Role of *Bacillus subtilis* DNA Glycosylase MutM in Counteracting Oxidative-Induced DNA Damage and in Stationary-Phase-Associated Mutagenesis


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Running Title: Role of MutM in stress-associated mutagenesis.

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Abstract

Reactive oxygen species (ROS) promote the synthesis of the DNA lesion 8-oxo-G whose mutagenic effects are counteracted in distinct organisms by the DNA glycosylase MutM. We report here that, in *B. subtilis*, *mutM* is expressed during the exponential and stationary phases of growth. In agreement with this expression pattern, results of a Western blot analysis confirmed the presence of MutM in both stages of growth. In comparison with a wild-type strain, cells of *B. subtilis* lacking MutM increased its spontaneous mutation frequency to Rif<sup>+</sup> and were more sensitive to the ROS-promoter agents hydrogen peroxide and Paraquat. However, despite its proven participation in preventing ROS-induced-DNA damage, the expression of *mutM* was not induced by hydrogen peroxide, Mitomycin-C, or NaCl suggesting that transcription of this gene is not under control of the RecA, PerR or σ<sup>b</sup> regulons. Finally, the role of MutM in stationary-phase-associated mutagenesis was investigated in the strain *B. subtilis* YB955 (hisC<sup>952</sup>, metB<sup>5</sup> and leuC<sup>427</sup>). Results revealed that under limiting growth conditions, a *mutM* knock out strain significantly increased the amount of stationary-phase-associated *his*, *met* and *leu* revertants produced in this strain. In summary, our results support the notion that the absence of MutM promotes mutagenesis allowing nutritionally stressed *B. subtilis* cells to escape from growth-limiting conditions.

Importance

The present study describes the role played by a DNA repair protein (MutM) in protecting the soil bacterium *Bacillus subtilis* from the genotoxic effects induced by reactive oxygen species (ROS)-promoter agents. Moreover, it reveals that the genetic inactivation of *mutM* allow
nutritionally stressed bacteria to escape from growth-limiting conditions, putatively by a mechanism that involves accumulation and error-prone processing of oxidized DNA bases.
Introduction

Reactive oxygen species (ROS), including hydrogen peroxide, superoxide and hydroxyl radicals are produced in all aerobic organisms as side products of oxidative metabolism or following exposure to environmental agents and are normally in balance with the cellular antioxidant defenses. Oxidative stress occurs when this critical balance is disrupted because of depletion of antioxidants or excess accumulation of ROS (1). Therefore, when antioxidant cellular defenses are deficient or overwhelmed, the damaging potential of ROS increases and targets different cellular biomolecules, including, lipids, proteins, carbohydrates and DNA (2). One of the most common events resulting from attack of DNA by the hydroxyl radical is the formation of 7,8-dihydro-8-oxodeoxyguanosine (8-oxo-G); a DNA lesion extensively studied due to its strong mutagenic and genotoxic properties (3). However, the hydroxyl radicals can also impact the deoxyribonucleotide and ribonucleotide pools generating the oxidized precursors 8-oxo-dGTP and 8-oxo-GTP, respectively (4, 5). The former is frequently incorporated opposite adenine during DNA synthesis, giving rise to G:C \(\rightarrow\) T:A transversions; whereas 8-oxo-GTP has the potential of being used as a substrate by the RNA polymerase generating oxidized mRNAs that may originate transcriptional errors (6, 7). In *E. coli*, the mutagenic effects of 8-oxo-G are prevented by MutM, a DNA glycosylase that recognizes and hydrolyzes this oxidized base from DNA (3). Following this event, the repair of the apurinic/apyrimidinic (AP) site generated and the restitution of the undamaged guanine are carried out by downstream components of the base excision repair pathway (BER) (8, 9).

It has been shown that oxidative stress is a crucial factor that promotes mutagenesis in nutritionally stressed bacteria (10–13) and that the oxidized guanine (GO) DNA repair system (composed of the DNA glycosylases MutM and MutY and the nucleotide diphosphohydrolase
MutTA) is involved in this type of mutagenesis in *B. subtilis* (13). However, the individual contribution of MutM in preventing mutagenesis and its role in conferring protection against the toxic effects of oxidative stress in this microorganism is currently unknown. Here, we report that disruption of *mutM* sensitized *B. subtilis* to the noxious effects of the oxidizing agents hydrogen peroxide and paraquat (PQ). Whereas in *E. coli* the superoxide radical induce the expression levels of *mutM* (14), our results showed that in *B. subtilis* the transcription of this gene is controlled in a temporal manner keeping active the expression of *mutM* during logarithmic and stationary phases of growth. Notably, the absence of this repair protein promoted the generation of mutations in nutritionally stressed cells of this bacterium.

Materials and Methods

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *B. subtilis* YB955 is a prophage-“cured” strain that contains the *hisC952*, *metB5*, and *leuC427* auxotrophic mutations (15–17). *B. subtilis* strains were maintained on tryptic blood agar base (TBAB) (Acumedia Manufacturers, Inc., Lansing, MI). Liquid cultures of *B. subtilis* strains were grown in Penassay broth (PAB) (antibiotic A3 medium; Difco Laboratories, Sparks, MD). *E. coli* cultures were grown in Luria-Bertani (LB) medium. When required, neomycin (Neo; 10 μg ml⁻¹), tetracycline (Tet; 10 μg ml⁻¹), spectinomycin (Sp; 100 μg ml⁻¹), kanamycin (Kn; 10 μg ml⁻¹), ampicillin (Amp; 100 μg ml⁻¹), chloramphenicol (Cm; 5 μg ml⁻¹), erythromycin (Ery; 1 μg ml⁻¹), rifampin (Rif; 10 μg ml⁻¹) or isopropyl-β-D-thiogalactopyranoside (IPTG; 1 mM) was added to media. Hydrogen peroxide (H₂O₂), and 1,1'-Dimethyl-4,4'-bipyridinium dichloride (Paraquat, PQ) were obtained from Sigma-Aldrich (St Louis MO).
Construction of mutant strains. To obtain a mutM mutant strain in the genetic background YB955, chromosomal DNA of strain B. subtilis PERM599 (PS832 ΔmutM::tet) was isolated and used to transform competent cells of strain B. subtilis YB955 generating the strain B. subtilis PERM571 (YB955; ΔmutM::tet).

For complementation of the mutM mutation a copy of this gene was placed ectopically at the amyE locus, under the control of the IPTG-inducible hyper-spank promoter (Phs). To this end, the open reading frame of mutM was amplified by PCR using genomic DNA of B. subtilis YB955 as a template and Vent DNA polymerase (New England Biolabs, Beverly, MA). Oligonucleotide primers 5'-AAGTCGACGAGATAGGAAGTGATG-3' (forward) and 5'-ATGCATGCGGGAAAGTGAAAAATC-3' (reverse) containing SalI and SphI sites, respectively (underlined) were used in the PCR reaction. The PCR product was ligated into pCR-Blunt-II-TOPO vector (Invitrogen Life Technologies, Carlsbad, CA), generating plasmids pPERM735 (Table 1). The mutM gene was released from this plasmid and inserted into the integrative vector pdr-111-amyE-Phyperspank (a gift from David Rudner). The resulting construct pPERM792 was introduced by transformation into the strain B. subtilis PERM571 and PERM573 to generate the strain B. subtilis PERM1199 and PERM794.

To obtain a mutM mutY double mutant, the strain PERM571 (YB955 ΔmutM::tet) was transformed with genomic DNA of PERM704 (YB955 mutY::sp) (18) generating the strain B. subtilis PERM572 (YB955 ΔmutM::tet mutY::sp) (Table 1). The double homologous recombination event resulting in inactivation of the gene of interest was confirmed by PCR with specific oligonucleotide primers (data not shown).

Design of mutM-lacZ and mutM-flag constructs. Construction of a transcriptional fusion between mutM and the lacZ gene was performed in the integrative plasmid pMUTIN4 (19).
this end, a 405-bp fragment, extending from 264 pb upstream to 141 pb downstream of the mutM ORF start codon was amplified using Vent DNA polymerase (New England Biolabs, Beverly, MA) and oligonucleotide primers 5’- CGCGAATTCCGATTCAAGGAAGCGCCG-3’ (forward) and GCCGGATCCTCGCGCAAATTCCTCCGG-3’ (reverse) with EcoRI/BamHI restriction sites, respectively. The PCR product was ligated into pCR-Blunt-II-TOPO (Invitrogen Life Technologies, Carlsbad, CA) generating plasmid PERM617. The EcoRI/BamHI fragment was ligated into pMUTIN4, previously digested with the same restriction enzymes. The resulting construct containing the mutM-lacZ fusion was designated pPERM657 and was introduced by transformation into competent cells of strains B. subtilis YB955 to generate the strain B. subtilis PERM659 (Table 1).

An in-frame translational fusion between mutM and the FLAG epitope was constructed in the vector pMUTIN-FLAG (20). To this end, a DNA fragment encompassing 15 bp upstream (including the Shine-Dalgarno sequence) of the translational start codon to the last codon of the mutM ORF was amplified by PCR, utilizing Vent DNA polymerase (New England Biolabs) and the oligonucleotide primers 5´-GGAAGCTTCAGAGATAGGAAGTCATGGAT-3´ (forward) and 5´-GGGGTACCGTTTTTTGTCTGGCACTTTG-3´ (reverse), which inserted HindIII and KpnI sites (underlined) into the cloned DNA. The PCR-amplified DNA fragment (849 pb) was first ligated into pCR-BluntII-TOPO (Invitrogen, Carlsbad, CA) and then replicated in E. coli XL10-GOLD Kan’ (Stratagene, Cedar Creek, TX). The resulting construct (PERM698) was treated with HindIII and KpnI, the 849 bp mutM insert was ligated into HindIII/KpnI-treated pMUTIN-FLAG, and the ligation products were introduced by transformation into competent cells of E. coli XL10-GOLD Kan’ (Stratagene, Cedar Creek, TX). This strategy generated plasmid pPERM748, which was used to transform B. subtilis YB955, generating strains B. subtilis PERM796 (Table 1). The crossover events leading to insertion of the mutM-lacZ and...
mutM-flag fusions into the corresponding loci were confirmed by PCR with specific oligonucleotide primers (data not shown).

**Beta-galactosidase assays.** B. subtilis strains PERM659, containing a transcriptional mutM-lacZ fusion, were propagated in liquid A3 medium. Aliquots of 1 ml were collected from cultures at exponential growth phase, stationary phase or sporulation. Cells were washed with 0.1 M Tris-HCl (pH 7.5), pelleted by centrifugation, and stored at 20°C until determination of β-galactosidase activity (21). Briefly, washed cell samples were first disrupted with lysozyme and subjected to centrifugation; the β-galactosidase activity present in the supernatant was then determined as previously described, using ortho-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate.

**RT-PCR experiments.** Total RNA from exponentially growing or stationary phase B. subtilis YB955 cells grown in A3 medium was isolated by using TRI reagent (Molecular Research Center, Inc. Cincinnati, OH). Reverse transcription-PCRs (RT-PCRs) were performed with the RNA samples and a Master AMP RT-PCR kit (Epicentre Technologies, Madison, WI) according to the manufacturer’s instructions. The primers used for RT-PCR were 5′-GGTCTTCATCAAGCCGAGGAG-3′ (forward) and 5′-ATTTCAGCGTGAAGGGTTTTG-3′ (reverse), which generated a 377 bp RT-PCR product extending from 249 bp downstream from the start codon of mutM to 626 bp downstream of this point. As a control, in each experiment, the absence of chromosomal DNA in the RNA samples was assessed by PCRs carried out with Vent DNA polymerase (New England Biolabs) and the set of primers described above. The size of the RT-PCR product was determined by utilizing the 1-kb-Plus DNA ladder (Life Technologies, Rockville, MD) during agarose gel electrophoresis.
Western blot assay. *B. subtilis* strain YB955 was cultivated with shaking in liquid antibiotic A3 medium at 37°C. Aliquots of 1.5 ml were collected from cultures during exponential, transition or stationary phases of growth. Cells were collected by centrifugation (16000 \times g; 1 min), washed twice with 25 mM Tris-HCl (pH 7.5) buffer, and stored at 20°C. Bacterial pellets were resuspended in 0.3 ml of the same buffer supplemented with a protease inhibitor cocktail (Roche, Mannheim, Germany) and were disrupted by sonication with a VCX130-PB Vibra Cell apparatus (Sonics and Materials Inc., Newton, CT). The cell lysate was subjected to centrifugation to eliminate undisrupted cells and cell debris. The supernatant was separated, and its protein concentration was determined with a Coomassie (Bradford) protein assay kit (Pierce, Rockford, IL). Protein aliquots (100 μg) were separated in SDS-12% polyacrylamide gels and then electrotransferred to polyvinylidene difluoride (PVDF) membranes. Western blot analyses were performed with a FLAG monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 10000-fold and then processed with an ECL (enhanced chemiluminescence) Western blotting system (Amersham Pharmacia, Buckinghamshire, United Kingdom).

Stationary-phase mutagenesis assays. Essentially, cultures were grown in flasks containing antibiotic A3 medium with aeration (250 r.p.m.) at 37°C to 90 min after the cessation of exponential growth (designated T90). Growth was monitored with a spectrophotometer measuring the optical density at 600 nm (OD_{600}). The stationary-phase mutagenesis assays were performed as previously described (22, 23), using solid Spizizen minimal medium (SMM; 1X Spizizen salts supplemented with 0.5% glucose and either 50 μg ml\(^{-1}\) or 200 ng ml\(^{-1}\) of the required amino acid and 50 μg ml\(^{-1}\) each of isoleucine and glutamic acid). The concentration of the amino acid used depended on the reversion tested. For instance, in selecting His\(^{+}\) revertants,
50 μg ml\(^{-1}\) (each) of methionine and leucine was added to the medium and 200 ng ml\(^{-1}\) of histidine was added. Isoleucine and glutamic acid were added as described previously (16) in order to protect the viability of the cells. The number of revertants was scored daily. The initial number of bacteria plated for each experiment was estimated by serial dilution of the bacterial cultures and then plating of the cells on LB medium. The experiments were repeated at least three times.

**Analysis of mutation frequencies.** Spontaneous mutation frequencies to rifampin resistance in growing cells were determined as previously described (13). Essentially, the appropriate strains were grown for 12 h at 37°C in antibiotic A3 medium with proper antibiotics. Mutation frequencies were determined by plating aliquots on six LB plates containing 10 μg ml\(^{-1}\) rifampin, and the rifampin resistant (Rif\(^{r}\)) colonies were counted after 1 day of incubation at 37°C. The number of cells used to calculate the frequency of mutation to Rif\(^{r}\) was determined by plating aliquots of appropriate dilutions on LB plates without rifampin and incubating the plates for 24 to 48 h at 37°C. These experiments were repeated at least three times (24).

**Assays of sensitivity to oxidative stress inducers.** *B. subtilis* strains YB955, PERM571 and PERM1199 were grown in LB medium with aeration (250 rpm.) at 37°C. Growth was monitored with a spectrophotometer measuring the optical density at 600 nm (OD\(_{600}\)). Before cessation of exponential growth (OD\(_{600}\) ~0.6) the cells were collected by centrifugation (6500 × g, 5 min), washed twice with phosphate buffered saline (PBS; pH 7.2) and resuspended in the same buffer. Cell aliquots of equal volumes were treated with different final concentrations of H\(_{2}\)O\(_{2}\) or PQ and incubated during 30 min at 37°C with shaking. The total viable cell numbers in each culture were determined by spotting serial dilutions of the cultures on LB-agar plates. The
number of colonies was counted after 24 h of incubation at 37°C.

**Statistical analysis.** For determination of mutation frequencies and oxidative stress sensitivity, differences were calculated by performing one-way ANOVA followed by a Tukey’s post-hoc analysis. Significance was set at P < 0.05.
Results and discussion

MutM confers protection to *B. subtilis* from the toxicity promoted by oxidant agents.

ROS-promoted DNA lesions including, 8-oxo-G, may potentially generate mutagenesis and cell death (25, 26). The 8-oxo-G lesion is processed through the BER pathway with the specific participation of MutM that eliminates this oxidized base from DNA (5, 27, 28). Thus, we analyzed whether MutM conferred protection to growing *B. subtilis* cells from the cytotoxic effects of oxidative stress. To this end, growing cells of a *mutM* knock out strain and its MutM-proficient parental strain were treated with increasing amounts of H$_2$O$_2$ or PQ. Results showed that disruption of *mutM* significantly sensitized exponentially growing cells of *B. subtilis* to these oxidizing agents (Fig. 1A-B). This result reveals a role for MutM in conferring protection to *B. subtilis* from the lethal effects of H$_2$O$_2$ and PQ. In support of this notion, the susceptibility to H$_2$O$_2$ and PQ of the *mutM* strain was restablished to the levels of the parental strain YB955 following expression of the wild type *mutM* gene from the IPTG-inducible *phs* promoter (Fig. 1A-B). Taken together, these results strongly suggest that MutM plays a significant role in preventing the cytotoxic effects of 8-oxo-G and possibly of other related lesions including the opened ring derivative formamidopyrimidine (FaPy) (29–32), thus contributing to *B. subtilis* survival. However, in addition to inducing the formation of oxidized bases, H$_2$O$_2$ and PQ may also promote other types of DNA lesions, including 8-OxoG:A mispairs, apurinic/apyrimidinic (AP) sites, as well as single- and/or double-strand DNA breaks (12). Therefore, in addition to MutM, other repair proteins including MutY, Nth and the AP-endonucleases Nfo and ExoA most probably contribute to protecting *B. subtilis* from the genotoxic effects of H$_2$O$_2$ (33).

Of note, the absence of MutM also decreased the H$_2$O$_2$ resistance of *E. coli* cells and such effect was associated with an increased amount of 8-oxo-G lesions in the genome of this
microorganism (34). However, the protective role conferred by MutM against H$_2$O$_2$-promoted DNA damage has also been described in other bacteria, including *Pseudomonas aeruginosa* and *Mycobacterium smegmatis*. Thus, cells of these strains lacking MutM were significantly more susceptible to H$_2$O$_2$ treatment than its MutM-proficient counterparts (35, 36).

**Spontaneous mutation frequencies in *B. subtilis* cells lacking MutM.** Due to an anticipated role of the MutM protein in preventing the mutagenic and cytotoxic effects of 8-oxo-G (13, 37), the mutation frequency to a Rif$^r$ phenotype was determined in growing cells of the MutM-deficient and parental YB955 strains. The results revealed that loss of the MutM increased the spontaneous mutation frequency to Rif$^r$ 5-fold in comparison with an isogenic strain that produced a functional MutM protein (Fig. 1C). From these results we proposed that MutM prevented the spontaneous mutagenic events promoted by oxidative stress in growing *B. subtilis* cells. Two observations supported this contention; the mutation levels to Rif$^r$ calculated in the MutM-deficient strain were restored to the levels of the parental strain following expression of *mutM* from the IPTG-inducible *Phs* promoter (Fig. 1C). Moreover, the overexpression of *mutM* induced a significant decrease in the mutation frequency to Rif$^r$ of a hypermutagenic strain that was deficient for MutM, MutY and MutT (Fig. 1D).

In agreement with a previous report (37), our results revealed that cells of the strain *B. subtilis* YB955 lacking MutM showed a slight but statistically significant increase in spontaneous mutation frequency to Rif$^r$ in reference to the MutM-proficient parental strain. It must be pointed that MutM-deficient strains of *E. coli* and *P. putida* also presented a mutagenic Rif$^r$ phenotype; however, in these bacteria, as well as in *B. subtilis* (13), the single MutY deficiency conferred a stronger mutagenic effect than that observed in the strains lacking MutM (5, 38, 39). These results suggest the existence of alternative repair pathways that compensate for the absence of
MutM; in agreement with this notion, the genome of the three microorganisms discussed above count with Nth a DNA glycosylase capable of processing 8-oxo-G and AP sites (40–42). In the case of \textit{B. subtilis}, it has been recently shown that the genetic inactivation of Nth not only increased its spontaneous Rif\textsuperscript{r} mutation frequency but also sensitized this bacterium to the ROS-promoter agent \textit{H}_{2}\text{O}_{2} (33).

\textbf{Stationary-phase mutagenesis in \textit{B. subtilis} cells deficient for MutM.} We next investigated the role played by MutM in stationary-phase-associated mutagenesis of \textit{B. subtilis}. These experiments were performed in strain \textit{B. subtilis} YB955, which is auxotrophic for three amino acids due to the chromosomal mutations \textit{hisC952} (amber), \textit{metB5} (ochre), and \textit{leuC427} (missense). This strain has been validated and widely used as a model system to understand how mutations are generated in amino-acid-starved cells (18, 23, 43). Analysis of frequencies of reversion to \textit{his}, \textit{met}, and \textit{leu} in cell cultures that were starved for each of these amino acids revealed that MutM contributes to mutagenesis in starved \textit{B. subtilis} cells. As shown in \textbf{Figure 2}, the MutM-deficient strain significantly increased the frequency of \textit{his met} and \textit{leu} reversions in reference to those generated by the parental strain YB955. These results strongly suggest that unrepaired 8-oxo-G lesions that accumulate in the MutM-deficient strain promote stationary-phase associated mutagenesis in \textit{B. subtilis}. In a marked contrast with our results, the single absence of MutM did not promote mutagenesis in starved cells of \textit{E. coli} and \textit{P. putida} (11, 44). However, in \textit{E. coli} and \textit{P. putida}, the lack of MutY did induce a significant increase in the production of stationary phase associated mutations (11, 45) suggesting that accumulation of non-processed 8-oxo-G lesions contribute to stationary-phase-associated mutagenesis in these strains. In support of this notion, when the \textit{mutM} mutation was combined with a deficiency in MutY, the mutation frequency was further enhanced in starved \textit{E. coli} cells (11). Thus, despite
that the lack of MutY also contributes to SPM in *B. subtilis* (18), our results clearly indicate that the sole disruption of *mutM* also favored this type of mutagenesis in this microorganism. In support of this contention, an ectopic copy of *mutM* expressed from the IPTG-inducible *P*hs promoter diminished the number of the His\(^+\), Met\(^+\) and Leu\(^+\) revertants to those produced by the parental strain YB955 (Fig. 2). Moreover, we corroborated that the genetic defect in *mutM* did not affect the survival of *B. subtilis* cells starved for *his*, *met* and *leu* during the ten days that lasted the SPM experiments (Fig. S1).

**Analysis of mutM expression during the life cycle of B. subtilis.** As described above, MutM confers protection to growing *B. subtilis* cells from the toxic effects of H\(_2\)O\(_2\) and its deficiency promotes adaptive mutagenesis in nutritionally stressed cells. These results suggest that *mutM* could be expressed in the exponential and stationary phases of growth of this microorganism. To explore this notion we analyzed the temporal pattern of expression of *mutM* and determined the levels of its encoded product during the life cycle of *B. subtilis*. The levels of transcription were determined employing the strain *B. subtilis* PERM659 that harbors a genomic copy of a transcriptional *mutM-lacZ* fusion (Table 1). The results showed that this strain expressed barely similar levels of β-galactosidase during the exponential, transition (from exponential to stationary phase) and first hours of stationary phases of growth (Fig. 3A). However, the expression levels of the reporter *lacZ* gene commenced to diminish during the late stationary phase of growth. Results from a RT-PCR experiment performed with RNA samples collected during exponential growth as well as during the transition and stationary phases of growth confirmed the presence of *mutM* mRNAs during the three developmental phases analyzed (Fig. 3B). In agreement with this result, we also detected a MutM-Flag protein in actively
growing and in the stationary phase of growth of a *B. subtilis* strain harboring an in-frame translational *mutM-flag* fusion (Fig. 3C). Based on these and previous results (46, 47), it is feasible to propose that *B. subtilis* expresses *mutM* during all its life cycle to contend with the genotoxic and cytotoxic effects of ROS. However, despite the role displayed by *mutM* in protecting *B. subtilis* from oxidative-induced DNA damage we did not find evidence that this gene is part of the gene circuitries that respond to distinct types of stressful conditions, including, DNA damage and oxidative or osmotic stresses (48–54). This conclusion was deduced from experiments showing that neither H$_2$O$_2$ (0.1%) nor MC (0.5 μg ml$^{-1}$) or NaCl (4%) turned on the transcription of a *mutM-lacZ* fusion inserted in the genome of strain *B. subtilis* YB955 (Fig. S2). Therefore, in conjunction with previous reports (48, 55–57), it is feasible to conclude that expression of *mutM* is not under control of the master regulators RecA/DinR, PerR or σ$^B$.

In contrast, in *E. coli*, *mutM* is under negative transcriptional control of the Fur, Fnr and ArcA regulators; thus, the mRNA levels of *mutM* are enhanced in this bacterium by ROS-producing chemicals including Paraquat. Noteworthy, the levels of expression of *mutY* are repressed under the stressful conditions that activate *mutM* (14, 58, 59).

Thus, the ability of *B. subtilis* to keep active the synthesis of MutM during the logarithmic and stationary phases is in agreement with our results that demonstrated anti-mutagenic roles of this repair protein in both stages of growth (Figs. 1 and 2). Moreover, the presence of MutM in the stationary phase of *B. subtilis* but apparently absent in *E. coli* (56), may explain why the single disruption of *mutM* did not promote mutagenesis in starved cells of *E. coli* unless combined with a mutation in MutY (11). Alternatively, the existence in *E. coli* of repair proteins that process 8-oxo-G lesions, including, Nth and Nei (11, 60) may suppress mutagenesis in starved *E. coli* cells deficient for MutM.
Our analysis of his, met and leu reversions in nutritionally stressed B. subtilis cells showed that deficiencies in mutM significantly increased the mutagenesis levels in the three alleles tested. However, the mechanisms involved in generating such reversions could be different; thus for the his and met alleles ROS-promoted synthesis of 8-oxo-G could be responsible of these reversions. In support of this contention, genetic inactivation of mutY in the MutM-deficient strain dramatically increased the production of His and Met revertants in the resulting mutM mutY mutant (Fig. 4). In contrast, the levels of reversion of the leu allele in the mutM/mutY strain were reduced compared to those observed in the mutM and parental YB955 strains (Fig. 4C). This result suggest that MutY promotes reversions in the leuC allele; in support of this notion, the levels of Leu⁺ revertants were almost completely abated in the MutY-deficient strain (Fig. 4C). Furthermore, a previous study demonstrated that processing of accumulated G:A mismatches in starved B. subtilis by MutY is involved in generating stationary-phase-associated Leu⁺ revertants (18).

As shown in this and previous reports (13, 18), DNA repair proteins that process ROS-induced DNA damage play prominent roles in modulating mutagenesis in starved bacterial cells. Nevertheless, current reports have shown that in B. subtilis this type of mutations is also dependent on Mfd, a protein that couples transcription with the DNA repair machinery (61, 62). It has been recently found that production of Leu⁺ prototrophs in MutY-deficient B. subtilis cells of strain YB955 were fully dependent on a functional Mfd protein (Gomez-Marroquín et al., Unpublished results). Therefore, we are currently investigating how Mfd coordinates the activity of repair proteins of the GO system to generate mutations that occur in nutritionally stressed B. subtilis cells.
Acknowledgements

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References


Table 1. *Bacillus subtilis* strains and plasmids used in this study.

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<th>Strain or plasmid</th>
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<td>PERM796</td>
<td>168 pMUTIN-FLAG mutM; Ery&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td>PERM1199</td>
<td>YB955 ΔmutM::tet with a Phs-mutM; Tet&lt;sup&gt;f&lt;/sup&gt;, Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<td>pdr-111-amyE-</td>
<td>bla- and spec-carrying Phyperspank promoter</td>
<td>43</td>
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<td>Phyperspank</td>
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<tr>
<td>pPERM617</td>
<td>pCR-Blunt-II-TOPO with a EcoRI-BamHI promoter region of mutM of 405 pb; Kan&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td>plasmid</td>
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<tr>
<td>pPERM657</td>
<td>pMUTIN4 carrying a 405 pb EcoRI-BamHI of DNA Fragment encompassing 264 bp upstream and 141 bp downstream of the mutM translational start codon; Amp&lt;sup&gt;+&lt;/sup&gt;, Ery&lt;sup&gt;+&lt;/sup&gt;.</td>
<td>This study</td>
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<td>pPERM698</td>
<td>pCR-Blunt II-TOPO with a 849-bp HindIII-KpnI PCR product containing mutM; Kn&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td>pPERM735</td>
<td>pCR-Blunt-II-TOPO containing 936 pb SalI-SphI of mutM; Kan&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td>pPERM748</td>
<td>pMUTIN-FLAG carrying a 849 bp HindIII-KpnI mutM fragment from pPERM707; Amp&lt;sup&gt;+&lt;/sup&gt;, Ery&lt;sup&gt;+&lt;/sup&gt;.</td>
<td>This study</td>
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<tr>
<td>pPERM792</td>
<td>pdr-111-amyE-PhyPerspank containing 936 pb SalI-SphI fragment of mutM; Amp&lt;sup&gt;+&lt;/sup&gt;, Cm&lt;sup&gt;+&lt;/sup&gt;.</td>
<td>This study</td>
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Figure legends

Fig. 1. Contribution of MutM in survival of *B. subtilis* to H$_2$O$_2$ and PQ treatment (A-B) and frequencies of spontaneous mutation to Rif$^r$ of different *B. subtilis* strains (B-C). (A-B). *B. subtilis* YB955 (parental; ●), PERM751 (ΔmutM; ○) and PERM1199 (ΔmutM; amyE::Phs-mutM; ▲) strains were treated with different amounts of H$_2$O$_2$ (A) or PQ (B) and cell viability was determined as described in Materials and Methods. The values shown represent the means and standard deviations of three independent experiments done in triplicate. (B-C). *B. subtilis* YB955 (parental), PERM751 (ΔmutM), PERM1199 (ΔmutM; amyE::Phs-mutM), PERM573 (ΔGO) and PERM794 (ΔGO; amyE::Phs-mutM) were grown overnight in PAB medium, and frequencies of mutation to Rif$^r$ were determined as described in Materials and Methods. Each bar represents the mean of data collected from three independent experiments done in sixuplicate, and the error bars represent SEM. The statistical differences (a, b, c, d) between the mutation frequencies of each strain and condition, as determined by ANOVA (P < 0.05) are shown above each bar.

Fig. 2. Stationary-phase-induced reversions to (A) his, (B) met and (C) leu of the YB955 (◊), PERM571 (ΔmutM) (▲) and PERM1199 (ΔmutM amyE::Phs-mutM) (●) *B. subtilis* strains were determined as described in Materials and Methods. Data represent counts from six plates averaged from three separate tests normalized to initial cell titers ± SD.

Fig. 3. (A). Levels of β-galactosidase in a mutM-lacZ transcriptional fusion during vegetative and stationary phases of growth. *B. subtilis* strain PERM659 was grown in liquid
antibiotic (A3) medium. Cell samples were collected at the indicated times and treated with lysozyme and the extracts were assayed for β-galactosidase as described in Materials and Methods. Data shown are average values for triplicate independent experiments ± SD for β-galactosidase specific activity (△) and for A600 values (○). (B) RT-PCR analysis of mutM transcription during vegetative and stationary phases of growth. RNA samples (1 µg) isolated from a B. subtilis YB955 A3 culture, at the steps indicated, were processed for RT-PCR analysis as described in Materials and Methods. The arrow shows the size of the expected RT-PCR product. 16S and 23S rRNA bands are shown in the lower panel. (C) Western blot analysis of MutM-FLAG synthesis during vegetative and stationary phases of growth. B. subtilis strain YB955 was grown in liquid A3 medium. Cell extract samples (~100 µg of protein; see Materials and Methods), harvested at the steps indicated, were separated by SDS-PAGE and transferred to PVDF membranes (Bio-Rad, Hercules, CA). The blots were stained with Ponceau red (top), probed with a FLAG monoclonal antibody diluted 10000-fold, and then processed with an ECL Western blot system (bottom). The positions of molecular mass markers are indicated to the left of the stained membrane. T0 is the time point in the culture when the slopes of the logarithmic and stationary phases of growth intercepted. T90, T180, and T270 indicate the time in minutes after T0. Veg., vegetative growth.

Fig. 4. Stationary-phase-induced reversions to (A) his, (B) met and (C) leu of the YB955 (○), PERM571 (ΔmutM) (◇), PERM704 (ΔmutY) (□) and PERM573 (ΔmutM ΔmutY) (△) B. subtilis strains were determined as described in Materials and Methods. Data represent counts from six plates averaged from three separate tests normalized to initial cell titers ± SD.