

Antimutator Role of the DNA Glycosylase *mutY* Gene in *Helicobacter pylori*

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***Helicobacter pylori* has a highly variable genome with ongoing diversification via inter- and intragenomic recombination and spontaneous mutation. DNA repair genes modulating mutation and recombination rates that influence diversification have not been well characterized for *H. pylori*. To examine the role of putative base excision repair *ung* and *mutY* glycosylase and *xthA* apurinic/aprimidinic endonuclease genes in *H. pylori*, mutants of each were constructed in strain JP26 by allelic exchange. Spontaneous mutation frequencies of JP26 *mutY* mutants, assessed by rifampin resistance, were consistently higher (26-fold) than that of the wild type, whereas the *ung* and *xthA* mutants showed smaller increases. In *trans* complementation of the JP26 *mutY* mutant restored spontaneous mutation frequencies to wild-type levels. In cross-species studies, *H. pylori mutY* complemented an *Escherichia coli mutY* mutant and vice versa. In contrast, the *ung* and *mutY* mutants did not show higher frequencies of intergenomic recombination or greater sensitivity to UV-induced DNA damage than the wild type. The *H. pylori mutY* open reading frame contains an eight-adenine homonucleotide tract; we provide evidence that this is subject to slipped-strand mispairing, leading to frameshifts that eliminate gene function. Our findings indicate that *H. pylori* possesses phase-variable base excision repair, consistent with a tension between repair and mutation.**

DNA bases may be damaged either spontaneously or by bioactive molecules, such as reactive oxygen species (3, 36). In *Escherichia coli*, base excision repair (BER) plays a major role in removing mutation-inducing DNA lesions produced by base modifications (9). In BER, a damage-specific DNA glycosylase first recognizes and excises the damaged bases, detaching them from the DNA backbone, leaving apurinic/aprimidinic (AP) sites. Then, an AP endonuclease cuts the DNA strand 5' of the AP site and removes the sugar, and a repair patch of nucleotides is catalyzed by general-purpose DNA polymerases and ligases (50). Some DNA glycosylases that recognize oxidative damage also have AP endonuclease activity (63).

E. coli possesses several DNA glycosylases, including Ung, MutY, Fpg, and Nei, that each recognize a specific type of damaged DNA base (12). The Ung glycosylase removes uracil that is accidentally incorporated into DNA when cytosine becomes deaminated (40). MutY is responsible for excising adenine in A · G and A · C mismatches and opposite 8-oxoguanine, one of the most stable products of oxidative DNA damage (2, 16, 24, 35). Although many prokaryotic species possess putative glycosylase and AP endonuclease homologs, BER pathways are not fully conserved across all species, and the specific abnormal bases that the systems repair differ greatly (12).

Helicobacter pylori is a species of gram-negative, microaerophilic bacteria that persistently colonize the human stomach (56); the inflammation induced predisposes to peptic ulceration and gastric adenocarcinomas (4, 34, 37). Compared to many other organisms, *H. pylori* has a high level of genetic diversity (23, 46, 53) and has high-frequency spontaneous mutation and intergenomic recombination (25, 57). The relative lack of DNA repair homologs in *H. pylori* (60) is consistent

with an intrinsic genomic plasticity that may be adaptive in relatively inhospitable human gastric niches (5, 30, 60). For example, the methyl-directed mismatch repair pathway is not present (60), and *H. pylori* MutS2 does not exhibit MutS1 functions (21).

High mutation rates may be advantageous to organisms under stressful conditions but can otherwise be costly (41, 54). Because of this tension, we studied the roles that DNA repair and, more specifically, base excision repair play in *H. pylori* survival. From phylogenomic analysis, putative *ung* and *mutY* glycosylase genes and an *xthA* AP endonuclease gene have been annotated in the *H. pylori* genome. In this report, we provide evidence of the primary importance of *mutY* in *H. pylori* DNA repair of spontaneous mutations, while *ung* and *xthA* play smaller roles. The *mutY* open reading frame (ORF) has a homonucleotide tract of eight adenines; we provide evidence that implies that frameshifts in this tract create subpopulations of cells that differ in mutation rates.

MATERIALS AND METHODS

Amino acid alignment and phylogenetic analyses of MutY homologs. Amino acid sequences of MutY homologs were retrieved from the Comprehensive Microbial Resource from the TIGR website (<http://cmr.tigr.org/tigr-scripts/CMR/CMRHomePage.cgi>). Conserved domains were identified with Pfam (14) and the Simple Modular Architectural Research Tool (SMART) program (<http://smart.embl-heidelberg.de/>) (28), which encompasses several protein domain databases. Amino acid sequences were aligned using CLUSTAL_X (58) and visualized with Genedoc (www.psc.edu/biomed/genedoc) in conservation shading mode. Phylogenetic trees were constructed using MEGA 2.1 (26) according to the neighbor-joining method (45), with 1,000 bootstrap replicates.

Bacterial strains and plasmids. The *E. coli* and *H. pylori* strains and plasmids used in this study are listed in Table 1. *E. coli* CC104 wild-type and CC104 *mutM mutY* mutant strains were routinely grown on LB agar plates at 37°C. *H. pylori* strains were grown at 37°C in 5% CO₂ on Trypticase soy agar (TSA), with antibiotics added as described below for individual experiments.

Construction of *H. pylori* mutants. Fragments of the HP0142 (*mutY* homolog), HP1347 (*ung* homolog), and HP1526 (*xthA* homolog) open reading frames from strain 26695 were amplified by PCR using primers based on sequenced *H. pylori* strain 26695 (Table 2). Products were cloned into pGEMT-Easy (Promega,

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TABLE 1. Plasmids and bacterial strains used in this study

Plasmid or strain	Relevant characteristic(s)	Source or reference
Plasmids		
pMutYKm	<i>mutY::aphA</i> in pGEMT-Easy	This work
pUngCat	<i>ung::cat</i> in pGEMT-Easy	This work
pXthACat	<i>xthA::cat</i> in pGEMT-Easy	This work
pADC	<i>ureAB</i> fragment in pUC18 with <i>cat</i> cassette	21
pADC-HpMutY	<i>ureAB</i> fragment in pUC18 with <i>H. pylori mutY</i> and <i>cat</i> cassette	This work
pADC-EcMutY	<i>ureAB</i> fragment in pUC18 with <i>E. coli mutY</i> and <i>cat</i> cassette	This work
Strains		
<i>H. pylori</i>		
JP26	Wild-type strain	22
JP26 <i>mutY::aphA</i>	Δ <i>mutY</i> with <i>aphA</i> insertion	This work
JP26 <i>mutYHpcomp</i>	<i>mutY::aphA</i> complemented with <i>H. pylori mutY</i> in <i>ureAB</i> locus	This work
JP26 <i>mutYEcomp</i>	<i>mutY::aphA</i> complemented with <i>E. coli mutY</i> in <i>ureAB</i> locus	This work
JP26 <i>ung::cat</i>	Δ <i>ung</i> with <i>cat</i> insertion	This work
JP26 <i>xthA::cat</i>	Δ <i>xthA</i> with <i>cat</i> insertion	This work
JP26 <i>mutY::aphA ung::cat</i>	Δ <i>mutY</i> with <i>aphA</i> insertion, Δ <i>ung</i> with <i>cat</i> insertion	This work
JP26 <i>mutY::aphA xthA::cat</i>	Δ <i>mutY</i> with <i>aphA</i> insertion, Δ <i>xthA</i> with <i>cat</i> insertion	This work
<i>E. coli</i>		
CC104	Wild-type strain	31
CC104 <i>mutM mutY</i>	Δ <i>mutM</i> with mini-Kan insertion, Δ <i>mutY</i> with mini-Tn10 insertion	31
CC104 <i>mutM mutY</i> + pADC	Δ <i>mutM</i> Δ <i>mutY</i> transformed with control plasmid pADC	This work
CC104 <i>mutM mutY</i> + pADC-EcMutY	Δ <i>mutM</i> Δ <i>mutY</i> transformed with pADC-EcMutY	This work
CC104 <i>mutM mutY</i> + pADC-HpMutY	Δ <i>mutM</i> Δ <i>mutY</i> transformed with pADC-HpMutY	This work

Madison, WI) to create pUng, pMutY, and pXthA, respectively. Next, pMutY, with an internal BamHI site, was digested with BamHI and ligated to an *aphA* cassette conferring kanamycin resistance to create pMutYKm. Inverse PCR was performed on pUng (using primers *ung*inVBamHI-F and *ung*inVBamHI-R) and pXthA (with primers *xthA*inVBamHI-F and *xthA*inVBamHI-R) to introduce BamHI sites. The PCR products were digested and each subsequently ligated

with a *cat* cassette conferring chloramphenicol resistance to create pUngCat and pXthACat. *H. pylori* strain JP26 was then transformed with these plasmids to achieve kanamycin resistance, chloramphenicol resistance, or both, and transformants were selected on appropriate media to obtain the JP26 *mutY::aphA*, JP26 *ung::cat*, and JP26 *xthA::cat* mutants and JP26 *mutY::aphA ung::cat* and JP26 *mutY::aphA xthA::cat* double mutants (Table 1). Chromosomal DNA was

TABLE 2. Oligonucleotide primers used in this study

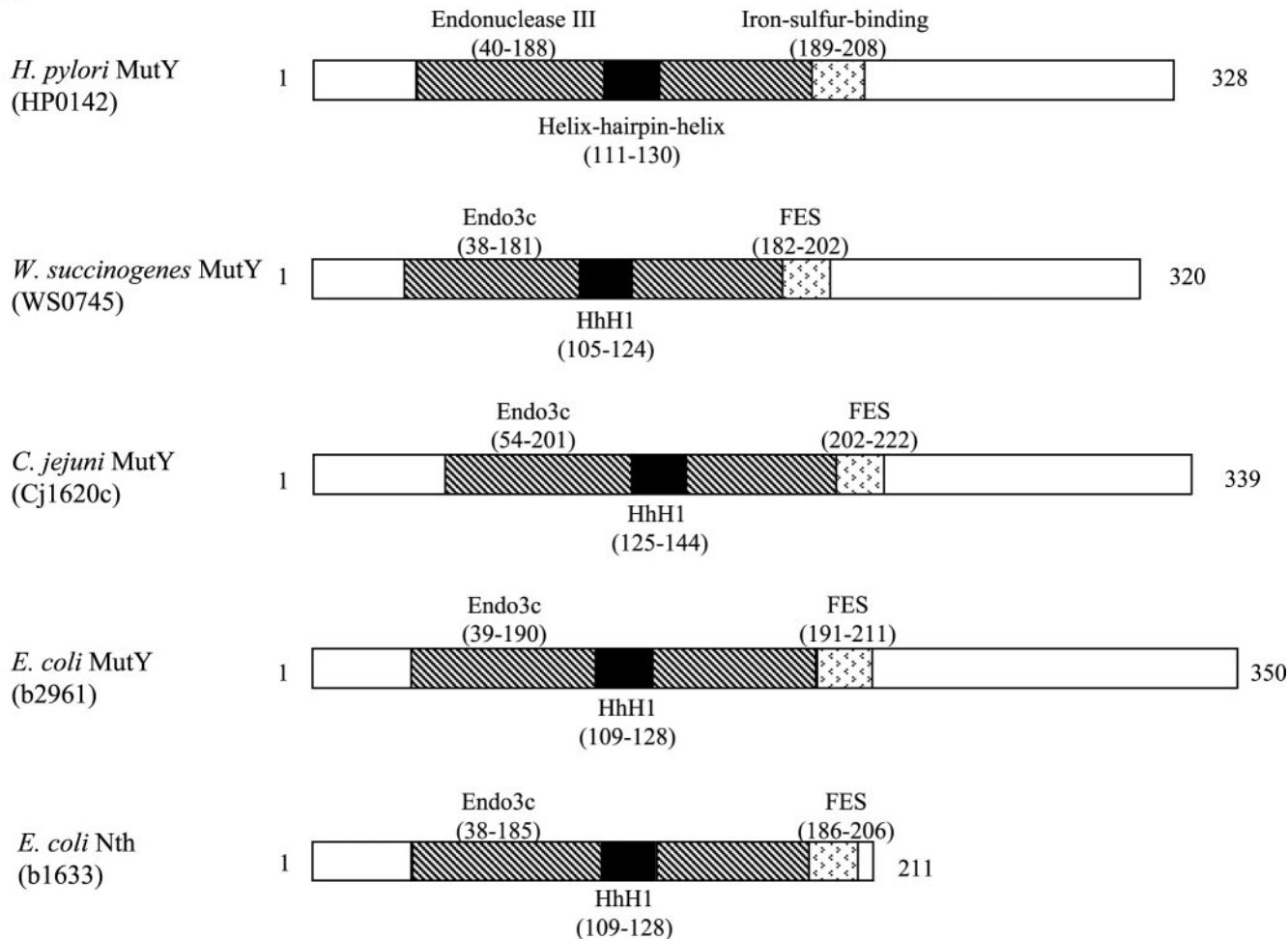
Organism and primer designation	Primer genomic location (nt) ^a	Primer sequence (5'→3') ^b	PCR product size (bp) ^c
<i>H. pylori</i>			
<i>mutY</i> -F	154662–154683	TGGTATGAAGAATTTGGGCGCA	911
<i>mutY</i> -R	153773–153794	AGCGTCATAGAGCTTATGGGTA	
<i>mutYXbaI</i> -F	154687–154712	<u>TCTAGATGGAACTTTACACAACGCCCTTTTA</u>	1,067
<i>mutYSmaI</i> -R	153646–153662	<u>CCCGGGAGTTGTGATCAGCGCGA</u>	
<i>mutYhomoA</i> -F	154470–154489	<u>AGTCCGAATTCCTTTATTGCTCTGGCGAGGGC</u>	112
<i>mutYhomoA</i> -R	154388–154410	<u>CGAGCTTCTAGATCATTGGGTAGTTGTGAGTGGTG</u>	
<i>mutYnoshift</i> -F	154349–154369	<u>AGTCCGAATTCACCTCCAGGGATTGGCGCATA</u>	112
<i>mutYnoshift</i> -R	154268–154291	<u>CGAGCTTCTAGACTTAAAAGCACGCGCTTGATATTA</u>	
<i>mutYnoshift-F+1</i>	154349–154370	<u>AGTCCGAATTCAACTCCAGGGATTGGCGCATA</u>	113
<i>mutYnoshift</i> -R	154268–154291	<u>CGAGCTTCTAGACTTAAAAGCACGCGCTTGATATTA</u>	
<i>ung</i> -F	1408140–1408161	<u>AAGGCACTAGAGGCAAAAGGAGG</u>	1,269
<i>ung</i> -R	1406893–1406915	<u>CGATATTTAGGGTTCTATACGC</u>	
<i>ung</i> inVBamHI-F	1407297–1407316	<u>AGCTGGATCCCCAAAACAAACACATCATC</u>	4,017
<i>ung</i> inVBamHI-R	1407604–1407621	<u>AGCTGGATCCACGCTAAAGCTCAACCCC</u>	
<i>xthA</i> -F	1605981–1606003	<u>CGTGTGGTATGTGTCAAAAAGGG</u>	1,230
<i>xthA</i> -R	1604774–1604793	<u>TTGCTGAAAACACATGGACG</u>	
<i>xthA</i> inVBamHI-F	1605263–1605284	<u>AGCTGGATCCGGCTTTAGCGATGAAGAGAGAG</u>	4,018
<i>xthA</i> inVBamHI-R	1605532–1605553	<u>AGCTGGATCCATATTAATACCATAGCTCACGC</u>	
<i>E. coli</i>			
<i>Ec-mutYXbaI</i> -F	3101002–3101020	<u>TCTAGATGCCCAACAACAGTGAA</u>	1,157
<i>Ec-mutYSmaI</i> -R	3102139–3102158	<u>CCCGGGACCTTCTGCTTCACGTTGCA</u>	

^a Locations for *H. pylori* primers are based on the sequenced *H. pylori* strain 26695 (60), and locations for *E. coli* primers are based on the sequenced *E. coli* strain K-12 MG1655 (6). nt, nucleotides.

^b The following restriction sites are underlined: BamHI (GGATCC), XbaI (TCTAGA), SmaI (CCCGGG), and EcoRI (GAATTC).

^c PCR product sizes apply to primer pairs.

A.



B.

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                                Endo3c
H. pylori 26695 : ..METLHNALLKYYEEFGRKGLDFRNLRKGINAPYEVYI SEVMSQQTQINIVIERFYSPFLEAFPTLKDLANA : 70
H. pylori J99   : ..METLHNALLKYYEEFGRKDLDFRNLRKGINAPYEVYI SEVMSQQTQISTVIERFYPPFLKAFPTLKDLANA : 70
E. coli K12-MG1655 : MQASQFSACVLDWYDQYGRKTLWQIDK...TPYKVMVLSVMTLQQTQVATVIP.YEERFMAREPPTVTDLANA : 68

                                HhH1
H. pylori 26695 : QLEEVLLWRGLGYYSRAKRLKRSABEICVKEHHSQLENDYQSLLKLPGLGAYTANAILCFGFREKRACVDAN : 142
H. pylori J99   : PLEEVLLWRGLGYYSRAKRLKRSABEICVKEHHSQLENDYQSLLKLPGLGAYTANAILCFGFREKSACVDAN : 142
E. coli K12-MG1655 : PLEEVLLHWITGLGYYSRAKRLKRAAQVATLGGKFEETFEVVAALPGVGRSTACAILSLSLGKHFPILDGN : 140

                                FES
H. pylori 26695 : IKRVLRLRGLDPNIHAKDLQIKANEFLNLNESFN...HNQALIDLGALIGS.PKPKCAICFFNPNYCLG.. : 207
H. pylori J99   : VKRVLRLRGLDPNIHAKDLQIKANDFLNLNESFN...HNQALIDLGALIGS.PKPKCAICFFNPNYCLG.. : 207
E. coli K12-MG1655 : VKRVLARCYAVSGWPGKKEVENKLSLSEQVTPAVGVVERFNQAMMDLGAMICTRSKPKCSLCLQNGCTAAA : 212

#
H. pylori 26695 : KNHLEKHTLKKKOEIIOBER..YLGVVIQNNQIALEKIEQKLYLGMHHPDLKEN.....LEFKL : 265
H. pylori J99   : KNHLEKHTLKKKOEIIOBER..YLGVVIQNNQIALEKIEQKLYLGMHHPDLKEN.....LEFKL : 265
E. coli K12-MG1655 : NNSWALYPGKPKOTLPERTGYFLLOHEDEVLLAQRPPSGLWCGELYCFEFADEESLRQWLQQRQIAADNL : 284

H. pylori 26695 : PFGAIKHSHTKSKLHLNLYSAAIKDLKNPVR....FVSLKDLLETLPSSMTLKLINFLKQKNLFGG : 328
H. pylori J99   : PFGTIKHSHTKSKLHLNLYLATTKDLKNPVR....FVSLKDLLETLPSSMTLKLINFLKQKNLFGG : 328
E. coli K12-MG1655 : TQITAFRHTFSSHLDIVPMWLPVSSFTGCMDEGNALWYNLAQPPSVGLAAPVERILQQRRTGAPV... : 350
    
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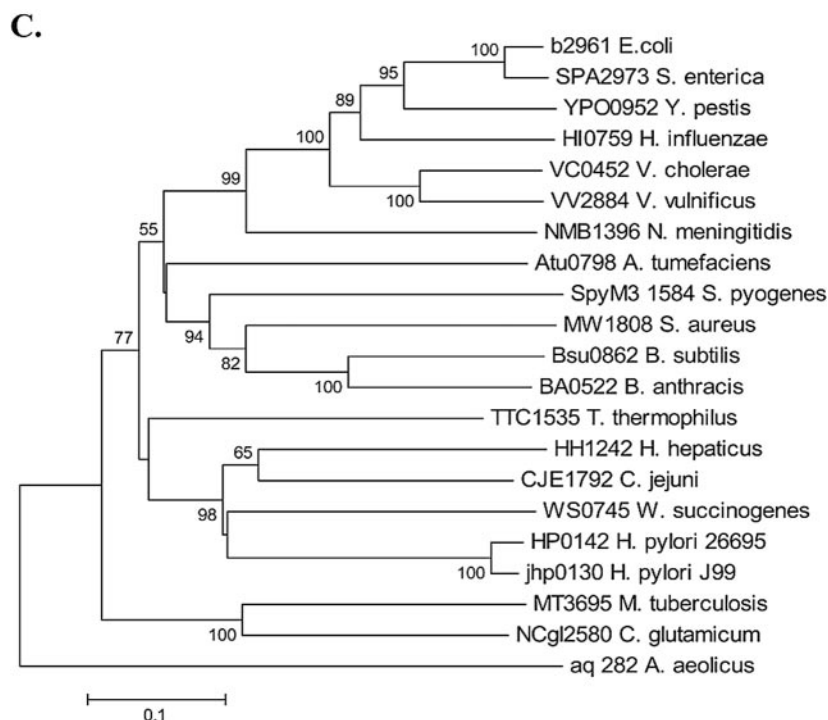


FIG. 1. Genetic analyses of MutY homologs. (A) Schematic of MutY homologs and *E. coli* endonuclease III (Nth). Conserved protein domains were identified using Pfam (14) and the SMART program (<http://smart.embl-heidelberg.de>) (28). (B) MutY amino acid alignment. *H. pylori* 26695 MutY and J99 MutY show 94.8% identity and 97.3% similarity, and *H. pylori* 26695 MutY and *E. coli* K-12 MG1655 MutY show 36.7% identity and 59.1% similarity. The helix-hairpin-helix (HhH1; ★), endonuclease (Endo3c; -), and iron-sulfur-binding (FES; #) domains of *H. pylori* MutY are labeled above the sequences. Residues are shaded as follows: black, 100% conservation; gray, similarity groups. (C) Phylogenetic analysis of MutY homologs. A phylogenetic tree was constructed with MutY amino acid sequences obtained from the Comprehensive Microbial Resource (<http://cmr.tigr.org>). The program MEGA 2.1 (26) was used to construct the phylogeny via the neighbor-joining method (45), with 1,000 bootstrap replicates. *S. enterica*, *Salmonella enterica*; *Y. pestis*, *Yersinia pestis*; *H. influenzae*, *Haemophilus influenzae*; *V. cholerae*, *Vibrio cholerae*; *V. vulnificus*, *Vibrio vulnificus*; *N. meningitidis*, *Neisseria meningitidis*; *A. tumefaciens*, *Agrobacterium tumefaciens*; *S. aureus*, *Staphylococcus aureus*; *B. subtilis*, *Bacillus subtilis*; *B. anthracis*, *Bacillus anthracis*; *C. glutamicum*, *Corynebacterium glutamicum*; *A. aeolicus*, *Aquifex aeolicus*.

isolated from the transformants, and the correct insertion of the *aphA* and/or *cat* cassette was confirmed by specific PCR in each case.

Assay to examine recovery from UV-induced DNA damage. *H. pylori* cells to be tested were grown on TSA plates for 48 h and suspended in phosphate-buffered saline (PBS), and 100 to 500 CFU was inoculated to TSA plates. Cells were exposed to UV radiation (0 to 1,380 kJ/m²) at a wavelength of 312 nm (Stratagene transilluminator) and incubated at 37°C in 5% CO₂ for 96 h. Colonies were counted and survival fractions calculated.

Assay to examine intergenomic recombination. *H. pylori* strains grown on TSA plates for 48 h were harvested in 1 ml of PBS, and 30 µl of the suspension was combined with 60 ng of donor DNA by spotting onto a TSA plate. The TSA plate was incubated for 18 h at 37°C in 5% CO₂. Donor DNA was an 800-bp PCR product of *H. pylori rpsL* from streptomycin-resistant strain JP26 with an A128G mutation (19). The transformation mixture was then harvested in 1 ml of PBS, and 100-µl portions of the appropriate serial dilutions were plated onto either TSA or brucella agar (BA) plates containing 10% newborn calf serum and 20 µg/ml streptomycin. The plates were incubated for 5 days at 37°C in 5% CO₂, and the total recombination frequency was determined by dividing the number of streptomycin-resistant colonies by the total CFU.

Assay of spontaneous mutation frequencies. Since single point mutations in *rpoB* confer *H. pylori* resistance to rifampin (18), rifampin resistance was used to assess spontaneous mutation frequencies. Rifampin-sensitive *H. pylori* strains were diluted onto TSA plates and grown for 5 days at 37°C in 5% CO₂. Colonies were picked and expanded onto nonselective TSA plates for 48 h of growth and then harvested in PBS and serially diluted onto TSA plates or BA plates containing 10% newborn calf serum and rifampin (10 µg/ml). Plates were incubated at 37°C in 5% CO₂ for 96 h, colonies counted, and spontaneous mutation frequencies calculated.

Complementation of the JP26 *mutY::aphA* mutant. Primers mutYXbaI-F and mutYSmaI-R (Table 2) were used to amplify ORF HP0142 (*mutY*) with JP26 genomic DNA as the template, and the products were ligated into pGEMT-Easy to create pMutYComp. Next, pMutYComp was digested with XbaI and SmaI to obtain the HP0142 fragment, which was ligated with XbaI- and SmaI-digested pADC containing *cat* in the *H. pylori ureAB* locus downstream of a *ureA* promoter (21). ORF HP0142 was thus inserted downstream of the *ureA* promoter, creating pADC-HpMutY. The HP0142 ORF was introduced *in trans* into the *ureAB* locus of JP26 *mutY::aphA* cells via natural transformation with pADC-HpMutY, creating JP26 mutYHpcomp. Transformants were selected for chloramphenicol resistance, and the correct insertion of HP0142 into *ureA* downstream of the *ureA* promoter was confirmed by PCR of the chromosomal DNA. The JP26 *mutY::aphA* strain complemented with *E. coli mutY* (JP26 mutYEccomp) was created using parallel methods. Primers Ec-mutYXbaI-F and Ec-mutYSmaI-R were used to amplify *mutY* from *E. coli* strain CC104, and then all other steps were completed as described above.

Cross-species complementation of MutY. To determine whether *H. pylori mutY* (HP0142) can functionally complement in *E. coli*, an *E. coli mutY mutM* mutant (31) was studied. *H. pylori* MutY from shuttle plasmid pADC-HpMutY was expressed using the *ureA* promoter. To assess spontaneous mutation frequencies, cells of rifampin-sensitive *E. coli* strains were diluted onto TSA plates and grown for 1 day at 37°C, and colonies were expanded onto TSA, allowing mutations to occur. After an additional 24 h of growth, cells were harvested into saline and serially diluted onto LB plates with and without rifampin (50 µg/ml). Plates were incubated at 37°C for 24 h, colonies counted, and spontaneous mutation frequencies calculated.

Shifts in *H. pylori mutY* homopolymeric tract. The 987-bp *H. pylori* 26695 and

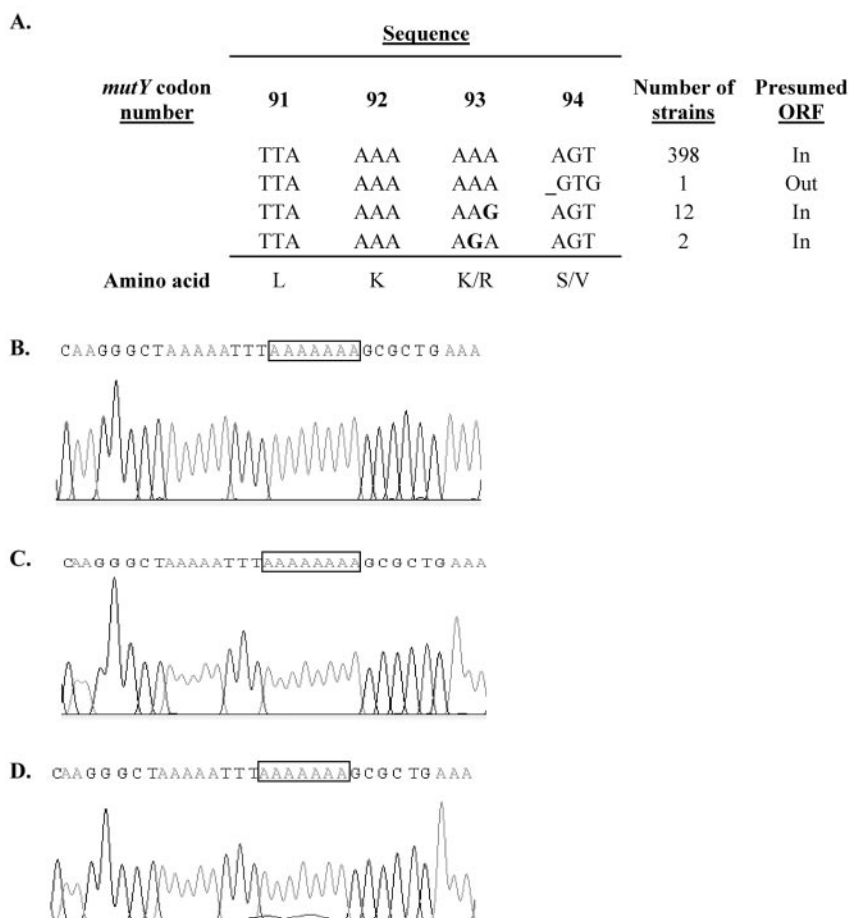


FIG. 2. Polymorphisms in *mutY*. (A) Genotypes of 413 *mutY* homonucleotide tracts from the *H. pylori* multilocus sequence typing database (<http://pubmlst.org/helicobacter/>). Substitutions are shown in bold, and the frameshift is depicted by _. (B) Chromatogram of *mutY* homonucleotide tract from strain ALA15. (C) Chromatogram of blue colony from *mutY* blue-white screen (eight adenines). (D) Chromatogram of white colony from *mutY* blue-white screen (seven adenines).

J99 *mutY* ORFs each include an eight-adenine homonucleotide tract beginning at position 273 (1, 60). Since partial *mutY* sequences of 413 *H. pylori* strains were available on <http://pubmlst.org/helicobacter/>, the homonucleotide region was examined for polymorphisms (database accessed March 2006). To determine whether frameshifts occur in the *mutY* homonucleotide tract in a population of cells growing on plate culture, a blue-white screen was developed. An ~100-bp fragment containing the *H. pylori mutY* homonucleotide tract was PCR amplified from JP26 genomic DNA by use of primers *mutY*homoA-F and *mutY*homoA-R with 5' EcoRI and XbaI sites, respectively (Table 2). The products were ligated into pUC18 to create pMutYhomoA-F, which was then transformed into DH5 α cells, inoculated to carbenicillin, IPTG (isopropyl- β -D-thiogalactopyranoside), and X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) plates, and incubated for 37°C overnight. After color development at 4°C, white and blue colonies, representative of out-of-frame and in-frame products, respectively, were counted. Representative white colonies were expanded, DNA prepared, and the pMutYhomoA-F inserts sequenced. A similarly sized fragment from *mutY*, but from a region without homopolymeric tracts, was amplified as a control. Primers *mutY*noshift-F and *mutY*noshift-R were used to generate the positive-control in-frame product, and primers *mutY*noshift-F+1 and *mutY*noshift-R were used to generate the negative-control out-of-frame product. The products were ligated into pUC18 to create pMutYnoshift and pMutYnoshift+1, and the plasmids were screened as described above.

Statistical analyses. Student's *t* test, unpaired with equal variance, was used to determine statistical significance in all cases. A *P* value of <0.05 was defined as statistically significant.

RESULTS

Analyses of MutY family homologs. The conserved domains in MutY, based on Pfam (14) and the SMART database (<http://smart.embl-heidelberg.de>) (28), are shown in schematic form for representative species in Fig. 1A. Protein structure is highly conserved. The 20-amino-acid helix-hairpin-helix is the canonical DNA binding domain shared by all BER glycosylases (10). Studies of *E. coli* MutY implicate the iron-sulfur-binding domain in substrate recognition and protein stability (31, 44). MutY orthologs possess an endonuclease III domain with homology to *E. coli* Nth, a glycosylase that has AP endonuclease activity, consistent with the reported MutY AP endonuclease activity (32, 33). On the amino acid level, the aligned *H. pylori* 26695 and J99 MutY deduced products show 94.8% identity and 97.3% similarity, and the *H. pylori* 26695 and *E. coli* K-12 MG1655 MutY deduced products share 36.7% identity and 59.1% similarity (Fig. 1B). The phylogenetic position of MutY from *H. pylori* is closest to that of other *Campylobacteriales* and is also related to that of *Thermus thermophilus*, an extremophile (Fig. 1C).

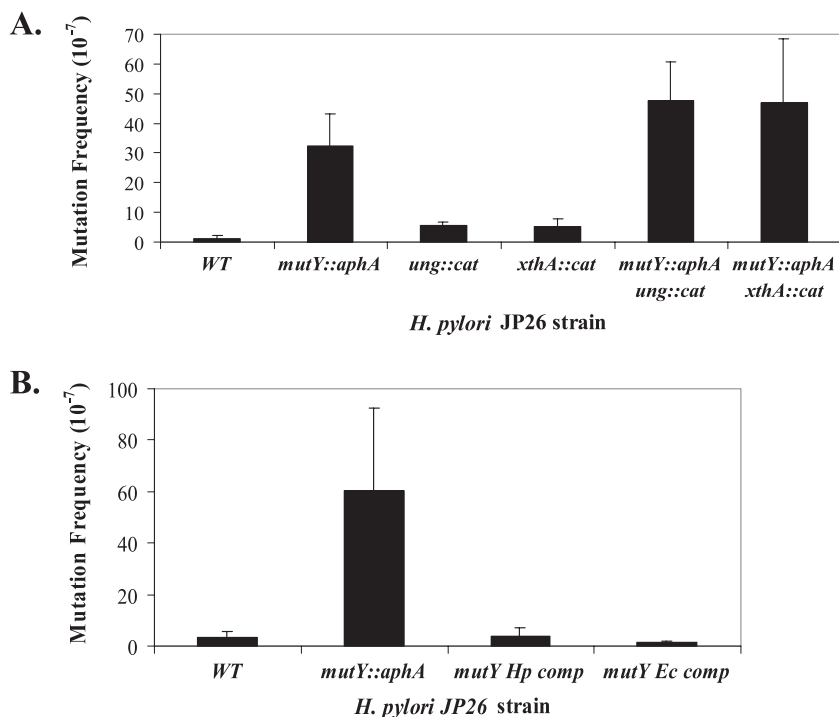


FIG. 3. Spontaneous mutation frequencies of *H. pylori* cells. (A) Wild-type (WT) and mutant strains. *H. pylori mutY* mutants showed a 26-fold increase in spontaneous mutation frequency ($P < 0.05$), and the *ung* and *xthA* mutants showed fourfold increases, as detected by resistance to rifampin. *mutY ung* and *mutY xthA* double mutants had 37-fold increases in the frequency of spontaneous mutation. Bars represent means \pm standard deviations for ≥ 6 replicate experiments. (B) Complementation of *H. pylori mutY* mutant phenotype. *H. pylori mutY* mutants were complemented by either *H. pylori* or *E. coli mutY*. Spontaneous mutation frequencies returned to wild-type levels. Bars represent means \pm standard deviations for ≥ 4 replicate experiments.

***H. pylori mutY* shifts in homonucleotide tract.** Of the 413 *mutY* sequences available in the *H. pylori* multilocus sequence typing database, polymorphisms involving the adenine homonucleotide tract were observed to occur in 15 strains (Fig. 2A). One (ALA15) of the 413 strains (2.4×10^{-3}) showed a frameshift with a 1-bp deletion in the eight-adenine *mutY* homonucleotide tract (Fig. 2B). There also were 14 (3.4×10^{-2}) substitution mutations at codon 93 (AAA) encoding lysine: 12 with synonymous (G) substitutions in the third position and 2 with nonsynonymous substitutions (G) in the second position, encoding arginine. In the blue-white screen for shifts in the *H. pylori mutY* homonucleotide tract, the positive control, with an in-frame nonhomonucleotide *mutY* fragment, gave 97% blue colonies and the negative control, with an out-of-frame nonhomonucleotide *mutY* fragment, gave 96.6% white colonies (data not shown). From the screen with the *mutY* homonucleotide fragment, two white colonies showed a shift from eight adenines (Fig. 2C) to seven (Fig. 2D), providing further evidence that natural phase variation occurs in *mutY*. Since these studies show that *H. pylori* subpopulations differing in expression of the product encoded by *mutY* exist, we sought to compare phenotypes of the on and off versions by creating defined mutants.

***H. pylori* glycosylase and AP endonuclease mutants show increased frequencies of spontaneous mutation.** Based on the homologies and phylogenies (Fig. 1 and data not shown), we hypothesized that the *ung* and *mutY* homologs in *H. pylori* encode DNA glycosylases, that *xthA* encodes an AP endonu-

lease, and that these genes play a role in *H. pylori* BER. To test these hypotheses, *H. pylori* mutants were created to examine frequencies of spontaneous rifampin mutations due to BER impairment, which permits retention of errors throughout the genome. Errors occurring in *rpoB* may lead to alterations in the rifampin binding site of RNA polymerase, rendering the cells resistant to rifampin (18). The *H. pylori ung* and *mutY* glycosylase mutants and the *xthA* AP endonuclease mutant each showed increased spontaneous mutation frequencies (Fig. 3A). Compared to the wild type, the *mutY* mutant showed an ~ 26 -fold increase, but for the *ung* and *xthA* mutants, the differences were fourfold. The *mutY ung* and *mutY xthA* double mutants had nearly identical increases in spontaneous mutations (37-fold) compared to that of wild-type cells. The results further indicate that MutY activity in *H. pylori* is not limited by lack of Ung or XthA base excision repair functions.

Complementation of the *H. pylori mutY* mutant with *mutY* in trans. The *mutY* homolog HP0142 is present in a putative dicistronic operon in strains 26695 and J99 with no downstream genes (60). To determine whether the spontaneous mutation phenotype was specific to the *mutY* mutation and not due to polar effects or artifact, the *H. pylori mutY::aphA* mutant was complemented in trans by expressing *mutY* downstream of a strong *ureA* promoter in the distant *ureAB* chromosomal locus. Complementation restored the spontaneous mutation frequency to wild-type levels (Fig. 3B), confirming that the observed phenotype is specific to the *mutY* mutation and not due to polar effects. The *H. pylori mutY::aphA* mutant was also

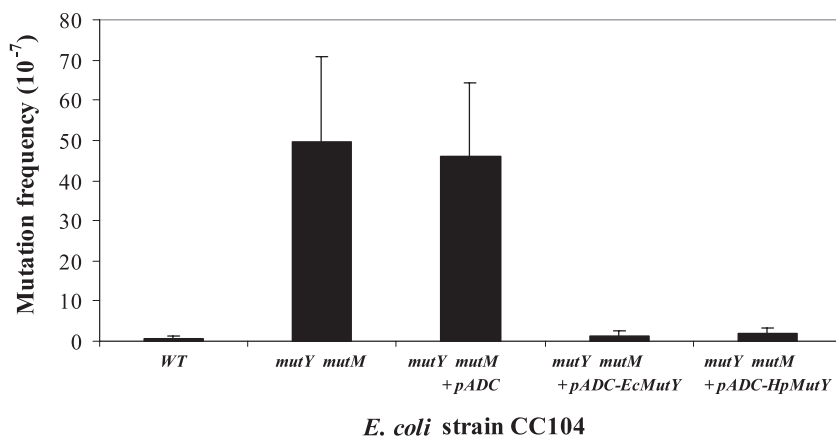


FIG. 4. Cross-species complementation of *E. coli mutY* mutant. The *E. coli mutY* mutant was complemented by *H. pylori mutY* expressed from plasmid pADC-HpMutY. Transformation with the control plasmid pADC had no effect, and the positive control (pADC-EcMutY) showed full complementation. Bars represent means \pm standard deviations for ≥ 11 replicate experiments. WT, wild type.

complemented in *trans* by *E. coli mutY* in the identical locus to a comparable degree.

***H. pylori MutY* complements *E. coli MutY* mutant.** To determine whether the *H. pylori MutY* protein could complement a MutY defect in *E. coli*, the *E. coli* CC104 *mutM mutY* mutant strain (31) was transformed with *H. pylori mutY* in a shuttle plasmid (pADC-HpMutY), with the vector alone (pADC), or with the *E. coli mutY* (pADC-EcMutY) as a positive control. Compared to the wild type, the *mutM mutY* mutant showed a 73-fold increase in the frequency of spontaneous mutation, which was not complemented by the vector alone. However, both *E. coli* and *H. pylori mutY* nearly completely complemented the defect (Fig. 4). In total, the cross-species complementation studies provide further evidence for MutY function encoded by HP0142.

***H. pylori ung* and *mutY* glycosylase and *xthA* AP endonuclease mutants are no more sensitive to UV-induced DNA damage than the wild type.** Exposure to UV generates primarily

cyclobutane pyrimidine dimers and 6-4 pyrimidine-pyrimidone photoproducts (7), substrates that are not recognized by *E. coli* Ung and MutY. As such, we hypothesized that the *H. pylori* glycosylase mutants are no more sensitive to UV-induced DNA damage than wild-type cells. Furthermore, UV radiation creates interstrand cross-links in DNA as well as strand breaks that can halt replication fork progression; the DNA template can be restored by recombination repair recognizing and replacing damaged DNA. As expected, *H. pylori recA* mutants were more susceptible to UV exposure than wild-type cells (Fig. 5) (59). However, the *ung*, *mutY*, and *xthA* mutants did not show increased susceptibility to UV, providing evidence that these constituents of BER are not involved in recombination repair of damaged DNA in *H. pylori*.

***H. pylori* glycosylase mutants do not show elevated frequencies of recombination.** Glycosylases function not only in BER but also in homologous recombination. Nitric oxide is a potent DNA damaging agent, and NO^- -induced homologous recom-

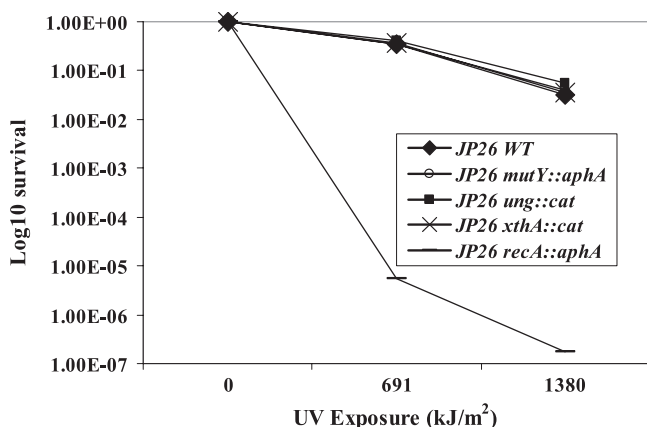


FIG. 5. Survival of *H. pylori* wild-type (WT) and mutant strains after UV exposure. *H. pylori* wild-type and mutant strains were exposed to 312-nm UV (0 to 1,380 kJ/m²), and survival fractions were determined. As expected, the positive control, the JP26 *recA* mutant, showed >4 -log₁₀ sensitivity. However, there were no significant differences between the wild type and the *mutY*, *ung*, and *xthA* mutants.

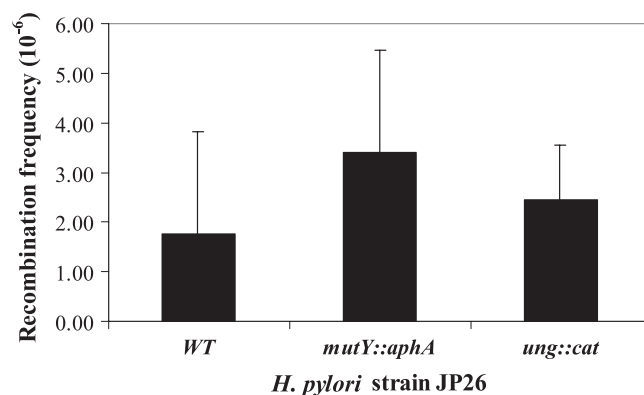


FIG. 6. Intergenomic recombination frequencies of *H. pylori* wild-type (WT) and mutant strains. Ability to be transformed by an 800-bp PCR product that confers streptomycin resistance was used to determine recombination frequency. *H. pylori* JP26 *mutY* and *ung* mutants did not show elevated frequencies of intergenomic recombination compared to the wild-type JP26 strain. Bars represent means \pm standard deviations for ≥ 4 replicate experiments.

TABLE 3. Homonucleotide tracts in glycosylase genes of different organisms

Phylum	Class	Order	Organism and strain	ORF designation/tract characteristic(s) ^a for glycosylase gene				
				<i>mutY</i>	<i>ung</i>	<i>hth</i>		
Proteobacteria	Epsilonproteobacteria	Campylobacteriales	<i>Helicobacter pylori</i> 26695	HP0142/A ₈ ; A ₇	HP1347/A ₇ × 3	HP0585/—		
			<i>Helicobacter pylori</i> 26695			HP0602/A ₈ ; A ₇ ; T ₇ × 2		
			<i>Helicobacter pylori</i> J99	jhp0130/A ₈ ; A ₇	jhp1266/A ₇ × 3	jhp0532/—		
			<i>Helicobacter pylori</i> J99			jhp0549/A ₇ × 2; T ₇ × 2		
			<i>Helicobacter hepaticus</i> ATCC 51449	HH1242/A ₈ ; A ₇	HH1249/—	HH1575/A ₈ ; A ₇		
			<i>Campylobacter jejuni</i> NCTC 11168	Cj1620c/A ₈ ; A ₇ × 3; T ₇ × 2	Cj0086c/A ₈ ; A ₇	Cj0595c/T ₇		
			<i>Campylobacter jejuni</i> RM1221	CJE1792/A ₈ ; A ₇ × 3; T ₇ × 2	CJE0081/A ₈ ; A ₇	CJE0698/T ₇		
			<i>Molihella succinogenes</i> DSMZ 1740	WS0745/—	WS0557/—	WS1060/A ₇		
			Gammaproteobacteria	Enterobacteriales	<i>Escherichia coli</i> K-12 MG1655	b2961/—	b2580/—	b1633/—
					<i>Salmonella enterica</i> serovar Paratyphi ATCC 9150	SPA2973/A ₇	SPA0271/—	SPA1400/—
<i>Yersinia pestis</i> KIM	y3339/A ₇	y1281/C ₇			y2080/—			
<i>Haemophilus influenzae</i> KW20 Rd	HI0759/—	HI0018/A ₈			HI1689/—			
<i>Vibrio cholerae</i> El Tor N16961	VC0452/—	VC2359/—			VC1101/—			
<i>Legionella pneumophila</i> Lens	lp10930/A ₆ ; A ₈ ; T ₈ ; T ₇	*			lp12793/A ₇			
<i>Neisseria meningitidis</i> MC58	NMB1396/A ₇	NMB1222/—			NMB0533/—			
Chlamydiae	Chlamydiales	<i>Chlamydia abortus</i> S26 3			CAB380/A ₇	CAB954/—	CAB898/A ₆ ; T ₇	
Deinococcus-Thermus	Deinococci	Thermales			<i>Thermus thermophilus</i> HB8	TTHA1898/—	*	TTHA0112/—
Actinobacteria	Actinobacteria	Actinomycetales	<i>Streptomyces avermitilis</i> MA-4680	SAV4707/—	SAV1527/—	SAV4593/—		
			<i>Mycobacterium tuberculosis</i> H37Rv	Rv3589/—	Rv2976c/—	Rv3674c/—		
Firmicutes	Bacilli	Bacillales	<i>Listeria innocua</i> CLIP 11262	lin1797/A ₇	lin0408/—	lin2008/A ₇ ; T ₇		
			<i>Bacillus subtilis</i> 168	*	Bsu3793/—	Bsu2233/—		
			<i>Bacillus anthracis</i> Ames	BA0522/—	BA5648/T ₇	BA1570/—		
			<i>Streptococcus pneumoniae</i> R6	spr1108/A ₇	spr1055/—	spr1157/A ₇		
			<i>Streptococcus pyogenes</i> MGAS315	SpvM3_1584/—	SpvM3_0621/—	SpvM3_0642/—		

^a Tract composition, size, and occurrence (e.g., × 3 denotes three occurrences) are shown. —, no tract with ≥ 7 of the same nucleotide; *, no annotated ORF.

ination in *E. coli* is promoted by DNA glycosylases (55). Processing of DNA base damage by glycosylases leads to the creation of AP sites and single-strand breaks, which in turn may be converted into double-strand breaks, providing substrate for recombination. However, the *H. pylori ung* and *mutY* mutants did not show elevated frequencies of intergenomic recombination (Fig. 6).

Homonucleotide tracts in other bacterial glycosylase genes.

To determine whether the presence of homonucleotide tracts in the *H. pylori* glycosylase genes may be more prevalent, using the whole-organism genomic sequences, we conducted informatic analysis of other members of *Campylobacterales* and other eubacteria (Table 3). Among the other *Campylobacterales*, homonucleotide tracts of ≥ 7 nucleotides were common in ORFs annotated as *mutY*, *ung*, or *nth* in *Campylobacter jejuni* and *Helicobacter hepaticus* but not *Wolinella succinogenes*. Similarly, although homonucleotide tracts were common in other phyla, they were not universally present, and in certain organisms, including *E. coli*, *Mycobacterium tuberculosis*, and *Streptococcus pyogenes*, none were observed.

DISCUSSION

HP0142 was annotated as *mutY* based on the strong homology of its deduced product with known MutY proteins. We show that the protein sequence possesses conserved domains and amino acid properties characteristic of MutY and that the cloned ORF complements the *mutY* deficiency in an *E. coli* mutant. Interrupting *mutY* in *H. pylori* resulted in elevated frequencies of spontaneous mutation, consistent with a role in BER, and in *trans* complementation with either the *H. pylori* or the *E. coli mutY* confirmed that this phenotype was not due to a polar or extraneous event. Moreover, the mutant's lack of an effect on UV repair, as expected, indicates the specificity of its role. Thus, HP0142 is an authentic *mutY*, but although *H. pylori mutY* was able to complement the *E. coli mutY* mutant, the two proteins may not have all of the same functions. The C-terminal domain of *E. coli* MutY is involved in both DNA binding and glycosylase activities (29), and future experiments should define the properties of the nonconserved C-terminal domain of *H. pylori* MutY. The close proximity of the phylogenetic position of *H. pylori* MutY with that of the extremophile *T. thermophilus* suggests convergent evolution due to parallel environmental constraints.

Exposure to UVB (wavelength range, 280 to 320 nm) is known to generate singlet oxygen, superoxide ions, and other free radicals, which subsequently increase 8-oxoguanine levels in cells (17, 42, 64). In mice lacking Ogg1, a mammalian glycosylase responsible for removing 8-oxoguanine from DNA, UVB exposure increases susceptibility to skin carcinogenesis (27). The *E. coli xthA* mutant is sensitive to inactivation by broad-spectrum near-UV (300- to 400-nm) radiation (49, 51). Therefore, the *H. pylori* BER mutants might be similarly sensitive to UV, as glycosylases and AP endonucleases repair the DNA damaged by oxidative by-products. Under our experimental conditions, in which 312-nm UV light was used, neither the *mutY* nor the *xthA H. pylori* mutant differed from wild-type cells in phenotype. It is possible that the experimental conditions did not generate sufficient 8-oxoguanine in the cells for a phenotype to be observed, that *H. pylori* has pathways to ca-

tabolize 8-oxoguanine, or that 8-oxoguanine is not a major substrate for *mutY* and *xthA* in *H. pylori*.

The elevated spontaneous mutation frequencies of *H. pylori* strains in which the genes encoding the glycosylases Ung and MutY and AP endonuclease XthA have been interrupted can be explained in several ways. First, loss of glycosylase and AP endonuclease function impairs the BER pathway, leading to increased spontaneous mutation frequency as errors are retained throughout the genome. However, the fact that the *mutY* strain has a greater effect (26-fold) than the *ung* or *xthA* strains (fourfold) implies that MutY has greater in vivo glycosylase activity than Ung in *H. pylori* and/or that the MutY substrate is generated more frequently than is the Ung substrate and that the BER products generated by *H. pylori* MutY activity may be substrates for AP endonucleases other than XthA. If the products generated by *H. pylori* MutY were substrates for XthA only, then mutations in the downstream gene in the pathway (*xthA*) also should yield a similar (26-fold) increase in spontaneous mutation frequency. That the *xthA* mutation resulted in a smaller (fourfold) difference in mutation frequency suggests the presence of other AP endonucleases in *H. pylori*. Alternatively, it is possible that *H. pylori* MutY has its own AP endonuclease activity, as has been shown for the *E. coli* MutY (32, 33), which has structural similarity to *H. pylori* MutY (Fig. 1B). Furthermore, that MutY may function independently of Ung and XthA is supported by the enhanced mutation frequency in the *mutY ung* and *mutY xthA* double mutants.

Based on phylogenomic studies, *H. pylori* has had loss of DNA repair pathway components, including the methyl-directed mismatch repair system found in *E. coli* (12). Nevertheless, it is possible that *H. pylori* has an unidentified mismatch repair system (8). *E. coli* MutY is responsible not only for repair of oxidative DNA damage but for mismatch repair as well. MutY processes at least some A · G mispairs in *E. coli* (2), and the lack of MutY activity generates G · C \rightarrow T · A transversions, resulting in *rpoB* mutations that confer rifampin resistance (24). That *H. pylori* MutY was capable of complementing the *E. coli mutY* mutant suggests that it can play a similar role in mismatch repair preventing G · C \rightarrow T · A transversion mutations, if such a system does exist in *H. pylori*.

Phase variation, a common mechanism used by gram-negative bacteria to generate intrastain diversity, permits phenotypic variation important to adaptation to niches or to changing environmental conditions (48, 62). Translational phase variation reversibly switches gene expression via insertion or deletion of DNA repeat sequences within the coding region, altering the reading frame, which ultimately leads to a premature stop codon (8, 62). *H. pylori* strains vary both in the presence of repeats in particular genes and in length (48). That homopolymeric tracts are present in both *mutY* and *ung* (Table 3) suggests the possibility of phase variation, and the existence of a wild-type *H. pylori* strain (ALA15) with a seven-adenine tract in *mutY*, along with experimental evidence of frameshift, indicates that this is an actual mechanism for *H. pylori* to control DNA repair rates. Variation in repeat length may be generated by polymerase slippage during PCR and/or sequencing reactions, and PCR has the greatest potential to artifactually generate such variation (20). However, studies of length variation in the homonucleotide tract of *H. pylori* ORF HP0619

in strain JP96-9, possessing nine cytosines, provide evidence that for repeats of <11 nucleotides the observed polymorphisms are attributable to repeats of different lengths in the template and not to a PCR artifact (48). The phenomenon of phase variation in *H. pylori* has been explored previously for genes *pldA*, *babB*, and *rfaJ* (47). In recent work using enzyme-linked immunosorbent assay techniques in addition to the previously described *E. coli* reporter assay, we have examined the phenotypes associated with phase variation involved with a homopolymeric tract in *futC*, which controls Lewis^x expression in *H. pylori*. This provides a measurable phenotype and illustrates the effect of the phase variation in an *H. pylori* background, as well as the efficacy of the *E. coli* assay (E. L. Sanabria-Valentín et al., Abstr. 106th Gen. Meet. Am. Soc. Microbiol., abstract D-001, 2006).

Phase variation of *mutY* offers a model for *H. pylori* adaptation. Although mutations are generally deleterious, spontaneous *mutY* frameshifts create subpopulations of hypermutators that can generate beneficial mutations under appropriate selection conditions. As hypermutators acquire the mutations needed to adapt to changing environments, the increasing number of deleterious mutations makes hypermutability selectively disadvantageous (11, 13, 15, 61). Therefore, persistent *H. pylori* colonization can be viewed as comprising two populations, mutator and nonmutator, living in an equilibrium determined by the fitness cost and benefit of particular mutations. Since phase variation may occur independently for *mutY* and *ung*, there may be at least four different populations (and possibly more in relation to other genes) in terms of "mutator" status. This is a more complex model than that appreciated for *E. coli* (38, 39, 52), but it might reflect the differing selective pressures on *H. pylori*.

Slipped-strand mispairing in homonucleotide tracts of DNA repair genes (e.g., *mutY*) also can allow for out-of-frame, hypermutator populations that have already acquired the beneficial mutations to regenerate nonhypermutator populations. Since the advantage of mutators is conditional, because mutator clones easily accumulate deleterious mutations after passage through bottlenecks (13), the reversion of an out-of-frame *mutY* to in frame after a beneficial mutation is acquired might be advantageous. Ultimately, the existence of homonucleotide tracts in *mutY* (and other BER genes) reflects second-order selection. More than just selection for better adaptation to a specific environment, second-order selection acts on the regulation of the processes of genetic adaptation to new environments (43). DNA repair genes with homonucleotide tracts reflect second-order selection because they give rise to hypermutator alleles that themselves are the substrates for selection. That *H. pylori mutY* possesses a phase-variable homonucleotide tract indicates that expression of the products of DNA repair genes is under the influence of replication genes, themselves subject to mutation. This nonlinear system for DNA repair, exemplified in *H. pylori*, could be a model for certain organisms but not others (Table 3).

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