in the ΔmazG mutant as well. In sum, these transcriptional changes thus reflect a metabolic state observed during cell cycle re-entry [1]. In this connection, it is worth noting that genes involved in iron acquisition (mibTCDEFGH and irTB) were significantly upregulated (Figure 1(e)). In addition, the upregulation of fumarate reductase (frdC, 7.2-fold) in the ΔmazG mutant may result in increased anaerobic respiration via fermentation of fumarate to succinate (Figure 1(f)), a biochemical process that was shown to be essential for viability of nongrowing Mtb by sustain membrane potential, ATP synthesis and providing of biosynthetic precursors [15]. In line with this observation, we found that under hypoxic condition both the isocitrate lyase and the frdC showed increased expression in the ΔmazG mutant compared with that of wild-type Mtb (Supplementary Figure S2).

Increasing evidence is revealing the strong impact of bacterial metabolism on the mycobacterial life cycle, pathogenicity and immunoreactivity in the host [1]. However, as an important aspect of cellular metabolism, the mechanism of nucleotide metabolism and its interplay with other metabolic pathways in nongrowing Mtb remains unclear (Figure 1(g)). Our study reveals that the housecleaning of pyrimidine nucleotide pool plays a unique role in mycobacterial metabolic adaptation and growth control under growth-limiting environments. These results, taken together with the previous transposon library-screening study showing that the inactivation of Mtb pyrF and cdd (all are involved in pyrimidine metabolism) resulted in failure to arrest growth under hypoxic dormancy model [5], provide evidence that pyrimidine metabolism is a metabolic checkpoint during mycobacterial adaptation to nongrowing state. Further studies were carried out in our laboratory to address the biochemistry and metabolic flux underlying the maintenance of pyrimidine nucleotide pool in Mtb and evaluate whether pyrimidine metabolism serves as a potential target for intervention of bacterial persistence.

Supplementary Information accompanies the manuscript on the Emerging Microbes & Infections website http://www.nature.com/emi

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

The work was supported in part by the National Key R&D Program of China [grant number 2018YFD0500900] and the National Science and Technology Major Project of China [grant number 2018ZX10302301] to L-D. L. and the National Natural Science Foundation of China [grant numbers 31830002, 81671988] to G-P.Z.

ORCID

Guo-Ping Zhao http://orcid.org/0000-0002-4691-3257
Liang-Dong Lyu http://orcid.org/0000-0001-6391-6030

References

[1] Ehrt S, Schnappinger D, Rhee KY. Metabolic principles of persistence and pathogenicity in Mycobacterium tuberculosis. Nat Rev Microbiol. 2018;16:496–507.
[2] Boshoff HI, Barry CE. 3rd Tuberculosis - metabolism and respiration in the absence of growth. Nat Rev Microbiol. 2005;3:70–80.
[3] Fan XY, Tang BK, Xu YY, et al. Oxidation of dCTP contributes to antibiotic lethality in stationary-phase mycobacteria. Proc Natl Acad Sci U S A. 2018;115:2210–2215.
[4] Lyu LD, Tang BK, Fan XY, et al. Mycobacterial MazG safeguards genetic stability via housecleaning of 5-OH-dCTP. PLoS Pathog. 2013;9:e1003814.
[5] Baek SH, Li AH, Sassetti CM. Metabolic regulation of mycobacterial growth and antibiotic sensitivity. PLoS Biol. 2011;9:e1001065.
[6] Manina G, Dhar N, McKinney JD. Stress and host immunity amplify Mycobacterium tuberculosis phenotypic heterogeneity and induce nongrowing metabolically active forms. Cell Host Microbe. 2015;17:32–46.
[7] Boshoff HI, Reed MB, Barry CE, et al. Dna2 polymerase contributes to in vivo survival and the emergence of drug resistance in Mycobacterium tuberculosis. Cell. 2003;113:183–193.
[8] Peters JE, Craig NL. Tn7 transposes proximal to DNA double-strand breaks and into regions where chromosomal DNA replication terminates. Mol Cell. 2000;6:573–582.
[9] Griffin JE, Gawronski JD, DeJesus MA, et al. High-resolution phenotypic profiling defines genes essential
for mycobacterial growth and cholesterol catabolism. PLoS Pathog. 2011;7:e1002251.

[10] Fields CJ, Switzer RL. Regulation of pyr gene expression in Mycobacterium smegmatis by PyrR-dependent translational repression. J Bacteriol. 2007;189:6236–6245.

[11] Lee W, VanderVen BC, Fahey RJ, et al. Intracellular Mycobacterium tuberculosis exploits host-derived fatty acids to limit metabolic stress. J Biol Chem. 2013;288:6788–6800.

[12] Kana BD, Gordhan BG, Downing KJ, et al. The resuscitation-promoting factors of Mycobacterium tuberculosis are required for virulence and resuscitation from dormancy but are collectively dispensable for growth in vitro. Mol Microbiol. 2008;67:672–684.

[13] Eoh H, Wang Z, Layre E, et al. Metabolic anticipation in Mycobacterium tuberculosis. Nat Microbiol. 2017;2: Article no. 17084.

[14] Casonato S, Cervantes Sánchez A, Haruki H, et al. WhiB5, a transcriptional regulator that contributes to Mycobacterium tuberculosis virulence and reactivation. Infect Immun. 2012;80:3132–3144.

[15] Watanabe S, Zimmermann M, Goodwin MB, et al. Fumarate reductase activity maintains an energized membrane in anaerobic Mycobacterium tuberculosis. PLoS Pathog. 2011;7:e1002287.