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MutY is Down-regulated by Oxidative Stress in *E. coli*

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In *Escherichia coli*, MutM (8-oxoG DNA glycosylase/lyase or Fpg protein), MutY (adenine DNA glycosylase) and MutT (8-oxodGTPase) function cooperatively to prevent mutation due to 7, 8-dihydro-8-oxoguanine (8-oxoG), a highly mutagenic oxidative DNA adduct. MutM activity has been demonstrated to be induced by oxidative stress. Its regulation is under the negative control of the global regulatory genes, *fur*, *fnr* and *arcA*. However, interestingly the presence of MutY increases the mutation frequency in *mutT*− background because of MutY removes adenine (A) from 8-oxoG:A which arises from the misincorporation of 8-oxoG against A during DNA replication. Accordingly we hypothesized that the response of MutY to oxidative stress is opposite to that of MutM and compared the regulation of MutY activity with MutM under various oxidative stimuli. Unlike MutM, MutY activity was reduced by oxidative stress. Its activity was reduced to 30% of that of the control when *E. coli* was treated with paraquat (0.5 mM) or H_2O_2 (0.1 mM) and induced under anaerobic conditions to more than twice that observed under aerobic conditions. The reduced mRNA level of MutY coincided with its reduced activity by paraquat treatment. Also, the increased activity of MutY in anaerobic conditions was reduced further in *E. coli* strains with mutations in *fur*, *fnr* and *arcA* and the maximum reduction in activity was when all mutations were present in combination, indicating that MutY is under the positive control of these regulatory genes. Therefore, the down-regulation of MutY suggests that there has been complementary mechanism for its mutagenic activity under special conditions. Moreover, the efficacy of anti-mutagenic action should be enhanced by the reciprocal co-regulation of MutM.

Keywords: 8-oxoG; Oxidative stress; *E. coli*; MutY; MutM; MutT

INTRODUCTION

Among many oxidative DNA adducts known, much attention has been paid to 7,8-dihydro-8-oxoguanine (8-oxoG) due to its facile formation and high mutagenic action.[1,2] During DNA synthesis, 8-oxoG is mismatched with adenine (A) instead of cytosine (C) and this lead to G:C → T:A transversion.[3] *Escherichia coli* is equipped with a triple defense system against this mutagenic action,[4–6] indicating that the threat 8-oxoG poses to genetic integrity. This defense system consists of MutM,[7] MutY[8] and MutT.[9] MutM is a DNA glycosylase/lyase, which removes 8-oxoG residues from DNA strand (glycosylase activity) and cleaves the DNA strand at two sites 3′ and 5′ to the resulting apurinic site (lyase activity).[7] However, MutM cannot remove an 8-oxoG residue mismatched to A (8-oxoG:A) in DNA. This mispairing is recognized by MutY,[8] which removes A from 8-oxoG:A by using its adenine glycosylase activity. 8-oxoG is also formed in the cytoplasmic guanine nucleotides. 8-oxodGTP is a typical product of cytoplasmic 8-oxoG, which unless removed is incorporated into DNA. MutT prevents 8-oxodGTP incorporation into DNA by hydrolyzing it to 8-oxodGMP using its 8-oxodGTPase activity.[9]

We previously found that the activity of MutM in *E. coli* is regulated by the redox state of cells and that this regulation is under the control of the *fur*, *fnr* and *arcA*, regulatory genes of the oxidative

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metabolism.[10,11] When *E. coli* is in an oxidative state, because of exposure to high oxygen tension or ROS-producing chemicals, such as, paraquat, the proteins of *fur*, *fnr* and *arcA* become inactive and are no longer able to repress the expression of MutM, and thus, MutM activity is enhanced.[11] On the other hand, in a less oxidative (or reduced) state, these regulatory proteins become active and can repress the expression of MutM, and therefore, MutM activity decreases.[11] These regulatory proteins are also known to be involved in the control of oxidative energy metabolism and superoxide dismutase (SOD) activity.[12,13] In summary, the above means that *E. coli* adapts to the oxidative milieu efficiently, by changing cell function at three levels simultaneously, i.e. by reducing energy metabolism (ROS production), by increasing ROS removal and by enhancing oxidative DNA damage repair.

In the *E. coli* repair system against 8-oxoG, MutY and MutT also play substantial roles. However, in a *mutT*$^-$ background, *E. coli* (*mutY*$^+$) is more mutagenic than the *E. coli* (*mutY*$^-$) because MutY excises A opposite 8-oxoG that has been misincorporated by DNA polymerase.[14] This means that MutY is anti-mutagenic for the G:C → T:A transversion pathway and mutagenic for the T:A → G:C transversion pathway.[15,16] In fact, considerable 8-oxoG is generated in the cellular nucleotide pool as well as in DNA by oxidative stress. Because nucleotide pool is mainly located in the cytoplasm, cytoplasmic dGTP can be more easily attacked by oxidative stress than the G residue of DNA in nucleus. Therefore, significant amounts of 8-oxodGTP are probably produced under conditions of oxidative stress and this causes MutY's mutagenic activity to exceed its anti-mutagenic activity. Accordingly, there is a high probability of MutY down-regulation under conditions of high oxidative stress oppositely with MutM to enhance cellular protection. Therefore, we examined the activity of MutY under various oxidative conditions and compared this with the corresponding MutM activity.[10,11]

MATERIALS AND METHODS

Bacterial Strains

The *E. coli* strains used in this study are listed in Table I.[11,13] GC4468 is the wild type with respect to the following regulatory genes: *fur*, *fnr* and *arcA*. Other strains derived from GC4468 have mutations in one or more of these genes.

Preparation of DNA Substrates

The sequences of oligodeoxynucleotides used as substrates for MutY and MutM are listed in Table II.

TABLE I *E. coli* strains used in this study

Strain	Genotype
GC4468	Δ(argF-Δlac169rpsL sup(Am))
NC442	Same as GC4468 but *fur* :: *Tn5*
NC504	Same as GC4468 but Δ*fnr*
NC505	Same as GC4468 but Δ*arcA*
NC515	Same as GC4468 but Δ*arcA fur* :: *Tn5*
NC521	Same as GC4468 but Δ*fnr fur* :: *Tn5*
NC522	Same as GC4468 but Δ*arcA*Δ*fnrfur* :: *Tn5*

See Refs. [11,13].

The oligodeoxynucleotide containing a single 8-oxoG (Seq. 1) was obtained from the Midland Certified Reagent Co. (Midland, TX, USA). The other oligodeoxynucleotides were synthesized locally. Seq. 1 is complementary to Seq. 2 and Seq. 3. Seq. 4 was an 11-mer segment of Seq. 2 from the 3′-end to the site cleaved by MutY, and was used as a marker for MutY product. The 3′-end of Seq.1 or Seq. 2 was radiolabeled with terminal transferase (Roche MB, Mannheim, Germany) for 60 min at 37°C in the presence of [α-^{32}P] ddATP (Amersham Pharmacia Biotech, Little Chalfont, UK)[11,17] and purified using a Micro Bio-Spin Chromatography Column (Bio-Rad, Hercules, CA, USA). To obtain duplex DNA substrates, the 3′-end labeled oligodeoxynucleotides were annealed with the unlabeled complementary oligodeoxynucleotides for 10 min at 65°C and then slowly cooled to room temperature. The resulting 8-oxoG:A substrate was used for the MutY nicking assay and the 8-oxoG:C substrate was used for the MutM nicking assay as described below.

Aerobic Culture and Oxygen Radical Treatments

The *E. coli* GC4468 strain was grown overnight in Luria–Bertani (LB) media, then inoculated (1% by volume) into fresh LB media, and allowed to grow, with shaking at 200 rpm, to an optical density of 0.2 at 600 nm. At this stage, the bacteria were further cultivated for 1–2 h in the absence or in the presence of 0.5 mM paraquat (Sigma Chemical Co., St. Louis, MO, USA) or in the absence or presence of 0.1 mM H_2O_2 and cells were harvested for nicking assay or RT-PCR. All experiments were performed at 37°C.

TABLE II Oligodeoxynucleotides used in MutY and MutM nicking assay

Name	Sequence	Size
Seq. 1	5′ – GGTGGCCTGAC$\overset{oxo}{\mathbf{G}}$*CATTCCCCAA – 3′	22-mer
Seq. 2	5′-TTGGGGAATG**A**GTCAGGCCACC-3′	22-mer
Seq. 3	5′-TTGGGGAATG**C**GTCAGGCCACC-3′	22-mer
Seq. 4	5′-GTCAGGCCACC-3′	11-mer

*8-oxoG.

Anaerobic Culture

Anaerobic culture of various *E. coli* strains was performed at 37°C in a Coy anaerobic glove chamber containing 10% CO_2, 10% H_2 and 80% N_2.[18] The LB medium was equilibrated in the chamber for 3 days beforehand. To ensure adaptation to the anaerobic conditions, each strain taken from a single colony was subcultured successively at least twice in the chamber, and was then used to inoculate fresh medium. After being grown to saturation, the cells were harvested for nicking assay. To avoid the effect of oxygen on protein synthesis, 200 mg/l chloramphenicol (Sigma) was added to the cultures 15 min before they were removed from the anaerobic chamber.

Preparation of Cell Extracts

Cultures were harvested by centrifugation at 4000*g* for 10 min and the cell pellets were resuspended in reaction buffer I (80 mM NaCl, 10 mM EDTA, 2.9% glycerol, 1 mM dithiothreitol and 20 mM Tris–HCl, pH 7.6,) for the MutY assay or reaction buffer II (50 mM KCl, 2 mM EDTA, 2.9% glycerol, 1 mM dithiothreitol and 50 mM Tris–HCl, pH 7.5) for the MutM assay. To disrupt the cells, suspensions were sonicated for 1 min, centrifuged at 13,000*g* for 20 min, and the supernatants obtained were used as crude enzyme extracts for the nicking assay. All procedures were performed below 4°C and protein content was determined by the bicinchonic acid method[19] using bovine serum albumin as a standard.

MutY Nicking Assay

The MutY assay was performed as described previously.[8] The 3′-end labeled 8-oxoG:A duplex (0.2 pmol) was incubated with each of the crude enzyme extracts (50 μg) in 25 μl of reaction buffer I at 37°C for 15 min. To cut the DNA strand at the apurinic site resulting from the removal of A, 5 μl of 1N NaOH was then added and the mixture heated at 90°C for 4 min. An equal volume of urea loading buffer (9 M urea, 0.05% xylene cyanol and 0.05% bromophenol blue) was then added and aliquot electrophoresed in a 20% polyacrylamide gels containing 7 M urea. Cleaved DNA products were visualized by autoradiography and quantified by a Microcomputer Imaging (Imaging Research Inc. Ontario, Canada). Percent cleavage was determined by dividing the intensity of the cleaved product by the total intensity, which was defined as the sum of the intensities of the intact substrate and of the cleaved product.

RNA Preparation and RT-PCR Analysis

All solutions and plasticware were rendered RNase-free by diethyl pyrocarbonate (DEPC) treatment. RNA was extracted from mid-logarithmic phase cultures of GC4468 as described above. After freeze-thaw treatment, the total RNA was isolated from GC4468 by using TRI Reagent (Molecular Research Center Inc. Ohio, USA) according to the manufacturer's instructions. Possible DNA contamination was eliminated by digestion with RNase-Free DNase (Promega, Madison, WI) and RNasin (Promega), for 30 min at 37°C, as described by supplier, and the RNA was then recovered by phenol–chloroform extraction followed by ethanol precipitation. The RNA was washed with 70% ethanol, pelleted, dried and finally resuspended in DEPC-treated distilled water.

RT-PCR was preformed by using two-step methods with RT PreMix and PCR PreMix kits (Bioneer Co., Seoul, Korea). Before reverse transcription (RT), various amounts (1–4 μg) of total RNA and random hexamer (Promega) were denatured at 65°C for 5 min and then rapidly cooled on ice. These solutions were added to RT PreMix kits and RT was carried out at 42°C for 60 min and 94°C for 5 min. These solutions were then added to PCR PreMix kit containing MutY primers, PCR was performed for 32 cycles of 92°C for 30 s, 55°C for 30 s and 72°C for 90 s, and a final extension at 72°C for 5 min. Aliquots of the PCR products were analyzed by agarose (1.5%) gel electrophoresis. DNA contamination was determined by performing PCR without RT. As a positive control for each sample, RT-PCR was performed using primers specific for 16S rRNA; chosen as a control because of its stability and abundance. Sequences of the oligodeoxynucleotide primers used are listed in Table III.

MutM Nicking Assay

The MutM was assayed as described previously.[11] Briefly, the 3′-end labeled 8-oxoG:C duplex

TABLE III Oligdeoxyonucleotide primer sequences used for RT-PCR analysis

Target gene	Sequence (5′ to 3′)[a]	Size of product (bp)
MutY	F: TACAAATCATCGCACCCAAA R: CGCTTTCTCTGGGTAAGCAC	198
16S rRNA	F: GTTAGCCGGTGCTTCTTCTG R: CAGCCACACTGGAACTGAGA	204

[a]F, forward; R, reverse.

(0.2 pmol) was incubated with each of the crude enzyme extracts (50 μg) in 25 μl of reaction buffer II at 37°C for 15 min. To cut the DNA strand at the apurinic site resulting from the removal of 8-oxoG, 5 μl of 1N NaOH was added and the mixture heated at 90°C for 4 min. The MutM product was detected as described above.

Statistical Analysis

Data were analyzed using the Student's *t*-test and are expressed as means ± SE of four independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ were considered to be significant.

RESULTS

Down-regulation of MutY Activity and its mRNA Level by Oxidative Stress

To determine whether the activity of MutY is reduced by oxidative stress, we cultured *E. coli* in the presence of paraquat (a superoxide generator). As expected, the MutY activity reduced to a third (Fig 1a, lane 1) of the control level (Fig 1a, lane C) and this returned to the control level when the *E. coli* was re-cultured in fresh medium (Fig 1a, lane 2), but was again lowered by adding paraquat (Fig 1a, lane 3), indicating that MutY responds reversibly to oxidative stress. MutY activity was also found to be reduced upon adding H_2O_2 (Fig 1a, lane 4), thus confirming that MutY is down-regulated by oxidative stress.

RT-PCR analysis was used to examine MutY mRNA level after culturing *E. coli* in the absence or in the presence of paraquat. The MutY mRNA levels of the control were generally 2–3 times higher than in *E. coli* treated with paraquat (Fig 1b), suggesting that the mRNA that encodes for MutY is also down-regulated by oxidative stress. Therefore, the mRNA level of MutY is coincident with its reduced activity.

In Fig 2, the activity of MutY was observed under anaerobic conditions and its response to this condition was compared with that of MutM. Figure 2a shows that MutY activity under anaerobic conditions (lane 2) was about two times its activity under aerobic conditions (lane 1). In contrast, the activity of MutM under aerobic conditions (Fig 2b, lane 1) was reduced to ca. 30% of that under anaerobic conditions (Fig 2b, lane 2).

Control of MutY Activity by *fur*, *fnr* and *arcA*

To determine whether *fur*, *fnr* or *arcA* are involved in the regulation of MutY activity, we examined MutY activity in mutant strains of *fur*, *fnr*, *arcA* and combinations thereof (Fig 3). Under anaerobic

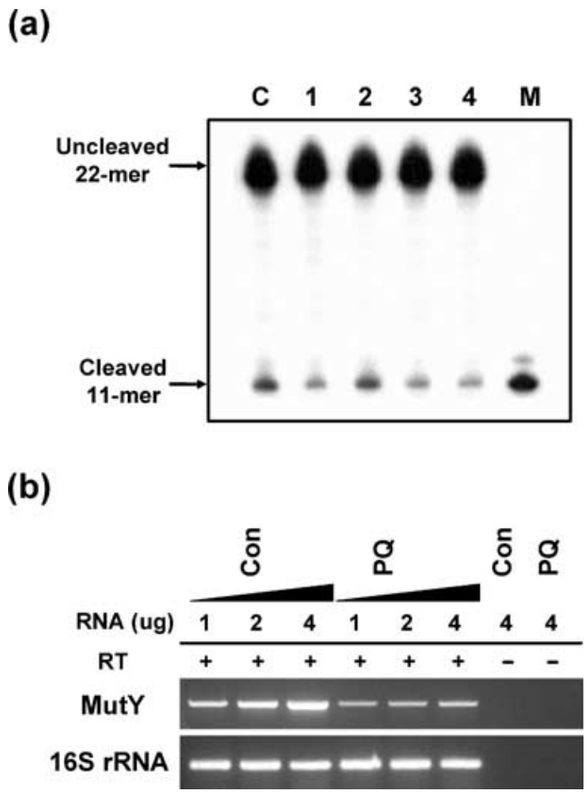

FIGURE 1 Effects of oxidative stress on MutY activity and its mRNA level. (a) MutY nicking assay. *E. coli* GC4468 was grown for 2 h in LB medium in the absence (lane C) and in the presence of 0.5 mM paraquat (lane 1) or 0.1 mM H_2O_2 (lane 4). GC4468 strain grown in the presence of 0.5 mM of paraquat (lane 1) was transferred to fresh medium and grown for a further 2 h (lane 2). The GC4468 re-grown in the fresh medium was reintroduced to the medium containing 0.5 mM paraquat and cultured for 2 h (lane 3). After harvesting, *E. coli* was disrupted by sonication and the cell extract obtained by centrifugation was used to MutY nicking assay. Lane M shows an 11-mer oligodeoxynuclotide used as a marker for the product of MutY. Arrows indicate the 22-mer substrate and the 11-mer product, respectively. (b) RT-PCR analysis of the MutY mRNA. RNA was isolated from *E. coli* GC4468 in the absence (Con) or in the presence of 0.5 mM paraquat (PQ). After the RT of serially diluted RNA to cDNA, the cDNA was amplified with specific primers for MutY and 16S rRNA by PCR. The PCR products prepared with (+) and without (−) RT were compared.

conditions, all of these mutants showed lower MutY activity than the wild type (GC4468). This effect was slight in the case of *fur* (NC442) mutant but was significant for *fnr* (NC504), *arcA* (NC505), *arcAfur* (NC515) and *fnrfur* (NC521) mutants. When all mutations were combined (NC522), the observed MutY decrease reached a maximum. These results for the mutants were contrary to that observed in the wild type (GC4468) under anaerobic conditions (Fig 2a). These results indicate that MutY is down-regulated by oxidative stress and that its regulation is under the positive control of *fur*, *fnr* and *arcA* and furthermore, this is opposite to that observed with MutM, of which activity was markedly increased in the triple mutant NC522 under anaerobic conditions

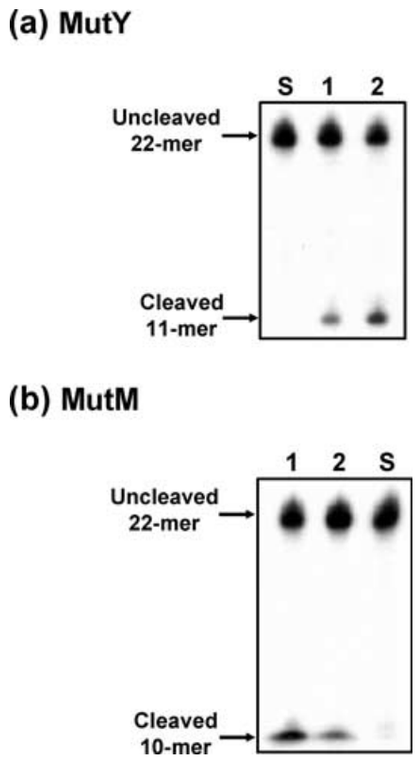

FIGURE 2 Effects of oxygen tensions on MutY and MutM activity. *E. coli* GC4468 was grown to saturation aerobically in air or anaerobically in a Coy chamber and cell extracts were prepared as described for Fig. 1a above and used to nicking assay. The cell extract obtained from the aerobic culture was assayed for MutY and MutM (lane 1), and the cell extract obtained from the anaerobic culture also assayed for MutY and MutM (lane 2). In lanes S, assay was performed with only DNA substrate as control. Arrows indicate the 22-mer substrate and the 11-mer MutY product, respectively.

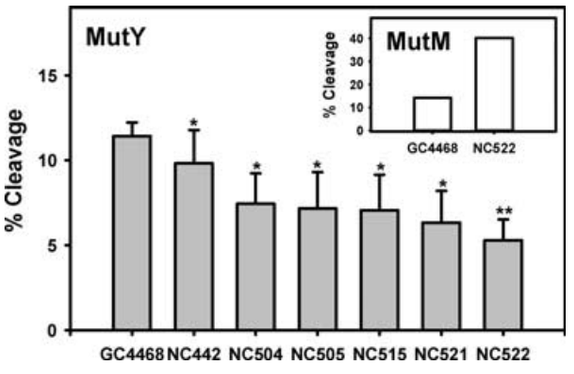

FIGURE 3 Comparison of the MutY activities of various *E. coli* stains in anaerobic conditions. The *E. coli* strains used and their genotypes are described in Table I. GC4468 is a wild type strain, and the other strains shown were derived from GC4468 with mutations in one or more of the three genes; *fur*, *fnr* and *arcA*. Each of these strains was cultured anaerobically to saturation in a Coy chamber and harvested. Bacteria were sonicated and the cell extracts, obtained by centrifugation, were assayed for MutY. The extracts of GC4468 and NC522 were also assayed for MutM (inset). Enzyme activity is expressed as % cleavage of substrate DNA. Details are described in "Materials and Methods" section.

(Fig 3, inset). Thus, these regulatory genes control MutY and MutM as activators and as repressors under conditions of oxidative stress, respectively.

DISCUSSION

The mutagenesis induced by 8-oxoG is more complex than originally envisioned, because 8-oxoG:A can be formed not only during DNA replication by the insertion of A opposite template 8-oxoG[3] but can also be generated by the incorporation of 8-oxoG opposite template A.[9] In either event, MutY removes A and finally gives rise to G:C formation. However, the removal of A by MutY from the 8-oxoG:A formed by the latter process becomes preferably mutagenic since the removal of A in such a situation would cause the complete loss of information pertaining to A and such a repair would bring about mutation. Therefore, MutY has two contrary effects. One is a beneficial effect that decreases G:C → T:A transversion, when MutY removes A from G:C-originated 8-oxoG:A. The other is a detrimental effect that increases T:A → G:C transversion when it removes A from T:A-originated 8-oxoG:A (Fig 4).

In this study, we found that the activity of MutY decreases under conditions of oxidative stress and that MutY is under the positive control of *fur*, *fnr* and *arcA*. This behavior of MutY is totally opposite to that of MutM. Moreover, this finding is in accord with the observations of a previous study,[14] which determined the numbers and types of mutations that occur in strains of *E. coli* defective in MutT and/or MutY repair. The high rates of G:C → T:A transversion in *mutY*$^-$ strains were unaffected by the status of *mutT*. However, *mutT*$^-$/*mutY*$^+$ strains had higher rates of T:A → G:C transversion than *mutT*$^-$/*mutY*$^+$ strains. These results support the notion that the activity of the MutY can be mutagenic, and that this is more pronounced in a *mutT*$^-$ background.[15,16]

Recently, dramatic progress has been made in the understanding of the 8-oxoG repair mechanisms in *E. coli* and higher organisms. In eukaryotes, homologues of *E. coli* MutT, MutM and MutY were cloned as MTH,[20] OGG1[21,22] and MYH.[23,24] Moreover, *E. coli* and eukaryotes have been reported to contain a unique glycosylase/AP lyase, OGG2,[25–27] which excises 8-oxoG residues opposite A; *E. coli* OGG2 was identified to be endonuclease VIII (Nei) and yeast OGG2 as Ntg1, while a mammalian OGG2 has not yet been cloned. OGG2 differs from MutM in that it removes 8-oxoG from 8-oxoG:A, but not from 8-oxoG:C, and it also differs from MutY because it removes 8-oxoG, but not A, from 8-oxoG:A. Therefore, OGG2 can compensate for the mutagenic effect

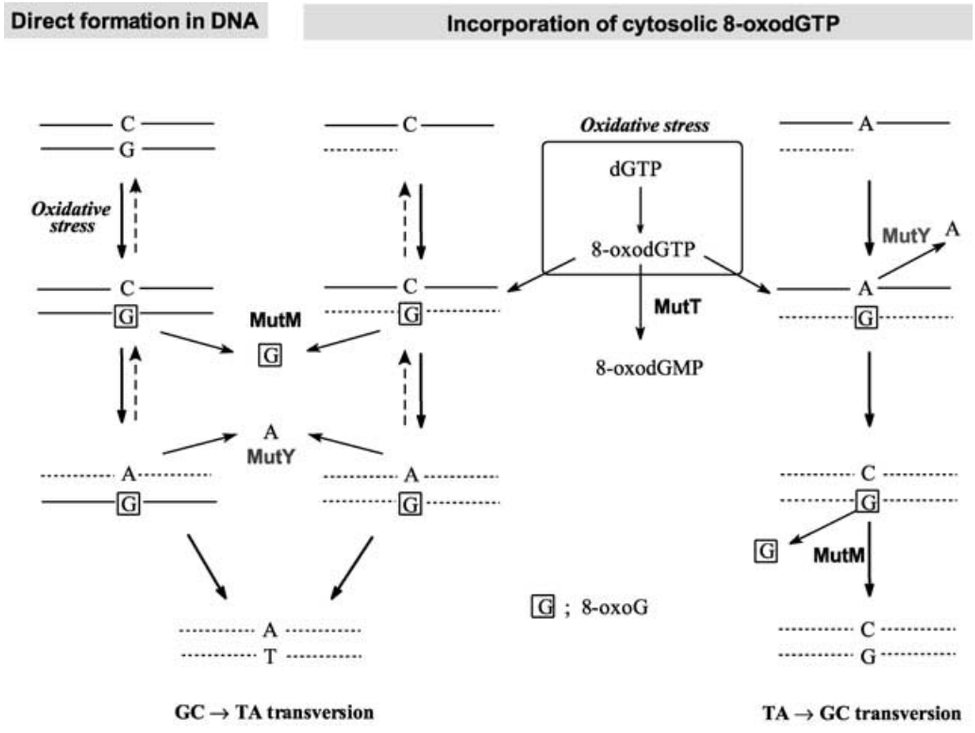

FIGURE 4 Various routes of 8-oxoG:A formation and the effect of MutY depending on the formation route. Under low oxidative stress, MutT can prevent the misincorporation of 8-oxoG opposite A so that MutM and MutY (blue color) cooperatively repair DNA damage. But under high oxidative stress, MutT can no longer remove 8-oxodGTP sufficiently and then MutY will then frequently encounter T:A-originated 8-oxoG:A. Under this condition, the activity of MutY (red color) is mutagenic, and therefore, its activity should be decreased. The direction of mutation is indicated by the bold arrows and the direction of repair by dotted arrows.

of MutY if OGG2 is directed towards T:A-originated 8-oxoG:A.

These reports and the results of the present study help us to understand the meaning of the down-regulation of MutY under conditions of oxidative stress (Fig 4). Under conditions of low oxidative stress, MutT can prevent the misincorporation of 8-oxoG opposite template A and then MutM and MutY can cooperatively repair 8-oxoG in DNA. However, under conditions of high oxidative stress, 8-oxodGTP which is primarily located in the cytoplasmic nucleotide pool and, is more likely to be oxidized, and may be formed in excess. Under such circumstances, it is likely that MutT cannot adequately remove 8-oxoG (8-oxodGTP) from the nucleotide pool. Therefore, MutY will frequently encounter T:A-originated 8-oxoG:A due to the increased availability of 8-oxodGTP. Accordingly, the activity of MutY should be reduced to counteract its potentially mutagenic effect. Unfortunately, the regulatory effect of MutT is not yet known. In this situation, however, the status of MutT does not appear to be important, becuase the high rates of G:C → T:A transversion in the *mutY*$^-$ strain were the same in the *mutT*$^-$ and *mutT*$^+$ strains. Regardless of MutT status, the down-regulation of MutY is beneficial to cellular defense against the effect of 8-oxoG formed by oxidative stress. A previous study reported that expression from the *mutY* promoter was rather higher under aerobic conditions than under anaerobic conditions.[28] Although their experimental approach was very different from ours to study the regulation of MutY under oxidative stress, it is in conflict with this finding. It is possible that their anaerobic culture condition was not enough. Compared with what we have done for general conditions of anaerobic culture as described in "Materials and Methods" section, they only cultured *E. coli* in filled bottle without shaking for anaerobic culture.

We also found that the regulation of MutY is under the control of *fur*, *fnr* and *arcA*, but that it occurs in the opposite direction to that of MutM. We excluded the involvement of *soxRS*[29] and *oxyR*[30] by using the mutants (BW831, BW847, BW949 and GS018) since MutY activity was unaltered in these mutants (data not shown). In addition to MutM, the global regulators, *fur, fnr* and *arcA* have control another member, namely MutY. Thus, we know that *E. coli* copes with oxidative DNA damage by using a reciprocal co-regulation involving MutY and MutM.

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