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# Identification of Nudix Hydrolase Family Members with an Antimutator Role in *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*†

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***Mycobacterium tuberculosis* and *Mycobacterium smegmatis* MutT1, MutT2, MutT3, and Rv3908 (MutT4) enzymes were screened for an antimutator role. Results indicate that both MutT1, in *M. tuberculosis* and *M. smegmatis*, and MutT4, in *M. smegmatis*, have that role. Furthermore, an 8-oxo-guanosine triphosphatase function for MutT1 and MutT2 is suggested.**

Oxidized guanine (8-oxo-G) is a potent mutagen because of its ambiguous pairing with cytosine and adenine. The *Escherichia coli* MutT protein specifically hydrolyzes both 8-oxo-deoxyguanosine triphosphate (8-oxo-dGTP) and 8-oxo-guanosine triphosphate (8-oxo-rGTP), preventing their misincorporation in DNA and RNA opposite template A (10, 23, 24, 26). The MutT *E. coli* protein has an antimutator function, and it was the first enzyme of the MutT/Nudix hydrolase family, characterized by a 23-amino-acid region, to be studied. Nudix hydrolases, are so named because the ones characterized so far all hydrolyze a *nu*cleoside *di*phosphate linked to some other moiety, *X*. Besides oxidized guanine, they were shown to degrade other substrates, such as NADH, GDP-mannose, or ADP-ribose (2, 3, 8, 9, 14, 15, 18).

Here we have investigated the role of the putative Nudix hydrolases MutT1, MutT2, MutT3, and Rv3908 (MutT4) of *M. tuberculosis* (5) and their putative *M. smegmatis* homologues (sequences were obtained from The Institute for Genomic Research [TIGR] website; www.tigr.org) as antimutators (Fig. 1). Sequence subunit analysis did not suggest that these proteins were members of any of the known subfamilies of Nudix hydrolases (6, 13, 28). The *mutT1 M. tuberculosis* knockout mutant (MT1K) was isolated from the transposon library described previously (12). Selection was done by plating aliquots of each independent insertional mutant onto 7H10 plates containing rifampin (Rif) at 2 μg/ml, two times the MIC of Rif for *M. tuberculosis* used by Morlock et al. (17). One of the clones giving a higher number of Rif-resistant (Rif$^r$) colonies than the wild-type strain harbored an insertion in *mutT1*. All other mutants were generated by allelic replacement using a replication temperature-sensitive vector harboring the counter-selectable marker *sacB* to carry a kanamycin cassette-disrupted copy of the genes of interest (20). The bacterial strains, plasmids, and primers employed in this study are provided in the supplemental material. DNA isolation, cloning, and Southern hybridization were performed according to standard techniques. Complemented strains of the *M. smegmatis* mutants were generated by electroporation of the pVV16-derived vectors (11) described in the supplemental material. In 20 independent experiments, we carried out an adapted Luria-Delbruck fluctuation test, as described by Morlock et al. (17). The results obtained are summarized in Table 1. MutT1 deficiency in *M. tuberculosis* resulted in a 15.5-fold spontaneous mutation frequency increase by rifampin resistance screening compared with the wild-type strain. A similar 12-fold increase was observed for the *mutT1* mutant of *M. smegmatis*. Furthermore, we observed a striking 48.1-fold increase in spontaneous Rif$^r$ colonies for the MutT4-deficient *M. smegmatis* strain. By contrast, we observed no increase for the MutT4-deficient *M. tuberculosis* strain. One may hypothesize the existence of enzymes with functions redundant to that of MutT4 in *M. tuberculosis*, thus masking the effect of MutT4 deficiency in these species. Defects in *mutT2* and *mutT3* genes resulted in no apparent differences between mutants and wild type. Moreover, similar results were obtained when screening for isoniazid resistance (data not shown). Complementation of the

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MutT1E.coli      FPGGKVEMGETPEQAVVRELQEEVGITPQHFSLFEKLEYEFP-DRHITLW
MutT1H37Rv       LPKGKVDPGETAPVGAVREILEETGHRANLGRRLLTVTYPTDSPFRGVKK
MutT1mc2155      LPKGKLDQGETEPVAAAREIHEETGHTAVLGRRLGRVTYPIP---QGTKR
MutT2H37Rv       LPGGKVAAGETERAALARELAEELGLEVADLAVGDRVGDDIALNGTTTLR
MutT2mc2155      LPGGKVTPGESDADALARELREELG---VDVAVGERLGADVALNDAMTLR
MutT3H37Rv       LPGGARDSHETPEQTAVRESSEEAGLSAERLEVRATVVTAEVCGVDDTHW
MutT3mc2155      LPGGARDSHETPEQAAVREAHEEAGLPAEQLTVRTTVVTAEVAGIGGTQW
MutT4H37Rv       LPKGHIELGETAEQTAIREVAEETGIRGSVLAALGRIDYWFVTDGRRVHK
MutT4mc2155      LPKGHIELGETAEQTAIREVAEETGIQGSVLAALGSIDYWFVTEGRRVHK
                 :* *     *:      **  ** *      :       .
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FIG. 1. MutT protein sequence alignment. *M. tuberculosis* Rv2985 (MutT1), Rv1160 (MutT2), Rv0413 (MutT3), and Rv3908 (MutT4) and *M. smegmatis* MSMEG2389 (MutT1), MSMEG5134(MutT2), MSMEG0784 (MutT3), and MSMEG6883 (MutT4) were selected from the *M. tuberculosis* (Tuberculist) and *M. smegmatis* (TIGR) genomes because of their annotation or after a BLAST analysis. These sequences were compared to *E. coli* MutT by using alignments available at http://www.ch.embnet.org/software/ClustalW.html. The detected region of similarity is shown. *, absolutely conserved residues that define the *mutT* or Nudix motif; :, residues that are strongly conserved.

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† Supplemental material for this article may be found at http://jb.asm.org/.

TABLE 1. Frequency and nature of spontaneous *rpoB* mutations

| Base substitution | No. of mutations[a] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | H37Rv | MT1K | MT2K | MT3K | MT4K | mc$^2$155 | MS1K | MS2K | MS3K | MS4K |
| A to C | 1 (0.1) | 9 (9.5) | 7 (0.7) | 0 (0.0) | 0 (0.0) | 1 (0.3) | 16 (49.4) | 22 (9.8) | 2 (1.0) | 0 (0.0) |
| A to G | 9 (0.6) | 20 (21.2) | 12 (1.2) | 12 (1.2) | 6 (0.6) | 7 (1.8) | 8 (24.7) | 5 (2.2) | 0 (0.0) | 4 (49.6) |
| A to T | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (3.1) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| C to A | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (0.5) | 0 (0.0) |
| C to G | 1 (0.1) | 0 (0.0) | 2 (0.2) | 5 (0.5) | 5 (0.5) | 0 (0.0) | 2 (6.2) | 1 (0.4) | 0 (0.0) | 0 (0.0) |
| C to T | 21 (1.4) | 3 (3.2) | 11 (1.1) | 13 (1.3) | 21 (2.0) | 22 (5.7) | 5 (15.4) | 4 (1.8) | 9 (4.3) | 4 (49.6) |
| AAC deletion | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 8 (3.8) | 0 (0.0) |
| TTCGGC deletion | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 7 (3.4) | 0 (0.0) |
| T to A | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 2 (24.8) |
| T to C | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 2 (0.5) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 22 (272.9) |
| T to G | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 5 (2.4) | 0 (0.0) |
| Total frequency of mutation[b] | 2.2 ± 3.1 | 33.9 ± 12.1 | 3.3 ± 4.1 | 3.2 ± 3.0 | 3.1 ± 3.0 | 8.3 ± 4.6 | 98.7 ± 24.2 | 14.3 ± 5.7 | 15.3 ± 5.4 | 397.0 ± 82.4 |
| Fold increase[c] | 1.0 | 15.5 | 1.5 | 1.5 | 1.4 | 1.0 | 12.0 | 1.7 | 1.9 | 48.1 |

[a] Parentheses ($10^{-8}$) indicate the mutation frequency of each subclass.
[b] The averages ± standard deviations are shown. In each experiment, the frequency of Rif$^r$ cells ($10^8$) was determined by measuring the number of Rif$^r$ cells in 20 independent cultures as described by Morlock et al. (17). Frequency of mutation represents the average of 20 independent cultures.
[c] In comparison with the wild-type strain.

*mutT1* and *mutT4* mutants of *M. smegmatis* with wild-type copies of the *M. smegmatis* or *M. tuberculosis mutT1* and *mutT4* genes reduced the mutation frequency to that seen in the wild type (see the supplemental material), indicating that the genes from both sources were capable of restoring wild-type mutation frequencies in the mutants.

In order to investigate the possible function of these mycobacterial MutT proteins, we sequenced the *rpoB* gene cluster I region of 32 randomly picked Rif$^r$ colonies derived from each mutant, as described by Rad et al. (22). The results are shown in Table 1. As described previously for MutT-defective *E. coli* (10), we observed 95- to 165- and 7- to 32-fold increases in A-to-C transversions for the *mutT1*- and *mutT2*-deficient *M. tuberculosis* and *M. smegmatis*, respectively, in comparison with the wild type. We found that *M. smegmatis* MutT3-deficient strains displayed 518-codon deletions and previously undescribed double 508/509-codon deletions (19). MutT4 deficiency in *M. smegmatis* was associated with a very high number of T-to-C mutations.

To assess the possible function of these proteins, enzyme assays were performed with 8-oxo-dGTP and other known Nudix hydrolase substrates. The strains for production of the recombinant proteins were obtained by transforming the pVV16-derived vectors in *M. smegmatis* mc$^2$155. Recombinant proteins, which carry a six-histidine tag at the carboxyl terminus, were partially purified using a Ni-nitrilotriacetic acid superflow QIAGEN resin as described by Stadthagen et al. (25). The standard reaction mixture was in 50 μl of a solution containing 50 mM Tris-Cl (pH 8.5), 5 mM $Mg^{2+}$, 25 mM NaCl, 2 mM substrate, 0.5 U of yeast inorganic pyrophosphatase for substrates such as (deoxy)nucleoside triphosphates and their derivatives (or 4 U of alkaline phosphatase for all other substrates), and the excess 5 μg of the partially purified extracts. The solution was incubated at 37°C for 30 min, and the reaction was stopped by the addition of 250 μl of 4 mM EDTA (or a Norit suspension to remove unreacted triphosphates). The liberated inorganic orthophosphate was assayed by the colorimetric procedure of Fiske and SubbaRow (7) as modified by Ames and Dubin (1). The results, normalized for a control reaction of an *M. smegmatis* strain carrying the empty vector, are shown in Fig. 2. As suggested by the *rpoB* sequencing, MutT1 and MutT2 displayed a clear 8-oxo-GTPase activity. Additionally, as reported for human and *E. coli* MutT, these proteins exhibited hydrolytic activity on dGTP. Although conclusions on the substrate specificities of our enzymes cannot readily be drawn from our experiments, MutT4 seemed to display a greater hydrolytic activity on dATP than on other substrates under the conditions used in our assay. No recom-

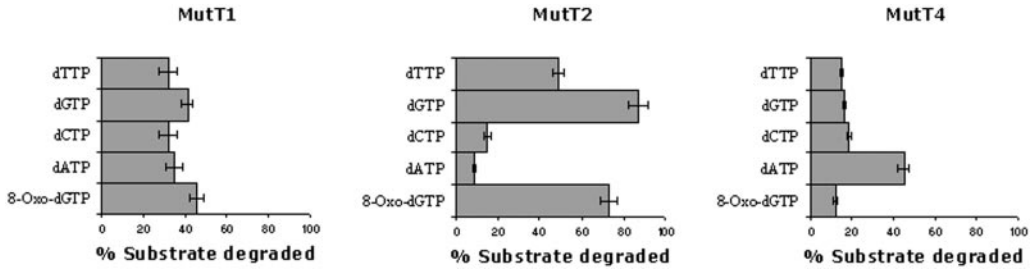


FIG. 2. Rate of hydrolysis of the various (deoxy)nucleoside triphosphate substrates for the partially purified extracts. Experiments were performed as described in the text.

binant MutT3 protein could be obtained in a suitable form for analysis. Because no antimutator phenotype was suggested for this gene, we did not pursue its analysis.

For *M. tuberculosis*, with the exception of the *dnaE2* role in inducible mutagenesis (4), no other gene was found to be associated with a mutator or antimutator phenotype (16). Strains of the *M. tuberculosis* W-Beijing family were linked with an increased risk of resistance (21). Sequencing studies revealed that W-Beijing strains could be divided into several branches according to the accumulation of unique missense alterations in three putative antimutator genes, including two of the oxidative damage-related *mutT* type, *mutT2* and *mutT4* (22). A previous study by Werngren and Hoffner (27) revealed no mutator phenotype for W-Beijing strains. However, those results do not specify which types of W-Beijing strains were used; hence no definite conclusions could yet be made. Here we describe an apparent antimutator role for the MutT1 enzyme of *M. tuberculosis* and *M. smegmatis*. Additionally, our results suggest that this enzyme shares with MutT2 the function of *E. coli* MutT, which is in apparent contrast with the observed antimutator role for MutT1, but not for MutT2, and this may account for the low number of $Rif^r$ colonies found for the mutator MutT1-deficient strains, compared with the numbers observed in other bacteria deficient in MutT enzymes. At present, we have no satisfactory explanation for the conflicting data. One may hypothesize that MutT1 has broader substrate specificity than MutT2. This is the first example of a gene with an antimutator role in *M. tuberculosis*. Besides 8-oxo-dGTPase activity, this gene's complete role is still unknown. Further studies might elucidate its predominance over MutT2.

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