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Catabolism of 8-oxo-purines is mainly routed *via* the guanine to xanthine interconversion pathway in *Mycobacterium smegmatis*

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ABSTRACT

Metabolism of purine bases remains poorly understood in the pathogenic bacterium *Mycobacterium tuberculosis* and closely related, nonpathogenic *Mycobacterium smegmatis* (Msm). To gain insight into the purine metabolism in mycobacteria, we tested uptake of purine bases with a *ΔpurF* Msm mutant with an inactive purine *de novo* biosynthesis pathway and confirmed that hypoxanthine and guanine, but not xanthine, can serve as nucleotide precursors for recycling in the salvage pathway. Further, we focused on purine catabolism in wild-type (wt) Msm. We found that only xanthine and guanine could serve as a sole nitrogen source for wt Msm. These data confirm that Msm catabolism of purines is directed mainly *via* oxidative guanine to xanthine interconversion and not through metabolic conversion of hypoxanthine to xanthine. Our data represent the first experimental evidence confirming the use of 8-oxo-purines as a nitrogen source by Msm.

1. Introduction

Purine bases are an abundant component of many biomolecules including nucleotides, cofactors and nucleic acids. In most organisms, purine nucleotides can be synthesized *de novo* from simpler precursors or reconverted from preexisting purine bases and nucleosides by the salvage pathway. In cultivable mycobacteria, genomic and transcriptomic analyses have identified active genes for both the *de novo* purine synthesis pathway and purine recycling/interconversion salvage pathway [1–3]. The first step in the *de novo* pathway is amido-phosphoribosyltransferase (PurF)-catalyzed formation of 5-phosphoribosylamine, in which the pyrophosphate group of 5-phosphoribosyl-1-pyrophosphate is replaced with an amino group from glutamine. Inactivation of the *purF* gene in *Mycobacterium smegmatis* (Msm) leads to purine auxotrophy, which is accompanied by decreased survival under hypoxic conditions [4]. The following nine steps in the *de novo* biosynthetic pathway lead to synthesis of inosine-5-monophosphate, a branching precursor of adenosine- and guanosine-5-monophosphate. Mycobacteria also express a fully competent battery of purine salvage pathway enzymes that can regulate levels of individual purine nucleotides in response to cellular needs, and can scavenge precursors to the purine bases from the environment [5,6]. Salvage pathway enzymes catalyze the turnover and interconversion of bases and nucleotides.

The pool of purine bases that is not salvaged can be catabolized to end-products and secreted or, in some bacterial species, re-used as a nitrogen source. Purine catabolism starts with oxidation of xanthine to uric acid, which is catalyzed by members of the xanthine oxidase/dehydrogenase enzyme family (XO/XDH). Uric acid is next oxidized by urate oxidase to allantoin, which is then decomposed to urea and glyoxalate *via* subsequent reactions catalyzed by allantoinase and ureidoglycolate lyase. Urea is finally converted to re-useable ammonium and carbon dioxide by urease (Fig. 1). In contrast to the *Mycobacterium tuberculosis* genome, the Msm genome encodes complete set of purine catabolic enzymes [7–9]. Transcriptomic analysis of Msm continuously cultivated under nitrogen-limited conditions indicated significant upregulation of purine nucleotide catabolic genes [8,9]. However, a more detailed analysis of purine catabolism in Msm has not yet been performed.

Here, we present experimental evidence confirming the ability of Msm to salvage some 8-oxo-purines and to catabolize guanine and xanthine and use them as a sole nitrogen source.

2. Material and methods

2.1. Construction of deletion plasmids and purF gene disruption

The *ΔpurF* Msm mutant was prepared according to the method described by Shenkerman et al., with some modifications [10]. The DNA

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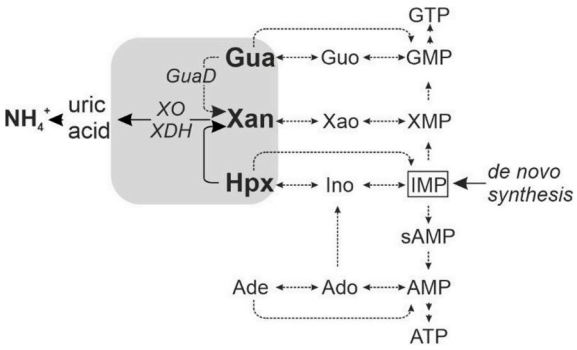

Fig. 1. Salvage and catabolism of purine metabolites. Ado – adenosine, Ade-adenine, Ino – inosine, Hpx – hypoxanthine, Xao - xanthosine, Xan – xanthine, Guo - Guanosine, Gua –guanine. GuaD – guanine deaminase, XAO – xanthine oxidase.

constructs pYS1, pYS2 and pML2714 were kindly provided by Dr. Eyal Gur (Ben-Gurion University of the Negev, Israel). Details about the pYS2-based *purF* deletion DNA construct and corresponding primers are listed in Table 1. The upstream (*ups*) and downstream (*dns*) regions of the *purF* coding sequence were sequentially inserted into the *loxP-gf-p-hyg*^{r}*-loxP* cassette of the pYS2 plasmid *via* *Spe*I/*Swa*I and *Pac*I/*Nsi*I sites, respectively, using T4 DNA ligase. The *ups* and *dns* regions were amplified by PCR with Q5 polymerase (NEB) using Msm chromosomal DNA as a template. *SpeI/NsiI*-linearized pYS2-ΔpurF was used to generate the *purF*::*loxP* strain (*ΔpurF*) as described [10]. Gene disruption was verified by PCR using a primer pair that anneals at the boundaries of the recombined region, and the resulting DNA amplicon was sequenced.

2.2. Media and growth conditions

Msm mc^2 155 wt and *ΔpurF* strains were inoculated into Middlebrook 7H9/ADC medium (Himedia) to a final O.D.$_{600}$ of 0.005. For growth of the *ΔpurF* strain, the medium was supplemented with 50 μM hypoxanthine. The bacterial cells were cultivated at 37 °C and 220 rpm until the O.D.$_{600}$ reached 0.5–1. For drop tests, solid HdB medium containing 1.5% agar, 0.5% glycerol, and 0.1% glucose was prepared according to Berney et al. [11]. Aliquots from the stock solutions (75 mM in DMSO) of xanthine, hypoxanthine, and guanine (Sigma-Aldrich) were added to agar media at 55 °C immediately before plate pouring. Cultured bacterial cells were suspended in sterile PBS. Serial 10-fold dilutions of cell suspensions were prepared in PBS, spotted on prepared plates, and incubated at 37 °C. Three independent experiments were performed.

2.3. Growth curve determination

Uptake of 8-oxo-purines by the *ΔpurF* strain and wt Msm in liquid medium was tested using Bioscreen C (Dynex, USA). A 200 μl aliquot of

Table 1
Primers used to construct the pYS2- purF deletion plasmid.

Construct name	Gene to delete	upstream region (from – to)	primer pair	downstream region (from – to)		primer pair
pYS2-ΔpurF	*purF*	5,869,979–5,869,280	1; 2	5,867,743–5,867,044		3; 4
primer	sequence (5′- to -3′)			orientation[a]	restriction site	
1	aaaACTAGTgaacgacacctggatgctgacggtg			F	*Spe*I	
2	tttATTTAAATgatatggctgctcccggatgggctg			R	*Swa*I	
3	aaaaTTAATTAAtacgggctcaccccgagaacagtgc			F	*Pac*I	
4	tttATGCATgcctgggacgtcggcgggcccgacg			R	*Nsi*I	

[a] – F – forward, R – reverse.

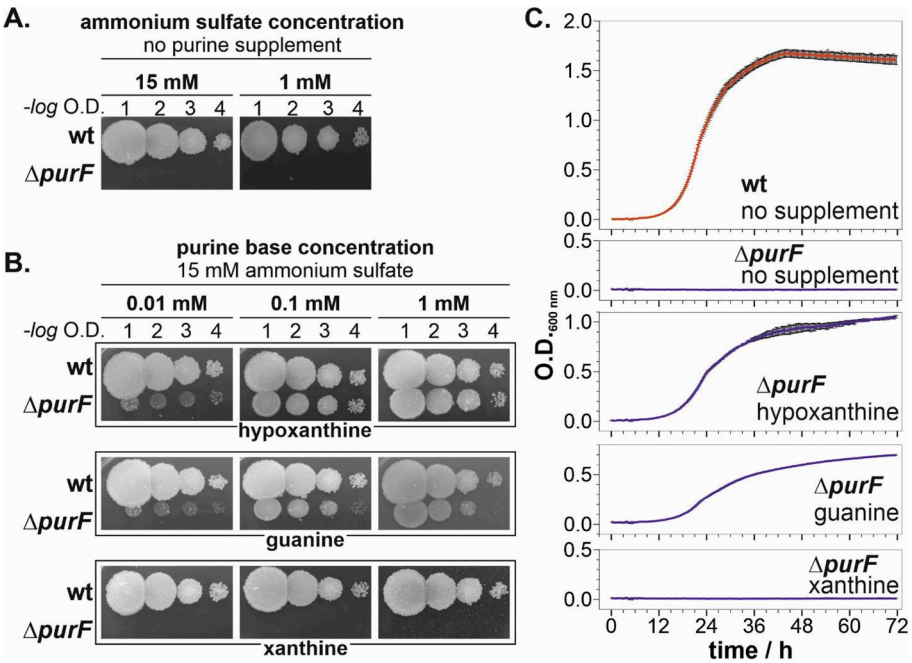

Fig. 2. Dependency of the *ΔpurF* and wt Msm growth on uptake of purines from environment. Serially diluted and spotted Msm *ΔpurF* and control wt strains were cultivated on minimal solid HdB medium without (A) or with (B) 8-oxo-purines for 3 days. (C) Bioscreen growth curves of the wt and *ΔpurF* strains in liquid HdB medium without or with 0.1 mM 8-oxo-purines. Grey lines represent standard deviation error bars. N = 4.

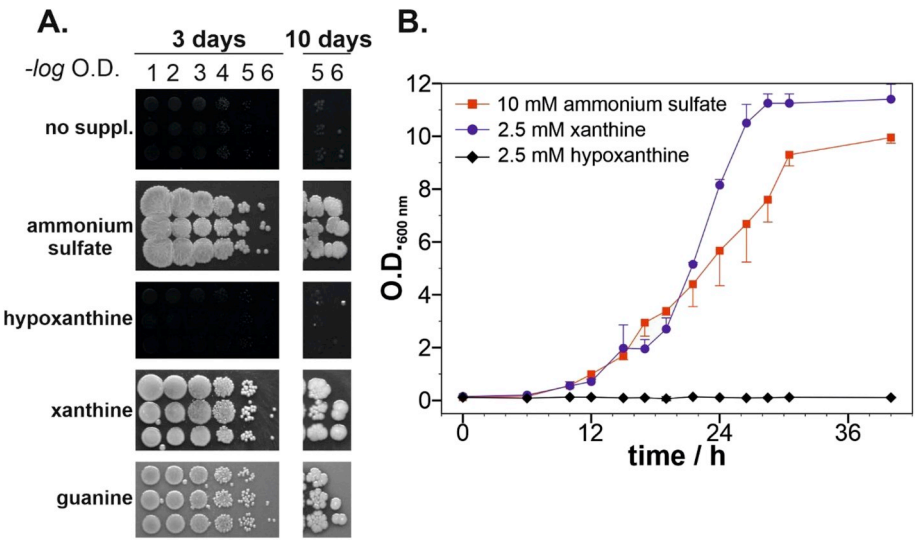

Fig. 3. Growth of the Msm wt strain on minimal HdB medium with 8-oxo-purines as a sole nitrogen source. (A) The Msm strain growth on minimal solid HdB medium with 2.5 mM 8-oxo-purines or 10 mM ammonium sulfate for 3- and 10-days. For each condition 3 independent dilutions and spots are shown. (B) Growth of the Msm wt strain in liquid minimal HdB medium containing 2.5 mM 8-oxo-purines or 10 mM ammonium sulfate. Error bars represents standard deviation. N = 4.

HdB medium containing 0.5% glycerol, 0.1% glucose, 10 mM ammonium sulfate and 0.05% tyloxapol was pipeted into individual wells of a 100-well polystyrene honeycomb plate. Purines were added from a 75 mM stock solution in DMSO up to a final concentration of 0.1 mM. Finally, 20 µl of washed cell suspension ($O.D._{600} = 0.1$) in PBS was added to the medium. Plates were cultivated at 37 °C and 400 rpm. The $O.D._{600}$ value was recorded at 15 min intervals. Four independent biological replicates were performed.

The growth curves of the wt strain in the presence of 2.5 mM xanthine, 2.5 mM hypoxanthine or 10 mM ammonium sulfate as sole nitrogen source were determined as follows: 50 ml of sterile HdB medium containing 0.5% glycerol, 0.1% glucose, and 0.1% tyloxapol, and purine base or ammonium sulfate was inoculated with the wt Msm strain to an initial $O.D._{600}$ of 0.1. Msm culture was incubated at 37 °C and 200 rpm in an Innova 44 shaker (New Brunswick, USA). The $O.D._{600}$ value was monitored during growth in a 1-cm cuvette using a BioPhotometer Plus spectrophotometer (Eppendorf, Germany). Four independent biological replicates were performed.

3. Results and discussion

First, we examined the ability of Msm to utilize purines from the cultivation medium using the purine *de novo* synthesis-deficient Msm mutant *ΔpurF,* which does not grow without purine supplementation. We deleted the *purF* coding sequence using the method described by Shenkerman et al. (2014) [10]. To rule out potential side effects of *purF* gene deletion, we performed a *purF* complementation experiment (Supplementary information). Exogenously plasmid-based expression of PurF in the *ΔpurF* strain restored growth to levels comparable to wt Msm (Fig. S1). The uptake of exogenous 8-oxo-purines and growth of *ΔpurF* strain was first tested on solid minimal HdB medium, containing ammonium sulfate as nitrogen source, and various concentrations of xanthine, hypoxanthine, and guanine, as precursors for purine salvage pathway. In contrast to the wt Msm strain, the *ΔpurF* mutant did not grow on solid medium without a purine supplement with 15 or 1 mM ammonium sulfate (Fig. 2A). In the presence of 0.01 mM guanine and hypoxanthine, we observed slight growth of the *ΔpurF* strain after 3 days (Fig. 2B, top and middle panel). Growth of the *ΔpurF* strain was restored at hypoxanthine concentrations higher than 0.1 mM. Growth of the *ΔpurF* strain in the presence of guanine was lower than with hypoxanthine, potentially due to the low solubility of guanine, which may cause decreased bioavailability. In general, the water solubility of 8-oxo-purines decreases in the following order: hypoxanthine (6 mM), xanthine (0.3 mM), and guanine (0.04 mM) [12,13]. Xanthine did not support growth of the *ΔpurF* strain on solid medium (Fig. 2B, bottom panel). Similar results were obtained for the *ΔpurF* strain cultivated in liquid HdB medium containing 8-oxopurines at a fixed concentration of 0.1 mM (Fig. 2C). The *ΔpurF* strain grew better in the presence of hypoxanthine than guanine, and no growth was observed in the presence of xanthine. Taken together, our results show that Msm can utilize hypoxanthine and guanine from the environment in the absence of *de novo* purine nucleotide biosynthesis, and these purines can serve as precursors for salvaging by hypoxanthine-guanine phosphoribosyltransferase (HGPRT). The inability of xanthine to restore growth of the *ΔpurF* strain could be linked to its poor uptake by Msm and/or restricted salvaging of this purine, which does not serve as a substrate for HGPRT. Previous characterization of Mtb HGPRT showed that this enzyme accepts only hypoxanthine and guanine as substrates *in vitro* [14,15].

Next, we focused on catabolism of 8-oxo-purines in Msm. We tested the growth of wt Msm cultivated on solid minimal HdB medium in the presence of 2.5 mM hypoxanthine, xanthine, and guanine, which served as a sole nitrogen source and compared these findings with growth on HdB medium in the presence of 10 mM ammonium sulfate. We evaluated colony growth after 3 and 10 days of cultivation. After 3 days, Msm formed colonies in the presence of ammonium, guanine, and xanthine (Fig. 3A). Interestingly, in the presence of hypoxanthine, which was efficiently salvaged (Fig. 2B and C) and has the highest solubility, colonies were not observed even in samples cultivated for 10 days (Fig. 3A). As expected, we did not observe colony formation for Msm cultivated on solid minimal HdB medium without addition of any nitrogen source.

Bacterial growth could be limited by slow diffusion of nutrients in solid medium, and therefore we examined Msm growth in HdB liquid medium supplemented with 10 mM ammonium sulfate, hypoxanthine or xanthine. Due to the extremely low solubility of guanine, accompanied by the high turbidity of liquid medium, we did not include guanine among the 8-oxo-purines tested. The results (summarized in Fig. 3B) revealed that Msm grew only in the presence of 10 mM ammonium sulfate and 2.5 mM xanthine. The growth of Msm with xanthine was slightly faster than with ammonium sulfate; however, both cultures reached a comparable final cell density (Fig. 3B). Taking into consideration the similar nitrogen content provided by 10 mM ammonium sulfate and 2.5 mM xanthine (xanthine is catabolized to four molecules of ammonium), our findings indicate that xanthine is a comparable or even better nitrogen source for Msm than ammonium. Our results

confirm that Msm can catabolize guanine and xanthine to ammonium, which can be further used as a sole nitrogen source. This active purine catabolic pathway in Msm might be connected to the occurrence of this species in aqueous environments and other ecological niches in which purines can be present from decomposition of living species [16]. Purine are rich nitrogen compounds and serve as a nitrogen source for a variety of bacterial species [17–19]. This purine catabolic pathway is likely not active in Mtb, although some contradictory results have been published [17]; however, a more detailed characterization of purine catabolism has not yet been performed. No homologous genes of GuaD, urate oxidase (MSMEG_1296), or allantoinase (MSMEG_1294) have been identified in the Mtb genome, which further supports the absence of this pathway in Mtb.

The inability of Msm to catabolize hypoxanthine was surprising. Previous transcriptomic study suggested that Msm can use xanthine as a nitrogen source [8]. Efficient catabolism of guanine on solid medium by Msm is in agreement with the previous observation that the MSMEG_1298 locus, which encodes a guanine deaminase that catalyzes guanine deamination to xanthine (GuaD, see Fig. 1), is upregulated during nitrogen starvation [8]. Baloni et al. found that guanosine can serve as a good nitrogen source [9]. They also, however, reported xanthine and guanine as poor nitrogen sources for Msm, but their experimental conditions likely did provide an effective concentration of these bases in media. In our experimental set-up, the xanthine was comparable nitrogen source with ammonium (Fig. 2C). Catabolism of xanthine requires xanthine oxidase/dehydrogenase (XO/XDH), which catalyzes the first reaction (Fig. 1). This enzyme has not yet been characterized in Msm. The substrate specificities of XO/XDH enzymes from different species are relatively broad, and in some prokaryotic species, this enzyme can efficiently catalyze the oxidative conversion of hypoxanthine to xanthine [20–22]. We therefore speculate that the Msm XO/XDH enzyme has a strict preference for xanthine, contributing to the inability of Msm to catabolize hypoxanthine.

Taken together, we have experimentally confirmed that Msm can salvage only hypoxanthine and guanine as nucleotide precursors and efficiently catabolize only guanine and xanthine.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tube.2019.101879.

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