JB Journals.ASM.org

Roles of Endonuclease V, Uracil-DNA Glycosylase, and Mismatch Repair in *Bacillus subtilis* DNA Base-Deamination-Induced Mutagenesis

Karina López-Olmos,[a] Martha P. Hernández,[a] Jorge A. Contreras-Garduño,[a] Eduardo A. Robleto,[b] Peter Setlow,[c] Ronald E. Yasbin,[d] and Mario Pedraza-Reyes[a]

Department of Biology, Division of Natural and Exact Sciences, University of Guanajuato, Guanajuato, México[a]; School of Life Sciences, University of Nevada—Las Vegas, Las Vegas, Nevada, USA[b]; Department of Molecular, Microbial, and Structural Biology, University of Connecticut Health Center, Farmington, Connecticut, USA[c]; and College of Arts and Sciences, University of Missouri—St. Louis, St. Louis, Missouri, USA[d]

The disruption of *ung*, the unique uracil-DNA-glycosylase-encoding gene in *Bacillus subtilis*, slightly increased the spontaneous mutation frequency to rifampin resistance (Rif^r), suggesting that additional repair pathways counteract the mutagenic effects of uracil in this microorganism. An alternative excision repair pathway is involved in this process, as the loss of YwqL, a putative endonuclease V homolog, significantly increased the mutation frequency of the *ung* null mutant, suggesting that Ung and YwqL both reduce the mutagenic effects of base deamination. Consistent with this notion, sodium bisulfite (SB) increased the Rif^r mutation frequency of the single *ung* and double *ung ywqL* strains, and the absence of Ung and/or YwqL decreased the ability of *B. subtilis* to eliminate uracil from DNA. Interestingly, the Rif^r mutation frequency of single *ung* and *mutSL* (mismatch repair [MMR] system) mutants was dramatically increased in a *ung* knockout strain that was also deficient in MutSL, suggesting that the MMR pathway also counteracts the mutagenic effects of uracil. Since the mutation frequency of the *ung mutSL* strain was significantly increased by SB, in addition to Ung, the mutagenic effects promoted by base deamination in growing *B. subtilis* cells are prevented not only by YwqL but also by MMR. Importantly, in nondividing cells of *B. subtilis*, the accumulations of mutations in three chromosomal alleles were significantly diminished following the disruption of *ung* and *ywqL*. Thus, under conditions of nutritional stress, the processing of deaminated bases in *B. subtilis* may normally occur in an error-prone manner to promote adaptive mutagenesis.

Three of the four bases usually present in DNA (cytosine, adenine, and guanine) contain an exocyclic amino group. The loss of this group by deamination occurs spontaneously under physiological conditions via a hydrolytic reaction (33, 34, 53) and leads to the production of the highly mutagenic lesions uracil, hypoxanthine, and xanthine, respectively. This process is greatly enhanced by oxygen free radicals and other agents, such as UV radiation, heat, ionizing radiation, nitrous acid, nitric oxide (NO), and sodium bisulfite (SB) (8, 9, 55, 56).

The spontaneous or induced deamination of cytosine creates a promutagenic U/G mispair that results in G·C-to-A·T transitions. Uracil in DNA may also occur through DNA polymerase's incorporation of dUMP instead of dTMP during replication, creating a U/A base pair that is not directly mutagenic but may be cytotoxic (26, 28, 29). The major pathway to remove uracil from DNA is thought to be base excision repair (BER), initiated by uracil-DNA glycosylase (Ung), first discovered in *Escherichia coli* in 1974 (32). Ung hydrolyzes the N-glycosylic bond between uracil and deoxyribose, leaving an abasic site (AP site) that is further processed by an apurinic/apyrimidinic (AP) endonuclease followed by DNA polymerase and DNA ligase (29, 33).

Ung is a ubiquitous enzyme that belongs to family 1 of the uracil-DNA glycosylases (UDGs) (39, 40, 67). UDGs are widely distributed among organisms of all domains of life and are grouped in at least eight families (15). For instance, in addition to Ung,
E. coli possesses a UDG family 2 member designated mismatch-specific uracil-DNA glycosylase (Mug) (16). A similar situation is found for the yeast *Schizosaccharomyces pombe*, whose genome encodes two UDGs belonging to families 1 and 2, respectively (15). The relevant role played by UDGs is clearly manifested in mammals and *Archaea*, where at least three and four different UDG activities, respectively, have been found for organisms of these kingdoms (15).

Endonuclease V (Endo V) from *E. coli*, encoded by the *nfi* gene, was also reported to be involved in an alternative excision pathway eliminating uracil and other deaminated bases from DNA (17, 22, 66). *In silico* analysis has revealed the existence of orthologs of this enzyme in several bacterial genomes, yeast, *Archaea*, and thermophilic organisms, and a homolog of this protein has even been identified in mammals (15). Endonuclease V activity is strictly dependent on $MgCl_2$ and does not function as a DNA glycosylase; instead, it catalyzes the incision of the second phosphodiester bond 3′ to the substrate lesion, generating 3′ OH and 5′ phosphate termini (15). The completion of repair following this pathway is not well understood, but for *E. coli*, it was proposed previously that the exonuclease activity of XthA and the subsequent action of DNA polymerase I and DNA ligase may be involved (15).

The ability of stressed cell populations to acquire mutations that favor cell growth after applying a nonlethal selection pressure has been termed adaptive or stationary-phase mutagenesis (48). This type of mutagenesis has been reported to occur in *E. coli* and other prokaryotic and eukaryotic models, including *Bacillus subtilis* (5, 20, 25, 60). It has been shown that repair pathways that

Received 26 August 2011 Accepted 25 October 2011
Published ahead of print 4 November 2011
Address correspondence to Mario Pedraza-Reyes, pedrama@ugto.mx.
Copyright © 2012, American Society for Microbiology. All Rights Reserved.
doi:10.1128/JB.06082-11

process oxidative stress-induced and mismatched DNA lesions are a key factor in generating potentially beneficial DNA alterations in nondividing *B. subtilis* cells (11, 41, 64).

B. subtilis relies on several repair mechanisms to counteract the adverse effects of intra- and extracellular factors that generate a myriad of insults to DNA (3, 7, 11, 24, 49, 64). DNA repair genes are differentially expressed during growth and/or sporulation, and the physiological roles played by their encoded products have been demonstrated in several cases (45, 47, 50, 62). Analysis of the genome of *B. subtilis* reveals the presence of a uracil-DNA-glycosylase-encoding gene (*ung*) that can be classified into family 1 of the UDGs. The biochemical properties of its encoding product were recently reported, showing that it is more active against uracil in single-stranded DNA than in double-stranded DNA and exhibits a greater preference for U/G than U/A mismatches (42). However, this microorganism lacks genes whose products have obvious homology to members of other UDG families. Here we report that the inactivation of the *ung* gene resulted in a moderate mutator phenotype, suggesting that additional mechanisms are involved in protecting the genome of *B. subtilis* from the mutagenic effects of uracil and/or other deaminated bases. In agreement with this suggestion, our results showed that in growing *B. subtilis* cells, Ung in collaboration with Endo V (YwqL) and the mismatch repair system (MutSL) counteracts the mutagenic effects of DNA base deamination. In contrast, in nondividing *B. subtilis* cells, Ung and YwqL were shown to exert a promutagenic role that may be important for generating adaptive mutations.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains and plasmids used in this work are listed in Table 1. The procedures for the transformation and isolation of chromosomal and plasmid DNAs were described previously (4, 10, 51). The growth medium used routinely was Penassay broth (PAB) (antibiotic medium 3; Difco Laboratories, Sparks, MD). When required, chloramphenicol (Cm; 5 μg/ml), erythromycin (Er; 5 μg/ml), kanamycin (Kan; 10 μg/ml), neomycin (Neo; 10 μg/ml), rifampin (Rif; 10 μg/ml), or tetracycline (Tet; 10 μg/ml) was added to the medium. *E. coli* cultures were grown in Luria-Bertani (LB) medium (36) supplemented with ampicillin (100 μg/ml). Liquid cultures were incubated at 37°C with vigorous aeration.

Construction of mutant strains and integration of *ung-lacZ* and *ywqL-lacZ* fusions. The construction of *ung* and *ywqL* mutant strains and transcriptional *ung-lacZ* and *ywqL-lacZ* fusions was performed with the integrative vector pMUTIN4 (63). To this end, a 561-bp NotI-BamHI internal fragment of the *ung* gene (from bp 99 to 660 downstream of the *ung* translational start codon) and a 375-bp EcoRI-BamHI internal fragment of the *ywqL* gene (from bp 154 to 529 downstream of the *ywqL* translational start codon) were amplified by PCR with Vent DNA polymerase (New England BioLabs, Ipswich, MA) using chromosomal DNA from *B. subtilis* 168 (a gift from Wayne Nicholson). The oligonucleotide primers used for the amplification of the *ung* fragment were 5′-GGCGCGGCCGCGGAGCAAACGATTTATCG-3′ (forward) and 5′-CCGGGATCCAATCAATCGGCGCCTC-3′ (reverse), and those used for the amplification of the *ywqL* fragment were 5′-CCGAATTCCAGGATGGAGAACCATACGG-3′ (forward) and 5′-GCGGATCCGTCCATATACCTCGCCGTC-3′ (reverse) (restriction sites are underlined). The *ung* and *ywqL* PCR fragments were inserted between the NotI and BamHI and the EcoRI and BamHI sites of pMUTIN4, respectively. The resulting constructs, designated pPERM633 (*ung*::*lacZ*) and pPERM800 (*ywqL*::*lacZ*), were propagated in *E. coli* DH5α cells. Plasmids pPERM633 and pPERM800 were independently used to transform *B. subtilis* 168, generating strains PERM640 (*ung-lacZ* Er^r) and PERM791 (*ywqL-lacZ* Er^r), respectively (Table 1). To obtain a *ung ywqL* double mutant, a 312-bp EcoRI-BamHI internal fragment of the *ung* gene (from bp 100 to 396 downstream of the *ung* translational start codon) was amplified by PCR with Vent DNA polymerase (New England BioLabs, Ipswich, MA) using chromosomal DNA from *B. subtilis* 168 and oligonucleotide primers 5′-GCGAATTCGCGGAGCAAACGATTTATCCG-3′ (forward) and 5′-GCGGATCCCTGTCCGCGCCTTACTGTCAG-3′ (reverse) (restriction sites are underlined). The PCR *ung* fragment was inserted into pJF751 (14) digested with EcoRI and BamHI. The resulting construct, pPERM788, was propagated in *E. coli* DH5α cells and was used to transform *B. subtilis* strain PERM791 (Δ*ywqL* Er^r), generating strain *B. subtilis* PERM793 (Δ*ung ywqL* Cm^r Er^r). Strains deficient in MutSL (mismatch repair [MMR]) were generated by transforming *B. subtilis* strains 168 (wild type [WT]) and PERM640 (Δ*ung* Er^r) with chromosomal DNA isolated from *B. subtilis* MPRYB151 (Δ*mutSL* Neo^r) (41), generating strains PERM739 (Δ*mutSL* Neo^r) and PERM737 (Δ*ung mutSL* Er^r Neo^r), respectively. The *nfo* and *exoA* genes of *B. subtilis* PERM640 (Δ*ung* Er^r) were interrupted by the transformation of this strain with chromosomal DNA isolated from strains PERM454 (Δ*nfo exoA* Neo^r Tet^r); this procedure generated strain PERM738 (Δ*ung nfo exoA* Er^r Neo^r Tet^r).

To assay DNA base deamination repair *in vivo*, the *ywqL* and *ung* mutations were transferred into *B. subtilis* 1A751 (*eglS*Δ*102 bglT*/*bglS*Δ*EV npr apr his*), a strain deficient in the production of extracellular cellulase and protease activities (*Bacillus* Genetic Stock Center, Columbus, OH). To accomplish this, competent cells of *B. subtilis* 1A751 were independently transformed with plasmids pPERM788 and pPERM800, generating strains PERM1055 (Δ*ung* Cm^r) and PERM1056 (Δ*ywqL* Er^r), respectively. To construct the *ung ywqL* double mutant in the same genetic background, plasmid pPERM800 was used to transform competent cells of *B. subtilis* PERM1055, generating strain PERM1057 (Δ*ung ywqL* Cm^r Er^r) (Table 1).

To carry out stationary-phase-associated-mutagenesis assays, the *ung* and *ywqL* mutations were transferred into *B. subtilis* strain YB955 (a prophage-"cured" strain that carries the *hisC952*, *metB5*, and *leuC427* alleles) (60). Strain YB955 was transformed with genomic DNA isolated from *B. subtilis* PERM793 (Δ*ung ywqL*), generating strain PERM1028 (Table 1). For all the strains generated, the single or double recombination events leading to the inactivation of the appropriate genes were confirmed by PCR using specific oligonucleotide primers (data not shown).

Constructs to overexpress *ung*, *ywqL*, and the *mutSL* operon. To overexpress *ung* or *ywqL* in the *ung ywqL* and *ung mutSL* genetic backgrounds, the open reading frames (ORFs) of the *ung* and *ywqL* genes were amplified by PCR with Vent DNA polymerase (New England BioLabs, Ipswich, MA) using chromosomal DNA from *B. subtilis* 168 and oligonucleotide primers 5′-GCGTCGACGTTCGTAAGGAGGCCTGAATC-3′ (forward) and 5′-CGGCTAGCTATTTGCCATTCGGCGGCGTC-3′ (reverse) for the *ung* gene and 5′-GCAAGCTTGGTCGAGAGTGAGCAATCGTT-3′ (forward) and 5′-GCGCATGCGAACTCAATTGGCAGAGCGAT-3′ (reverse) for the *ywqL* gene (restriction sites are underlined). The ORFs of *ung* (905 bp) and *ywqL* (943 bp) were cloned between the SalI-NheI and HindIII-SphI sites, respectively, of pdr111-*amyE*-hyper-SPANK (a gift from David Rudner, Harvard Medical School, Boston, MA), placing the genes under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible P*hs* promoter, generating plasmids pPERM924 (P*hs-ung-lacI*) and pPERM925 (P*hs-ywqL-lacI*). A construct termed pMPR002 (P*spac-mutSL-lacI*) that overexpresses *mutSL* from the IPTG-inducible P*spac* promoter was previously described (64). Plasmids pPERM924 and pPERM925 were introduced by transformation into *B. subtilis* strain PERM793 (Δ*ung ywqL*), generating strains PERM1047 and PERM944, respectively (Table 1). Plasmids pPERM924 and pMPR002 (P*spac-mutSL-lacI*) were independently transformed into competent cells of *B. subtilis* PERM737 (Δ*ung mutSL*) to generate strains PERM942 and PERM868, respectively (Table 1).

***β*-Galactosidase assays.** Strains PERM640 (*ung-lacZ*) and PERM791 (*ywqL-lacZ*) were grown in liquid PAB medium. Samples of 1 ml were collected every 30 min until 4 h after the cessation of exponential growth

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Genotype and/or description[e]	Source or reference[f]
B. subtilis strains		
168	Wild type; *trpC2*	Laboratory stock
1A751	*eglS*Δ*102 bglT*/*bglS*Δ*EV npr apr hi*	BGSC
YB955	*hisC952 metB5 leuC427 xin-1 Sp*β^{SENS}	60
PERM640[a]	Δ*ung*::*lacZ* Em^r	pPERM633→168[d]
PERM454[a]	Δ*nfo*::*neo* Δ*exoA*::*tet* Neo^r Tet^r	49
PERM738[a]	Δ*ung*::*lacZ* Δ*nfo*::*neo* Δ*exoA*::*tet* Em^r Neo^r Tet^r	PERM454→PERM640[c]
MPRYB151	YB955 carrying *mutSL*::*neo*	41
PERM739[a]	Δ*mutSL*::*neo* Neo^r	MPRYB151→168[c]
PERM737[a]	Δ*ung*::*lacZ* Δ*mutSL*::*neo* Em^r Neo^r	MPRYB151→PERM640[c]
PERM791[a]	Δ*ywqL*::*lacZ* Em^r	pPERM800→168[d]
PERM793[a]	Δ*ung*::*lacZ* Δ*ywqL*::*lacZ* Cm^r Em^r	pPERM788→PERM791[d]
PERM868[a]	Δ*ung*::*lacZ* Δ*mutSL*::*neo* with a P*spac-mutSL-lacI* construct from pMPR002 inserted into the *amyE* locus; Em^r Neo^r Spc^r	pMPR002→PERM737[d]
PERM942[a]	Δ*ung*::*lacZ* Δ*mutSL*::*neo* with a P*hs-ung-lacI* construct from pPERM924 inserted into the *amyE* locus; Em^r Neo^r Spc^r	pPERM924→PERM737[d]
PERM944[a]	Δ*ung*::*lacZ* Δ*ywqL*::*lacZ* with a P*hs-ywqL-lacI* construct from pPERM925 inserted into the *amyE* locus; Cm^r Em^r Spc^r	pPERM925→PERM793[d]
PERM1047[a]	Δ*ung*::*lacZ* Δ*ywqL*::*lacZ* with a P*hs-ung-lacI* construct from pPERM924 inserted into the *amyE* locus; Cm^r Em^r Spc^r	pPERM924→PERM793[d]
PERM956[a]	Δ*ung*::*lacZ* Δ*ywqL*::*lacZ* with a P*hs-lacI* construct inserted into the *amyE* locus; Cm^r Em^r Spc^r	pdr111 *amyE*-hyper-SPANK→PERM793[d]
PERM992[a]	Δ*ung*::*lacZ* Δ*mutSL*::*neo* with a P*hs-lacI* construct inserted into the *amyE* locus; Em^r Neo^r Spc^r	pdr111 *amyE*-hyper-SPANK→PERM737[d]
PERM1055[b]	IA751 carrying Δ*ung*::*lacZ* Cm^r	pPERM788→1A751[d]
PERM1056[b]	1A751 carrying Δ*ywqL*::*lacZ* Em^r	pPERM800→1A751[d]
PERM1057[b]	1A751 carrying Δ*ung*::*lacZ* Δ*ywqL*::*lacZ* Cm^r Em^r	pPERM800→PERM1055[d]
PERM1028	YB955 carrying Δ*ung*::*lacZ* Δ*ywqL*::*lacZ* Cm^r Em^r	PERM793→YB955[c]
Plasmids		
pMUTIN4	Integration vector; Amp^r Em^r	63
pJF751	Integration vector; Amp^r Cm^r	14
pdr111 *amyE*-hyper-SPANK	Hyper-SPANK expression vector; Amp^r Spc^r	44
pMPR002	pDG364 containing the P*spac-mutSL-lacI* construct; Amp^r Cm^r	64
pPERM270	pDG148 containing the P*spac-cel9-lacI* construct; Amp^r Kan^r	1
pPERM633	561-bp NotI-BamHI fragment (internal region of ORF) of *ung* cloned into pMUTIN4; Amp^r Em^r	This study
pPERM788	312-bp EcoRI-BamHI fragment (internal region of ORF) of *ung* cloned into pJF751; Amp^r Cm^r	This study
pPERM800	375-bp EcoRI-BamHI fragment (internal region of ORF) of *ywqL* cloned into pMUTIN4; Amp^r Em^r	This study
pPERM924	904-bp *B. subtilis ung* open reading frame cloned into the SalI-NheI sites of pdr111 *amyE*-hyper-SPANK; Amp^r Spc^r	This study
pPERM925	943-bp *B. subtilis ywqL* open reading frame cloned into the HindIII-SphI sites of pdr111 *amyE*-hyper-SPANK; Amp^r Spc^r	This study

[a] The background for this strain is 168.
[b] The background for this strain is IA751.
[c] Chromosomal DNA from the strain to left of the arrow was used to transform the strain to the right of the arrow.
[d] DNA of the plasmid to the left of the arrow was used to transform the strain to the right of the arrow.
[e] Amp, ampicillin; Cm, chloramphenicol; Er, erythromycin; Kan, kanamycin; Neo, neomycin; Spc, spectinomycin; Tet, tetracycline.
[f] BGSC, *Bacillus* Genetic Stock Center.

and washed with 0.025 M Tris-HCl (pH 7.4), and the cell pellet was stored at −20°C. Cell extracts were obtained by disruption with lysozyme followed by centrifugation, and the β-galactosidase activity in the supernatant fluid was determined according to a previously described protocol, using *ortho*-nitro-phenyl-β-D-galactopyranoside (ONPG) as a substrate (35, 38). The basal values of the ONPGase activity expressed by the parental strain during the logarithmic and stationary phases of growth were subtracted from the β-galactosidase values of the *ywqL-lacZ*- and *ung-lacZ*-containing strains at each time point.

RT-PCR experiments. Total RNA from exponentially growing or stationary-phase *B. subtilis* 168 cells grown in PAB medium was isolated by using Tri reagent (RNA/DNA/Protein Isolation reagent; Molecular Research Center, Inc., Cincinnati, OH). Reverse transcription-PCRs (RT-PCRs) were performed with the RNA samples and the Master AMP RT-PCR kit (Epicentre Technologies, Madison, WI) according to the instructions provided by the manufacturer. The primers used for RT-PCR were 5′-GGCGCGGCCGCGGAGCAAACGATTTATCG-3′ (forward) and 5′-CCGGGATCCAATCAATCGGCGCCTC-3′ (reverse), which amplified a 561-bp *ung* fragment encompassing bp 99 to 660 downstream of the *ung* translational start codon. The primers used to generate a 375-bp RT-PCR product of *ywqL* encompassing bp 154 to 529 downstream of the *ywqL* translational start codon were 5′-CCGAATTCCAGGATGGAGAACCAT

ACGG-3′ (forward) and 5′-GCGGATCCGTCCATATACCTCGCCGT C-3′ (reverse). The absence of chromosomal DNA in the RNA samples was assessed by PCRs carried out with Vent DNA polymerase (New England BioLabs, Ipswich, MA) and the set of primers described above. The size of the RT-PCR product was assessed with reference to the migration of a 1-kbp Plus DNA ladder (Life Technologies, Rockville, MD) during agarose gel electrophoresis.

Analysis of spontaneous mutation frequencies. Spontaneous mutations to rifampin resistance (Rif^r) in cultures were determined as follows. Strains were grown for 24 h at 37°C in PAB medium supplemented with the proper antibiotics, and aliquots of cells were plated onto six LB plates containing 10 μg/ml rifampin. Rif^r colonies were counted after 2 days 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 onto LB medium plates without rifampin and incubating the plates for 24 h at 37°C. Selection medium for strains overexpressing *ung*, *ywqL*, or *mutSL* was supplemented with 2 mM IPTG. Mutation frequencies are reported as the average numbers of Rif^r colonies per 10^9 viable cells, and all these experiments were repeated at least three times.

Analysis of mutagenesis induced by SB. Mutations to Rif^r in *B. subtilis* cells treated with agents that promote the deamination of bases in DNA were determined as follows. To determine the mutagenesis generated by SB, PAB cultures of each strain grown overnight were inoculated into flasks containing fresh PAB medium to an optical density at 600 nm (OD_{600}) of 0.5, each culture was divided in half, and the two halves were transferred into different flasks. One of the cultures was untreated, and the other was treated with 0.25 mM SB. The flasks of untreated and SB-treated cultures were shaken at 37°C for 12 h. Mutation frequencies were determined by plating aliquots of each culture onto six LB plates containing 10 μg/ml rifampin as well as plating aliquots of appropriate dilutions onto LB plates without rifampin. Rif^r colonies were counted after 24 h of incubation at 37°C.

DNA base deamination repair assay. Assays of the repair of deaminated DNA bases *in vivo* were performed as follows. Plasmid pPERM270 containing an IPTG-inducible *spac-cel9* construct (P*spac-cel9*) (1, 46, 61) was mutagenized, as previously described (12), by dilution 10-fold into 4 M $NaHSO_3$-40 mM mercaptoethanol (pH 5.8) and incubation at 37°C for 120 min. The treated plasmid was dialyzed at 4°C against 2,000 volumes of 20 mM Tris-HCl buffer (pH 8.0)–20 mM $MgSO_4$-1 mM mercaptoethanol for 24 h with three buffer changes. The isogenic strains *B. subtilis* 1A751, *B. subtilis* PERM1055 (Δ*ung*), *B. subtilis* PERM1056 (Δ*ywqL*), and *B. subtilis* PERM1057 (Δ*ung ywqL*) were transformed to Kan^r with either SB-treated or untreated pPERM270, and transformants were screened by using Congo red for the production of cellulase activity as previously described (6, 18). In brief, the transformed colonies were grown at 37°C on LB agar plates supplemented with Kan, 0.2% carboxymethyl-cellulose (CMC) as a substrate, and 5 mM IPTG. The presence of cellulolytic activity was indicated by the formation of a clear zone on the agar plate after staining with 0.2% Congo red for 10 min and two destaining washes with 0.5 M NaCl. The DNA repair activities of the different strains were determined by calculating the mutation frequency as the number of colonies that lost the ability to produce cellulase activity among the total Kan^r colonies.

Stationary-phase mutagenesis assays. The stationary-phase mutagenesis assays were performed as previously described (41, 60). Briefly, 10 ml of cells was grown with vigorous aeration in PAB medium at 37°C to 90 min after the cessation of exponential growth (designated T_{90}). Cultures were harvested by centrifugation at 10,000 × *g* for 10 min at room temperature and then resuspended in 10 ml of Spizizen minimal salts (SMS). One hundred microliters of cells was plated in sextuplicates onto solid Spizizen minimal medium (SMM) (58) (1× Spizizen salts supplemented with 0.5% glucose and either 50 μg or 200 ng of the required amino acid/ml and 50 μg each of isoleucine and glutamic acid/ml). The concentration of the amino acid used depended on the reversion that was being selected. For instance, when selecting for His$^+$ revertants, 50 μg of methionine and leucine/ml was added to the medium, and 200 ng of histidine/ml was added. Isoleucine and glutamic acid were added as described previously (59), in order to maintain the viability of the cells. The number of revertants was scored daily. The initial number of bacteria plated for each experiment was determined by the serial dilution of the bacterial cultures and plating of the cells onto LB plates. The number of colonies was then determined following 48 h of growth at 37°C. These experiments were repeated at least three times.

The survival rates of the bacteria plated onto the minimal selective medium were determined as follows. Three agar plugs were removed from each selection plate every 2 days. The plugs were removed with sterile Pasteur pipettes and taken from areas of the plates where no growth was observed. The plugs were suspended in 1 ml of 1× Spizizen salts, diluted, and plated onto LB plates. Again, the number of colonies was determined following 48 h of growth at 37°C.

The growth-dependent reversion rates for His$^+$, Met$^+$, and Leu$^+$ revertants were measured by fluctuation tests with the Lea-Coulson formula, $r/m - \ln(m) = 1.24$ (31). Three parallel cultures were used to determine the total number of CFU plated onto each plate (Nt) by titration. The mutation rates were calculated as previously described, with the formula $m/2Nt$ (41, 60).

Statistical analyses. Statistical tests were performed by using Statistica 8 for Windows (StatSoft, Inc., Tulsa, OK). Wilcoxon tests were performed during the analysis of mutagenesis induced by SB. For analyses of spontaneous mutation frequencies, a generalized model (GLM) was carried out, employing a Poisson distribution of data and long-link function. When we had unequal *n*'s between treatments, we used the likelihood type 3 test in the model, and we used likelihood type 1 for equal *n*'s between treatments. Differences with P values of ≤ 0.05 were considered statistically significant.

RESULTS

Spontaneous mutation rates in a *B. subtilis ung* null mutant. The deamination of cytosine occurs spontaneously in cells generating U/G promutagenic mispairs that are processed by the BER pathway with the aid of uracil-DNA glycosylases. The genome of *B. subtilis* contains only a single uracil-DNA-glycosylase-encoding gene, termed *ung*. Therefore, it was expected that a *ung* disruption should induce a strong mutator phenotype in this microorganism. Surprisingly, in comparison with the wild-type parental strain, the loss of *ung* function increased the spontaneous mutation frequency to Rif^r by only ~2-fold (Fig. 1A). As expected, the mutation frequency of the Ung-deficient strain was significantly increased by SB treatment (Fig. 1B). In addition, a modest increase (~2.5-fold) in the Rif^r mutation frequency was observed when null mutations in *nfo* and *exoA*, encoding the major AP endonucleases in *B. subtilis* (50, 57), were introduced into the Δ*ung* genetic background (data not shown). Therefore, we investigated whether, in addition to the BER pathway that employs Ung, *B. subtilis* has additional mechanisms to contend with the mutagenic effects of uracil and other deaminated bases.

Antimutagenic role of YwqL. Although *B. subtilis* seems to possess a single UDG, the results of a BLAST analysis revealed the existence of a genomic open reading frame, designated *ywqL*, that encodes a putative ortholog of endonuclease V (Endo V) of *E. coli* (30). Amino acid sequence analysis revealed that YwqL is 31% identical and 51% homologous to *E. coli* Endo V, encoded by the *nfi* gene (data not shown). The product of *nfi* has been associated with the processing of a wide spectrum of DNA lesions and structures, including uracil, hypoxanthine, xanthine, base mismatches, AP sites, hairpins, unpaired loops, and pseudo-Y and flap structures (17, 22, 65, 66).

To investigate the contribution of YwqL to DNA repair in *B.*

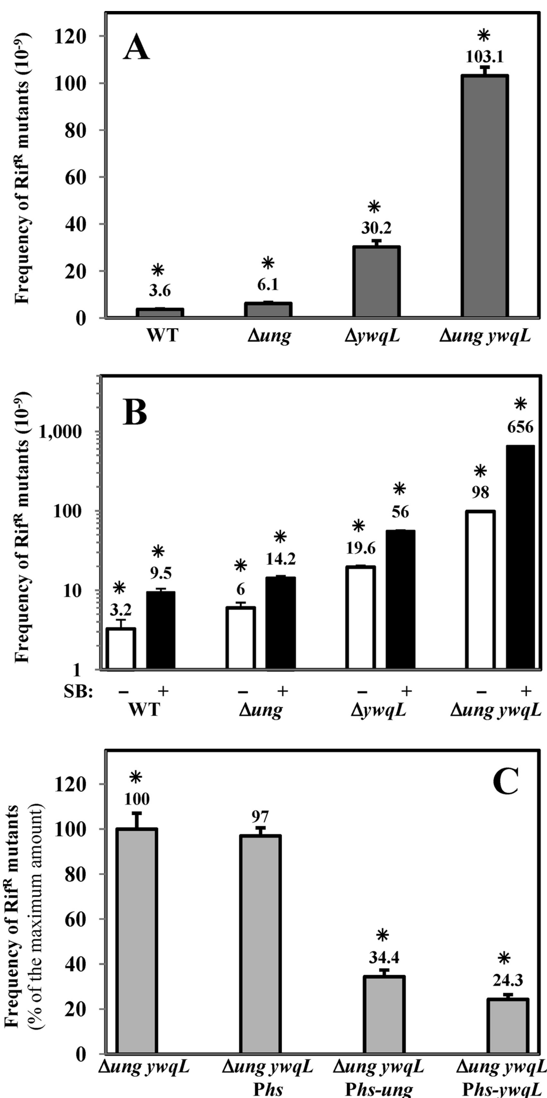

FIG 1 Frequencies of spontaneous mutation to Rif^r of different *B. subtilis* strains. (A) *B. subtilis* strains 168 (parental strain), PERM640 (Δ*ung*), PERM791 (Δ*ywqL*), and PERM793 (Δ*ung ywqL*) were grown overnight in PAB medium, and frequencies of mutation to Rif^r were determined as described in Materials and Methods. A GLM revealed significant differences between compared values ($\chi^2 = 1{,}997.549$; df = 3; $P < 0.0001$). (B) Frequencies of mutation to Rif^r of different *B. subtilis* strains treated with SB. *B. subtilis* strains 168 (parental strain), PERM640 (Δ*ung*), PERM791 (Δ*ywqL*), and PERM793 (Δ*ung ywqL*) were grown at 37°C in PAB medium to an OD$_{600}$ of 0.5 and then divided into two Erlenmeyer flasks; one of the flasks was left as an untreated control (white bars), and the other was supplemented with 0.25 mM SB (black bars). Cultures were shaken for 12 additional hours at 37°C, and frequencies of mutation to Rif^r were determined as described in Materials and Methods. Results are expressed as averages ± standard deviations (SD) from at least two independent experiments. Mutation frequencies of untreated and SB-treated strains were compared with a Wilcoxon test, revealing significant differences ($P < 0.027$). (C) Effects of *ung* or *ywqL* overexpression on the frequency of spontaneous mutation to Rif^r of a Δ*ung ywqL* strain. *B. subtilis* strains PERM793 (Δ*ung ywqL*), PERM956 (Δ*ung ywqL* P*hs*), PERM1047 (Δ*ung ywqL* P*hs-ung*), and PERM944 (Δ*ung ywqL* P*hs-ywqL*) were grown overnight at 37°C in PAB medium supplemented with the proper antibiotics and IPTG (2 mM), when required. Frequencies of mutation to Rif^r were determined as described in Materials and Methods. Results are expressed as percentages of the maximum value calculated for the Δ*ung ywqL* strain. Values are expressed as averages ± standard deviations from at least two independent experiments. A GLM revealed significant differences between compared values ($\chi^2 = 240.3844$; df = 3; $P < 0.0001$). Asterisks indicate values that were significantly different.

subtilis, a null *ywqL* mutant was generated and used to study the prevention of deamination-induced DNA damage. The frequencies of spontaneous mutation to Rif^r in the *ywqL* strain were ~8-fold higher than those in the YwqL-proficient strain; this response was significantly exacerbated in the YwqL-deficient strain by SB treatment (Fig. 1A and B). Together, these results strongly suggest that in addition to Ung, *B. subtilis* YwqL prevents mutagenic effects of cytosine (and perhaps guanine and adenine) deamination through the so-called alternative excision repair pathway (15, 27).

Base-deamination-induced mutagenesis is enhanced in a Ung YwqL-deficient *B. subtilis* strain. Instead of relying on additional UDGs, *B. subtilis* uses Ung and YwqL to prevent the genotoxic effects of base deamination. To investigate whether both proteins operate on the same type of lesions, a *ung ywqL* strain was constructed. Notably, the mutation frequencies expressed by strains with single defects in *ung* and *ywqL* were significantly lower than those exhibited by the strain with defects in both genes, as the mutation frequency of this strain was ~3-fold higher than the additive values of the single *ung* or *ywqL* strain (Fig. 1A and B). These results suggest that Ung and YwqL counteract the mutagenic effects of the base analog uracil. Two experimental approaches were undertaken in attempts to further support this suggestion. First, the *ung ywqL* strain was treated with the base deamination inducer SB, and the effects of this agent on mutation frequencies were determined (9, 21, 54). With respect to a control culture, SB increased the mutation frequency of the *ung ywqL* strain ~6-fold (Fig. 1B). Significantly, the effect of SB in the *ung ywqL* mutant was several times greater than that observed for strains harboring single *ung* or *ywqL* mutations (Fig. 1B). We also generated constructs to overexpress *ung* or *ywqL* from the IPTG-inducible P*hs* promoter. These constructs were used to recombine single copies of the P*hs-ung* and P*hs-ywqL* cassettes into the *amyE* locus of the *ung ywqL* strain. Consistent with their suggested antimutagenic roles, the overexpression of either Ung or YwqL from the P*hs* promoter significantly decreased the mutation frequency of the *ung ywqL* strain, while the P*hs* vector alone did not (Fig. 1C).

Strains lacking Ung and/or YwqL are also deficient in repair of deaminated DNA. The strains that lacked Ung and/or YwqL exhibited increased frequencies of mutation to Rif^r. Therefore, we investigated whether the mutagenic phenotypes exhibited by these strains were correlated with a deficiency in the repair of deaminated DNA *in vivo*. We treated *in vitro* plasmid preparations of pPERM270, which can direct the synthesis of an extracellular cellulase in *B. subtilis* in an IPTG-dependent manner (1, 46), with the deamination inducer SB (21, 54). Both the treated and untreated plasmids were transformed into cellulase-deficient *B. subtilis* 1A751 (*eglS*Δ*102 bglT/bglS*Δ*EV npr aprE his*) lacking Ung and/or YwqL. Deficiencies in the repair of deaminated bases in the different strains were then determined by calculating the mutation frequency from the number of colonies that had no cellulase activity among the total Kan^r colonies. As shown in Fig. 2, the absence of Ung or YwqL decreased the capability of *B. subtilis* to process deaminated DNA, and the absence of both repair proteins increased this effect even further. In contrast, no deficiencies in the processing of the deaminated plasmid were found for the Ung YwqL-proficient strain (data not shown). From these results, we conclude that the increased Rif^r mutagenesis in strains lacking Ung and/or YwqL is due to deficiencies in the repair of uracil and presumably other deaminated bases in DNA.

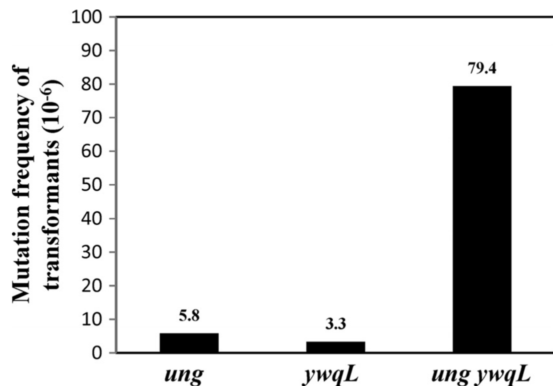

FIG 2 Abilities of different *B. subtilis* strains to repair a deaminated P*spac-cel9* expression plasmid. Competent cells of *B. subtilis* strains 1A751 (parental strain), PERM1055 (Δ*ung*), PERM1056 (Δ*ywqL*), and PERM1057 (Δ*ung ywqL*) were transformed with a P*spac-cel9* construct treated or not with SB as described in Materials and Methods. Around 300 Kan^r colonies of each strain were tested for the ability to produce cellulase activity *in situ*, as previously described (6, 18). The DNA repair activity is expressed as the mutation frequency of transformant colonies that lost the ability to produce cellulase activity among the total Kan^r colonies analyzed.

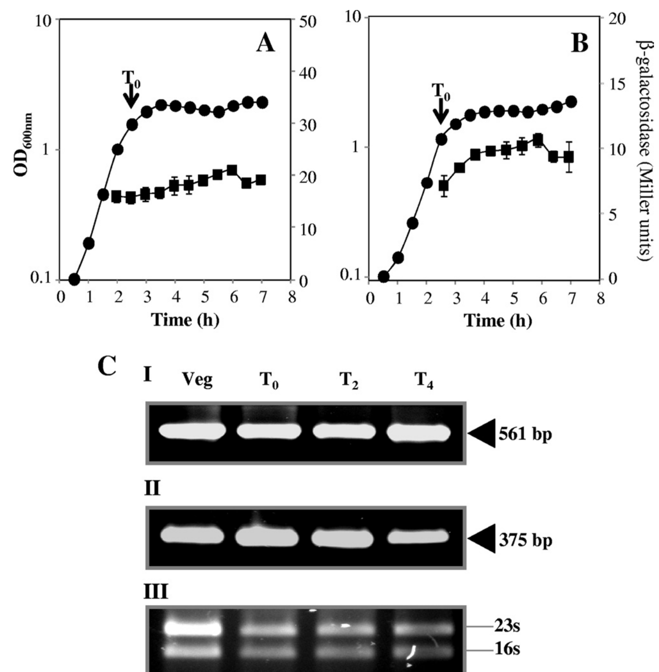

FIG 3 (A and B) Levels of β-galactosidase from *B. subtilis* strains containing *ung-lacZ* (A) or *ywqL-lacZ* (B) transcriptional fusions. *B. subtilis* strains PERM640 and PERM791 were independently grown at 37°C in PAB medium. Cell samples were collected at the indicated times and treated with lysozyme, and the extracts were assayed for β-galactosidase activity as described in Materials and Methods. Shown are averages of data from triplicate independent experiments ± SD of the β-galactosidase specific activity (■) and A_{600} (●). (CI and II) RT-PCR analysis of *ung* (I) and *ywqL* (II) transcription during vegetative and stationary phases of growth. RNA samples (~1 μg) isolated from a *B. subtilis* 168 PAB culture, at the times indicated, were processed for RT-PCR analysis as described in Materials and Methods. Arrowheads show the sizes of the expected RT-PCR products. (III) 16S and 23S rRNA bands. T_0 is the time point for the culture when the slopes of the logarithmic and stationary phases of growth intercepted. T_2 and T_4 indicate the times in hours after T_0. Veg, vegetative growth.

Analysis of *ung* and *ywqL* expression during *B. subtilis* growth. As noted above, Ung and YwqL may both act to prevent the genotoxic effects of uracil. Therefore, it is reasonable to expect that *ung* and *ywqL* will exhibit similar patterns of expression. To investigate this notion, we utilized *B. subtilis* strains PERM640 and PERM791, which harbor single copies of transcriptional *ung-lacZ* and *ywqL-lacZ* fusions, respectively. To avoid sporulation, the temporal patterns of expression of both *lacZ* fusions in PAB medium were analyzed. Under these conditions, *ywqL*-directed β-galactosidase activity was detected in exponentially growing cells, and levels remained constant during the transition from the exponential to the stationary phase and until at least 4.5 h beyond the latter point (Fig. 3A). Under the same conditions, the *ung-lacZ* transcriptional fusion showed a similar pattern of expression (Fig. 3B).

To further show that *ywqL* and *ung* exhibited similar patterns of gene expression, total RNA samples isolated from different stages of the *B. subtilis* life cycle were analyzed by qualitative RT-PCR to detect the mRNAs of these two genes. As shown in Fig. 3C, the temporal patterns of expression exhibited by the *ung*- and *ywqL-lacZ* fusions were paralleled by the RT-PCR results, since mRNAs of both *ywqL* and *ung* were detected not only in exponentially growing cells but also during the transition and stationary phases of growth.

Ung and MutSL counteract the mutagenic effects of base deamination. Previous studies also implicated the mismatch repair (MMR) pathway in the processing of U/G mispairs in mammals (52). To investigate if this DNA repair pathway also contributes to the prevention of the mutagenic effects of uracil on *B. subtilis* DNA, a null *mutSL* mutation was recombined into the genome of the *ung* strain to generate a *ung mutSL* strain, and the mutation frequency of these strains was determined (Fig. 4A). The spontaneous mutation frequency of the *ung mutSL* strain was ~4-fold higher than the sum of the frequencies for the single mutant strains. These results suggest that in addition to Ung, the MMR pathway also prevents the mutagenic effects of base deamination. In support of the latter contention, a dramatic increase in the mutation frequency of the *ung mutSL* strain was observed following its treatment with SB (Fig. 4B), an agent that preferentially promotes cytosine deamination (21, 54). To further investigate the contribution of Ung and MutSL to counteracting the mutagenic effects observed for the *ung mutSL* strain, we independently inserted P*hs-ung* or P*spac-mutSL* cassettes into the *amyE* locus of the *ung mutSL* strain. The IPTG induction of either *ung* or *mutSL* from the P*hs* or P*spac* promoter, respectively, partially complemented the mutagenic phenotype of the *ung mutSL* strain, although a higher level of complementation was obtained with the P*spac-mutSL* cassette (Fig. 4C). Together, these results suggest that U/G promutagenic mispairs not only are subject to Ung processing but also are corrected with the participation of the MMR pathway.

Adaptive mutagenesis in *B. subtilis* cells lacking Ung and YwqL. As demonstrated in this work, Ung, YwqL, and MMR are all involved in protecting the genome of growing *B. subtilis* cells from the mutagenic effects induced by DNA base deamination. However, as noted above, *ung* and *ywqL* exhibited high levels of expression during the stationary phase of growth as well, suggesting that their protein products may protect cells from base-deamination-induced mutagenesis under conditions of no actual

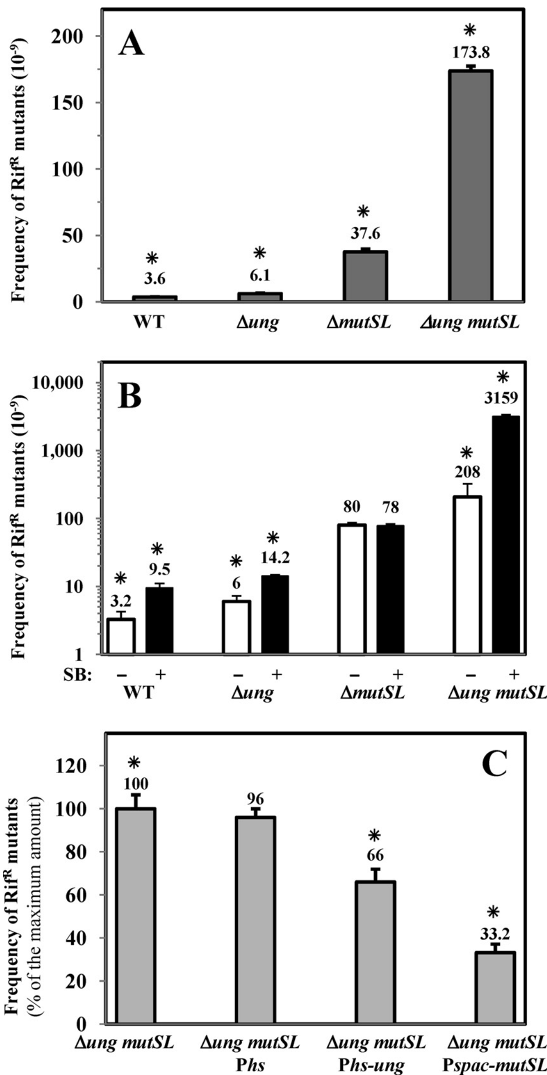

FIG 4 (A) Frequencies of spontaneous mutation to Rif^r of different *B. subtilis* strains. *B. subtilis* strains 168 (parental strain), PERM640 (Δ*ung*), PERM739 (Δ*mutSL*), and PERM737 (Δ*ung mutSL*) were grown overnight in PAB medium, and frequencies of mutation to Rif^r were determined as described in Materials and Methods. A GLM revealed significant differences between compared values ($\chi^2 = 3{,}791.829$; df = 3; $P < 0.0001$). (B) Frequencies of mutation to Rif^r of different *B. subtilis* strains treated with SB. *B. subtilis* strains 168 (parental strain), PERM640 (Δ*ung*), PERM739 (Δ*mutSL*), and PERM737 (Δ*ung mutSL*) were grown at 37°C in PAB medium to an OD_{600} of 0.5 and then divided into two flasks, one left as an untreated control (white bars) and the other supplemented with 0.25 mM SB (black bars). Cultures were shaken for 12 additional hours at 37°C, and frequencies of mutation to Rif^r were determined as described in Materials and Methods. Results are expressed as averages ± standard deviations from at least two independent experiments. Except for the Δ*mutSL* strain, a Wilcoxon test revealed significant differences ($P < 0.027$) between mutation frequencies of untreated and SB-treated strains. (C) Effects of *ung* or *mutSL* overexpression on the frequency of spontaneous mutation to Rif^r of a Δ*ung mutSL* strain. *B. subtilis* strains PERM737 (Δ*ung mutSL*), PERM992 (Δ*ung mutSL* P*hs*), PERM942 (Δ*ung mutSL* P*hs-ung*), and PERM868 (Δ*ung mutSL* P*spac mutSL*) were grown overnight at 37°C in PAB medium supplemented with the proper antibiotics and IPTG (2 mM), when required. Frequencies of mutation to Rif^r were determined as described in Materials and Methods. Results are expressed as percentages of the maximum value calculated for the Δ*ung mutSL* strain. Values are expressed as averages ± standard deviations from at least two independent experiments. A GLM revealed significant differences between compared values ($\chi^2 = 1{,}120.381$; df = 3; $P < 0.0001$). Asterisks indicate values that were significantly different.

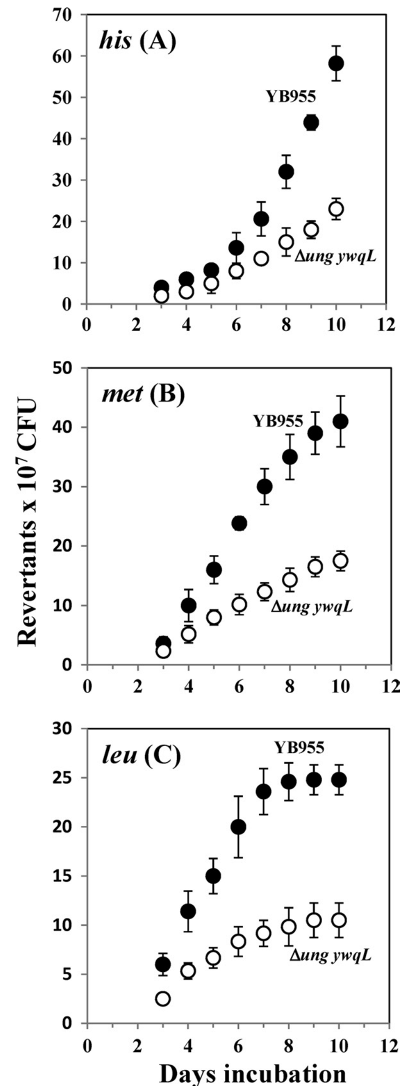

FIG 5 Stationary-phase-induced reversions for the *his* (A), *met* (B), and *leu* (C) mutant alleles of *B. subtilis* strains YB955 (●) and PERM1028 (Δ*ung ywqL*) (■), as described in Materials and Methods. Results are the average numbers of accumulated revertants in six different selection plates. This experiment was performed at least three times.

growth. To test this idea, the *ung* and *ywqL* mutations were transferred into *B. subtilis* strain YB955, a model system widely employed to understand how mutations are generated in amino-acid-starved cells (11, 41, 60, 64). This strain is auxotrophic for three amino acids due to the mutations *hisC952* (amber), *metB5* (ochre), and *leuC427* (missense) (60). Surprisingly, the results of the stationary-phase-associated experiments revealed that with respect to parental strain YB955, there was a significant reduction in the number of His$^+$, Met$^+$, and Leu$^+$ revertants generated by the Ung YwqL-deficient strain (Fig. 5). Thus, with respect to the parental strain, during day 8, the level of production of revertants in the three alleles tested was around three times lower for the *ung ywqL* mutant (Fig. 5). Importantly, during the course of the experiment, there were no significant differences in the survival rates of the two strains (data not shown). Thus, the decrease in the number of His$^+$, Met$^+$, and Leu$^+$ revertant colonies observed for

the *ung ywqL* strain was not due to differences in growth or survival with respect to the parental strain. The growth-dependent mutation rates of the *ung ywqL* strain for the generation of His$^+$, Met$^+$, and Leu$^+$ colonies were also determined and compared with those of parental strain YB955. The results revealed that in exponentially growing cells, the *his*, *met*, and *leu* reversion rates were not significantly different between the Ung YwqL-deficient strain and parental strain YB955 (data not shown). Taken together, these results suggest that the processing of uracil and perhaps other deaminated bases in nondividing *B. subtilis* cells takes place in an error-prone manner and further suggest that Ung and/or YwqL is involved in determining the error frequency of this process.

DISCUSSION

E. coli and other organisms rely on redundant UDGs to counteract the mutagenic effects of uracil generated from cytosine deamination (16, 23, 37, 39). The disruption of *ung* in *E. coli* increases spontaneous Rif^r mutations ~5-fold (13), although a UDG (Mug) can compensate for the loss of the Ung function in this microorganism to some degree. In contrast, a previously reported analysis of the *B. subtilis* genome revealed the existence of a single open reading frame encoding a uracil-DNA glycosylase termed *ung* (30). Unexpectedly, the disruption of this gene increased the spontaneous Rif^r mutation frequency of *B. subtilis* cells only 1.7-fold, although this effect was enhanced by the uracil inducer SB. These results suggest that the BER pathway that employs Ung plays a minor role in eliminating uracils that are spontaneously generated in the *B. subtilis* genome. As noted above, the *B. subtilis* genome lacks additional UDG-encoding genes; therefore, we speculated that alternative DNA repair proteins that may recognize a broader spectrum of DNA lesions could participate in counteracting the mutagenic effects of base deamination in *B. subtilis*. This appears to be the case, as the disruption of *ywqL*, which encodes a putative endonuclease V (30), induced a stronger Rif^r phenotype, increasing its mutation frequency ~8-fold in comparison with that of the parental strain. It was reported previously that Endo V works as a promiscuous enzyme in *E. coli*, as in addition to processing uracils, it is also involved in the repair of hypoxanthine, xanthine, and AP sites, among other types of lesions (17, 22, 65, 66). Our results suggest that YwqL plays a similar role in *B. subtilis*, as the mutation frequency of a *ywqL* strain was enhanced by the base deamination promoter SB. Thus, the higher level of Rif^r mutagenesis exhibited by the Δ*ywqL* mutant than that exhibited by the Δ*ung* strain is in agreement with its promiscuous ability to process several types of DNA damage, in addition to U/G mispairs.

Interestingly, in reference to the single *ung* and *ywqL* strains, a significant increase in the spontaneous mutation frequency of the Ung YwqL-deficient strain was observed, confirming that both proteins contend with the mutagenic effects of DNA base deamination. The results supporting this notion were that SB, which primarily induces cytosine deamination, significantly increased the mutation frequency of the *ung ywqL* strain. In addition, the ability to repair uracils *in vivo* that were generated by SB in a plasmid *in vitro* was significantly diminished in the strain that lacked both Ung and YwqL. Together, these results strongly suggest that the repair of uracils in *B. subtilis* occurs with the participation of both Ung and YwqL. However, YwqL may process not only uracils but also other types of DNA lesions, including AP sites. In support of this notion, our results showed that the Rif^r phenotype exhibited by the *ung ywqL* strain was complemented not only by the overexpression of *ung* but also by the increase of the transcription of *ywqL* in this genetic background. Therefore, the specificity of Ung to repair uracils and the ability of YwqL to process a wider spectrum of DNA lesions, including deaminated bases, may explain the partial complementation generated by the P*hs-ung* and P*hs-ywqL* cassettes. As the elimination of uracils from DNA with the participation of Ung proceeds through the BER pathway, it remains to be investigated how YwqL processes uracil and other deaminated bases through this alternative excision repair pathway (27). As previously shown (15), Endo V catalyzes the rupture of the second phosphodiester bond 3′ to the damaged base; however, the factors that complete this repair process are poorly understood. For instance, the 3′→5′ exonuclease activities of Xth and DNA polymerase I in *E. coli* and AP endonuclease I (APEX I) together with a 3′ flap endonuclease in mammals have been implicated in postincisional events (15).

It was reported previously that the Msh2/Msh6 heterodimer (MutSα) is able to recognize U/G mispairs (52), although this type of lesion may not be processed by the MMR machinery in human cells (52). However, analyses of Rif^r mutation frequencies in *ung*, *mutSL*, and *ung mutSL* strains suggested that MMR and Ung are both involved in the processing of uracils. Therefore, in contrast to mammals, the MMR system of *B. subtilis* seems to be involved in the repair of U/G mispairs; this idea was supported by the increase in the mutation frequency of the Ung MMR-deficient strain by SB and the complementation of the Rif^r phenotype of this strain by the overexpression of either *ung* or *mutSL*.

The ability of the MMR system to interact with components of the BER pathway has been demonstrated to occur in human cells as well as in bacteria (2, 43). For instance, the interference between Ung and MMR during the processing of adjacent U/G mispairs was proposed previously to control somatic hypermutation (SHM) in lymphocytes (52). Similarly, a physical interaction between MutS and MutY was demonstrated to occur in *E. coli* and human cells (2, 19). The possibility of a physical interaction between Ung and MutS that may regulate the antimutagenic activity of both pathways is under investigation in our laboratory.

The results described in this work revealed that in growing *B. subtilis* cells, the action of not only Ung but also YwqL minimizes the mutagenic effects of base deamination. Consistent with this idea, our results revealed that *ung* and *ywqL* are expressed during the exponential growth of this bacterium. However, both genes maintained high levels of expression during stationary phase. Therefore, we investigated the impact of base deamination in stationary-phase-associated mutagenesis by utilizing *B. subtilis* strain YB955 (*hisC952 metB5 leuC427*) deficient in both Ung and YwqL. A significant reduction in the *his*, *met*, and *leu* reversion frequencies was observed for the *ung ywqL* mutant with respect to the values for the strain containing both Ung and YwqL. These results suggest that the processing of deaminated bases and perhaps other AP sites contributes to adaptive mutagenesis in nutritionally stressed, nondividing *B. subtilis* cells. However, during growth, the *his*, *met*, and *leu* reversion rates of the *ung ywqL* mutant did not differ from those calculated for the parental strain, suggesting that another DNA repair system(s) avoids the mutagenic effects of base deamination in this strain. In support of this notion, the results described in this work indicate that the MMR pathway is involved in counteracting the mutagenic effects of ura-

cil. In contrast, in nondividing cells, where a saturation of the MMR system occurs (11, 41, 64), a different situation was found to occur. Therefore, experiments aimed at an understanding of the mechanism(s) involved in the generation reversions in *B. subtilis* YB955 due to the actions of YwqL and Ung under conditions of no cell growth are currently in progress. The promutagenic effect of Ung and YwqL described in this work is not unprecedented, as it was postulated previously that in pathogen-activated B cells, the repair of uracils occurs in an error-prone manner (52). Moreover, it was recently proposed that in starved *B. subtilis* cells, the saturation of the MMR system may induce the expression of *mutY*, disturbing the balance between MutY and MMR proteins to produce adaptive Leu^+ revertants (11). An inspection of the *B. subtilis* genome indicated that *ung* and *ywqL* are the distal cistrons of the putative *ywdDEF-ung* and *ywqGHIJKL* operons, respectively (30). Therefore, the phenotypic effects exhibited by the single and double *ung* and *ywqL* mutants cannot be due to polar effects caused by the integration mutagenesis of these genes.

ACKNOWLEDGMENTS

This work was supported by the University of Guanajuato and by the Consejo Nacional de Ciencia y Tecnología (CONACYT) (grant 84482) of México. K.L.-O. and M.P.H. were supported by scholarships from the CONACYT.

We are grateful for the excellent technical assistance of Norma Ramírez-Ramírez.

REFERENCES

1. **Avitia CI, et al.** 2000. Temporal secretion of a multicellulolytic system in *Myxobacter* sp. AL-1. Molecular cloning and heterologous expression of *cel9* encoding a modular endocellulase clustered in an operon with *cel48*, an exocellobiohydrolase gene. Eur. J. Biochem. **267**:7058–7064.
2. **Bai H, Lu AL.** 2007. Physical and functional interactions between *Escherichia coli* MutY glycosylase and mismatch repair protein MutS. J. Bacteriol. **189**:902–910.
3. **Barraza-Salas M, et al.** 2009. Effects of forespore-specific overexpression of apurinic/apyrimidinic endonuclease Nfo on the DNA-damage resistance properties of *Bacillus subtilis* spores. FEMS Microbiol. Lett. **302**: 159–165.
4. **Boylan RJ, Mendelson NH, Brooks D, Young FE.** 1972. Regulation of the bacterial cell wall: analysis of a mutant of *Bacillus subtilis* defective in biosynthesis of teichoic acid. J. Bacteriol. **110**:281–290.
5. **Cairns J, Overbaugh J, Miller S.** 1988. The origin of mutants. Nature **335**:142–145.
6. **Castellanos-Juárez FX, Sandoval AA, Urtiz-Estrada N, Pedraza-Reyes M.** 2006. Obtención de variantes hiperactivas e inactivas de la endocelulasa Cel9 de *Myxobacter* sp. AL-1. Acta Univ. **16**:22–28.
7. **Castellanos-Juárez FX, et al.** 2006. YtkD and MutT protect vegetative cells but not spores of *Bacillus subtilis* from oxidative stress. J. Bacteriol. **188**:2285–2289.
8. **Chen H, Shaw BR.** 1993. Kinetics of bisulfite-induced cytosine deamination in single-stranded DNA. Biochemistry **32**:3535–3539.
9. **Chernikov AV, Gudkov SV, Shtarkman IN, Bruskov VI.** 2007. Oxygen effect in heat-induced DNA damage. Biophysics **52**:185–190.
10. **Cutting SM, Vander Horn PB.** 1990. Genetic analysis, p 27–74. *In* Harwood CM, Cutting SM (ed), Molecular biological methods for *Bacillus*. John Wiley & Sons, Sussex, England.
11. **Debora BN, et al.** 2011. Mismatch repair modulation of MutY activity drives *Bacillus subtilis* stationary-phase mutagenesis. J. Bacteriol. **193**: 236–245.
12. **Duncan BK, Weiss B.** 1982. Specific mutator effects of *ung* (uracil-DNA glycosylase) mutations in *Escherichia coli*. J. Bacteriol. **151**:750–755.
13. **Duncan BK, Rockstroh PA, Warner HR.** 1978. *Escherichia coli* K-12 mutants deficient in uracil-DNA glycosylase. J. Bacteriol. **134**:1039–1045.
14. **Ferrari E, Haward SMH, Hoch JA.** 1985. Effect of sporulation mutations on subtilisin expression assayed using a subtilisin-β-galactosidase gene fusion, p 180–184. *In* Hoch JA, Setlow P (ed), Molecular biology of microbial differentiation. American Society for Microbiology, Washington, DC.
15. **Friedberg EC, et al.** 2006. DNA repair and mutagenesis, 2nd ed. American Society for Microbiology, Washington, DC.
16. **Gallinari P, Jiricny J.** 1996. A new class of uracil-DNA glycosylases related to human thymine-DNA glycosylase. Nature **383**:735–738.
17. **Gates FT, Linn S.** 1977. Endonuclease V of *Escherichia coli*. J. Biol. Chem. **252**:1647–1653.
18. **Gilkes NR, Langsford ML, Kilburn DG, Miller RC, Warren RAJ.** 1984. Mode of action and substrate specificities of cellulases from cloned bacterial genes. J. Biol. Chem. **259**:10455–10459.
19. **Gu Y, et al.** 2002. Human MutY homolog, a DNA glycosylase involved in base excision repair, physically and functionally interacts with mismatch repair proteins human MutS homolog 2/human MutS homolog 6. J. Biol. Chem. **277**:11135–11142.
20. **Halas A, Baranowska H, Policinska Z.** 2002. The influence of the mismatch repair system on stationary-phase mutagenesis in the yeast *Saccharomyces cerevisiae*. Curr. Genet. **42**:140–146.
21. **Hayatsu H, Wataya Y, Kai K, Iida S.** 1970. Reaction of sodium bisulfite with uracil, cytosine, and their derivatives. Biochemistry **7**:2858–2865.
22. **He B, Qing H, Kow YW.** 2000. Deoxyxanthosine in DNA is repaired by *Escherichia coli* endonuclease V. Mutat. Res. **459**:109–114.
23. **Hendrich B, Bird A.** 1998. Identification and characterization of a family of mammalian methyl-CpG binding proteins. Mol. Cell. Biol. **18**: 6538–6547.
24. **Ibarra JR, et al.** 2008. Role of the Nfo and ExoA apurinic/apyrimidinic endonucleases in repair of DNA damage during outgrowth of *Bacillus subtilis* spores. J. Bacteriol. **190**:2031–2038.
25. **Kasak L, Horak R, Kivisaar M.** 1997. Promoter-creating mutations in *Pseudomonas putida*: a model system for the study of mutation in starving bacteria. Proc. Natl. Acad. Sci. U. S. A. **94**:3134–3139.
26. **Kouzminova EA, Kuzminov A.** 2006. Fragmentation of replicating chromosomes triggered by uracil in DNA. J. Mol. Biol. **355**:20–33.
27. **Kow YW.** 2002. Repair of deaminated bases in DNA. Free Radic. Biol. Med. **33**:886–893.
28. **Krokan HE, Drablos F, Slupphaug G.** 2002. Uracil in DNA—occurrence, consequences and repair. Oncogene **21**:8935–8948.
29. **Krokan HE, Standal R, Slupphaug G.** 1997. DNA glycosylases in the base excision repair of DNA. Biochem. J. **325**:1–16.
30. **Kunst F, et al.** 1997. The complete genome sequence of the Gram positive bacterium *Bacillus subtilis*. Nature **390**:249–256.
31. **Lea DE, Coulson CA.** 1949. The distribution of the numbers of mutants in bacterial populations. J. Genet. **49**:264–285.
32. **Lindahl T.** 1974. An N-glycosidase from *Escherichia coli* that releases free uracil from DNA containing deaminated cytosine residues. Proc. Natl. Acad. Sci. U. S. A. **71**:3649–3653.
33. **Lindahl T.** 1979. DNA glycosylases, endonucleases for apurinic/apirimidinic sites and base excision repair. Prog. Nucleic Acid Res. Mol. Biol. **22**:135–192.
34. **Lindahl T.** 1993. Instability and decay of the primary structure of DNA. Nature **362**:709–715.
35. **Mason JM, Hackett RH, Setlow P.** 1988. Regulation of expression of genes coding for small, acid-soluble proteins of *Bacillus subtilis* spores: studies using *lacZ* gene fusions. J. Bacteriol. **170**:239–244.
36. **Miller JH.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
37. **Neddermann P, Jiricny J.** 1994. Efficient removal of uracil from GU mispairs by the mismatch-specific thymine DNA glycosylase from HeLa cells. Proc. Natl. Acad. Sci. U. S. A. **91**:1642–1646.
38. **Nicholson WL, Setlow P.** 1990. Sporulation, germination and outgrowth, p 391–550. *In* Harwood CR, Cutting SM (ed), Molecular biological methods for *Bacillus*. John Wiley & Sons, Sussex, England.
39. **Nilsen H, et al.** 2001. Excision of deaminated cytosine from the vertebrate genome: role of the SMUG1 uracil-DNA glycosylase. EMBO J. **20**: 4278–4286.
40. **Olsen LC, Aasland R, Krokan HE, Helland DE.** 1991. Human uracil-DNA glycosylase complements *E. coli ung* mutants. Nucleic Acids Res. **19**:4473–4478.
41. **Pedraza-Reyes M, Yasbin RE.** 2004. Contribution of the mismatch DNA repair system to the generation of stationary-phase-induced mutants of *Bacillus subtilis*. J. Bacteriol. **186**:6485–6491.
42. **Pérez-Lago L, et al.** 2011. Characterization of *Bacillus subtilis* uracil-DNA

glycosylase and its inhibition by phage ϕ29 protein p56. Mol. Microbiol. **80**:1657–1666.
43. **Pitsikas P, Polosina YY, Cupples CG.** 2009. Interaction between the mismatch repair and nucleotide excision repair pathways in the prevention of 5-azacytidine-induced CG-to-GC mutations in *Escherichia coli.* DNA Repair **8**:354–359.
44. **Pybus C, et al.** 2010. Transcription-associated mutation in *Bacillus subtilis* cells under stress. J. Bacteriol. **192**:3321–3328.
45. **Ramírez MI, Castellanos FX, Yasbin RE, Pedraza-Reyes M.** 2004. The *ytkD* (*mutTA*) gene of *Bacillus subtilis* encodes a functional antimutator 8-oxo-(dGTP/GTP)ase and is under dual control of sigma A and sigma F RNA polymerase. J. Bacteriol. **186**:1050–1059.
46. **Ramírez-Ramírez N, et al.** 2008. Expression, characterization and synergistic interactions of *Myxobacter* sp. AL-1 Cel9 and Cel48 glycosyl hydrolases. Int. J. Mol. Sci. **9**:247–257.
47. **Rivas-Castillo AM, Yasbin RE, Robleto E, Nicholson WL, Pedraza-Reyes M.** 2010. Role of the Y-family DNA polymerases YqjH and YqjW in protecting sporulating *Bacillus subtilis* cells from DNA damage. Curr. Microbiol. **60**:263–267.
48. **Rosenberg SM, Thunlin C, Harris RS.** 1998. Transient and heritable mutators in adaptative evolution in the lab and in nature. Genetics **148**: 1559–1566.
49. **Salas-Pacheco JM, Setlow B, Setlow P, Pedraza-Reyes M.** 2005. Role of the Nfo (YqfS) and ExoA apurinic/apyrimidinic endonucleases in protecting *Bacillus subtilis* spores from DNA damage. J. Bacteriol. **187**: 7374–7381.
50. **Salas-Pacheco JM, Urtiz-Estrada N, Martínez-Cadena G, Yasbin RE, Pedraza-Reyes M.** 2003. YqfS from *Bacillus subtilis* is a spore protein and a new functional member of the type IV apurinic/apyrimidinic-endonuclease family. J. Bacteriol. **185**:5380–5390.
51. **Sambrook J, Fritsch EF, Maniatis T.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
52. **Schanz S, Castor D, Fischer F, Jiricny J.** 2009. Interference of mismatch and base excision repair during the processing of adjacent UG mispairs may play a key role in somatic hypermutation. Proc. Natl. Acad. Sci. U. S. A. **106**:5593–5598.
53. **Shapiro R.** 1981. Damage to DNA caused by hydrolysis, p 3–12. *In* Seeberg E, Kleppe K (ed), Chromosome damage and repair. Plenum Publishing Corp, New York, NY.
54. **Shapiro R, Weisgras JM.** 1970. Bisulfite-catalyzed transamination of cytosine and cytidine. Biochem. Biophys. Res. Commun. **24**:839–843.
55. **Shapiro R, Pohl SH.** 1968. The reaction of ribonucleosides with nitrous acid. Side products and kinetics. Biochemistry **7**:448–455.
56. **Shapiro R, Shiuey SJ.** 1969. Reaction of nitrous acid with alkylaminopurines. Biochim. Biophys. Acta **174**:403–405.
57. **Shida T, Ogawa T, Ogasawara N, Sekiguchi J.** 1999. Characterization of *Bacillus subtilis* ExoA protein: a multifunctional DNA-repair enzyme similar to the *Escherichia coli* exonuclease III. Biosci. Biotechnol. Biochem. **63**:1528–1534.
58. **Spizizen J.** 1958. Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. Proc. Natl. Acad. Sci. U. S. A. **44**: 1072–1078.
59. **Sung HM, Yasbin RE.** 2000. Transient growth requirement in *Bacillus subtilis* following the cessation of exponential growth. Appl. Environ. Microbiol. **66**:1220–1222.
60. **Sung HM, Yasbin RE.** 2002. Adaptative, or stationary-phase, mutagenesis, a component of bacterial differentiation in *Bacillus subtilis.* J. Bacteriol. **184**:5641–5653.
61. **Téllez-Valencia A, Sandoval AA, Pedraza-Reyes M.** 2003. The non-catalytic amino acid Asp446 is essential for enzyme activity of the modular endocellulase Cel9 from *Myxobacter* sp. AL-1. Curr. Microbiol. **46**: 307–310.
62. **Urtiz-Estrada N, Salas-Pacheco JM, Yasbin RE, Pedraza-Reyes M.** 2003. Forespore-specific expression of *Bacillus subtilis yqfS*, which encodes type IV apurinic/apyrimidinic endonuclease, a component of the base excision repair pathway. J. Bacteriol. **185**:340–348.
63. **Vagner V, Dervyn E, Ehrlich D.** 1998. A vector for systematic gene inactivation in *Bacillus subtilis.* Microbiology **144**:3097–3104.
64. **Vidales LE, Cárdenas LC, Robleto E, Yasbin RE, Pedraza-Reyes M.** 2009. Defects in the error prevention oxidized guanine system potentiate stationary-phase mutagenesis in *Bacillus subtilis.* J. Bacteriol. **191**: 506–513.
65. **Yao M, Kow YW.** 1996. Cleavage of insertion/deletion mismatches, flap and pseudo-Y DNA structures by deoxyinosine 3′-endonuclease from *Escherichia coli.* J. Biol. Chem. **271**:30672–30676.
66. **Yao M, Hatahet Z, Melamede RJ, Kow YW.** 1994. Purification and characterization of a novel deoxyinosine-specific enzyme, deoxyinosine 3′ endonuclease, from *Escherichia coli.* J. Biol. Chem. **269**:16260–16268.
67. **Yonekura S, Nakamura N, Yonei S, Zhang-Akiyama Q.** 2009. Generation, biological consequences and repair mechanisms of cytosine deamination in DNA. J. Radiat. Res. **50**:19–26.