A distinct physiological role of MutY in mutation prevention in mycobacteria

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Oxidative damage to DNA results in the occurrence of 7,8-dihydro-8-oxoguanine (8-oxoG) in the genome. In eubacteria, repair of such damage is initiated by two major base-excision repair enzymes, MutM and MutY. We generated a MutY-deficient strain of *Mycobacterium smegmatis* to investigate the role of this enzyme in DNA repair. The MutY deficiency in *M. smegmatis* did not result in either a noteworthy susceptibility to oxidative stress or an increase in the mutation rate. However, rifampicin-resistant isolates of the MutY-deficient strain showed distinct mutations in the rifampicin-resistance-determining region of *rpoB*. Besides the expected C to A (or G to T) mutations, an increase in A to C (or T to G) mutations was also observed. Biochemical characterization of mycobacterial MutY (*M. smegmatis* and *M. tuberculosis*) revealed an expected excision of A opposite 8-oxoG in DNA. Additionally, excision of G and T opposite 8-oxoG was detected. MutY formed complexes with DNA containing 8-oxoG : A, 8-oxoG : G or 8-oxoG : T but not 8-oxoG : C pairs. Primer extension reactions in cell-free extracts of *M. smegmatis* suggested error-prone incorporation of nucleotides into the DNA. Based on these observations, we discuss the physiological role of MutY in specific mutation prevention in mycobacteria.

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INTRODUCTION

Reactive oxygen species produced during cellular metabolism or encountered as environmental pollutants inflict severe damage on the integrity of DNA. Of the four bases in DNA, guanine is highly susceptible to oxidative stress, resulting in the formation of 7,8-dihydro-8-oxoguanine (8-oxoG) or its derivatives (Fraga *et al.*, 1990; Farr & Kogoma, 1991; Delaney *et al.*, 2007; David *et al.*, 2007). The presence of oxidized G in DNA is mutagenic because of its pairing with A or G. Eubacteria possess an elaborate 8-oxoG (GO) repair system consisting of Fpg (MutM), MutY and MutT enzymes to deal with such threats to genomic integrity (Michaels & Miller, 1992; Tajiri *et al.*, 1995; Fowler *et al.*, 2003; Bruner *et al.*, 2000). Fpg is known to excise 8-oxoG paired with C or T and initiate the base-excision repair pathway. MutY is an atypical DNA glycosylase whose predominant activity removes A or G incorporated against 8-oxoG during replication (Au *et al.*, 1989; Lu & Chang, 1988; Tsai-Wu *et al.*, 1992). Such MutY activity increases the chances of incorporating the correct base (C) against 8-

oxoG, as well as the probability of further repair of the lesion (8-oxoG) by Fpg. Thus, the action of MutY prior to the second round of DNA replication provides an efficient mechanism to decrease fixation of A or G in place of C in the genome, and prevents C to A (or G to T) and to a lesser extent C to G (G to C) mutations. In addition, *Escherichia coli* MutY has been reported to possess a weak activity of excising A from C : A mismatch (Tsai-Wu *et al.*, 1992). The third enzyme of the GO repair pathway, MutT, cleaves 8-oxo-dGTP (to 8-oxo-dGMP) from the nucleotide pool to minimize its direct incorporation into DNA (Michaels & Miller, 1992; Mo *et al.*, 1992).

The capacity to repair 8-oxoG damage is considered to be crucial in G+C-rich bacteria such as *Mycobacterium tuberculosis*, an important human pathogen which is exposed to reactive oxygen species within the host macrophages. Studies on the DNA repair mechanisms of this group of organisms are in early stages but they are believed to be important during dormancy or the active multiplication phase of the pathogen. However, the genome sequence of *M. tuberculosis* revealed that this organism lacks the mismatch repair enzymes (Cole *et al.*, 1998; Mizrahi & Andersen, 1998). Thus, the base-excision repair and nucleotide-excision repair pathways must contribute significantly to the maintenance of genomic integrity in these bacteria.

Abbreviations: EMSA, electrophoretic mobility shift assay; GO or 8-oxoG, 7,8-dihydro-8-oxoguanine; RRDR, rifampicin resistance determining region.

Supplementary material is available with the online version of this paper.

We have been using *Mycobacterium smegmatis* as a model to carry out genetic analysis of various DNA repair enzymes in mycobacteria (Venkatesh *et al.*, 2003; Jain *et al.*, 2007; Kurthkoti *et al.*, 2008). It had been observed that in mycobacteria, C to A (or G to T) mutations occurred minimally (Boshoff *et al.*, 2003). Further, unlike an *E. coli* *fpg* strain, an *M. smegmatis* *fpg* strain showed no detectable increase in C to A mutations. However, the strain suffered a significant increase in A to G (or T to C) and C to G (or G to C) mutations (Jain *et al.*, 2007).

Recently, it has been reported that in *Pseudomonas aeruginosa*, another G+C rich organism, loss of *mutY* had a greater impact on spontaneous mutation rate than loss of *fpg* (Sanders *et al.*, 2009). A distinct impact of *Fpg* deficiency in *M. smegmatis* (Jain *et al.*, 2007) encouraged us to further our knowledge of the physiological significance of the GO repair pathway in mycobacteria. In this study, we generated a MutY-deficient strain of *M. smegmatis* and assessed the role of MutY in the GO repair pathway. We then biochemically characterized *M. tuberculosis* MutY, which shows high similarity with the *M. smegmatis* counterpart, to understand the mechanism underlying the mutation spectrum in the MutY-deficient strain.

METHODS

DNA oligomers, plasmids, strains, media and growth conditions. A list of DNA oligomers and plasmids is provided in Supplementary Table S1 (available with the online version of this paper); the strains used are summarized in Table 1. Media components were from Difco. *M. smegmatis* mc²155 (Snapper *et al.*, 1990) was grown in Luria-Bertani (LB) containing 0.2% (v/v) Tween 80 (LBT), or in Middlebrook 7H9 containing 0.2% (v/v) glycerol and 0.2% Tween 80. LBT agar contained 1.5% (w/v) agar. Genetically engineered strains of *M. smegmatis* were selected on Middlebrook 7H10 agar containing 0.5% glycerol, 0.05% Tween 80 and 10% (w/v) sucrose. Media were supplemented with hygromycin, gentamicin and kanamycin at 50 µg ml⁻¹, 5 µg ml⁻¹ and 50 µg ml⁻¹, respectively, as needed.

Generation of *mutY* disruption allele, screening and Southern blot analysis. *M. smegmatis* *mutY* (Msm-*mutY*, MSMEG_6083) was identified by its homology with the *M. tuberculosis* *mutY* (Rv3589; Cole *et al.*, 1998) from the *M. smegmatis* sequence retrieved from The

Institute of Genomics Research (<http://www.tigr.org>); it was disrupted in *M. smegmatis* mc²155 by a Kan^R marker to generate the *mutY::kan* strain (for details see supplementary online material).

MutY activity assays. Double-stranded DNA oligomers were generated by annealing 5' ³²P end-labelled Comp-G, Comp-A, Comp-T, and Comp-C oligomers with the 8-oxoG oligomer (Supplementary Table S1) and used for MutY activity assays essentially as described by Jain *et al.* (2007); see Supplementary online material for details.

Generation of an *M. smegmatis* *mutY* strain complemented with ectopically expressed *mutY*. The Msm-*mutY* gene was amplified from *M. smegmatis* using a forward (Msm-*mutY*UP-Fp) and a reverse (Msm-*mutY*DN2-Rp) primer as a ~1.6 kb amplicon, and subcloned into pMV361 to generate pMV-Msm-*mutY*, wherein the expression of *mutY* would be driven by its own promoter (see Supplementary online material for details). pMV-Msm-*mutY* was introduced into the *M. smegmatis* *mutY::kan* strain by electroporation and the complemented strain was named *mutY* (L5att::*mutY*). A control strain, *mutY* (L5att::vector), referred to as the *mutY* strain, was generated by transformation of the empty vector, pMV361, into the *mutY::kan* strain (other strain designations are summarized in Table 1).

Effect of hydrogen peroxide on bacterial growth. Growth of *M. smegmatis* strains was monitored in a Bioscreen C kinetic growth reader (OY Growth) at 37 °C with constant shaking as described previously (Kurthkoti *et al.*, 2008).

Survival of *M. smegmatis* strains in RAW 264.7 cells. Murine macrophage cell line RAW 264.7 was maintained in DMEM (Sigma) with glucose (4.5 mg ml⁻¹) and 10% fetal calf serum (Gibco). Cells were seeded in 24-well tissue culture plates (0.2 × 10⁶ per well) in 1 ml complete medium and allowed to adhere for 14 h. Bacteria were grown in LBT to an OD₆₀₀ of ~0.4 from a 48 h old pre-culture. The bacterial cultures (0.15 ml) were mixed with 1.35 ml tissue culture medium at an m.o.i. of ~10. The infections were done in triplicate in 0.2 ml at 37 °C in 5% CO₂ for 5 h, and cells were washed twice with PBS and treated with gentamicin (100 µg ml⁻¹) for 1 h 30 min to kill the extracellular bacteria. Cells were washed again with PBS and incubated in 2 ml DMEM containing 20 µg gentamicin ml⁻¹ (0 h time point). Cells were lysed at 0, 24, 48 and 72 h in PBS containing 0.5% Triton X-100 (Sigma) and the total viable counts of the recovered bacteria were determined by dilution plating on LBT agar. Counts from triplicate samples were analysed and plotted.

Determination of mutation rates. Mutation rates were determined as described before (David, 1970; Kurthkoti *et al.*, 2008).

Table 1. *M. smegmatis* strains

Strain	Relevant details	Reference
mc ² 155	A high-efficiency transformation strain of <i>M. smegmatis</i>	Snapper <i>et al.</i> (1990)
WT	mc ² 155 harbouring an integrative vector, pDK20 (Kan ^R) at the L5 attachment (<i>att</i>) site of the chromosome	Venkatesh <i>et al.</i> (2003)
<i>mutY::kan</i>	mc ² 155 wherein <i>mutY</i> gene has been disrupted with a <i>kan</i> cassette	This study
<i>mutY</i>	<i>mutY::kan</i> strain harbouring the integrative vector pMV361 (Hyg ^R) at the L5 attachment (<i>att</i>) site of the chromosome	
<i>mutY</i> (L5att:: <i>mutY</i>)	<i>mutY</i> strain harbouring the integrative plasmid pMV-Msm- <i>mutY</i> at the L5 <i>att</i> site of the chromosome	
<i>fpg::hyg</i>	mc ² 155 wherein <i>fpg</i> gene has been disrupted with a <i>hyg</i> marker	Jain <i>et al.</i> (2007)
<i>fpg</i>	<i>fpg::hyg</i> strain harbouring a replicative plasmid, pTKmx (Kan ^R)	Jain <i>et al.</i> (2007)

Analysis of mutation spectrum. *M. smegmatis* cultures were plated on LBT agar containing rifampicin ($50 \mu\text{g ml}^{-1}$) and incubated at 37°C for 96 h. The isolated colonies were suspended in $20 \mu\text{l}$ water, incubated at 90°C for 5 min, spun at 13 000 r.p.m. for 5 min in a table-top centrifuge and the supernatant was used as template to PCR amplify the rifampicin-resistance determining region (RRDR) with *Taq* DNA polymerase and the 306-*rpoB*-Fp and 306-*rpoB*-Rp primers (Jain *et al.*, 2007). The PCR products were eluted from agarose gel and sequenced using the *Msm-rpoB*-seq-Fp primer (Macrogen).

Cloning of *MtuMutY* into pET11d and purification of *MtuMutY*. The *MtuMutY* (Rv3589) ORF was amplified by PCR from *M. tuberculosis* DNA using primers *Mtu-mutY*-Fp (beginning with the second codon in the ORF) and *Mtu-mutY*-Rp (which anneals ~ 0.15 kb downstream of the termination codon) and Dynazyme EXT polymerase. The reaction was heated at 94°C for 4 min followed by 30 cycles of incubation at 94°C for 1 min, 50°C for 45 s and 70°C for 1 min. The PCR product was cloned into pGEM-T Easy vector. The sequence of the insert was confirmed by DNA sequencing, released by *EcoRI* digestion and subcloned into pTrc99c linearized with *EcoRI*. After confirming the correct orientation, the *mutY* fragment was released by *NcoI* and *BamHI* and mobilized into similarly digested pET11d to give pET*MtuMutY*. During these manipulations, a Gly-Asn-Ser-Ile sequence was added between the initiator methionine and the second amino acid of the MutY sequence. Details of the purification of *MtuMutY* using the pET*MtuMutY* expression construct are provided in the supplementary online material.

Analysis of complex formation of *MtuMutY* with 8-oxoG-containing DNA. Electrophoretic mobility shift assays (EMSAs) were carried out as described previously (Srinath *et al.*, 2007). Briefly, *MtuMutY* (200 ng) was incubated with 10 000 c.p.m. of radiolabelled ssDNA or dsDNA (~ 0.1 pmol) on ice for 15 min in EMSA buffer [20 mM Tris/HCl (pH 8.0), 50 mM NaCl, 5% (v/v) glycerol, 1 mM β -mercaptoethanol and $50 \mu\text{g BSA ml}^{-1}$] in $20 \mu\text{l}$ volumes, and resolved on 10% native polyacrylamide (19:1) gels of 0.75 mm thickness and 160 mm length at 150 V in cold room (8°C) using $0.5 \times$ TBE. Bands corresponding to the complex and the free DNA were identified by BioImage Analyser.

Incorporation of G and 8-oxoG into DNA by *M. smegmatis* cell-free extracts. Cell-free extracts from *M. smegmatis* mc²155 were prepared as described previously (Jain *et al.*, 2007) and used in the primer extension assays. The substrates for primer extension were prepared by annealing of a 5' ³²P-end labelled Extension-oligo (Supplementary Table S1) as primer with Comp-G, Comp-A, Comp-T and Comp-C oligomers (Table 1). The substrates allow for extension of the primer by incorporation of the nucleotide(s) against G, A, T or C in the template. The reaction ($10 \mu\text{l}$) contained 5 μg cell-free extract, 12 000 c.p.m. of the primer-extension substrate, 5 pmol FpKRS, 50 or 100 μM 8-oxo-dGTP or dGTP in 10 mM Tris/HCl (pH 7.9), 10 mM MgCl_2 , 50 mM NaCl and 1 mM DTT. The reactions were incubated at 37°C for 5 min, stopped with 10 μl 80% formamide dye, heated at 90°C for 5 min, then analysed on 8 M urea/15% polyacrylamide gel and visualized by BioImage Analyser.

RESULTS

Disruption of *mutY* in *M. smegmatis*

M. smegmatis has served as a faithful model in genetic analyses of the fundamental processes of DNA repair in mycobacteria. Hence, to understand the physiological

significance of MutY in this important group of bacteria, we identified *M. smegmatis mutY* (MSMEG_6083) by its homology to *M. tuberculosis mutY* (Rv3589). Comparison of the genomic contexts (Fig. 1a) also suggested that MSMEG_6083 and Rv3589 are orthologues. To generate a *mutY* null allele of *M. smegmatis*, we disrupted a cloned copy of *M. smegmatis mutY* by replacing ~ 0.1 kb of its ORF with a *kan* cassette and introduced the generated construct, pPR-Msm-*mutY*::*kan*, into *M. smegmatis* to effect allelic exchange of the chromosomal copy with the disrupted allele (Pelicic *et al.*, 1997). PCR-based screening showed single amplicons of ~ 1.3 and 2.5 kb, respectively, from the parent *M. smegmatis* mc²155 (defined as wild-type) and the derived mutant strains (Fig. 1c, lanes 1 and 2). This observation agreed with the expected pattern (Fig. 1b) and indicated a successful exchange of the wild-type *mutY* allele with the disrupted (*mutY*::*kan*) allele. Genomic blot analysis also revealed bands of the expected sizes of ~ 4.4 and ~ 5.6 kb upon *EcoRV* digestion of the genomic DNA from the two strains (Fig. 1d, lanes 1 and 2).

To ensure that the disruption of the chromosomal copy of *mutY* resulted in the deficiency of MutY activity, we used cell-free extracts from the *mutY*::*kan* strain and its derivative harbouring pMV-Msm-*mutY* at the *L5att* site [strain *mutY* (*L5att*::*mutY*)] for the activity assays. A duplex containing a ³²P 5'-labelled oligomer (Comp-A oligo, Table S1) with an A at position 15, opposite 8-oxoG in the complementary strand, was used as substrate, S (Fig. 1e). In the assay, excision of the A residue from the Comp-A oligo followed by the cleavage of the backbone results in two fragments, one of which (³²P-labelled) is detected as a faster-migrating product (P) band upon gel electrophoresis. As seen in Fig. 1(e), the cell-free extracts prepared from the parent strain (wild-type) resulted in processing of the 30-mer duplex DNA (lane 3), as in the control reaction with the *M. tuberculosis* MutY (*MtuMutY*, lane 2). This activity was largely missing from the extracts of the MutY-deficient strain (*mutY*::*kan*, lane 4). However, the activity was restored in the extracts prepared from the complemented strain (lane 5) harbouring an intact copy of *mutY* at the *L5att* site [*mutY* (*L5att*::*mutY*)]. These experiments show that disruption of *mutY* with the *kan* cassette results in a deficiency of MutY activity, and the deficiency is rescued by an ectopically inserted copy of the *mutY* gene.

Determination of mutation rates

The effect of loss of *mutY* on DNA repair was investigated by determining mutation rates by spontaneous appearance of rifampicin resistance (Rif^R). As shown in Table 2, the deficiency of MutY did not result in a significant change in the mutation rate of the *mutY*::*kan* strain over that of the parent strain (~ 1.3 -fold). However, consistent with our earlier report (Kurthkoti *et al.*, 2008), deficiency of Fpg (strain *fpg*::*hyg*) led to a moderate (~ 3 -fold) increase in the mutation rate.

Fig. 1. Generation and characterization of the *M. smegmatis* *mutY::kan* strain. (a) Schematic representation (not to scale) of the genomic context of *mutY* in *M. smegmatis* (Msm) between positions 6142417 and 6151165, and its comparison with that from the *M. tuberculosis* (Mtu) genome between positions 4026444 and 4035710. Annotations for *M. smegmatis* genome (MSMEG numbers) are as follows: 6079, DNA repair protein RadA; 6080 and 6081, conserved hypothetical proteins; 6082, carbonic anhydrase (homologous to Rv3588c); 6083, probable adenine glycosylase (*MutY*); 6084, AraC-family transcriptional regulator; 6085, hydrolase; 6086, antibiotic biosynthesis monooxygenase domain protein; 6087, β -lactamase. Annotations for *M. tuberculosis* (Rv numbers) are as follows: RadA, DNA repair protein; 3586, conserved hypothetical protein; 3587c, probable conserved membrane protein; 3588c, carbonic anhydrase; 3589, probable adenine DNA glycosylase (*MutY*); 3590, PE_PGRS family protein; 3591c, possible hydrolase; 3592, conserved hypothetical protein (gene TB11.2); and 3593, probable conserved lipoprotein, LpqF. (b) Schematic representation of the expected sizes of the amplicons in PCR, and fragments in genomic blots from the wild-type (upper panel) and the disrupted *mutY::kan* (lower panel) loci. The location of the *mutY* gene-specific probe used in the genomic blot (panel c) is indicated by a dotted line above the *mutY* gene in the upper panel. (c) Agarose gel electrophoresis of the PCR amplicons from the wild-type (lane 1) and the *mutY* null strains (*mutY::kan*, lane 2). Lane M shows λ DNA HindIII/HindIII size markers. (d) Genomic blots of wild-type and *mutY::kan* *M. smegmatis* strains. Sizes of DNA bands are indicated. (e) MutY activity assays with cell-free extracts (25 μ g) prepared from various strains (as indicated) or purified *M. tuberculosis* MutY (lane 2). The Comp-A strand was 5'-³²P labelled, and ~20 000 c.p.m. (~0.1 pmol) of the duplex DNA containing 8-oxoG:A pairs were used. The reaction contents were analysed on 8 M urea-15% polyacrylamide gel. Lane 1, is a substrate alone negative control (c). The bands corresponding to product and substrate are indicated as P and S, respectively.

Analysis of mutation spectrum

The effect of MutY deficiency on the mutation spectrum was determined by DNA sequence analysis of the RRDR from Rif^R isolates of the *mutY::kan* strain and comparison with that of the parent strain (Table 3, Supplementary Fig. S1). It was observed that loss of *mutY* resulted in an increase in C to A (G to T) mutations over the wild-type

(to 15% over an undetectable value). Furthermore, an increase in the A to C (or T to G) mutations from 2% to 28%, and an increase in A to G (or T to C) mutations from 34% to 48% also occurred. These increases are compensated for by a decrease in the frequency of other mutations. The contribution of the C to T (or G to A) mutations, which accounted for 52% of the mutations in the parent background, decreased to ~4% in the *mutY::kan* strain

Table 2. Effect of deficiency in DNA repair enzymes on mutation rate

Strain	$10^{-9} \times$ Total no. of viable bacteria plated*	$10^{10} \times$ Mutation rate†	Fold increase
WT	7.3 ± 0.63	2.1	1.0
<i>mutY::kan</i>	6.3 ± 1.6	2.82	1.34
<i>fpg::hyg</i>	7.9 ± 1.8	6.46	3.07

*Mean $\pm$ SD of the total viable counts (determined by dilution plating of an aliquot) of bacteria in the 6 day cultures used to plate on rifampicin plates.

†Mutation rates were calculated according to David (1970) using the equation $a = 2 \times \ln 2 [(M_t/N_t) - (M_0/N_0)]/n$ [a , mutation rate; n , number of generations; M_t , number of Rif^R colonies obtained from the 6 day culture (Methods); N_t , number of bacteria in the 6 day culture; M_0 , number of Rif^R colonies in the starter diluted culture used to make 6 day culture; N_0 , number of bacteria in the starter culture used to make the 6 day culture]. The value of n was calculated as $(\log_{10} N_t - \log_{10} N_0)/0.301$. As the value of M_0 was 0, the simplified equation $a = 2 \times \ln 2 (M_t/N_t)/n$ was used to calculate mutation rates. Mutation rates were calculated for all 12 replicates of the 6 day cultures, and the mean mutation rates shown were calculated from ten replicates, eliminating the two with the highest and the lowest rates.

(see Discussion). High occurrence of C to T mutations in the parent strain in our studies as well as those reported earlier (Boshoff *et al.*, 2003) suggests that in mycobacteria, with a G+C-rich genome, there is a significant level of background cytosine deamination, and when the bacteria are challenged with Rif, frequently it is the C to T mutations that are selected to confer Rif^R.

Table 3. Spectrum of mutations in the RRDR locus of rifampicin-resistant isolates of *M. smegmatis* (wild-type) and *M. smegmatis mutY::kan*

Information on the positions of the changes is summarized in Supplementary Fig. S1.

Mutations detected	Strain	
	WT*	<i>mutY::kan</i> †
C→T/G→A	52 % (30/58)	4 % (2/46)
C→G/G→C	7 % (4/58)	4 % (2/46)
C→A/G→T	None	15 % (7/46)
A→G/T→C	34 % (20/58)	48 % (22/46)
A→C/T→G	2 % (1/58)	28 % (13/46)
A→T/T→A	5 % (3/58)	None
No mutations in RRDR‡	21/79	2/48

*A total of 79 samples were sequenced, of which 58 samples showed mutations in RRDR.

†A total of 48 samples were sequenced, of which 46 showed mutations in RRDR.

‡Mutations elsewhere resulted in resistance to rifampicin.

Effect of hydrogen peroxide on *mutY* strain

We have shown previously that Fpg deficiency in *M. smegmatis* resulted in susceptibility of the mutant (*fpg::hyg*) to H₂O₂. Hence, it was of interest to analyse the effect of H₂O₂ on the growth of *M. smegmatis* deficient in MutY and compare it with the growth of the parent *M. smegmatis*, and the strain deficient in Fpg (MutM). All the strains showed comparable growth in the absence of peroxide (Fig. 2a, panel i), indicating that the loss of DNA repair enzymes (Fpg and MutY) did not affect the general growth kinetics of the bacteria. However, with the addition of H₂O₂ in the growth medium (Fig. 2a, panel iii), while the growth of the *fpg* strain was seen to decrease, the growth profiles of the *mutY* and the *mutY* (L5att::*mutY*) strains were similar to that of the parent strain.

Cell infection assays

M. smegmatis, a saprophytic organism, does not survive for prolonged periods in an intracellular environment. However, cell infection assays have been used to assess the effect of various mutations on the intracellular survival of the bacterium (Lagier *et al.*, 1998, Dheenadhayalan *et al.*, 2006). Also, it is known that macrophage cell lines inflict oxidative-stress-related damage on the DNA of the intracellular bacteria (Schlosser-Silverman *et al.*, 2000). Hence, we studied the consequence of MutY deficiency on the survival of the *M. smegmatis* strains in RAW 264.7 macrophage cells of mouse origin (Fig. 2b). As expected, all the strains were gradually cleared from the RAW 264.7 cells. However, the *fpg* strain was cleared relatively faster than the parent or the *mutY* strains (see the 48 and 72 h time points). Although, when compared with the parent strain, there is a detectable impact of MutY deficiency on the survival of the *mutY* strain, this is minor (~2-fold, see 48 and 72 h time points). When the *mutY* strain was complemented with a copy of *mutY* gene in its L5 locus, the survival of the complemented strain was rescued. These results indicate that MutY contributes to counteracting the intracellular stress, although under the conditions used, its contribution is less than that of Fpg.

Substrate specificity of mycobacterial MutY

We have previously analysed the base-excision activity of mycobacterial MutY using substrates containing different nucleotides against 8-oxoG and *M. smegmatis* cell-free extracts, and shown that it possessed a predominant activity of processing A against 8-oxoG and a minor activity of processing G against 8-oxoG (Jain *et al.*, 2007). In this study, use of the *M. smegmatis mutY* cell-free extract as a negative control allowed us to further confirm our earlier observations. In addition, we could infer a very weak activity of excision of T against 8-oxoG (data not shown). However, to further verify the substrate specificity of mycobacterial MutY, we developed an expression

Fig. 2. (a) Effect of H₂O₂ on growth. Saturated cultures of *M. smegmatis* strains WT (■), *fpg* (▼), *mutY* (▲) and *mutY* (L5att::mutY) (◆) were diluted 100-fold in LBT. H₂O₂ was either not added (i) or added at 1.0 mM (ii) and 1.5 mM (iii) concentrations. Cultures were grown at 37 °C for 36 h in a Bioscreen C kinetic growth reader in honeycomb plates. Growth was monitored by measuring OD₆₀₀. Growth curves were prepared from three independent colonies for each strain, and mean values (±SD) were plotted against time. (b) Survival of *M. smegmatis* strains in RAW 264.7 macrophage cells. The macrophage cells were infected with the *M. smegmatis* strains indicated, in triplicate. Viability of intracellular bacteria at 0, 24, 48 and 72 h post-infection was determined by dilution plating. Counts from triplicate samples were averaged and the mean values (±SD) were plotted. Student's unpaired *t* test was used to determine the statistical significance of differences between the values (***, *P*<0.0001; n.s., not significant).

construct for *M. tuberculosis* MutY (*MtuMutY*, which shows 70% identity and 78% similarity with the *M. smegmatis* MutY). Use of the purified *MtuMutY* substantiated the A-excision activity of the mycobacterial MutY (Fig. 3a, compare lanes 6–8 with 5). And, consistent with the observations made from the cell-free extracts of *M. smegmatis*, excision of G and T (present as 8-oxoG:G and 8-oxoG:T pairs) was also detectable (compare lanes 2–4 with 1, and lanes 10–12 with 9, respectively). However, these activities were weak and required extended periods of incubation with the enzyme. Not unexpectedly, excision of C (which forms the correct base pair) from an 8-oxoG:C pair was not detectable (compare lanes 14–16 with 13).

To further our understanding of the specificity of the recognition of the base pairs containing 8-oxoG, we carried out EMSAs using native gels to resolve the complexes of MutY with various duplexes (Fig. 3b). As expected, MutY did not form stable complexes with ssDNA (compare lanes 2 and 1) or with an 8-oxoG:C pair containing dsDNA (compare lanes 10 and 9), both of which are not utilized by it as substrates, and it formed complexes with dsDNA harbouring 8-oxoG:A quite efficiently. Interestingly, MutY also efficiently complexed with dsDNA harbouring 8-oxoG:G or 8-oxoG:T pairs, which are utilized poorly as substrates (lanes 3–8). It is unclear whether the property of MutY complexation with the latter two DNAs represents a physiologically important phenomenon (see Discussion).

Fig. 3. (a) Time-course of the base-excision activity of *MtuMutY* on different oligonucleotide substrates. The duplex used in each assay is denoted at the top and the star at the end of one of the strands denotes the 5' ^{32}P -end labelling. Radiolabelled substrate (50 000 c.p.m.) in a 40 μl reaction containing 1.25 $\times$ reaction buffer was incubated at 37 °C for 5 min. An aliquot (8 μl) was taken and mixed with 2 μl H_2O for use as 0 h time point. The reaction was then started in the remainder of the mix by adding 800 ng (8 μl) of *MtuMutY*. Aliquots of 10 μl were taken at 1, 6 and 24 h. All the samples including the 0 h time point were then treated with NaOH and resolved on 8 M urea/15% polyacrylamide gel and visualized using BioImage Analyser. Substrate and product bands are indicated with S and P, respectively. (b) DNA oligomers (10 000 c.p.m.; ~0.1 pmol) as indicated were incubated with *MtuMutY* (200 ng) for 15 min on ice in EMSA buffer and resolved on the native PAGE. Bands corresponding to free ssDNA, dsDNA and complex are indicated.

Error-prone incorporation of 8-oxoG into DNA

To understand the mutation spectrum in MutY-deficient *M. smegmatis*, we designed substrates in which template-dependent extension of a 5' ^{32}P -end labelled primer by incorporation of G or 8-oxoG against any of the four standard nucleotides in the template by the DNA polymerase activities of *M. smegmatis* cell-free extracts was analysed (Fig. 4). As a control, extension of the primer in the absence of any added dNTPs was not detected (Fig. 4a, b, compare lanes 2, 6, 10 and 14 with 1, 5, 9 and 13, respectively). As seen from the band corresponding to its extension with one nucleotide ($n+1$ product), near-total incorporation of G occurred against C in the template in the presence of either 50 μM or 100 μM dGTP in the reaction (Fig. 4a, compare lanes 15 and 16 with 14). Similarly, an expected incorporation of 8-oxoG occurred against A in the template (Fig. 4b, compare lanes 7 and 8 with 6). Interestingly, a weak incorporation of G (as $n+1$

product) against A or T could also be detected (Fig. 4a, lanes 7 and 8; and 11 and 12, respectively). In addition, we observed a band corresponding to $n+3$ product (lanes 3, 4, 7, 8, 11, 12, 15 and 16). While the origin of this band is not clear, it could represent an error-prone incorporation of G by the mycobacterial DNA polymerase(s). The $n+3$ band was undetectable in the reactions not supplemented with dGTP (Fig. 4a, lanes 2, 6, 10 and 14). Importantly, under the same conditions, we observed that incorporation of 8-oxoG against T, and not unexpectedly against C, in the template occurred somewhat more significantly (Fig. 4b, lanes 11, 12, 15 and 16). Such an incorporation of 8-oxoG was further highlighted by the presence of $n+2$ and $n+3$ bands of gradually decreasing intensities (Fig. 4b, lanes 11, 12, 15 and 16). Although a better understanding of the erroneous incorporation of nucleotides in DNA in mycobacteria will have to await detailed studies on purification and biochemical analyses of the various DNA polymerases, the experiments in Fig. 4 using cell-free

Fig. 4. Primer extension assays in cell-free extracts of *M. smegmatis*. The duplex DNA substrates containing 5' overhanging sequence in the template (as shown above the lanes) were prepared by annealing Comp-G (lanes 1–4), Comp-A (lanes 5–8), Comp-T (lanes 9–12) and Comp-C (lanes 13–16) oligomers with the 5' ^{32}P -end labelled primer (Extension-oligo). The assays using 5 µg cell-free extract (indicated with '+') were carried out in the absence (lanes 2, 6, 10 and 14), and in the presence of 50 µM (lanes 3, 7, 11 and 15) or 100 µM (lanes 4, 8, 12 and 16) of added dGTP (a) or 8-oxo-dGTP (b), at 37 °C for 3 min, analysed on 8 M urea/15 % polyacrylamide gel and visualized on BioImage Analyser. Lanes 1, 5, 9 and 13 are no cell-free extract and no dNTP, substrate alone controls. The primer size (n) and the extension products (n + 1, n + 2 and n + 3) are as indicated with the arrows on the right hand side margins.

extracts do provide us with a strong first indication of the possibility of incorporation of 8-oxoG into DNA not only against A and C but also against T (Fig. 4b).

DISCUSSION

The redox potentials of G, A, C and T are 1.3, 1.4, 1.6 and 1.7 V, respectively. Guanine, with the lowest redox potential, is thus chemically the most susceptible base in DNA to oxidative stress, resulting in the formation of 8-oxoG (Steenken & Jovanovic, 1997). The G + C richness of the genome of mycobacteria and the habitat of the pathogenic species in macrophages, where they are exposed to reactive oxygen species, suggests that their DNA is naturally at a high risk of incurring oxidative damage. The base-excision repair enzymes Fpg (MutM) and MutY are crucial guardians of bacterial genomes against such oxidative damage. We have previously analysed the effects

of Fpg deficiency in *M. smegmatis* (Jain *et al.*, 2007). Here, we have investigated the effects of MutY deficiency on various properties of this bacterium.

We noticed that unlike Fpg deficiency, MutY deficiency did not result in a detectable phenotype in response to treatment with H_2O_2 . And although the MutY deficiency resulted in a decrease in the survival of the *mutY* strain in a mouse macrophage cell line, the impact was minor (Fig. 2b). Also, unlike the earlier studies in *E. coli* (Michaels *et al.*, 1992) or *P. aeruginosa* (Sanders *et al.*, 2009), loss of MutY in *M. smegmatis* did not significantly affect the mutation rate (Table 2). It may be mentioned that mycobacteria possess a number of MutM activities (Sidorenko *et al.*, 2008). It has also been shown before that increased levels of MutM activity compensate for the deficiency of MutY (Michaels *et al.*, 1992). This could be a reason for the smaller impact of MutY deficiency in mycobacteria. However, investigation of the mutation

spectrum in the RRDR of the *rpoB* locus did show a significant increase in C to A (or G to T) mutations, which otherwise occur at an extremely low frequency in mycobacteria (Boshoff *et al.*, 2003; Jain *et al.*, 2007). The MutY deficiency also resulted in a considerable increase in A to C (T to G) and a smaller increase in A to G (T to C) mutations (Table 3).

The biochemical activity assays reveal that similar to the *E. coli* MutY, the mycobacterial MutY excised A from the 8-oxoG:A pair. Excisions of G or T from their respective pairs with 8-oxoG also occurred, but these activities were poor (Fig. 3a). We have earlier shown that mycobacterial DNA polymerase(s) result in incorporation of A (in addition to G and C) against 8-oxoG (Jain *et al.*, 2007). Hence, a detectable increase in C to A mutations upon MutY deficiency in mycobacteria is readily understood due to its conventional role as adenosine DNA glycosylase in the GO repair pathway. However, an atypical increase in A to C (or T to G) and to some extent A to G (or T to C) mutations requires an explanation.

Fig. 5 summarizes the major steps in the GO repair pathway as established in *E. coli* (Michaels & Miller, 1992)

except that based on our earlier study (Jain *et al.*, 2007), misincorporation of G (in addition to A) has also been included if the replication of 8-oxoG containing template occurred prior to its excision by Fpg (steps 4 and 6, *right*). Steps 1 to 9 in this pathway are either involved in excision repair of 8-oxoG to maintain the integrity of the genome (step 3) or lead to mutations (step 9). Absence of an increase in C to G mutations as a consequence of MutY deficiency, and a relatively low (15 %) occurrence of C to A mutations, suggests that much of the repair of the 8-oxoG arising in DNA by its oxidative damage occurs by Fpg prior to replication (step 2).

However, the presence of 8-oxoG in DNA may also arise from an alternative route of direct incorporation of 8-oxo-dGTP from the cellular pool (which may accumulate because of the oxidative damage of dGTP, step 10) during replication by an error-prone DNA polymerase (step 12). Although the levels of 8-oxo-dGTP are kept to a minimum by the enzymic activity of MutT, which hydrolyses 8-oxo-dGTP to 8-oxo-dGMP and PP_i (step 11), incorporation of the residual 8-oxo-dGTP (e.g. against A and T) in DNA could occur (Fig. 4b) by error-prone DNA polymerases

Fig. 5. GO repair pathway in mycobacteria, highlighting the incorporation of G against 8-oxoG (steps 4 and 6, *right*). Also shown is a scheme for generation of mutations following misincorporation of 8-oxoG during replication (steps 12–16). For details of various steps refer to the Discussion.

(Davis & Forse, 2009; Shimizu *et al.*, 2003). Paradoxically, MutY activity on these base pairs (step 14) would lead to excision of the correct base from DNA with severe consequences of misincorporations during gap filling, and fixation of a mutation through steps 14–16. Additionally, the mycobacterial Fpg-mediated excision of the 8-oxoG (step 16, Jain *et al.*, 2007) may also contribute to the fixation of the mutations. In fact, occurrence of such a phenomenon would explain the high background (34 %, Table 3) of A to G (or T to C) mutations. And, while in steps 14–16, the process is shown to be facilitated by MutY, such a fixation of mutations could occur even in the absence of MutY by direct replication (step 13). As is evident from the data in Table 3, the absence of MutY would lead to preferential processing of DNA (with misincorporated 8-oxoG) via step 13 and result in even more mutations. How MutY lowers mutation fixation by acting at step 14 remains unclear. However, it may well be that complexation of MutY with DNA containing mispaired 8-oxoG (Fig. 3b) somehow facilitates 8-oxoG excision by Fpg (Jain *et al.*, 2007) or other uncharacterized Fpg-like proteins found in mycobacteria. Such a proposal is supported by our earlier observation that Fpg deficiency also resulted in an increase in A to G (T to C) mutations.

Thus, a high-level occurrence of 8-oxo-dGTP in the cellular pool for the G + C-rich mycobacteria could have severe consequences. Genome sequence analysis reports the presence of four probable MutT proteins in *M. tuberculosis* and another five Nudix-box-containing proteins to which MutT belongs. However, unlike the *E. coli* counterpart (Maki & Sekiguchi, 1992), none of the four MutTs shows a high specificity towards 8-oxo-dGTP (Dos Vultos *et al.*, 2006). It has also been reported (Moreland *et al.*, 2009) that the MutT2 of *M. tuberculosis*, which is the most similar to *E. coli* MutT, is actually a dCTPase. In fact, in *M. smegmatis* the orthologue of *M. tuberculosis* MutT2 is annotated as a dCTPase. However, at present, it cannot be ruled out that one or more of the remaining MutTs could actually possess 8-oxo-dGTPase activity. Nevertheless, the occurrence of A to G mutations in the wild-type and an increase in A to G and A to C mutations indicates that the steady-state levels of 8-oxo-dGTP may be high in mycobacteria. Clearly, understanding of the distinctive features of the DNA repair pathways in mycobacteria will be an exciting area of research to unravel the mechanisms that lead to development of multiple drug resistance as well as to design novel strategies to control the growth of this important class of pathogenic bacteria.

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REFERENCES

- Au, K. G., Clark, S., Miller, J. H. & Modrich, P. (1989). *Escherichia coli* mutY gene encodes an adenine glycosylase active on G/A mispairs. *Proc Natl Acad Sci U S A* **86**, 8877–8881.
- Boshoff, H. I., Reed, M. B., Barry, C. E., III & Mizrahi, V. (2003). DnaE2 polymerase contributes to in vivo survival and the emergence of drug resistance in *Mycobacterium tuberculosis*. *Cell* **113**, 183–193.
- Bruner, S. D., Norman, D. P. G., Fromme, J. C. & Verdine, G. L. (2000). Structural and mechanistic studies on repair of 8-oxoguanine in mammalian cells. *Cold Spring Harb Symp Quant Biol* **65**, 103–111.
- Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S. V., Eiglmeier, K., Gas, S. & other authors (1998). Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393**, 537–544.
- David, H. L. (1970). Probability distribution of drug-resistant mutants in unselected populations of *Mycobacterium tuberculosis*. *Appl Microbiol* **20**, 810–814.
- David, S. S., O'Shea, L. O. & Kundu, S. (2007). Base excision repair of oxidative DNA damage. *Nature* **447**, 941–950.
- Davis, E. O. & Forse, L. N. (2009). DNA repair: key to survival? In *Mycobacterium: Genomics and Molecular Biology*, pp. 79–119. Edited by T. Parish & A. Brown. London: Caister Academic Press.
- Delaney, S., Neeley, W. L., Delaney, J. C. & Essigmann, J. M. (2007). The substrate specificity of MutY for hyperoxidized guanine lesions in vivo. *Biochemistry* **46**, 1448–1455.
- Dheenadhayalan, V., Delogu, G. & Brennan, M. J. (2006). Expression of the PE_PGRS 33 protein in *Mycobacterium smegmatis* triggers necrosis in macrophages and enhanced mycobacterial survival. *Microbes Infect* **8**, 262–272.
- Dos Vultos, T., Blazquez, J., Rauzier, J., Matic, I. & Gicquel, B. (2006). Identification of nudix hydrolase family members with an anti-mutator role in *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*. *J Bacteriol* **188**, 3159–3161.
- Farr, S. B. & Kogoma, T. (1991). Oxidative stress response in *Escherichia coli* and *Salmonella typhimurium*. *Microbiol Rev* **55**, 561–585.
- Fowler, R. G., White, S. J., Koyama, C., Moore, S. C., Dunn, R. L. & Schaaper, R. M. (2003). Interactions among the *Escherichia coli* mutT, mutM and mutY damage prevention pathways. *DNA Repair (Amst)* **2**, 159–173.
- Fraga, C. G., Shigenaga, M. K., Park, J.-W., Degan, P. & Ames, B. N. (1990). Oxidative damage to DNA during aging: 8-hydroxy-2'-deoxyguanosine in rat organ DNA and urine. *Proc Natl Acad Sci U S A* **87**, 4533–4537.
- Jain, R., Kumar, P. & Varshney, U. (2007). A distinct role of formamidopyrimidine DNA glycosylase (MutM) in down-regulation of accumulation of G, C mutations and protection against oxidative stress in mycobacteria. *DNA Repair (Amst)* **6**, 1774–1785.
- Kurthkoti, K., Kumar, P., Jain, R. & Varshney, U. (2008). Important role of the nucleotide excision repair pathway in *Mycobacterium smegmatis* in conferring protection against commonly encountered DNA-damaging agents. *Microbiology* **154**, 2776–2785.
- Lagier, B., Pelicic, V., Lecossier, D., Prod'homme, G., Rauzier, J., Guilhot, C., Gicquel, B. & Hance, A. J. (1998). Identification of genetic loci implicated in the survival of *Mycobacterium smegmatis* in human mononuclear phagocytes. *Mol Microbiol* **29**, 465–475.
- Lu, A. L. & Chang, D. Y. (1988). Repair of single base pair transversion mismatches of *Escherichia coli* in vitro: correction of certain A/G mismatch is independent of dam methylation and host mutHLS gene function. *Genetics* **118**, 593–600.

- Maki, H. & Sekiguchi, M. (1992).** MutT protein specifically hydrolyses a potent mutagenic substrate for DNA synthesis. *Nature* **355**, 273–275.
- Michaels, M. L. & Miller, J. H. (1992).** The GO system protects organisms from the mutagenic effect of the spontaneous lesion 8-hydroxyguanine (7,8-dihydro-8-oxoguanine). *J Bacteriol* **174**, 6321–6325.
- Michaels, M. L., Cruz, C., Grollmant, A. P. & Miller, J. H. (1992).** Evidence that MutY and MutM combine to prevent mutations by an oxidatively damaged form of guanine in DNA. *Proc Natl Acad Sci U S A* **89**, 7022–7025.
- Mizrahi, V. S. & Andersen, S. J. (1998).** DNA repair in *Mycobacterium tuberculosis*. What have we learnt from the genome sequence? *Mol Microbiol* **29**, 1331–1339.
- Mo, J.-Y., Maki, H. & Sekiguchi, M. (1992).** Hydrolytic elimination of a mutagenic nucleotide, 8-oxodGTP, by human 18-kilodalton protein: sanitization of nucleotide pool. *Proc Natl Acad Sci U S A* **89**, 11021–11025.
- Moreland, N. J., Charlier, C., Dingley, A. J., Baker, E. N. & Lott, J. S. (2009).** Making sense of a missense mutation: characterization of MutT2, a Nudix hydrolase from *Mycobacterium tuberculosis*, and the G58R mutant encoded in W-Beijing strains of *M. tuberculosis*. *Biochemistry* **48**, 699–708.
- Pellicic, V., Jackson, M., Reyrat, J. M., Jacobs, W. R., Jr, Gicquel, B. & Guilhot, C. (1997).** Efficient allelic exchange and transposon mutagenesis in *Mycobacterium tuberculosis*. *Proc Natl Acad Sci U S A* **94**, 10955–10960.
- Sanders, L. H., Sudhakaran, J. & Sutton, M. D. (2009).** The GO repair system prevents ROS-induced mutagenesis and killing in *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* **294**, 89–96.
- Schlosser-Silverman, E., Elgrably-Weiss, M., Rosenshine, I., Kohen, R. & Altuvia, S. (2000).** Characterization of *Escherichia coli* DNA lesions generated within J774 macrophages. *J Bacteriol* **182**, 5225–5230.
- Shimizu, M., Gruz, P., Kamiya, H., Kim, S. R., Pisani, F. M., Masutani, C., Kanke, Y., Harashima, H., Hanaoka, F. & Nohmi, T. (2003).** Erroneous incorporation of oxidized DNA precursors by Y-family DNA polymerases. *EMBO Rep* **4**, 269–273.
- Sidorenko, V. S., Rot, M. A., Filipenko, M. L., Nevinsky, G. A. & Zharkov, D. O. (2008).** Novel DNA glycosylases from *Mycobacterium tuberculosis*. *Biochemistry (Mosc)* **73**, 442–450.
- Snapper, S. B., Melton, R. E., Mustafa, S., Kieser, T. & Jacobs, W. R., Jr (1990).** Isolation and characterization of efficient plasmid transformation mutants of *Mycobacterium smegmatis*. *Mol Microbiol* **4**, 1911–1919.
- Srinath, T., Bharti, S. K. & Varshney, U. (2007).** Substrate specificities and functional characterization of a thermo-tolerant uracil DNA glycosylase (UdgB) from *Mycobacterium tuberculosis*. *DNA Repair (Amst)* **6**, 1517–1528.
- Steenken, S. & Jovanovic, S. V. (1997).** How easily oxidizable is DNA? One-electron reduction potentials of adenosine and guanosine radicals in aqueous solution. *J Am Chem Soc* **119**, 617–618.
- Tajiri, T., Maki, H. & Sekiguchi, M. (1995).** Functional cooperation of MutT, MutM and MutY proteins in preventing mutations caused by spontaneous oxidation of guanine nucleotide in *Escherichia coli*. *Mutat Res* **336**, 257–267.
- Tsai-Wu, J. J., Liu, H. F. & Lu, A. L. (1992).** *Escherichia coli* MutY protein has both N-glycosylase and apurinic/apyrimidinic endonuclease activities on A.C and A.G mispairs. *Proc Natl Acad Sci U S A* **89**, 8779–8783.
- Venkatesh, J., Kumar, P., Krishna, P. S., Manjunath, R. & Varshney, U. (2003).** Importance of uracil DNA glycosylase in *Pseudomonas aeruginosa* and *Mycobacterium smegmatis*, G+C-rich bacteria, in mutation prevention, tolerance to acidified nitrite, and endurance in mouse macrophages. *J Biol Chem* **278**, 24350–24358.

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