Biochemical Properties of MutT2 Proteins from *Mycobacterium tuberculosis* and *M. smegmatis* and Their Contrasting Antimutator Roles in *Escherichia coli*

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Mycobacterium tuberculosis, the causative agent of tuberculosis, is at increased risk of accumulating damaged guanine nucleotides such as 8-oxo-dGTP and 8-oxo-GTP because of its residency in the oxidative environment of the host macrophages. By hydrolyzing the oxidized guanine nucleotides before their incorporation into nucleic acids, MutT proteins play a critical role in allowing organisms to avoid their deleterious effects. Mycobacteria possess several MutT proteins. Here, we purified recombinant *M. tuberculosis* MutT2 (*Mtu*MutT2) and *M. smegmatis* MutT2 (*Msm*MutT2) proteins from *M. tuberculosis* (a slow grower) and *M. smegmatis* (fast growing model mycobacteria), respectively, for their biochemical characterization. Distinct from the *Escherichia coli* MutT, which hydrolyzes 8-oxo-dGTP and 8-oxo-GTP, the mycobacterial proteins hydrolyze not only 8-oxo-dGTP and 8-oxo-GTP but also dCTP and 5-methyl-dCTP. Determination of kinetic parameters (K_m and V_{max}) revealed that while *Mtu*MutT2 hydrolyzes dCTP nearly four times better than it does 8-oxo-dGTP, *Msm*MutT2 hydrolyzes them nearly equally. Also, *Msm*MutT2 is about 14 times more efficient than *Mtu*MutT2 in its catalytic activity of hydrolyzing 8-oxo-dGTP. Consistent with these observations, *Msm*MutT2 but not *Mtu*MutT2 rescues *E. coli* for MutT deficiency by decreasing both the mutation frequency and A-to-C mutations (a hallmark of MutT deficiency). We discuss these findings in the context of the physiological significance of MutT proteins.

The pathogenic bacterium *Mycobacterium tuberculosis* has been a focus of major research efforts because of the severe loss of human life that it inflicts in causing tuberculosis, a highly contagious disease. The primary site of infection of *M. tuberculosis* is the alveolar macrophages. In an effort to eliminate the pathogen, the macrophages retort by producing reactive nitrogen intermediates (RNI) and reactive oxygen species (ROS), such as hydrogen peroxide, superoxides, and hydroxyl radicals (OH[•]), as part of the host's innate immune response against the infection. While *M. tuberculosis* has developed several mechanisms to protect itself against RNI and ROS (1–3), any residual levels of these reactive species are harmful to the pathogen. RNI and ROS are known to damage DNA, proteins, and lipids (4, 5). Another important target of ROS is the nucleotide pool (6), the substrates for DNA synthesis. Incorporation of oxidized/damaged nucleotides into the DNA during DNA replication is detrimental to the genomic integrity. Guanine is the DNA base most susceptible to oxidation because of its low redox potential, and the major product of guanine oxidation is 7,8-dihydro-8-oxo-guanine (8-oxo-G) (6, 7). The 8-oxo-dGTP present in the nucleotide pool can get incorporated in the DNA against adenine or cytosine, resulting in AT-to-CG and GC-to-TA transversion mutations (8, 9). In addition, the mycobacterial genome, which has a high G+C content (~65%), is in itself at a greater risk of accumulating 7,8-dihydro-8-oxo-deoxyguanine (8-oxo-dG) due to oxidation of the G in its DNA.

To prevent mutations arising from the occurrence of 8-oxo-G in the genome, the organisms possess a GO (8-oxo-G) repair pathway consisting of Fpg (MutM), MutY, and MutT proteins (10, 11). Fpg excises 8-oxo-G from DNA and initiates base excision repair (BER) to replace the damaged base with a normal base (12, 13). If a lapse in the removal of 8-oxo-G leads to incorporation of A against 8-oxo-G, another BER enzyme, MutY, removes the A

from the 8-oxo-G–A mispairs to incorporate C against 8-oxo-G to allow Fpg to work again (14). MutT proteins hydrolyze the 8-oxo-G-containing nucleoside triphosphates to prevent their direct incorporation during DNA replication.

Mycobacterium possesses BER and nucleotide excision repair (NER) pathways to remove damaged bases present in the DNA (10, 11). DNA glycosylases like those belonging to the uracil DNA glycosylase (UDG) family, Fpg (MutM)/Nei family, Nth family, and MutY are the key players in BER (14, 15). However, unlike *Escherichia coli*, mycobacteria investigated so far lack the mismatch repair pathway (16), DNA damage responses are independent of RecA (17), and the role of MutT, a Nudix hydrolase responsible for sanitizing 8-oxo-dGTP, is not fully elucidated (11).

E. coli MutT (*Eco*MutT) hydrolyzes 8-oxo-dGTP into its monophosphate with a K_m of 0.48 μ M (18). MutT also hydrolyzes 8-oxo-GTP to its monophosphate form. Thus, MutT prevents misincorporation of 8-oxo-dG in DNA and 8-oxo-G in RNA opposite an A in the template and decreases errors during replication and transcription (19, 20). The *E. coli* MutT has an antimutator role, and its absence leads to a severe increase in AT-to-CG transversion mutations (9, 21). MutT belongs to a family of Nudix hydrolases which catalyze the hydrolysis of nucleoside diphosphate linked to other moieties, X-like deoxynucleoside triphos-

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TABLE 1 Strains and plasmids

Strain or plasmid	Details	Reference or source
<i>E. coli</i> strains		
MG1655	An <i>E. coli</i> K strain, F ⁻ λ ⁻ <i>rph-1</i>	25
DY330	W3110 Δ <i>lacU169 gal490</i> λ <i>cl857</i> Δ(<i>cro-bioA</i>)	26
JW0097	Δ <i>mutT790::kan</i> LAM ⁻ <i>rph-1</i>	27
Δ <i>mutT::kan</i>	<i>E. coli</i> MG1655 containing Δ <i>mutT::kan</i> from <i>E. coli</i> JW0097	This study
Δ <i>mutT::Mtu-mutT2</i>	<i>E. coli</i> MG1655 wherein the <i>mutT</i> locus is replaced with <i>Mtu-mutT2</i> (Amp ^r)	This study
CC101	F' <i>ara-600</i> Δ(<i>gpt-lac</i>)5 λ ⁻ <i>relA1 spoT1 thiE1</i> F128 (used to screen for A → C or T → G mutations)	28
CC102	F' <i>ara-600</i> Δ(<i>gpt-lac</i>)5 λ ⁻ <i>relA1 spoT1 thiE1</i> F128 (used to screen for C → T or G → A mutations)	28
CC103	F' <i>ara-600</i> Δ(<i>gpt-lac</i>)5 λ ⁻ <i>relA1 spoT1 thiE1</i> F128 (used to screen for C → G or G → C mutations)	28
CC104	F' <i>ara-600</i> Δ(<i>gpt-lac</i>)5 λ ⁻ <i>relA1 spoT1 thiE1</i> F128 (used to screen for C → A or G → T mutations)	28
CC105	F' <i>ara-600</i> Δ(<i>gpt-lac</i>)5 λ ⁻ <i>relA1 spoT1 thiE1</i> F128 (used to screen for A → T or T → A mutations)	28
CC106	F' <i>ara-600</i> Δ(<i>gpt-lac</i>)5 λ ⁻ <i>relA1 spoT1 thiE1</i> F128 (used to screen for A → G or T → C mutations)	28
CC101 to CC106 Δ <i>mutT::kan</i>	<i>E. coli</i> CC101 to CC106 containing Δ <i>mutT::kan</i> from JW0097	This study
CC101 to CC106 Δ <i>mutT::Mtu-mutT2</i>	<i>E. coli</i> CC101 to CC106 wherein the <i>mutT</i> locus is replaced with <i>Mtu-mutT2::amp</i>	This study
Plasmids		
pBADHisB	pBADHisB plasmid (ColE1 <i>ori</i> Amp ^r); an expression vector containing an arabinose-inducible promoter	Invitrogen
pET14b <i>Mtu-mutT2</i>	pET14b plasmid with <i>Mtu-mutT2</i> cloned in its NdeI/HindIII site	This study
pTrc <i>Mtu-mutT2</i>	pTrc99c plasmid with <i>Mtu-mutT2</i> cloned in its NcoI/HindIII site (from pET14b <i>Mtu-mutT2</i>)	This study
pBAD <i>Mtu-mutT2</i>	pBADHisB plasmid with <i>Mtu-mutT2</i> cloned in its NcoI/HindIII site (from pET14b <i>Mtu-mutT2</i>)	This study
pET14b <i>Msm-mutT2</i>	pET14b vector with <i>Msm-mutT2</i> cloned in its NdeI/HindIII site	This study
pTrc <i>Msm-mutT2</i>	pTrc99c plasmid with <i>Msm-mutT2</i> cloned in its NcoI/HindIII site (from pET14b <i>Msm-mutT2</i>)	This study
pBAD <i>Mtu-mutT2</i>	pBADHisB plasmid with <i>Msm-mutT2</i> cloned in its NcoI/HindIII site (from pET14b <i>Msm-mutT2</i>)	This study
pET14b <i>Eco-mutT</i>	pET14b vector with <i>Eco-mutT</i> cloned in its NdeI/BamHI site	This study
pTrc <i>Eco-mutT</i>	pTrc99c plasmid with <i>Eco-mutT</i> cloned in its NcoI/NheI site (from pET14b <i>Eco-mutT</i>)	This study
pBAD <i>Eco-mutT</i>	pBADHisB plasmid with <i>Eco-mutT</i> cloned in its NcoI/NheI site (from pET14b <i>Eco-mutT</i>)	This study

phates (dNTPs), ADP-ribose (ADPR), NADH, GDP-mannose, etc., and contain the sequence motif (Nudix box) **GX₅EX₇REUX EEXGU**, wherein the conserved residues are in bold, U is a bulky hydrophobic residue, and X is any residue (22).

Bioinformatics analyses have revealed the presence of nine putative Nudix hydrolases in *M. tuberculosis*. Four of these, which have been annotated as putative MutT proteins, are Rv2985 (*M. tuberculosis* MutT1 [*MtuMutT1*]), Rv1160 (*MtuMutT2*), Rv0413 (*MtuMutT3*), and Rv3908 (*MtuMutT4*). Their corresponding homologues in *M. smegmatis* are MSMEG_2390 (*M. smegmatis* MutT1 [*MsmMutT1*]), MSMEG_5148 (*MsmMutT2*), MSMEG_0790 (*MsmMutT3*), and MSMEG_6927 (*MsmMutT4*). The other Nudix hydrolases are Rv2609c (GDP-mannose hydrolase), Rv3199c (NADH pyrophosphatase), Rv3733c, Rv1700 (ADPR pyrophosphatase), and Rv3672c. The mycobacterial Nudix hydrolases have not been extensively studied. *MtuMutT2*, which shares 27% identity with *E. coli* MutT, has been annotated as a probable 8-oxo-dGTPase (<http://genolist.pasteur.fr/TubercuList/>).

Recent studies on the four MutTs in *M. tuberculosis* and *M. smegmatis* (23) using partially purified proteins showed that *MtuMutT2* has significant activity against dGTP (~90%) and 8-oxo-dGTP (~70%) and poor activity against dCTP (~10%). Later, another study with purified *MtuMutT2* showed higher activity against dCTP (~90%) and modified dCTP (~65%) but very low activity against dGTP (~10%) (24). The latter study, however, did not investigate the efficiency of 8-oxo-dGTP hydrolysis by *MtuMutT2*. Besides, a homologue of mycobacterial MutT with the antimutator role of *E. coli* MutT has not yet been identified.

To better understand the physiological role of MutT2 proteins, in this study, we have carried out kinetic analysis of the utilization of various substrates by MutT2 proteins from *M. tuberculosis* and *M. smegmatis*. We show that, despite their high conservation, the two proteins show contrasting antimutator activities in a MutT-deficient *E. coli* strain.

MATERIALS AND METHODS

Bacterial strains, plasmids, modified nucleotides, media, and growth conditions. The bacterial strains and plasmids used are listed in Table 1. 8-oxo-dGTP, 8-oxo-GTP, and 5-methyl-dCTP (5-Me-dCTP) were purchased from TriLink BioTechnologies, San Diego, CA. Other dNTPs were from Fermentas. *E. coli* CC101 to CC106 (28) and *E. coli* JW0097 *mutT::kan* were from the Coli Genetic Stock Center (CGSC). *E. coli* strains were grown in Luria-Bertani broth (LB) or LB containing 1.5% (wt/vol) agar (Difco). Media were supplemented with ampicillin, rifampin, and kanamycin, as needed, at 100 μg ml⁻¹, 50 μg ml⁻¹, and 25 μg ml⁻¹, respectively.

Cloning of *MtuMutT2*, *MsmMutT2*, and *EcoMutT*. The open reading frame (ORF) of *Mtu-mutT2* (Rv1160) was PCR amplified using forward primer *Mtu-mutT2* Fp (5'-GACATATGCTGAATCAGATCGTG-3') containing an NdeI site (underlined) and reverse primer *Mtu-mutT2* Rp (5'-TAAGCTTCTAACAGCGACGGTGG-3') having an HindIII site (underlined) using Dynazyme EXT DNA polymerase (1 U per reaction; MBI Fermentas). The PCR product was ligated into the pJET1.2 cloning vector, excised by NdeI/HindIII digestion, and ligated to a similarly digested pET14b vector, forming pET14b*Mtu-mutT2*. The *Mtu-mutT2* ORF was then mobilized into the pBADHisB and pTrc99c vectors between the NcoI and HindIII sites, generating pBAD*Mtu-mutT2* and pTrc*Mtu-mutT2*, respectively, taking the 20-amino-acid long N-terminal His tag sequence (MGSSHHHHHHSSGLVPRGSH) present in pET14b along with it. Sim-

FIG 1 (A) Sequence comparison of *EcoMutT*, *MtuMutT2*, and *MsmMutT2*. The amino acid sequences were aligned by using the ClustalW program and the BOX SHADE (version 3.21) server. Identical residues are shown in black, whereas similar residues are shown in light gray boxes. The Nudix box sequence motif is underlined. (B) Analysis of purified MutT proteins (~3 µg each) on 17% SDS-polyacrylamide gels. Lanes: 1, protein size markers (M; as indicated); 2, *MtuMutT2*; and 3, *MsmMutT2*.

ilarly, the ORF of *Msm-mutT2* (MSMEG_5148) was PCR amplified using a primer set, *Msm-mutT2orfFp* (5'-AGGCCATATGACAAAGCAGAT-3') and *Msm-mutT2 Rp* (5'-AGTGAAGCTTCTACCGTCCTG-3'), containing *NdeI* and *HindIII* sites (underlined), respectively, using *Pfu* DNA polymerase. The PCR product was cloned into the pJET1.2 vector and then subcloned into the pBADHisB and pTrc99c vectors as described previously for *Mtu-mutT2*. The *E. coli mutT* ORF was also PCR amplified using primer *Eco-mutTorfFp* (5'-TAAGCATATGAAAAAGCTGCAAAATG-3') with an *NdeI* site (underlined) and primer *Eco-mutTorfRp* (5'-GCCGGATCCGACCTACAGACGTTTAAG-3') having a *BamHI* site (underlined) using *Pfu* DNA polymerase. The PCR product was ligated into the pJET1.2 cloning vector, excised by *NdeI* and *BamHI* digestion, and ligated to a similarly digested pET14b vector. The *Eco-mutT* ORF was then subcloned into the pBADHisB vector using *NcoI* and *NheI*, generating pBAD*Eco-mutT*. The sequences of all constructs were confirmed by DNA sequence analysis.

Expression and purification of *MtuMutT2*, *MsmMutT2*, and *EcoMutT*. N-terminally His-tagged *MtuMutT2*, *MsmMutT2*, and *EcoMutT* were purified using either the pTrc99c or pBADHisB construct in an *E. coli* Δ *mutT*::*kan* strain. *E. coli* JW0097 competent cells were transformed with either pTrc*Mtu-mutT2*, pTrc*Msm-mutT2*, or pBAD*Eco-mutT*. Isolated colonies were inoculated into 50 ml LB medium with ampicillin and grown until saturation (or overnight). Inoculum (1%) was added into 3 liters LB medium containing ampicillin, grown to an optical density at 595 nm of 0.6 at 37°C under shaking, supplemented with 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG) or 0.02% arabinose for pTrc99c constructs and pBADHisB constructs, respectively, and allowed to grow further for 3 h. Cells were harvested by centrifugation, suspended in buffer A (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 10% glycerol [vol/vol], 2 mM β -mercaptoethanol, 20 mM imidazole), lysed by sonication, and centrifuged at 29,000 rpm (Avanti J-30i, JA30.50 Ti rotor) for 2 h 30 min at 4°C. The supernatant was loaded onto a 5-ml Ni-nitrilotriacetic acid (NTA) column preequilibrated with buffer A, washed with 5 ml of buffer A, and eluted with a gradient of imidazole (20 to 500 mM) in the

same buffer. The fractions were analyzed on 15% SDS-polyacrylamide gels, and those containing the desired protein were pooled and loaded onto a Superdex-G75 gel filtration column and eluted in buffer B (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% glycerol [vol/vol], 2 mM β -mercaptoethanol). The purity of the proteins was checked by running on 15% SDS-polyacrylamide gels, the fractions containing the desired protein were pooled and concentrated by using a Centricon filter (cutoff, 10 kDa; Millipore), and the concentration of the protein was estimated by Bradford's method using bovine serum albumin (BSA) as the standard (29).

Enzymatic activity assay of *MtuMutT2* and *MsmMutT2*. *MtuMutT2* and *MsmMutT2* activity assays (30) were done in 10-µl reaction volumes containing 20 mM Tris-HCl (pH 8.0), 8 mM MgCl₂, 5 mM dithiothreitol, 40 mM NaCl, and 2% glycerol. dNTPs (dATP, dTTP, dCTP, dGTP, 8-oxo-dGTP, 8-oxo-GTP, 5-methyl-dCTP) were added at a concentration of 250 µM (or more) with 100 ng of the enzymes. The reaction was done at 37°C for 10 min and stopped by adding 10 µl of 0.1% SDS. The sample was separated by high-pressure liquid chromatography (HPLC) (31) with some modification using a C₁₈ column (Acclaim C₁₈, 120A, 3 µm, 4.6 by 150 mm) in an isocratic flow of 73 mM KH₂PO₄, 5 mM tetrabutylammonium hydroxide, and 25% methanol at a flow rate of 0.5 ml min⁻¹. For separations, on a DNAPac PA200 analytical column (4 by 250 mm), buffer consisting of 25 mM Tris-HCl (pH 9.0), and a gradient of 1 M LiCl from 0% to 40% for 25 min at a flow rate of 0.5 ml min⁻¹ were used. Nucleotides were detected by UV light absorbance, and the peaks were quantified by measuring the areas of UV absorbance using Chromeleon software (Dionex). Peaks were detected at 264 nm for dATP and dTTP, 271 nm for dCTP and 5-Me-dCTP, and 252 nm for dGTP, 8-oxo-dGTP, and 8-oxo-GTP. For kinetics studies, the substrates were taken at 100 µM to 1,200 µM for dCTP and 100 µM to 800 µM for 8-oxo-dGTP with *MtuMutT2* and at 50 µM to 500 µM for dCTP and 50 µM to 400 µM for 8-oxo-dGTP with *MsmMutT2*. The enzyme was taken at a concentration of 50 ng per reaction, and the reaction was done in a time course manner from 5 min to 20 min. The amount of product formation was maintained within 30% of the amount of starting substrate. The kinetic

FIG 2 HPLC separation of the substrates (dNTPs) and products (deoxynucleoside monophosphates) formed by the action of *MtuMutT2*. All dNTPs were taken at a concentration of ~ 250 μ M with 100 ng of the enzyme, and the reaction was done at 37°C for 10 min. The product and substrates were separated by HPLC using a C_{18} column (Acclaim C_{18} , 120A, $3\ \mu\text{m}$, 4.6 by 150 mm) in an isocratic flow of 73 mM KH_2PO_4 , 5 mM tetrabutylammonium hydroxide, and 25% methanol at a flow rate of $0.5\ \text{ml min}^{-1}$. The retention times of the peaks (in minutes) are shown on the x axes, and the peak intensities in milli-absorbance units (mAU) are shown on the y axes. (A) Activity of *MtuMutT2* on different dNTP substrates at 0 min and 10 min: 8-oxo-dGTP (i), 8-oxo-GTP (ii), dCTP (iii), and 5-Me-dCTP (iv). (B) Activity of *MtuMutT2* on mixtures of different dNTP substrates at 0 min and 10 min: mixture of 8-oxo-dGTP and dCTP (i) and mixture of 8-oxo-dGTP, dGTP, dATP, dCTP, and dTTP (ii). WVL, wavelength.

parameters were determined by fitting the data to a Michaelis-Menten plot in GraphPad Prism (version 5) software.

Generation of *mutT* knockout strains of *E. coli*. To generate $\Delta\text{mutT}::\text{kan}$ derivatives, P1 phage lysate was raised on *E. coli* JW0097 $\Delta\text{mutT}::\text{kan}$ and used to transduce the $\Delta\text{mutT}::\text{kan}$ allele into *E. coli* MG1655 and *E. coli* CC101 to CC106. The strains were then plated on LB agar containing kanamycin. Transductants were verified for the transfer of the $\Delta\text{mutT}::\text{kan}$ allele by diagnostic PCR using primers Eco-*mutT* Up (5'-TACAAGC AGTGCCATGGCCGCTG-3') and Eco-*mutT* Dn (5'-GATGCGGCGA AAACGCCTTATCTG-3') flanking the *mutT* locus. The presence of an ~ 1.2 -kb product confirmed the knockout of the *mutT* locus.

Mutation frequency analysis of *E. coli* MG1655 and *E. coli* $\Delta\text{mutT}::\text{kan}$ strains complemented with pBAD Eco-mutT , pBAD Mtu-mutT2 , and pBAD Msm-mutT2 plasmids. Specified replicates of the isolated colonies of the various *E. coli* strains carrying the required plasmid were grown at 37°C for 14 to 16 h in LB containing 0.02% arabinose and ampicillin. Serial dilutions of the culture were made, 100 μl of the 10^{-6} dilution was plated on LB agar for viable counts, and 1 ml of the culture was plated on LB agar containing rifampin and incubated at 37°C until

colonies appeared (for the *E. coli* MG1655 $\Delta\text{mutT}::\text{kan}$ strain, only 100 μl of the culture was plated). Mutation frequency was calculated by dividing the number of mutants that appeared on the rifampin plate by the number of bacteria plated.

Assay for reversion of Lac^{-} to Lac^{+} phenotype in *E. coli*. The *E. coli* CC101 to CC106 strains (Table 1), which have a mutation in the *lacZ* gene, were used to assay for reversions leading to the Lac^{+} phenotype (28). These strains and their $\Delta\text{mutT}::\text{kan}$ derivatives (harboring a vector or the different plasmid-based expression constructs of the MutT proteins) were taken in specified replicates. The strains were streaked on LB agar plates containing X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) (50 $\mu\text{g ml}^{-1}$), and a single white colony was inoculated into 2 ml LB with 0.5 mM IPTG and grown at 37°C for 14 h. Serial dilutions of the culture were made, and 100- μl volumes of the 10^{-6} dilution were plated in minimal medium containing 0.2% glucose for viable count. Also, 1 ml of the culture was spun down and plated in minimal medium containing 0.2% lactose for Lac^{+} revertants. Plates were incubated at 37°C . The number of revertants and viable counts were enumerated after 24 h and 72 h for glucose and lactose plates, respectively.

FIG 3 HPLC separation of the substrates (dNTPs) and products (deoxynucleoside monophosphates) formed by the action of *MsmMutT2* and *EcoMutT*. The products and substrates were separated by HPLC using s DNAPac PA200 analytical column (4 by 250 mm) in buffer consisting of 25 mM Tris-HCl (pH 9.0) and a gradient of 1 M LiCl from 0% to 40% for 25 min at a flow rate of 0.5 ml min⁻¹. The retention times of the peaks (in minutes) are shown on the x axes, and the peak intensities in milli-absorbance units are shown on the y axes. The activities of *MsmMutT2* on 8-oxo-dGTP (i), 8-oxo-dGTP with *EcoMutT* as a control (ii), dCTP (iii), and 5-Me-dCTP (iv) are shown.

RESULTS

Comparison of *MtuMutT2* and *MsmMutT2* sequences to *EcoMutT* sequence and their purification. A comparison of the *EcoMutT*, *MtuMutT2*, and *MsmMutT2* sequences is shown in Fig. 1A. Compared individually with *EcoMutT*, *MtuMutT2* and *MsmMutT2* showed 27.1% and 24.6% identities, respectively, and 50.4 and 47.6% similarities, respectively. However, the sequence identity and similarity scores between *MtuMutT2* and *MsmMutT2* were 60.6% and 94.0%, respectively.

N-terminally His-tagged *MtuMutT2* and *MsmMutT2* were expressed from pTrc*Mtu-mutT2* and pTrc*Msm-mutT2* constructs in the *E. coli* Δ *mutT::kan* strain to avoid any contamination with *E. coli* MutT protein and purified using a series of Ni-NTA affinity and G-75 gel filtration chromatography to near homogeneity (Fig. 1B). The calculated molecular masses of *MtuMutT2* and *MsmMutT2* are 15.1 kDa and 13.9 kDa, respectively. However, as the proteins were purified with an N-terminal tag containing a hexahistidine sequence, the expected molecular masses were 17.23 kDa and 15.8 kDa, respectively. The observed mobilities of the two proteins are consistent with their expected molecular masses (compare lanes 2 and 3 with lane 1).

Activity assays of *MtuMutT2* and *MsmMutT2*. The enzymatic activity of the purified *MtuMutT2* was assayed using different dNTPs and modified dNTPs, as described in Materials and Meth-

ods. As reported earlier (23, 24), we observed that *MtuMutT2* hydrolyzed 8-oxo-dGTP, 8-oxo-GTP, dCTP, and 5-Me-dCTP (Fig. 2A, panels i to iv). Also, when all the dNTPs (including 8-oxo-dGTP) were taken together in a mixed nucleotide pool to investigate the effect of other dNTPs (as would be the situation in the cell), the specificity of *MtuMutT2* toward dCTP and 8-oxo-dGTP was maintained. Other dNTPs (dATP, dGTP, and dTTP) were not hydrolyzed, nor did they have any apparent impact on the hydrolysis of dCTP/8-oxo-dGTP (Fig. 2B, panels i and ii). Similar to *MtuMutT2*, *MsmMutT2* also hydrolyzed 8-oxo-dGTP to 8-oxo-dGMP (Fig. 3i). *EcoMutT* was used as a control and, as expected, hydrolyzed 8-oxo-dGTP to 8-oxo-dGMP (Fig. 3ii). *MsmMutT2* also hydrolyzed dCTP and 5-Me-dCTP to their respective monophosphates (Fig. 3iii and iv).

Kinetic analysis of *MtuMutT2* and *MsmMutT2*. As *MtuMutT2* and *MsmMutT2* showed activity against dCTP and 8-oxo-dGTP, we determined the kinetic parameters of their hydrolysis with the mycobacterial proteins. We observed that although *MtuMutT2* has comparable K_m s for dCTP and 8-oxo-dGTP (484 μ M and 591 μ M, respectively), it shows a 4.6-fold higher k_{cat}/K_m for dCTP (11.5×10^2 M⁻¹ s⁻¹) than for 8-oxo-dGTP (2.5×10^2 M⁻¹ s⁻¹) (Fig. 4A; Table 2). We then determined the kinetic parameters for the same two substrates for *MsmMutT2*. It also showed comparable K_m s for dCTP and 8-oxo-

FIG 4 Michaelis-Menten plot of the kinetics of *MtuMutT2* (A) and *MsmMutT2* (B) on 8-oxo-dGTP and dCTP. The velocity at each substrate concentration was calculated from an independent time course reaction using 5 to 8 time points.

dGTP (47.5 μM and 51.5 μM , respectively). Interestingly, we observed that *MsmMutT2* shows only a 1.2-fold higher k_{cat}/K_m for dCTP than for 8-oxo-dGTP (Fig. 4B; Table 2). Comparison of kinetic parameters between *MtuMutT2* and *MsmMutT2* showed that *MsmMutT2* has a K_m for both the substrates about 10-fold lower than that of *MtuMutT2*, resulting in a k_{cat}/K_m value for 8-oxo-dGTP hydrolysis by *MsmMutT2* about 14-fold higher than that by *MtuMutT2*. This observation suggests that *MsmMutT2* may play a more efficient antimutator role.

Mutation frequency analysis of *E. coli* MG1655 *mutT::kan* and its rescue by *EcoMutT*, *MtuMutT2*, and *MsmMutT2*. To investigate if the mycobacterial MutT2 proteins performed a physiological role similar to that of *EcoMutT*, we carried out mutation frequency analysis of the $\Delta\text{mutT}::\text{kan}$ derivative of *E. coli* MG1655 (*E. coli* $\Delta\text{mutT}::\text{kan}$) having a plasmid-based support of *EcoMutT*, *MtuMutT2*, or *MsmMutT2* (Fig. 5). The mutation frequency of the *E. coli* $\Delta\text{mutT}::\text{kan}$ strain was ~ 68 -fold higher than that of *E. coli* MG1655 (compare bars 1 and 2), and as expected, introduction of *EcoMutT* rescued this mutation frequency to ~ 6 -fold (bar 3). Interestingly, *MsmMutT2* also rescued the $\Delta\text{mutT}::\text{kan}$ strain to a similar level of ~ 7 -fold. However, *MtuMutT2* failed to rescue the strain to any significant level (there was still a 39-fold increase in mutation frequency). In another effort, we integrated

MtuMutT2 into the *E. coli* MG1655 genome (at the *mutT* locus) to generate *E. coli* $\Delta\text{mutT}::\text{Mtu-mutT2}::\text{amp}$ (also referred to as *E. coli* $\Delta\text{mutT}::\text{Mtu-mutT2}$) by replacing the native *mutT* gene of *E. coli* with the *Mtu-mutT2* construct with the help of an Amp^r marker. In this strain, the P_{Trc} promoter drove the expression of *MtuMutT2*. As shown in Fig. S1 in the supplemental material, expression of *MtuMutT2* in this strain was inducible by IPTG. Also in this strain, not unexpectedly, we did not observe any significant rescue of the mutation rate by *MtuMutT2* (see Fig. S2 in the supplemental material).

Assay for rescue of A-to-C mutations in *E. coli*. To analyze if either of the mycobacterial MutT2 proteins rescued *E. coli* for MutT deficiency by decreasing A-to-C (or T-to-G) mutations, we made use of the *E. coli* CC101 to CC106 strains (28). These *E. coli* strains possess specific mutations at the active-site E461 codon (GAG) of the *lacZ* gene to TAG, GGG, CAG, GCG, GTG, or AAG in strains CC101, CC102, CC103, CC104, CC105, and CC106, respectively, and require the T-to-G (A-to-C), G-to-A (C-to-T), C-to-G (G-to-C), C-to-A (G-to-T), T-to-A (A-to-T), or A-to-G (T-to-C) mutation, respectively, to grow on minimal lactose plates. As expected, in our preliminary experiments, deficiency of MutT ($\Delta\text{mutT}::\text{kan}$), which is known to increase the incidence of A-to-C mutations, resulted in heightened reversion of the Lac[−] to Lac⁺ phenotype in the $\Delta\text{mutT}::\text{kan}$ derivative of CC101 but not the $\Delta\text{mutT}::\text{kan}$ derivatives of the CC102 to CC106 strains (which report on non-A-to-C or non-T-to-G mutations) (see Table S1 in the supplemental material). Therefore, further experiments to investigate for rescue of A-to-C mutations were carried out using CC101. As shown in Fig. 6, while introduction of *EcoMutT* and *MsmMutT2* resulted in a decrease of the reversion frequencies, introduction of *MtuMutT2* did not rescue the reversion frequency to any considerable level. These observations not only support the findings of rescue of the mutation frequency analysis (as determined by the appearance of Rif^r) in the $\Delta\text{mutT}::\text{kan}$ derivative of *E. coli* MG1655 (Fig. 5) but also support the idea that *MsmMutT2*

TABLE 2 Kinetics parameters of *MtuMutT2* and *MsmMutT2*^a

Enzyme	Substrate	K_m (μM)	V_{max} (pmol/min/mg protein)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1} \text{s}^{-1}$ [10^2])
<i>MtuMutT2</i>	dCTP	484 $\pm$ 43	1.94 $\pm$ 0.07	0.56	11.5
	8-oxo-dGTP	591 $\pm$ 123	0.52 $\pm$ 0.06	0.15	2.5
<i>MsmMutT2</i>	dCTP	47.5 $\pm$ 10	0.82 $\pm$ 0.04	0.21	44.2
	8-oxo-dGTP	51.5 $\pm$ 18	0.71 $\pm$ 0.05	0.18	34.9

^a K_m and V_{max} were determined by least-squares fitting to Michaelis-Menten plots, and the values of means $\pm$ standard errors of the means are shown. k_{cat} was calculated from V_{max} .

FIG 5 Mutation frequency of *E. coli* MG1655 and its $\Delta \text{mutT}::\text{kan}$ derivative harboring plasmid pBADHisB or its derivatives harboring *Eco-mutT*, *Msm-mutT2*, and *Mtu-mutT2*. Mutation frequency was calculated by dividing the number of colonies that appear on LB agar containing rifampin by the number of colonies that appear on LB agar. The values are the means $\pm$ SDs of 6 independent colonies.

functions in *E. coli* to replace the function of *EcoMutT* by decreasing the occurrence of A-to-C (or T-to-G) mutations. This observation suggests that, at least, *MsmMutT2* may carry out the physiological role of MutT in *M. smegmatis*.

DISCUSSION

Reactive oxygen species (ROS) are produced not only during oxidative stress but also during normal cellular metabolism. One important target of ROS is the nucleotide pool, and the most commonly oxidized nucleotide products are 8-oxo-dGTP and 8-oxo-GTP. Incorporation of 8-oxo-dG in DNA and of 8-oxo-G in RNA may result in severe physiological consequences during the processes of replication, transcription, and translation. Recent studies have suggested that the oxidative damage of the guanine nucleotide pool may be a common mechanism of cell killing by bactericidal antibiotics. In *E. coli*, it has been shown that the cytotoxicities of commonly used antibiotics such as beta-lactams and quinolones are caused by double-stranded DNA breaks (32). Overexpression of *E. coli* MutT, which hydrolyzes 8-oxo-dGTP and 8-oxo-GTP into its monophosphates and prevents their incorporation into DNA and RNA, respectively (19), was shown to confer a protective role during antibiotic treatment (32). Thus, cotargeting of MutT proteins during antibiotic treatment may present an effective way to kill various bacteria.

Pathogenic mycobacteria, which home in macrophages, are exposed to ROS produced by these cells as part of the host's innate immune response. Thus, for mycobacteria to be sustained in a niche of high ROS, they must possess robust mechanisms to san-

itize the oxidized forms of GTP/dGTP. Multiple MutT proteins may be present in mycobacteria to fulfill such a role to enhance their fitness in an oxidative environment. In this study, we chose to characterize the MutT2 protein in *M. tuberculosis*, a slow-growing mycobacterium, with a view to investigate mycobacterial MutT with 8-oxo-dGTPase activity and an antimutator role like that of *E. coli* MutT. *MtuMutT2* shows the closest match in its sequence and size with *EcoMutT*. In parallel, we have investigated MutT2 from *M. smegmatis*, a fast-growing mycobacterium but a bacterium that still grows much slower than *E. coli*. As shown in Table 2, the mycobacterial MutT2 proteins from *M. tuberculosis* and *M. smegmatis* hydrolyze 8-oxo-dGTP with K_m s of $\sim 591 \mu\text{M}$ and $51.5 \mu\text{M}$, respectively. The reported K_m for the *E. coli* MutT for the same activity is $0.48 \mu\text{M}$ (18). Likewise, there is a good correlation (anticorrelation) of the enzymatic efficiencies of these proteins (for hydrolysis of 8-oxo-dGTP) with the growth rates of the organisms, where *MtuMutT2* is the least efficient, *MsmMutT2* is intermediate, and *EcoMutT* is the most efficient among the three MutT proteins (Table 2) (18). Interestingly, in our antimutator analysis, while *MsmMutT2* rescues *E. coli* strain from its deficiency of MutT, *MtuMutT2* does so very poorly (Fig. 5). Similarly, when we investigated for the rescue of a specific mutation spectrum (A to C or T to G) using *E. coli* CC101, while rescue by *MtuMutT2* was very poor, rescue by *MsmMutT2* was nearly as good as that by the cloned copy of *EcoMutT* (Fig. 6). However, it may be noted that knockouts of MutT2 genes in *M. tuberculosis* and *M. smegmatis* resulted in an overall increase of similar mutation frequencies of about 1.5- and 1.7-fold, respectively (23). Although it should also

FIG 6 Frequency of Lac⁺ reversion of *E. coli* CC101, its $\Delta \text{mutT}::\text{kan}$ derivative harboring plasmid pBADHisB, or its derivatives harboring *Eco-mutT*, *Msm-mutT2*, and *Mtu-mutT2* to score for A-to-C mutations. Reversion frequencies were calculated by dividing the number of colonies that appear on a minimal lactose plate by the number of colonies that appear on a minimal glucose plate. The data are represented as means $\pm$ SDs of 10 independent colonies.

be said that as mycobacteria have evolved with a number of MutT proteins, it might well be that in mycobacteria other MutT proteins contribute to and/or carry out the primary function of hydrolyzing 8-oxo-GTP/8-oxo-dGTP to deal with the oxidative stress that the organisms encounter in the host. A parallel between the activities of MutT2 proteins of *M. tuberculosis* and *M. smegmatis* and the activities described in a recent report (33) of another Nudix box protein, NudC, an NADH pyrophosphatase which hydrolyzes NAD(H) to AMP and nicotinamide mononucleotide [NMN(H)], may also be drawn. While NudC (Rv3199c) from *M. tuberculosis* H37Rv shows very poor NADH-hydrolyzing activity, that from *M. bovis* BCG (an isoform of *M. smegmatis* allele) shows substantial hydrolysis of NADH as well as the active forms of isoniazid (NAD-INH) and ethionamide (NAD-ETH). The property of hydrolysis of NAD-INH and NAD-ETH was also revealed by the conferment of INH and ETH tolerance by both the *M. bovis* and *M. smegmatis* alleles of NudC. It remains to be seen if there is a physiological significance of downregulation of the activities at least two (i.e., MutT2 and NudC) of the Nudix box proteins in *M. tuberculosis*.

Interestingly, a polymorphism (P237Q) in *M. tuberculosis* NudC has been shown to be responsible for its inability to function as an NADH pyrophosphatase (33). It would be interesting to see if the poor activity of *Mtu*MutT2 toward hydrolyzing 8-oxo-dGTP (compared to that of *Msm*MutT2) is also a consequence of such a polymorphism/mutation. Nevertheless, the activity of the two mycobacterial MutT2 proteins as a dCTPase as well as an 8-oxo-dGTPase raises a question about the physiological significance of their dual function. One possibility for this linked activity could be that under heavy oxidative stress, the simultaneous presence of dCTPase activity also brings down the concentration of dCTP. Such activity in a G+C-rich organism may slow down DNA synthesis to an extent to allow sufficient time to rid the nucleotide pool of 8-oxo-dGTP to avoid its misincorporation. However, it is unclear how the two enzymatic activities may be regulated.

Further, while the *E. coli* MutT does not hydrolyze dCTP (18), the mycobacterial MutT2 proteins hydrolyze dCTP (in fact, they do so more efficiently than the 8-oxo-dGTP). In *E. coli*, Orf135 is known to possess dCTPase activity (34). The active-site residues important for recognition of substrates are also conserved (24). *Mtu*MutT2 shows overall sequence identity and similarity with *E. coli* Orf135, another MutT-type enzyme, of about 39% and 56%, respectively (see Fig. S3 in the supplemental material). In addition to the dCTPase and 5-Me-dCTPase as the major activities, Orf135 has poor activity against 8-OH-dGTP (8-oxo-dGTP) (34–36). These are also the enzymatic activities shared by *Mtu*MutT2. Both *Mtu*MutT2 and *Msm*MutT2 retain the Asp residue (D119 and D116, respectively) corresponding to D118 of Orf135 (see Fig. S3 in the supplemental material), which plays a role in substrate binding and specificity (36, 37). The corresponding amino acid in *E. coli* MutT is N119. Thus, the mutational analyses of *Mtu*MutT2 and *Msm*MutT2 may present us with a useful system to investigate the evolutionary aspects of Nudix box proteins in conferring distinct dNTPase activities. Such experiments may also provide a mechanistic basis for the distinct kinetic properties of *Mtu*MutT2 and *Msm*MutT2 toward hydrolysis of 8-oxo-dGTP and dCTP. Furthermore, the multiple Nudix box proteins in mycobacteria may have evolved to accommodate newer sub-

strate specificities in response to selective pressures that the organisms encounter, and these proteins may allow us to better understand the mechanism of drug resistance in mycobacteria (33).

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