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Role of *Bacillus subtilis* DNA Glycosylase MutM in Counteracting Oxidatively Induced DNA Damage and in Stationary-Phase-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 *Bacillus 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 cells of a wild-type strain, cells of *B. subtilis* lacking MutM increased their spontaneous mutation frequency to Rif^r and were more sensitive to the ROS promoter agents hydrogen peroxide and 1,1′-dimethyl-4,4′-bipyridinium dichloride (Paraquat). However, despite MutM's 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 the control of the RecA, PerR, or σ^B regulons. Finally, the role of MutM in stationary-phase-associated mutagenesis (SPM) was investigated in the strain *B. subtilis* YB955 (*hisC952 metB5 leuC427*). Results revealed that under limiting growth conditions, a *mutM* knockout strain significantly increased the amount of stationary-phase-associated *his*, *met*, and *leu* revertants produced. In summary, our results support the notion that the absence of MutM promotes mutagenesis that allows 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* allows nutritionally stressed bacteria to escape from growth-limiting conditions, putatively by a mechanism that involves the accumulation and error-prone processing of oxidized DNA bases.

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 they target 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 →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 *Escherichia 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 (BER) pathway (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

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TABLE 1 *Bacillus subtilis* strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
Strains		
YB955	*hisC952 metB5 leuC427 xin-1*	23
PS832	Wild type; Trp^r revertant of strain 168	P. Setlow
PERM311	Wild type; *trpC2*	Laboratory stock
PERM571	YB955 Δ*mutM*::*tet* Tet^r	Laboratory stock
PERM572	YB955 Δ*mutM*::*tet* Δ*mutY*::*sp* Tet^r Sp^r	This study
PERM573	YB955 Δ*ytkD*::*neo* Δ*mutM*::*tet* Δ*mutY*::*sp* Neo^r Sp^r Tet^r	13
PERM599	PS832 Δ*mutM*::*tet* Tet^r	P. Setlow
PERM659	PERM311 containing pMUTIN4::*mutM*; Ery^r	This study
PERM704	YB955 Δ*mutY*::*sp* Sp^r	18
PERM794	YB955 Δ*ytkD*::*neo* Δ*mutM*::*tet* Δ*mutY*::*sp* with P*hs-mutM* inserted into the *amyE* locus; Neo^r Sp^r Tet^r Cm^r	This study
PERM796	168 pMUTIN-FLAG *mutM* Ery^r	This study
PERM1199	YB955 Δ*mutM*::*tet* with P*hs-mutM*; Tet^r Cm^r	This study
Plasmids		
pdr-111-amyE-Phyperspank	*bla*- and *sp*-carrying P*hs*	43
pPERM617	pCR-Blunt-II-TOPO with an EcoRI-BamHI promoter region in *mutM* of 405 bp; Kan^r	This study
pPERM657	pMUTIN4 carrying a 405-bp EcoRI-BamHI DNA fragment encompassing 264 bp upstream and 141 bp downstream of the *mutM* translational start codon; Amp^r Ery^r	This study
pPERM698	pCR-Blunt II-TOPO with an 849-bp HindIII-KpnI PCR product containing *mutM*; Kan^r	This study
pPERM735	pCR-Blunt-II-TOPO containing the 936-bp SalI-SphI region of *mutM*; Kan^r	This study
pPERM748	pMUTIN-FLAG carrying an 849-bp HindIII-KpnI *mutM* fragment from pPERM707; Amp^r Ery^r	This study
pPERM792	pdr-111-amyE-Phyperspank containing the 936-bp SalI-SphI fragment of *mutM*; Amp^r Cm^r	This study

this microorganism are currently unknown. Here, we report that disruption of *mutM* sensitized *B. subtilis* to the noxious effects of the oxidizing agents hydrogen peroxide and Paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride [PQ]). Whereas in *E. coli* the superoxide radical induces the expression of *mutM* (14), our results showed that in *B. subtilis* the transcription of this gene is controlled in a temporal manner that keeps active the expression of *mutM* during the 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 (Kan; 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 (H_2O_2) 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 Δ*mutM*::*tet*) was isolated and used to transform competent cells of strain *B. subtilis* YB955, generating the strain *B. subtilis* PERM571 (YB955 Δ*mutM*::*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 hyperspank promoter (P*hs*). 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 (underlined), respectively, were used in the PCR. The PCR product was ligated into the pCR-Blunt-II-TOPO vector (Invitrogen Life Technologies, Carlsbad, CA), generating plasmid 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 *B. subtilis* strains PERM571 and PERM573 to generate the strains *B. subtilis* PERM1199 and PERM794.

To obtain a *mutM mutY* double mutant, the strain PERM571 (YB955 Δ*mutM*::*tet*) was transformed with genomic DNA of PERM704 (YB955 *mutY*::*sp*) (18), generating the strain *B. subtilis* PERM572 (YB955 Δ*mutM*::*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). To this end, a 405-bp fragment, extending from 264 bp upstream to 141 bp downstream of the *mutM* open reading frame (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 (underlined), 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, which had previously been digested with the same restriction enzymes. The resulting construct containing the *mutM-lacZ* fusion was designated pPERM657 and was introduced by transformation into compe-

tent cells of strain *B. subtilis* YB955 to generate 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 from 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 by utilizing Vent DNA polymerase (New England BioLabs) and the oligonucleotide primers 5′-GGAAGCTTCAG AGATAGGAAGTCATGGAT-3′ (forward) and 5′-GGGGTACCGTTTT TTGTCTGGCACTTTCG-3′ (reverse), which inserted HindIII and KpnI sites (underlined) into the cloned DNA. The PCR-amplified DNA fragment (849 bp) was first ligated into pCR-Blunt-II-TOPO (Invitrogen, Carlsbad, CA) and then replicated in *E. coli* XL10-GOLD Kan^r (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^r (Stratagene, Cedar Creek, TX). This strategy generated plasmid pPERM748, which was used to transform *B. subtilis* YB955, generating strain *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).

β-Galactosidase assays. *B. subtilis* strain PERM659, containing a transcriptional *mutM-lacZ* fusion, was 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 of 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 the exponential, transition, or stationary phase of growth. Cells were collected by centrifugation (16,000 × *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 10,000-fold and then processed with an enhanced-chemiluminescence (ECL) Western blotting system (Amersham Pharmacia, Little Chalfont, Buckinghamshire, United Kingdom).

Stationary-phase mutagenesis assays. Essentially, cultures were grown in flasks containing antibiotic A3 medium with aeration (250 rpm) at 37°C until 90 min after the cessation of exponential growth (designated T_{90} [90 min after the time point in the culture when the slopes of the logarithmic and stationary phases of growth intercepted]). 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; 1× Spizizen salts supplemented with 0.5% glucose and either 50 μg or 200 ng of the required amino acid per ml and 50 μg each of isoleucine and glutamic acid per ml). The concentration of the amino acid used depended on the reversion tested. For instance, in selecting His$^+$ revertants, 50 μg (each) of methionine and leucine ml^{-1} was added to the medium and 200 ng of histidine ml^{-1} 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. Frequencies of spontaneous mutation 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 (6,500 × *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_2O_2 or PQ and incubated for 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 analysis of variance (ANOVA) followed by a Tukey's *post hoc* analysis. Significance was set at a P of <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, which 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* knockout strain and its MutM-proficient parental strain were treated with increasing amounts of H_2O_2 or PQ. Results showed that disruption of *mutM* significantly sensitized exponentially growing cells of *B. subtilis* to these oxidizing agents (Fig. 1A and B). This result reveals a role for MutM in conferring to *B. subtilis* protection from the lethal effects of H_2O_2 and PQ. In support of this notion, the susceptibility to H_2O_2 and PQ of the *mutM* strain was reestablished to the level of the parental strain, YB955,

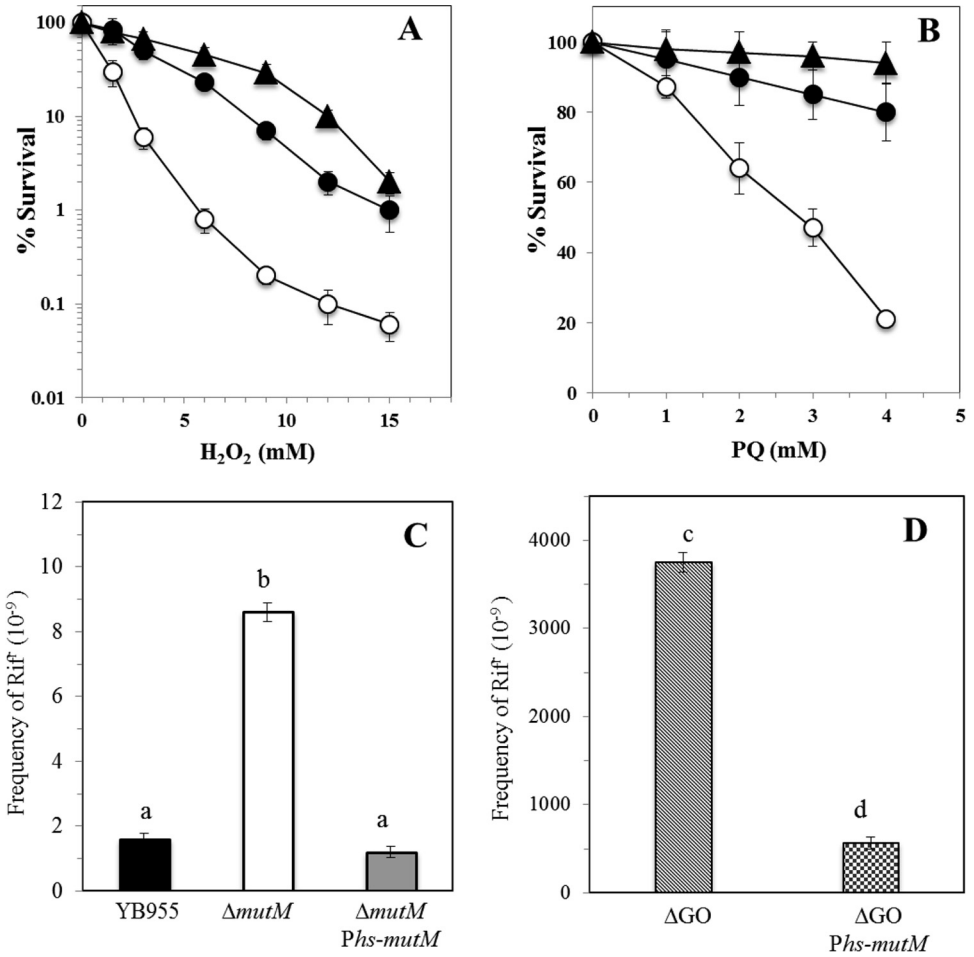

FIG 1 Contribution of MutM in the survival of *B. subtilis* to H_2O_2 and PQ treatment (A and B) and frequencies of spontaneous mutation to Rif^r of different *B. subtilis* strains (C and D). (A and B) *B. subtilis* YB955 (parental; ●), PERM751 (Δ*mutM*; ○), and PERM1199 (Δ*mutM amyE*::P*hs-mutM*; ▲) strains were treated with different amounts of H_2O_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 from three independent experiments done in triplicate. (C and D) *B. subtilis* YB955 (parental), PERM751 (Δ*mutM*), PERM1199 (Δ*mutM amyE*::P*hs-mutM*), PERM573 (GO system deletion [ΔGO]), and PERM794 (ΔGO *amyE*::P*hs-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 sextuplicate, and the error bars represent standard errors of the means (SEM). The statistical differences (a, b, c, and d) between the mutation frequencies of each strain and condition, as determined by ANOVA ($P < 0.05$), are shown above each bar.

following expression of the wild-type *mutM* gene from the IPTG-inducible P*hs* promoter (Fig. 1A and 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_2O_2 and PQ may promote other types of DNA lesions, including 8-OxoG·A mispairs and apurinic/apyrimidinic (AP) sites, as well as single- and/or double-strand DNA breaks (33). 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_2O_2 (33).

Of note, the absence of MutM also decreased the H_2O_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_2O_2-promoted DNA damage has also been described to occur in other bacteria, including *Pseudomonas aeruginosa* and *Mycobacterium smegmatis*. Thus, cells of these strains lacking MutM were significantly more susceptible to H_2O_2 treatment than their 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 the loss of MutM increased the spontaneous mutation frequency to Rif^r 5-fold in comparison with that of an isogenic strain that produced a functional MutM protein (Fig. 1C). From these results, we propose that MutM prevents the spontaneous mutagenic events promoted by oxidative stress in growing *B. subtilis* cells. Two observations support this contention: the levels of mutation 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 P*hs* promoter (Fig. 1C), and the overexpression of *mutM* induced a significant decrease in the frequency of mutation 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 *B. subtilis* strain YB955 lacking MutM showed a slight but statistically significant increase in their frequency of spontaneous mutation to Rif^r relative to that of the MutM-proficient parental strain. It must be pointed out that MutM-deficient strains of *E. coli* and *Pseudomonas 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, 36, 38, 39). These results suggest the existence of alternative repair pathways that compensate for the absence of MutM; in agreement with this notion, the genomes of the three microorganisms discussed above contain the gene for Nth, a DNA glycosylase capable of processing 8-oxo-G and AP sites (40–42). In the case of *B. subtilis*, it was recently shown that the genetic inactivation of Nth not only increases this bacterium's spontaneous Rif^r mutation frequency but also sensitizes it to the ROS promoter agent H_2O_2 (33).

Stationary-phase mutagenesis in *B. subtilis* cells deficient for MutM. We next investigated the role played by MutM in the stationary-phase-associated mutagenesis (SPM) of *B. subtilis*. These experiments were performed in strain *B. subtilis* YB955, which is auxotrophic for three amino acids due to the chromosomal mutations *hisC952* (amber), *metB5* (ochre), and *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 *his*, *met*, and *leu* in cell cultures that were starved for each of these amino acids revealed that MutM contributes to mutagenesis in starved *B. subtilis* cells. As shown in Fig. 2, the MutM-deficient strain significantly increased the frequency of *his*, *met*, and *leu* reversions in reference to those generated by 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 *B. subtilis*. In a marked contrast with our results, the single absence of MutM did not promote mutagenesis in starved cells of *E. coli* and *P. putida* (11, 12). However, in *E. coli* and *P. putida*, the lack of MutY did induce a significant increase in the production of stationary-phase-associated mutations (11, 44), suggesting that accumulation of nonprocessed 8-oxo-G lesions contribute to stationary-phase-associated mutagenesis in these strains. In support of this notion, when the *mutM* mutation was combined with a deficiency in MutY, the mutation frequency was further enhanced in starved *E. coli* cells (11). Thus, despite the fact 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 numbers of His$^+$, Met$^+$, and Leu$^+$ revertants relative to those produced by parental strain YB955 (Fig. 2). Moreover, we corroborated the finding that the genetic defect in *mutM* did not affect the survival of *B. subtilis* cells starved for *his*, *met*, and *leu* during the 10 days that the SPM experiments lasted (see Fig. S1 in the supplemental material).

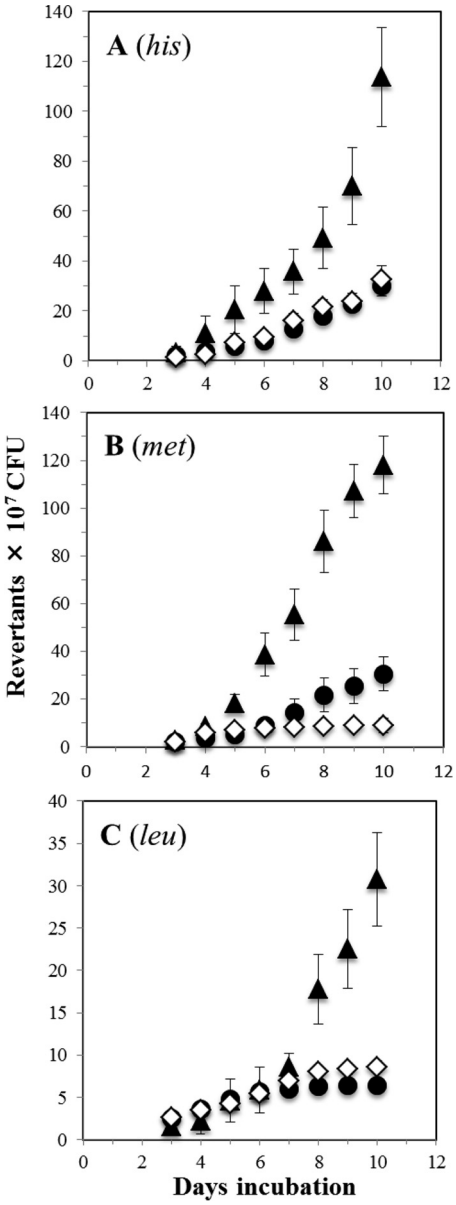

FIG 2 Stationary-phase-induced reversions to *his* (A), *met* (B), and *leu* (C) of the YB955 (◇), PERM571 (Δ*mutM*) (▲), and PERM1199 (Δ*mutM amyE*::P*hs-mutM*) (●) *B. subtilis* strains were determined as described in Materials and Methods. Data represent counts from six plates averaged from three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

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_2O_2, and its deficiency promotes adaptive mutagenesis in nutritionally stressed cells. These results suggest that *mutM* may 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 by employing *B. subtilis* strain PERM659, which harbors a genomic copy of a transcriptional *mutM-lacZ* fusion (Table 1). The results showed that this strain expressed barely similar levels of β-galac-

tosidase during the exponential transition (from exponential to stationary phase) and the 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 an 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 cells of a *B. subtilis* strain and in cells in the stationary phase of growth harboring an in-frame translational *mutM*-FLAG fusion (Fig. 3C). Based on these and previous results (45, 46), it is feasible to propose that *B. subtilis* expresses *mutM* during its entire life cycle to contend with the genotoxic and cytotoxic effects of ROS. However, despite the role displayed by *mutM* in protecting *B. subtilis* from oxidatively 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 stress (47–53). This conclusion was deduced from experiments showing that H_2O_2 (0.1%), mitomycin C (0.5 μg ml^{-1}), and NaCl (4%) did not turn on the transcription of a *mutM-lacZ* fusion inserted into the genome of strain *B. subtilis* YB955 (Fig. S2). Therefore, in conjunction with previous reports (47, 54–56), it is feasible to conclude that expression of *mutM* is not under the control of the master regulator RecA/DinR, PerR, or σ^B.

In contrast, in *E. coli*, *mutM* is under the 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. It is noteworthy that the levels of expression of *mutY* are repressed under the stressful conditions that activate *mutM* (14, 57, 58).

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 antimutagenic roles of this repair protein in both stages of growth (Fig. 1 and 2). Moreover, the presence of MutM in the stationary phase of *B. subtilis* but its apparent absence in *E. coli* (57) 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, 59), 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 may be different; thus, for the *his* and *met* alleles, ROS-promoted synthesis of 8-oxo-G may be responsible for 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 ablated in the MutY-deficient strain (Fig. 4C). Furthermore, a previous study demonstrated that processing of accumulated G·A

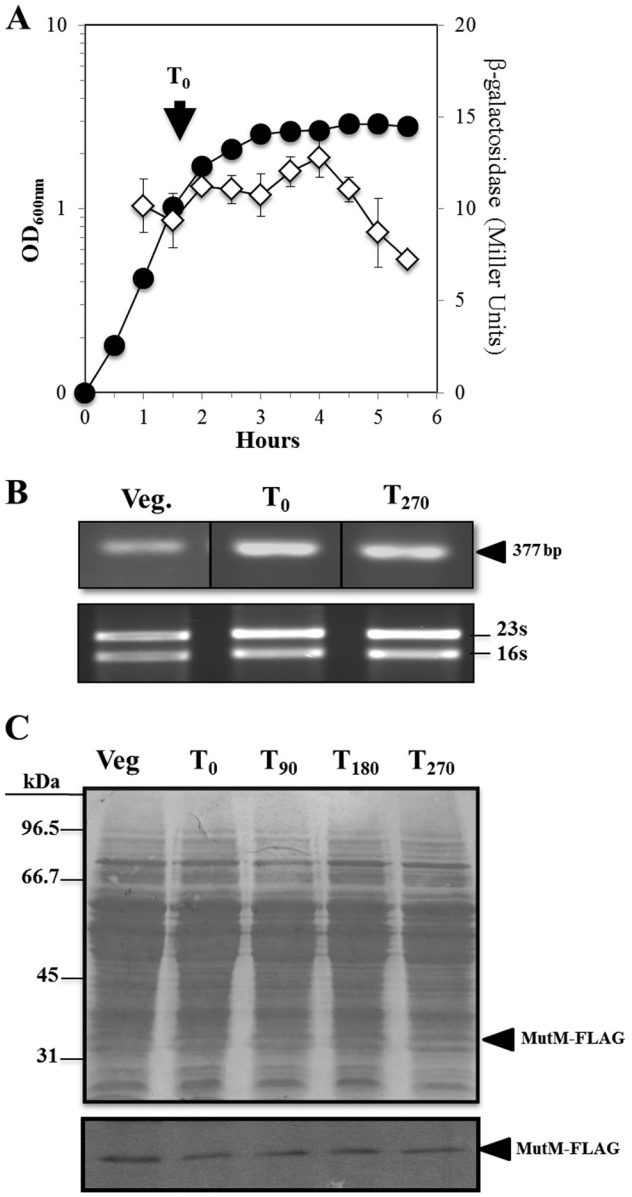

FIG 3 (A). Levels of β-galactosidase in a *mutM-lacZ* transcriptional fusion during the vegetative and stationary phases of growth. *B. subtilis* strain PERM659 was grown in liquid antibiotic (A3) medium. Cell samples were collected at the indicated times and treated with lysozyme, and the extracts were assayed for β-galactosidase as described in Materials and Methods. Data shown are average values from triplicate independent experiments ± SD for β-galactosidase specific activity (◇) and for A_{600} values (●). (B) RT-PCR analysis of *mutM* transcription during the 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 arrowhead 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 the 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 10,000-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. T_0 is the time point in the culture when the slopes of the logarithmic and stationary phases of growth intercepted. T_{90}, T_{180}, and T_{270} indicate times in minutes after T_0. Veg., vegetative growth.

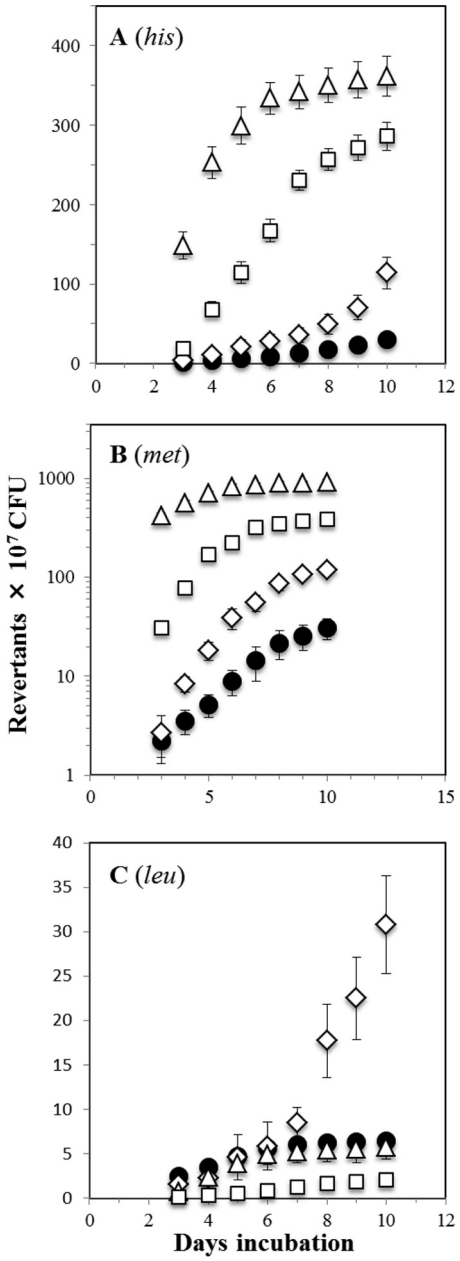

FIG 4 Stationary-phase-induced reversions to *his* (A), *met* (B), and *leu* (C) of the YB955 (●), PERM571 (Δ*mutM*) (◇), PERM704 (Δ*mutY*) (□), and PERM573 (Δ*mutM* Δ*mutY*) (△) *B. subtilis* strains were determined as described in Materials and Methods. Data represent counts from six plates averaged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

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 mutation is also dependent on Mfd, a protein that couples transcription with the DNA repair machinery (43, 60). It was recently found that production of Leu$^+$ prototrophs in MutY-deficient *B. subtilis* cells of strain YB955 are fully dependent on a functional Mfd protein (M. Gómez-Marroquín, E. A. Robleto, and M. Pedraza-Reyes, unpublished results). Therefore, we are currently investigating how Mfd coordinates the activities of repair proteins of the GO system to generate mutations that occur in nutritionally stressed *B. subtilis* cells.

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