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Structural and Functional Divergence of MutS2 from Bacterial MutS1 and Eukaryotic MSH4-MSH5 Homologs†

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MutS homologs, identified in nearly all bacteria and eukaryotes, include the bacterial proteins MutS1 and MutS2 and the eukaryotic MutS homologs 1 to 7, and they often are involved in recognition and repair of mismatched bases and small insertion/deletions, thereby limiting illegitimate recombination and spontaneous mutation. To explore the relationship of MutS2 to other MutS homologs, we examined conserved protein domains. Fundamental differences in structure between MutS2 and other MutS homologs suggest that MutS1 and MutS2 diverged early during evolution, with all eukaryotic homologs arising from a MutS1 ancestor. Data from MutS1 crystal structures, biochemical results from MutS2 analyses, and our phylogenetic studies suggest that MutS2 has functions distinct from other members of the MutS family. A *mutS2* mutant was constructed in *Helicobacter pylori*, which lacks *mutS1* and mismatch repair genes *mutL* and *mutH*. We show that MutS2 plays no role in mismatch or recombinational repair or deletion between direct DNA repeats. In contrast, MutS2 plays a significant role in limiting intergenomic recombination across a range of donor DNA tested. This phenotypic analysis is consistent with the phylogenetic and biochemical data suggesting that MutS1 and MutS2 have divergent functions.

MutS homologs (MSH) have been identified in most prokaryotic and all eukaryotic organisms examined. Prokaryotes have two homologs (MutS1 and MutS2), whereas seven MSH proteins (MSH1 to MSH7) have been identified in eukaryotes (16, 19, 23). The homodimer MutS1 and heterodimers MSH2-MSH3 and MSH2-MSH6 are primarily involved in mitotic mismatch repair, whereas MSH4-MSH5 is involved in resolution of Holliday junctions during meiosis (1, 64). All members of the MutS family contain the highly conserved Walker A/B ATPase domain (16), and many share a common mechanism of action. MutS1, MSH2-MSH3, MSH2-MSH6, and MSH4-MSH5 dimerize to form sliding clamps, and recognition of specific DNA structures or lesions results in ADP/ATP exchange (27, 45, 49, 64).

The function of the second prokaryotic homolog, MutS2, is unknown. Sequence analyses reveal fundamental differences between MutS2 and other MutS family members (19). MutS2 proteins contain a conserved C-terminal domain of ~250 amino acid residues not found in other MutS homologs and lack the conserved N-terminal region present in most of the other MutS family members (43). According to one hypothesis, MutS2 is more closely related to the meiotic recombination proteins MSH4 and MSH5, while MutS1 is more closely related to MSH2, -3, and -6 (19). This hypothesis suggests a gene duplication event early in the evolution of MutS, resulting in the two main MutS lineages, with MSH4 and MSH5 branching with MutS2 and MSH2, -3, and -6 branching with MutS1. Consistent with this hypothesis, MutS2 has been shown to not play a role in mismatch repair (13, 59, 69). However, arguing against this hypothesis is the lack of homology between MutS2 and MSH4-MSH5. According to another hypothesis, all eukaryotic MutS homologs evolved from one ancestor, MutS1, and MutS2 diverged well before the MutS1 homolog was introduced into eukaryotes (16). Based on that hypothesis, MutS2 may have a function distinct from that of MutS1 as well as MSH4-MSH5.

To explore these hypotheses further, we examined the domains conserved between all MutS homologs. To study the function of MutS2, we constructed a *mutS2* mutant in the gram-negative bacterium *Helicobacter pylori. H. pylori* is a common colonizer of the human gastric mucosa (52) and has a high level of genetic diversity among isolates from unrelated hosts, as well as among isolates from a single host (35, 61, 63). Consistent with this diversity, *H. pylori* lacks homologs to the mismatch repair protein MutS1 and interacting components MutH and MutL (4, 68), making it an ideal candidate for study of MutS2 function.

In this report, we provide evidence that the MutS2 is substantially diverged from MutS1 and the eukaryotic MSH proteins, and we confirm that MutS2 is not involved in mismatch repair nor in recombinational repair and deletions between direct repeats. We demonstrate that *mutS2* mutants have a significantly increased frequency of intergenomic recombination compared to wild-type strains. Although MutS2 and other MutS homologs share a common overall function of preserving genomic integrity, they have substantial differences not only in structure but also in function. Our results provide evidence against previous hypotheses proposing that MutS2 is closely related to MSH4-MSH5 and instead suggest that MutS2 has a separate function.

MATERIALS AND METHODS

Amino acid alignment and phylogenetic analyses of MutS homologs. Amino acid sequences of MutS1, MutS2, and eukaryotic MutS homologs were retrieved

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† Supplemental material for this article may be found at http://jb.asm.org/.

TABLE 1. Plasmids and *H. pylori* strains used in this study

Plasmid or strain	Relevant characteristics	Source or reference
Plasmid		
pVacAKm	*vacA*::*aphA* in pGEMT-Easy	33
pRecAKm	*recA*::*aphA* in pGEMT-Easy	33
pMutS2	HP0621 in pGEMT-Easy	This work
pMutS2Km	*mutS2*::*aphA* in pGEMT-Easy	This work
pVacA0	*vacA* with 0-bp deletion cassette	6
pVacA50	*vacA* with 50-bp deletion cassette	6
pVacA100	*vacA* with 100-bp deletion cassette	6
pMutS2-0	*mutS2* with 0-bp deletion cassette	This work
pMutS25-0	*mutS2* with 50-bp deletion cassette	This work
pMutS2-100	*mutS2* with 100-bp deletion cassette	This work
pAD1	*ureAB* fragment in pUC18	4
pADC-MutS2	pAD1 with *mutS2* and CAT cassette	This work
pRpsLWT	800-bp *rpsL* fragment in pGEM3-ZF	This work
pRpsLSS	800-bp *rpsL* fragment (A128G) in pGEM3-ZF	This work
pAD1-Cat	pAD1 with CAT cassette	This work
H. pylori strain		
JP26	Wild-type strain	33
JP26/*vacA*::*aphA*	Δ*vacA* with *aphA* insertion	33
JP26/*recA*::*aphA*	Δ*recA* with *aphA* insertion	33
JP26/*mutS2*::*aphA*	Δ*mutS2* with *aphA* insertion	This work
JP26 *mutS2*comp	*mutS2*::*aphA* complemented with *mutS2* in *ureAB* locus	This work
JP26/*vacA*::0	*vacA* interrupted with 0-bp deletion cassette	6
JP26/*vacA*::50	*vacA* interrupted with 50-bp deletion cassette	6
JP26/*vacA*::100	*vacA* interrupted with 100-bp deletion cassette	6
JP26/mutS2::0	*mutS2* interrupted with 0-bp deletion cassette	This work
JP26/mutS2::50	*mutS2* interrupted with 50-bp deletion cassette	This work
JP26/mutS2:100	*mutS2* interrupted with 100-bp deletion cassette	This work

from GenBank (www.ncbi.nih.gov). Conserved domains of MutS1, MutS2, and eukaryotic MutS homologs were identified with the Simple Modular Architectural Research Tool (SMART) program (http://smart.embl-heidelberg.de) (42), which encompasses several protein domain databases. Sequences with homology to the small MutS-related (SMR) domain were retrieved using PSI-BLAST (5). Amino acid sequences were aligned using ClustalX (66) with the Gonnet 250 protein weight matrix and visualized with Genedoc (www.psc.edu/biomed/genedoc). Phylogenetic trees were constructed with the program Mega 2.1 (38, 39), by using the neighbor-joining method (60) with 1,000 bootstrap replicates.

Bacterial strains and plasmids. The *H. pylori* strains used in this study (Table 1) were routinely grown on Trypticase soy agar (TSA) plates at 37°C in a 5% CO_2 incubator. *Escherichia coli* strain HB101 was used for construction and cloning of the plasmids (Table 1) and was grown in Luria-Bertani media at 37°C.

Construction of *H. pylori* mutants used to assess susceptibility to UV, intergenomic recombination frequencies, and spontaneous mutation frequencies. A fragment of HP0621 (*mutS2* homolog) was amplified by PCR using primers MutS3 and MutS5, based on sequenced strain 26695 (Table 2) and cloned into pGEMT-Easy (Promega, Madison, WI) to create pMutS2. Inverse PCR using primers MutinvF1 and MutinvR1 (Table 2) created BamHI restriction sites, and the *aphA* cassette, conferring kanamycin resistance (Km^r), was used to interrupt the open reading frame (ORF) to create pMutS2Km. Since it is not involved in recombination, the *vacA* locus (HP0887) was chosen as a control for the presence of the *aphA* cassette (8, 33). The *recA* locus (HP0153) was interrupted as another control, since several phenotypes related to this study have been characterized (7, 67). pVacAKm and pRecAKm, as previously constructed (33), were used to transform HP strain JP26, as described previously (71), to create JP26 *vacA*::*aphA* and JP26 *recA*::*aphA*. Strain JP26 also was transformed to Km^r with pMutS2Km to create JP26 *mutS2*::*aphA*. Chromosomal DNA was isolated from the putative mutant strains, and the correct insertion of the *aphA* cassette into the expected ORF was confirmed by PCR in each case, as described previously (33).

Construction of *H. pylori* mutants to assess intragenomic deletion frequencies. A unique BamHI site was created in pMutS2 and pVacA using inverse PCR, with primers based on sequenced strain 26695 (Table 2), and each ORF was subsequently interrupted with a deletion cassette containing either 0 (control)-, 50-, or 100-bp repeats to create pMutS2-0, -50, and -100 and pVacA-0, -50, and -100. The construction and use of the deletion cassettes has been described previously (8). *H. pylori* strain JP26 was subsequently transformed to chloramphenicol resistance (Cat^r) with these plasmids by natural transformation to create JP26 MutS2::0, -50, and -100 and JP26 *vacA*::0, -50, and -100. Chromosomal DNA was isolated from mutant strains, and the correct insertion of the deletion cassette into the expected ORF was confirmed by PCR in each case.

Assay of intragenomic deletion frequencies. To assess recombination frequencies in the *H. pylori* strains containing the deletion or control cassettes, the cells were grown on TSA plates for 48 h at 37°C (5% CO_2), allowing for deletions to occur, and then harvested, washed twice in phosphate-buffered saline (PBS), and

TABLE 2. Oligonucleotide primers used in this study

Primer designation	Genomic location[a]	Primer sequence (5′ → 3′)[b]
MutS5	668833–668855	AAGAGATCGCTTTGCAAAGAGC
MutS3	666264–666287	GCTAAAGATTTTAAGGGTTTTGG
MutinvF1	667275–667296	CGGGATCCTGAAGAAAAAGAACGGCCCAC
MutinvR1	667337–667354	CGGGATCCCTGCCATTAACACGCTCAAGC
MutSXbaI	668698–668679	GCTCTAGATGTCAGACGCTCCAAAAAG
MutSSmaI	666281–666296	TCCCCCGGGTTTTGGCTCAATAGC

[a] Location based on the sequence of strain 26695 (68).
[b] Restriction sites underlined; EcoRI (GGATCC), XbaI (TCTAGA), and SmaI (CCCGGG).

spread in 25-, 100-, and 200-μl aliquots on brucella agar (BA) plates supplemented with newborn calf serum (NCS) and 25 μg/ml kanamycin, as described previously (8, 33). As further controls, 200 ul from each suspension was inoculated to BA plates containing NCS, kanamycin (25 μg/ml), and chloramphenicol (20 μg/ml); as expected, in no experiments were strains with double resistance identified, confirming the specificity of the deletion process (8). Total CFU and numbers of Km^r deletion mutants were determined by plating serial dilutions onto TSA plates or TSA plates with kanamycin (25 μg/ml), respectively. Plates were incubated at 37°C in 5% CO_2 for 96 h, colonies were counted, and deletion frequencies were calculated.

Assay of spontaneous mutation rates. To assess spontaneous mutation rates in *H. pylori*, rifampin-sensitive *H. pylori* strains were diluted onto TSA plates and grown for 5 days at 37°C (5% CO_2). Since a point mutation in *rpoB* confers resistance to rifampin (29), rifampin resistance was chosen as a marker for spontaneous mutation. For each strain, nine colonies were chosen and expanded onto new TSA plates, allowing mutations to occur. After an additional 48 h of growth, strains were harvested into saline and serially diluted onto either BA plates containing 10% NCS and rifampin (7.5 μg/ml) or TSA plates. Plates were incubated at 37°C (5% CO_2) for 96 h, colonies were counted, and spontaneous mutation frequencies were calculated. A total of nine spontaneous mutation frequencies were calculated for both wild-type and *mutS2*::*aphA H. pylori* strains, and an algorithm based on the method of Lea and Coulson was used to determine rates (41).

Assay of recovery from DNA damage. *H. pylori* cells to be tested were grown on TSA plates for 48 h and suspended in 1 ml brucella broth. Equal amounts of suspension were inoculated on TSA plates to produce 100 to 500 CFU per plate. Cells then were exposed to UV at 312 nm (Stratagene Transluminator; Stratagene, La Jolla, CA) for 0 to 90 s and then incubated at 37°C in 5% CO_2 for 96 h. Colonies were counted and percent survival was calculated. Ciprofloxacin E-test strips (AB Biodisk, Solna, Sweden) were used to determine MICs for both wild-type and mutant *H. pylori* strains, according to the manufacturer's instructions. Plates were incubated for 48 h, and MIC determinations were repeated at least five times for each sample.

Assays of intergenomic recombination using PCR fragments and plasmids as donor DNA. *H. pylori* strains were grown on TSA plates at 37°C (5% CO_2). After 48 h, the recipient *H. pylori* cells were harvested into 1 ml of PBS, and 50 μl of the resulting suspension was combined with 100 ng donor DNA by spotting onto a TSA plate. Donor DNA was either an 800-bp PCR product of *H. pylori rpsL* from streptomycin-resistant (St^r) strain JP26 with A128G, a plasmid (pRpsLSR) containing the 800-bp *rpsL* sequence with A128G, a plasmid (pRpsLSS) containing the 800-bp wild-type *rpsL* sequence as a negative control, or with a plasmid (pAD1-Cat) containing the chloramphenicol resistance cassette (*cat*) in the middle of the *ureAB* fragment, as described previously (33). After incubation for 24 h at 37°C in 5% CO_2, the transformation mixture was harvested into 1 ml PBS, and 100 μl of the appropriate serial dilutions were plated onto either TSA plates or BA plates containing 10% NCS and 25 μg/μl streptomycin (for cells transformed with *rpsL* fragment or plasmid) or 20 μg/μl chloramphenicol (for cells transformed with pAD1-Cat). The plates were incubated for 4 days at 37°C in 5% CO_2, and the total recombination frequency was determined by the number of St^r or Cm^r colonies divided by the total CFU. As a negative control, *H. pylori* strains with no DNA added were also tested in parallel with each experiment; no colonies were seen in any case.

Assay of intergenomic recombination using chromosomal DNA. Assays were performed as described above, with chromosomal DNA from a 26695 St^r strain (A128G in *rpsL*) as the donor DNA. Chromosomal DNA was prepared using a standard phenol-chloroform extraction protocol, and 1 μg of donor DNA was used for each transformation.

Complementation of the JP26 *mutS2*::*aphA* mutant. Plasmid pADC-MutS2, with ORF HP0621 placed downstream of the *ureAB* promoter, was constructed by the same methods as described previously (6) and then used to introduce HP0621 in *trans* into the genome of mutant JP26 *mutS2*::*aphA* via natural transformation to create JP26 *mutS2*comp (see Fig. S1A in the supplemental material). Primers MutSXbaI and MutSSmaI, used to amplify the *mutS2* gene, are listed in Table 2. Transformants were selected based on Cat^r, and the correct insertion of *mutS2* and flanking regions into *ureA* downstream of the *ureAB* promoter was confirmed by PCR of the chromosomal DNA.

RESULTS

Analyses of MutS family homologs. The conserved domains found in MutS1, MutS2, and eukaryotic MutS homologs, based on the SMART database (http://smart.embl-heidelberg.de) (42), with cross-references to PFam data (26), are shown in schematic form for representative organisms (Fig. 1A). *E. coli* and *Saccharomyces cerevisiae* were selected as representative species for MutS1 and MSH, respectively. *H. pylori*, *Campylobacter jejuni*, *Thermus thermophilus*, and *Streptococcus pyogenes* were selected as representative species for MutS2. MutS2 from *H. pylori* has strong homology to MutS2 from the closely related species *C. jejuni* as well as to those of *S. pyogenes* and *T. thermophilus*, where MutS2 nicking endonuclease activity has been previously demonstrated (25). MutS2 lacks the conserved N-terminal region found in other MutS homologs, which contain MutS domains I and II (Fig. 1A). MutS domain I is critical for mismatch recognition, as evidenced by the crystal structures of *E. coli* and *Thermus aquaticus* MutS-DNA complexes (11, 40, 51), and is present in all MutS homologs that play a role in mitotic mismatch repair (32) (Fig. 1A). The absence of MutS domain I in MutS2 is consistent with the lack of mismatch repair activity shown in prior studies (13, 46). MutS domain II is a conserved intervening region hypothesized to have a structural role, linking MutS domain I to the DNA association domain (11, 40, 51).

The DNA association domain and ATPase domain are conserved in all MutS homologs, including MutS2 (Fig. 1A). Alignment of the ATPase domain containing the helix-turn-helix motif, a region of dimer contact in MutS1 and eukaryotic MSH, shows substantial conservation between homologs (32) (Fig. 1B). That MutS2 also contains this motif suggests that it may function as a dimer as well. The DNA association domain shows a lesser degree of conservation between homologs (data not shown). Crystal structures of MutS1 suggest that this domain forms a major portion of the sliding clamp, with positive residues forming loose hydrophilic associations with DNA. That the sliding clamp structure, common to MutS1, MSH2-MSH3, MSH2-MSH6, and MSH4-MSH5 dimers, shows less conservation is consistent with evidence that MutS dimers associate with a variety of DNA structures, including DNA mismatches, nucleotide loops, and Holliday junctions, and this may reflect differences in DNA structural recognition (27, 45, 49, 64).

Since the DNA association domain is important in the recognition of various DNA structures (32), we hypothesized that if these proteins were linked, MutS2 would associate with MSH4-MSH5. To test this hypothesis, a phylogeny was constructed using the DNA association domain of representative MutS1, MutS2, and MSH1-MSH6 sequences (Fig. 2A). Our results show that MSH4-MSH5 clusters with MutS1 and other eukaryotic MSH proteins and not with MutS2, indicating substantial differences. Phylogenetic analyses of the MutS ATPase domain (Fig. 2B) also show a deep branching of MutS2 from the MutS1/MSH family. These results support the hypothesis that MutS2 and MutS1 diverged early during prokaryotic evolution and that MSH1-MSH7 subsequently evolved from a MutS1 ancestor. Therefore, the function of MutS2 cannot be inferred based on phylogeny.

MutS2 contains a SMR domain at its C terminus that is not present in other MutS family members (Fig. 1A). The SMR domain is highly conserved (43, 48), even between distantly related species, and is present as either a small protein, represented by *E. coli* Smr (Fig. 1A), or as the C terminus of MutS2. The fact that the species with Smr does not possess

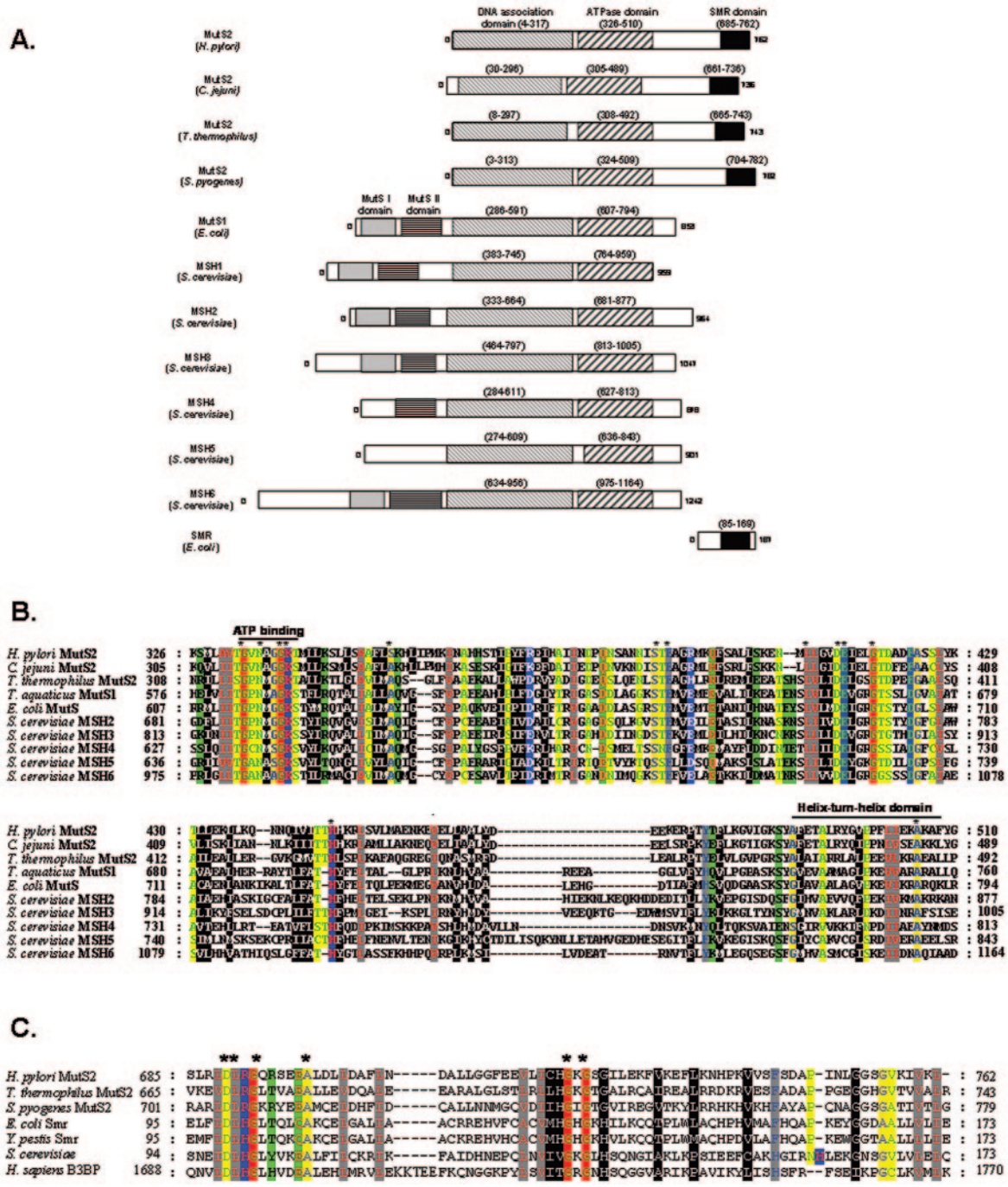

FIG. 1. Genetic analyses of *mutS1* and *mutS2* homologs. A. Schematic of MutS homologs. All sequences were obtained from GenBank, and conserved protein domains were identified using PFam (26) and the SMART program (http://smart.embl-heidelberg.de) (42). B. Amino acid alignment of the ATPase domain of MutS homologs. The ATPase domain of MutS1 from *E. coli* and *T. aquaticus*, MSH2, -3, -4, and -6 from *S. cerevisiae*, and MutS2 from *Listeria monocytogenes* and *H. pylori* were aligned with ClustalX and visualized using the program Genedoc (www.psc.edu/biomed/genedoc) in physiochemical properties shading mode, which divides residues into 12 groups representing three hierarchies. The first hierarchy is based on size, the second is based electrical charge for the polar amino acids, and the third, for nonpolar amino acids, is based on aromaticity. The highly conserved ATP binding domain (73) and putative helix-turn-helix domain (12, 54) are indicated with a black line. C. Amino acid alignment of SMR domains. The SMR domain of *H. pylori* MutS2 was aligned with SMR domains from other MutS2 and Smr proteins. Also included were SMR domains from hypothetical proteins in *Caenorhabditis elegans* and *Drosophila melanogaster* and the human Bcl-3 binding protein (B3BP), which has recently been shown to possess nicking endonuclease activity (72). Alignments were constructed and visualized as described for panel B. Asterisks indicate residues with 100% conservation.

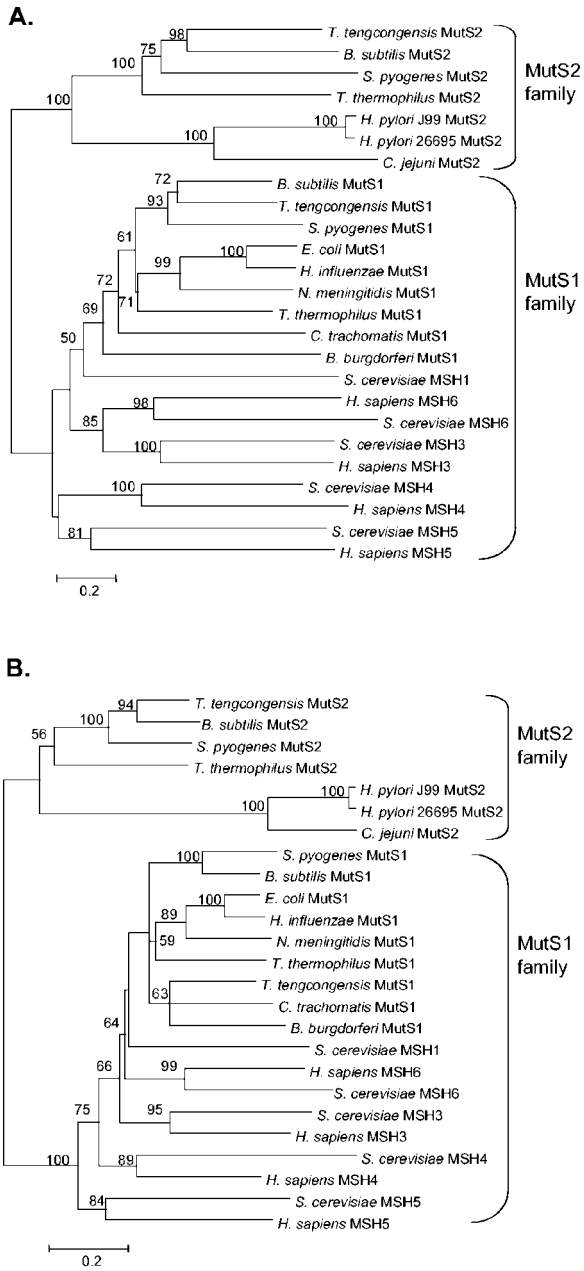

FIG. 2. Phylogenetic analysis of MutS family domains. A. The DNA association domain, conserved throughout MutS homologs, was identified using the SMART database (42), where it is annotated as MutSd. A phylogenetic tree was constructed using the amino acid sequences of MutS1 from nine representative bacterial species, MutS2 from six representative bacterial species, and MSH2 to MSH6 from *S. cerevisiae* and *H. sapiens* (Table 3). *B. subtilis*, *S. pyogenes*, and *T. tengcongensis* were selected, since they possess both MutS1 and MutS2. The program Mega 2.1 (38) was used to construct the phylogeny, by using the neighbor-joining method with 1,000 bootstrap replicates. B. The ATPase/dimerization domain, highly conserved throughout MutS homologs, was identified by using the SMART database (42), where it is annotated as MutSac. A phylogenetic tree was constructed as in panel A, with 1,000 bootstrap replicates.

MutS2, and vice versa (43), was supported by a more extensive sequence analyses (Table 3). The SMR domain has nicking endonuclease activity in *T. thermophilus* (25), and a human protein, B3BP, containing a SMR domain has similar function (72). Alignment of the Smr domain, using sequences identified through PSI-BLAST, indicates that *H. pylori* MutS2 possesses the SMR domain (Fig. 1C), suggesting similar function for *H. pylori* MutS2.

With the exception of the obligate intracellular organisms *Rickettsia prowazekii* and *Chlamydia pneumoniae*, organisms with *mutS1* also possess either *smr* or *mutS2* but not both (27), and our analysis of 33 bacterial species found no exceptions to this rule (Table 3). The *Mycobacterium* and *Mycoplasma* species were the only organisms with no homologs of either *mutS1*, *mutS2*, or *smr*. The closely related *Wolinella succinogenes* and *Helicobacter* and *Campylobacter* species (20) were the only organisms found to possess MutS2 but not MutS1.

Role of MutS2 in mismatch repair. To determine whether MutS2 is involved in mismatch repair in *H. pylori*, a *mutS2* mutant was constructed (see Fig. S1B in the supplemental material), and mutation rates for wild-type and *mutS2* mutant *H. pylori* strains were calculated. Our results show no difference in mutation rates between wild-type and *mutS2 H. pylori* strains (see Fig. S1B in the supplemental material), consistent with the studies of mutation frequencies in wild-type and *mutS2 H. pylori* strains by Bjorkholm et al. (13).

Effect of MutS2 on recombinational repair. We hypothesize that *H. pylori* MutS2 has the ability to bind DNA through its DNA association domain and to introduce nicks through the endonuclease activity present in its SMR domain. Since nicking endonucleases have been identified in several DNA repair pathways involving DNA damage (31, 47, 70), we asked whether MutS2 could recognize and process damaged DNA structures. Wild-type and *mutS2* strains were examined for susceptibility to two types of DNA damaging agents, UV and ciprofloxacin (see Fig. S2 in the supplemental material; also Table 4). UV radiation creates interstrand cross-links in DNA as well as strand breaks that can halt progression of the replication fork. Ciprofloxacin binds to DNA and creates ternary complexes with either DNA gyrase or topoisomerase (18, 36), resulting in lesions that impede replication (14, 44). Recombinational repair restores the DNA template by recognizing and replacing damaged DNA via recombination. Mutations in recombination proteins, such as *recA* and *ruvABC*, display high susceptibility to UV and fluoroquinolones (67). As expected, *H. pylori recA* mutants were more susceptible than the wild type to both UV and fluoroquinolone-induced DNA damage (see Fig. S2 in the supplemental material; also Table 4). The fact that the *mutS2* mutant did not show increased susceptibility to UV or fluoroquinolone-induced DNA damage provides evidence that MutS2 is not involved in recombinational repair of damaged DNA.

Effect of MutS2 on deletions between flanking DNA repeats. In silico analysis of the *H. pylori* genome reveals the existence of numerous, nonrandomly distributed, proximate, direct DNA repeat sequences that are "hot spots" for intragenomic DNA rearrangements (2, 8, 9, 57). Generation of loose DNA ends, that can participate in recombination events, are believed to promote deletion and duplication of intervening sequences between such direct DNA repeats. Certain recombination proteins, such as *H. pylori* RecG, reduce the frequency of deletion events between direct repeats, presumably by recognizing and unwinding intermediary DNA structures before deletion events reach completion (33). We examined whether MutS2

TABLE 3. Conservation of *mutS1* and *mutS2* homologs in representative prokaryotic species

Species	Presence[a]		
	MutS1	Smr fragment	MutS2
Mycoplasma genitalium	—	—	—
Mycoplasma pneumoniae	—	—	—
Mycobacterium bovis	—	—	—
Mycobacterium tuberculosis	—	—	—
Campylobacter jejuni	—	—	NP_282202
Helicobacter pylori 26695	—	—	O25338
Helicobacter pylori J99	—	—	Q9ZLL4
Wolinella succinogenes	—	—	Q7M8Q5
Chlamydia pneumoniae	Q9Z6W5	—	—
Rickettsia prowazekii	CAA14759	—	—
Bordetella pertussis	Q7VY01	Q7VW03	—
Neisseria meningitidis	Q9JWT7	NC_283999	—
Escherichia coli	NP_417213	P77458	—
Salmonella enterica serovar Typhi	P10339	P67246	—
Yersinia pestis	Q8ZBQ3	NP_993742	—
Haemophilus influenzae	Q6Q131	NP_43958	—
Pasteurella multocida	P57952	NP_245328	—
Vibrio cholerae	Q9KUI6	NP_231750	—
Treponema pallidum	O83348	O83680	—
Aquifex aeolicus	O66652	—	O67287
Bacillus subtilis	P49849	—	P94545
Borrelia burgdorferi	O51737	—	O51125
Clostridium perfringens	Q8XL87	—	Q8XJ80
Geobacter sulfurreducens	AAR35199	—	NP_951605
Lactobacillus plantarum	CAD64627	—	CAD64608
Listeria innocua	Q92BV3	—	NP_470532
Staphylococcus aureus	BAB57458	—	NP_645844
Streptococcus pyogenes	Q8K5J5	—	AAK34559
Synechocystis sp. (strain PCC6803)	P73769	—	P73625
Symbiobacterium thermophilum	YP_075586	—	YP_074944
Thermotoga maritima	P74926	—	Q9X105
Thermoanaerobacter tengcongensis	Q8R9D0	—	NP_622975
Thermus thermophilus	P61671	—	YP_005251

[a] Genbank accession numbers are provided if the homolog is present; —, not present.

also could influence such recombination by using a previously validated deletion cassette with identical DNA sequence (IDS) repeats of 0 (control), 50, or 100 bp (8). Although insertion of the complete cassette into a host *H. pylori* strain confers resistance to chloramphenicol, recombination between the flanking identical DNA repeats may delete the chloramphenicol cassette, changing the phenotype to kanamycin resistance and chloramphenicol susceptibility (8). To assess the effect of *mutS2* on intragenomic recombination between direct repeats, the deletion cassette was introduced into *mutS2* or *vacA* (as a control). Compared to the control strain, the *mutS2* mutant showed no significant difference in deletion frequencies (Fig. 3). These results suggest that MutS2 does not recognize or interfere with DNA intermediates formed during a deletion event.

TABLE 4. Susceptibility of wild-type and mutant *H. pylori* strains to ciprofloxacin

Strain designation	Ciprofloxacin MIC (μg/ml)[a]
JP26	0.11 ± 0.03
JP26 *mutS2*::*aphA*	0.12 ± 0.03
JP26 *recA*::*aphA*	0.04 ± 0.01

[a] *H. pylori* strains were assayed for inhibition within lawns on TSA plates containing a ciprofloxacin E-test strip (AB Biodisk), in concentrations from 0.002 to 32 μg/ml. Results shown are the means (± standard deviations) of five replicate determinations.

Effect of *mutS2* on intergenomic recombination. To explore the role of MutS2 in intergenomic recombination events in the naturally competent *H. pylori* (6), several forms of donor DNA were used to transform *H. pylori* wild-type and mutant strains. First, we examined transformation frequencies using donor chromosomal DNA with a point mutation in *rpsL* (A128G) to confer streptomycin resistance (Fig. 4A). The *mutS2*::*aphA* strain had a fivefold higher transformation frequency than the wild type ($P < 0.05$).

To determine whether the higher transformation frequencies noted in the *mutS2*::*aphA* strains with chromosomal donor DNA were more widely applicable, plasmid DNA was used to transform *H. pylori* (Fig. 4B). A plasmid (pSS) with an 800-bp *rpsL* fragment containing the same A128G mutation at its midpoint was used to transform *H. pylori* wild-type and *mutS2* mutant strains. The *mutS2*::*aphA* strain displayed a significantly higher (16-fold) transformation frequency than wild-

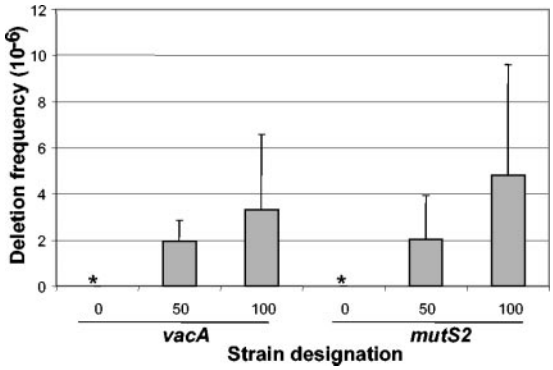

FIG. 3. Deletion frequency in *H. pylori* wild-type and mutant strains. The chloramphenicol/kanamycin resistance cassette flanked by identical repeats (IDS) of 0, 50, or 100 bp was inserted into *vacA* (control) or *mutS2*, insertion was confirmed, and deletion frequencies were calculated, as described previously (8). Asterisks indicate that no deletions were detected (frequency $< 10^{-8}$). As expected (8), *H. pylori* strains with the cassette in *vacA* or *mutS2* showed progressively higher deletion frequencies with increasing size of the IDS. Strains with the deletion cassette in *mutS2* do not show any significant difference in deletion frequency between flanking DNA repeats of 50 and 100 bp compared to control (*vacA*) mutants with comparable cassettes. Bars represent means ± standard deviations for four to six replicate experiments.

type JP26 ($P < 0.05$). Plasmid pWT, which contains the same 800-bp sequence without the A128G mutation, was used as a control and no transformants were detected, as expected (data not shown). Another form of donor DNA used was pAD1-Cat, which contains the 585-bp *ureAB* promoter region and the 312-bp downstream regions interrupted by a CAT cassette. Again, the *mutS2*::*aphA* strain had a significantly higher (520-fold) transformation frequency than the wild type ($P < 0.05$).

Examining recombination frequencies with a linear 800-bp *rpsL* PCR product showed that, as for other forms of donor DNA, the *mutS2*::*aphA* strain had a significantly higher (137-fold) transformation frequency than wild-type JP26 ($P < 0.05$) (Fig. 4C). To determine whether the results were specific to mutation of *mutS2* and not due to polar effects, the *mutS2*::*aphA* mutant strain was complemented in *trans* by expressing *mutS2* downstream of a strong (*ureA*) promoter (Fig. 4A). Complementation restored recombination frequency to wild-type levels, confirming that the observed phenotype is specific to the MutS2 mutation and not due to polar effects (Fig. 4C).

DISCUSSION

Our analysis of conserved protein domains in MutS family members reveals substantial differences between MutS2 and other MutS homologs, suggesting corresponding differences in biological function. By targeting phylogenetic analyses to a region specific for DNA association, the large differences in protein sequence that confound construction of a phylogenetic tree could be overcome. The results provide further evidence that MutS2 and MutS1 diverged early during prokaryotic evolution and that MutS1 was introduced into eukaryotes subsequent to this event, resulting in the evolution of MSH1 to MSH7. Phylogenetic analyses of the DNA association domains, and phenotypic analyses of a MutS2 mutant, argue against the hypothesis that MSH4-MSH5 and MutS2 evolved together. MSH4 and MSH5 are involved in promoting crossover of DNA during recombination (30, 37, 64). Our results for MutS2 in *H. pylori* indicate an opposing role, limiting recombination. In total, neither the phylogenetic or experimental results support previous hypotheses (19) suggesting that MSH4-MSH5 arose from an ancestral MutS2.

The SMR domain of MutS2, which is not present in other MutS homologs (Fig. 1A), has extensive sequence conservation, suggesting an important conserved function. In *T. thermophilus* MutS2, the SMR domain binds double-stranded DNA (dsDNA) and possesses endonuclease activity (25). In humans, the Bcl-3 binding protein (B3BP) has a single C-terminal SMR domain with nicking endonuclease activity, and it is believed to be involved in DNA repair and/or recombination (72).

Nicking endonucleases participate in multiple repair processes, including excision of damaged DNA, mismatch repair, and possibly class-switch immunoglobulin gene recombination and somatic hypermutation (31, 47, 70). These enzymes also participate in strand displacement reactions, introducing a site-specific nick into DNA, and a polymerase is used to displace the parental strand (50). We hypothesize that MutS2 acts similarly (Fig. 5), with the DNA association domain forming a loose clamp-like structure that recognizes recombination intermediates. Recent evidence shows that MutS2 has the ability to recognize and bind dsDNA (25, 53). The presence of a conserved helix-turn-helix domain in MutS2 (Fig. 2B) suggests that, as with other MutS homologs, it functions as a dimer. We propose that once recognition occurs, the Smr domain introduces a nick into the DNA, and the nick is subsequently extended, possibly by an exonuclease, resulting in displacement of the invading strand and abortion of potentially illegitimate recombination events.

That MutS2 mutants are hyperrecombinant regardless of the type of donor DNA (fragment, plasmid, or chromosome) supports the hypothesis that it recognizes a DNA structure common to all intergenomic recombination events (Fig. 5). That this hyperrecombinant phenotype is not dependent on the extent of homology in the donor DNA (point mutation versus nonhomologous cassette [Fig. 4B; also reference 53]) further suggests that MutS2 acts at an early step in recombination, possibly during strand invasion, to recognize and abort illegitimate recombination events; in this view, MutS2 mutation allows recombination to proceed unhindered, regardless of homology. That MutS2 is not involved in deletions between direct repeats suggests that it does not inhibit slipped-strand mispairing of homologous DNA, which is a common mechanism of deletion formation and does not require DNA strand exchange (3, 8); rather, MutS2 function may be specific to RecA-mediated recombination events (53). MutS2 inhibition of initiation of DNA strand exchange reactions (53) is consistent with this hypothesis. Since illegitimate recombination events also can be aborted by MutS1, through a separate mechanism (74), MutS1 presence may mask the hyperrecombinant phenotype of a MutS2 mutant. *H. pylori*, one of few organisms with MutS2 but not MutS1, is an ideal candidate for the study of MutS2 function.

That organisms lack MutS1, including the closely related *W.*

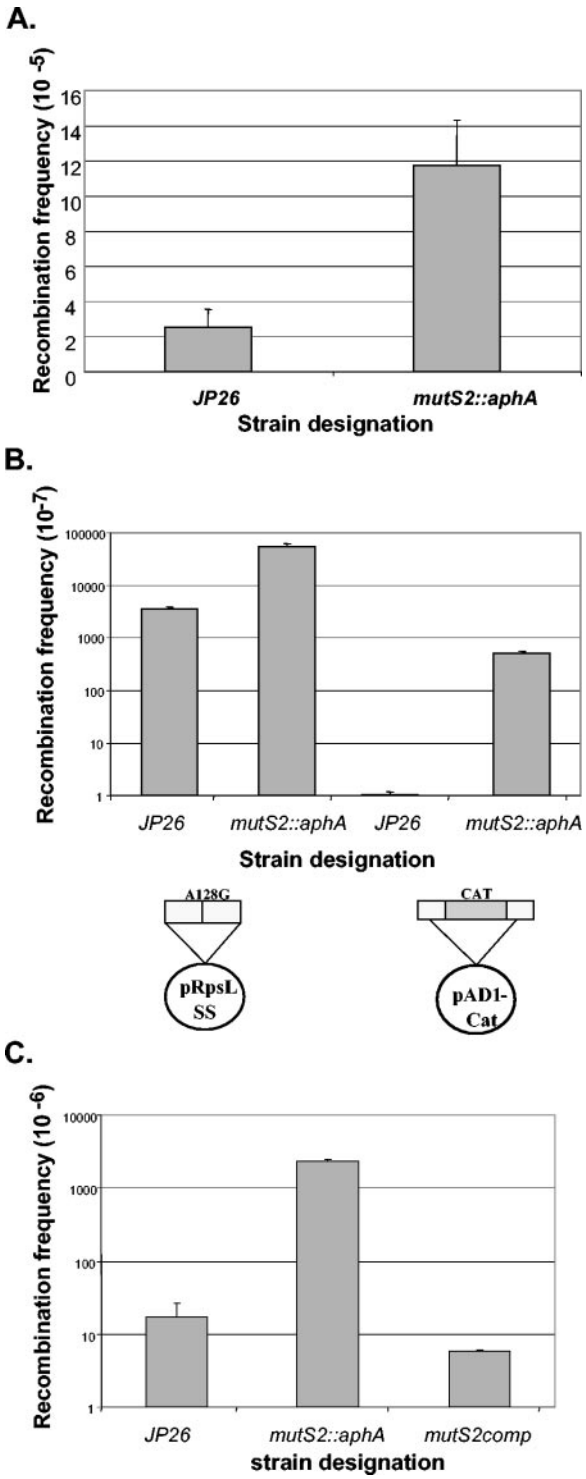

FIG. 4. Intergenomic recombination frequencies of *H. pylori* wild-type and mutant strains using chromosomal, plasmid, or PCR product DNA. A. *H. pylori* strains JP26 and JP26/*mutS2*::*aphA* were transformed to streptomycin resistance by using chromosomal DNA from St^r 26696, which has a point mutation (A128G) in the *rpsL* gene. Bars represent the means of four experiments. The *mutS2*::*aphA* mutant shows a significantly ($P < 0.05$) higher transformation frequency than wild-type JP26. B. *H. pylori* strains JP26 and *mutS2*::*aphA* were transformed with either pRpsLSS, which contains the 800-bp A128 *rpsL* fragment, or pAD1-CAT, which contains the *ureAB* promoter and downstream regions interrupted by a chloramphenicol resistance cassette, as shown in the schematic. For both pRpsLSS and pAD1-Cat donor DNA, the *mutS2*::*aphA* strain was transformed at a significantly ($P < 0.05$) higher frequency than wild-type JP26. Overall, pRpsLSS transforms both the wild-type and *mutS2* mutant strains at 2 to 3 $\log_{10}$ higher frequency ($P < 0.05$) than pAD1-Cat. C. *H. pylori* strains JP26, *mutS2*::*aphA*, and *mutS2*comp were transformed to streptomycin resistance with an 800-bp A128G *rpsL* PCR product; bars represent the means of four to eight replicate experiments. The *mutS2*::*aphA* strain shows a 2 $\log_{10}$ higher transformation frequency ($P < 0.05$) than wild-type JP26. Complementation of JP26 *mutS2*::*aphA* with *mutS2* in *trans* restored the recombination frequency to a level not significantly different from wild-type JP26.

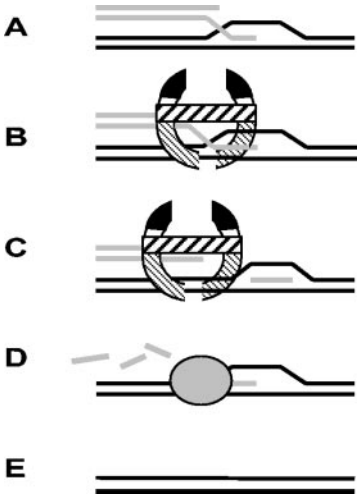

FIG. 5. Model of how MutS2 might limit intergenomic recombination. A. RecA-mediated strand invasion of donor DNA, which is essential for recombination events, forms a D-loop. B. MutS2, shown as a dimer via interaction at its ATPase/dimerization domains (thick gray lines), recognizes the primary recombination structure via its DNA association domains (thin gray lines). C. SMR endonuclease activity (represented in black) introduces a nick into the DNA. D. Recruitment of other proteins, possibly a repair polymerase such as PolA, with exonuclease activity, removes the invading DNA and fills in the gap. E. Restoration of original dsDNA.

succinogenes and *Campylobacter* and *Helicobacter* species (20), and the *Mycoplasma* and *Mycobacterium* species (Table 3), suggests that they may gain a selective advantage from the absence of MutS1-directed mismatch repair. Numerous phase-variable loci are present in these organisms, facilitating variation of antigenic structures at the cell surface (10, 15, 20, 28, 55, 56, 58, 62). As such, *H. pylori*, *C. jejuni*, *W. succinogenes*, and the *Mycoplasma* species may be regarded as multicellular populations that continually generate variants, particularly through phase variation events, that would be inhibited if mismatch repair were present. The *Mycobacterium* genome also contains an unusually high number of frameshifts, as well as repetitive sequence elements and repeat regions (8, 65). Clinical isolates of *M. tuberculosis* indicate that chromosomal rearrangements such as duplications, deletions, and inversions contribute significantly to variation of this species within its obligate human host (21, 22, 24, 34). Such events are facilitated by a lack of MutS1-directed mismatch repair (32, 74). Furthermore, *M. tuberculosis* is similar to *H. pylori* in that both species occupy niches in which other microbiota are underrepresented

or absent, resulting in limited opportunities for lateral gene transfer from exogenous sources (65). Therefore, loss of mismatch repair and the subsequent increased opportunity for chromosomal rearrangements and diversification within the population can facilitate microevolution. Since a high degree of mutation may decrease overall fitness (17), by possessing MutS2 function, *H. pylori* and its related campylobacters can limit the amount of diversification that occurs. In this respect, MutS1 and MutS2 may both function to preserve genomic integrity.

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