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Suppression of Homologous and Homeologous Recombination by the Bacterial MutS2 Protein

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Summary

In addition to their role in DNA repair, recombination events are associated with processes aimed at providing the genetic variability needed for adaptation and evolution of a population. In bacteria, recombination is involved in the appearance of new variants by allowing the incorporation of exogenous DNA or the reshuffling of endogenous sequences. Here we show that HpMutS2, a protein belonging to the MutS2 family in *Helicobacter pylori*, is not involved in mismatch repair but inhibits homologous and homeologous recombination. Disruption of HpmutS2 leads to an increased efficiency of exogenous DNA incorporation. HpMutS2 has a selective affinity for DNA structures mimicking recombination intermediates with no specificity for homoduplex DNA or mismatches. The purified protein has an ATPase activity stimulated by the same DNA structures. Finally, we show that HpMutS2 inhibits its DNA strand exchange reactions in vitro. Thus, MutS2 proteins are candidates for controlling recombination and therefore genetic diversity in bacteria.

Introduction

Successful adaptation of prokaryotes to specific niches and changing environments is achieved through phenotypic selection from a pool of genetic variants present in their population. The adaptation capacity of bacterial species is therefore related to the genetic diversity from which the most-fit variants are selected. At the origin of this variability are point mutations, horizontal gene transfer, and intragenomic rearrangements. The two latter processes require recombination events.

H. pylori colonizes the stomach mucosa of about half the human population, resulting in chronic gastritis, leading in some patients to peptic ulcers and, in a small fraction of cases, to cancer. The adaptation of *H. pylori* to the changing gastric environment within a host, or to new hosts, suggests an enhanced ability of this pathogen to change. Indeed, *H. pylori* is one of the most genetically diverse bacterial species. Because of its genome plasticity and its clinical importance, it constitutes a model for microbial phenotype evolution.

Numerous studies have shown the importance of different pathways in achieving genome plasticity. At the origin of most allelic variants are point mutations, but there is strong evidence for recombination playing a crucial role in the genetic diversity found in *H. pylori* (Suerbaum and Achtman, 1999). It is now clear that cocolonization and natural competence allow frequent transfer of genetic material between bacteria in a single host (Israel et al., 2001). However, the presence of extensive, nonrandomly distributed repetitive genomic sequences in *H. pylori* prompted experiments that suggested that recombination between direct repeats constitutes also a mechanism of genome diversification that might be widely conserved (Aras et al., 2003).

In vivo and in vitro experiments, computer simulations, analyses of natural isolates, and molecular evolution studies showed that selection initially favors bacterial strains having increased mutation rates (Radman et al., 2000). In *Escherichia coli*, inactivation of mismatch repair (MMR) provides two sources of genetic variability: (1) it increases the frequency of base substitutions and small deletion/insertions by the lack of postreplicative DNA repair (Taddei et al., 1997); and (2) it eliminates a barrier against recombination between divergent (homeologous) sequences (Rayssiguier et al., 1989). The MutS protein plays an essential role in these two functions. No mutants dissociating the inhibition of recombination between homeologous sequences from the MMR capacity of the protein have so far been found. This suggests that it is the recognition by MutS of heterologies in the recombination intermediate that impedes the completion of recombination (Worth et al., 1994).

In *H. pylori*, analysis of the complete genomic sequences (Alm et al., 1999; Tomb et al., 1997) failed to identify putative components of an MMR system other than open reading frame (ORF) *HP0621* encoding a protein with homology to MutS. Phylogenetic studies have divided the MutS homologs into two distinct families (Eisen, 1998): MutS-I, involved in MMR, and MutS-II, which includes the MSH4 and MSH5 groups found in yeast and other eukaryotes and the prokaryotic MutS2 proteins. The regrouping of the eukaryote and prokaryote MutS-II is based essentially in the similarity they share in the ATPase domain without any other significant homology throughout the rest of their sequences. Yeast strains in which *MSH4* or *MSH5* has been disrupted do not have an impaired MMR phenotype but present defects in meiotic recombination (Hollingsworth et al., 1995; Ross-Macdonald and Roeder, 1994). No function has been established for the members of prokaryotic MutS2 group to which HpMutS2 belongs. Large-scale

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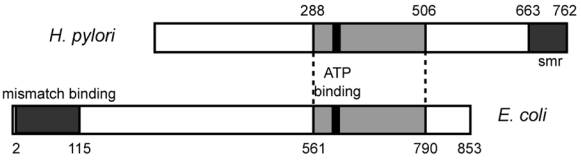

Figure 1. The *H. pylori* MutS Protein

Sequence alignment of the *E. coli* MutS (a MutS1 protein) and *H. pylori* MutS (a MutS2 protein). The light gray boxes correspond to the region homologous between the two proteins, including the ATP binding domain in black. The MutS1 mismatch binding domain and the MutS2 SMR domains are in dark gray.

genome sequencing efforts showed that the MutS2 proteins include proteins from eubacterial, archaea, and plant sources, the latter most likely derived from a cyanobacterial source and the result of a horizontal transfer from their plastids (Malik and Henikoff, 2000). A MutS2-encoding gene seems to always be present in those organisms that lack a *mutL* gene (Moreira and Philippe, 1999). The lack of clear phenotype changes in the absence of a functional MutS2 in the cases analyzed makes it difficult to speculate on the in vivo role of the protein in bacteria. However, there remains the intriguing possibility that MutS2 prokaryotic proteins are evolutionary precursors of the MSH4 and MSH5 proteins involved in meiotic recombination in eukaryotes. We therefore investigated the function of the *H. pylori* member of the MutS2 group, HpMutS2, both in vivo and in vitro and find that HpMutS2 plays a significant role in controlling homologous recombination.

Results

HpMutS Is Not Involved in Mismatch Repair

ORF *HP0621* codes for a 762 amino acid protein with limited homology to the MutS protein from *E. coli*. The homology region, spanning residues 288–506, shares 27% identity (41% similarity) to the *E. coli* protein and includes a conserved potential ATP binding motif (TGXNXXGK) between amino acids 333 and 340. Alignment of the sequences shows that the *E. coli* and *H. pylori* proteins do not share homology outside the region described above (Figure 1). Moreover, they seem to have very different domains in their terminal regions. HpMutS2 has a 100 amino acid C-terminal domain not present in the MutS1 proteins but found as a family of conserved bacterial and eukaryotic small proteins, two members of which are found in *E. coli*. Because this domain is present at the C terminus of many MutS2 proteins, it has been named *smr* (for small mutS-related) (Moreira and Philippe, 1999). It is noteworthy that HpMutS2 lacks the N-terminal domain from the *E. coli* and other MutS1 proteins shown to be responsible for the recognition and binding of mismatched bases in DNA (Sixma, 2001), suggesting that it may not participate in MMR.

To genetically test this hypothesis, we constructed several *H. pylori* strains where ORF *HP0621* was deleted and compared them to their parental isolates with respect to spontaneous mutation rates to rifampicin resistance (Table 1). The mutation rates of the different isolates, generally higher than those of *Enterobacteriaceae*, were not significantly modified by the disruption of the *HpmutS2* gene. Because mutations giving rise to rifampicin resistance are on an essential gene and are mostly base substitutions, these results confirmed that MutS does not participate in the repair of simple mismatches (Bjorkholm et al., 2001). To include a larger spectrum of mutation mechanisms, we also tested the mutation rate at the RdxA locus, a nonessential gene. Inactivation of *rdxA*, coding a nitroreductase, renders *H. pylori* resistant to metronidazole (Jeong et al., 2001). Therefore, any kind of sequence change leading to inactivation of the gene would be detected, including frameshifts, which are normally prevented by MMR. Among the strains available, 26695 was sensitive to metronidazole. As shown in Table 1, although mutation rates to metronidazole resistance are extremely high, 26695 *mutS2* did not show a higher mutation rate than the parental strain, thus confirming that HpMutS2 is not involved in MMR.

HpMutS Suppresses Homologous and Homeologous Recombination

In *Saccharomyces cerevisiae, MSH4* and *MSH5* have been implicated in the control of meiotic crossing-over (Hollingsworth et al., 1995; Ross-Macdonald and Roeder, 1994). Because HpMutS2 belongs to the same large family of proteins, we hypothesized that it could influence homologous recombination (HR) in *H. pylori*. In this pathogen, exogenous DNA sequences are readily integrated into the bacterial chromosome by HR. Therefore, we compared the recombination proficiencies of the X47-2AL *mutS2* and wild-type strains by monitoring the incorporation of a selectable marker into a nonessential locus. The exogenous DNA used was total chromosomal DNA from a N6 strain in which ORF *HP0645* is disrupted by a cassette conferring gentamicin resistance. The frequency of recombination of N6 DNA into X47-2AL *mutS2* chromosomes was 5-fold higher than into the parental strain (Figure 2A). When the experiment was repeated using DNA from an N6 derivative strain with a marker inserted into another nonessential locus, *HP1186*, a 21-fold increase in recombinant colonies was observed in the *mutS2* strain (Figure 2B). Very similar results were obtained when receptor strains 26695 and 26695 *mutS2* were compared (data not shown). To rule out an effect of MutS2 on restriction systems, we also compared the HR proficiency of the parental and *mutS2* strains when the exogenous DNA was obtained from isogenic bacteria. As a consequence of the lack of restriction, incorporation frequencies of X47-2AL DNA carrying a gentamicin cassette within the *HP0645* or *HP1186* locus into X47 chromosomes were two orders of magnitude higher than for transformation with N6 DNA (Figures 2C and 2D). However, the impact of disabling the *mutS2* gene was also evident. Indeed, the lack of a functional MutS2 resulted in 3- and 13-fold increases of recombination for the *HP0645* and *HP1186* loci, respectively. The results described above are consistent with MutS2 acting to inhibit HR.

To test whether the inhibition of recombination by HpMutS2 was dependent on the presence of sequence divergence between the recombination substrates, we

Table 1. Spontaneous Mutation Rates of *H. pylori* Parental and *mutS* Strains

Strain	X47-2AL	J99	26695		ADM1
Marker	rif	rif	rif	mtz	rif
Parental	3.6 (0.9)	9.2 (2.1)	9.7 (2.5)	1293 (264)	1.4 (0.4)
mutS	1.4 (0.4)	9.1 (2.2)	2.6 (0.4)	526 (100)	2.0 (0.4)

Mutation rates are expressed as mutations to rifampicin (rif) or metronidazole (mtz) resistance per locus and per generation ($\times 10^8$). Numbers correspond to the mutation rates derived from an experimentally determined median number of mutants from at least ten independent cultures. The standard deviation for each data set is shown in parentheses.

created three DNA constructs designed to integrate into another nonessential locus, *vacA*, of X47-2AL or X47-2AL *mutS2*. The gentamicin resistance cassette was flanked upstream by the first 404 or 430 nucleotides of *vacA*, a region containing the *s1* or the *s2* variable sequences, respectively, and downstream by 448 nucleotides associated with the *m* variable sequences of *vacA* from X47-2AL, 26695, or F8 *H. pylori* isolates (Figure 3A). Because all constructs were amplified in *E. coli,* this approach avoids any possible bias due to restriction by the transformed bacteria. Sequencing of these constructs showed that they differ markedly from each other, with the divergence of their flanking regions with respect to the X47-2AL recipient chromosome ranging from 0% (for the X47-2AL construct) to 26% (Figure 3A). The differences not only included base substitutions but also insertions or deletions of up to 5 bases and in one case an insertion of 57 bp. Surprisingly, in spite of their high degree of divergence, no significant differences were observed in the efficiency of integration into wild-type X47-2AL of the DNA derived from X47-2AL and the other strains. However, inactivation of the *mutS2* gene in the recipient strain resulted in 5- to 8-fold increases in recombination frequencies (Figure 3B).

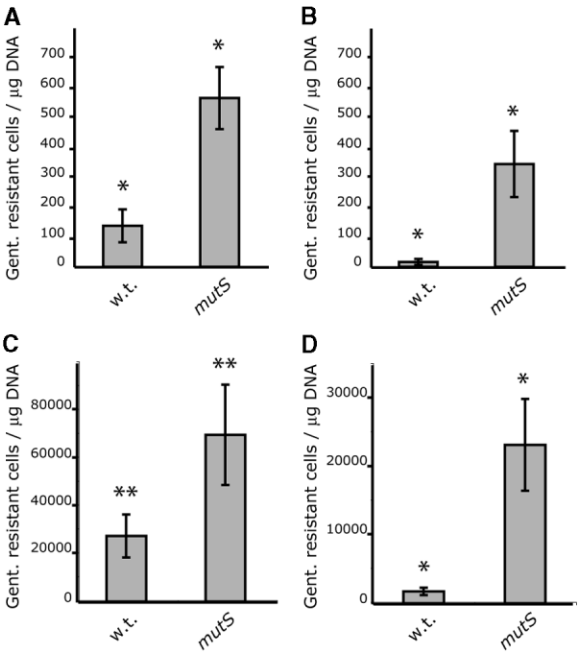

Figure 2. The Absence of HpMutS2 Facilitates Recombination of Exogenous DNA into the Bacterial Chromosome

Integration events were calculated as gentamicin-resistant colonies per microgram of chromosomal DNA. Values correspond to the average of ten independent transformations, and errors bars represent the corresponding standard deviation.

(A and B) Chromosomal DNA from N6 strains carrying a gentamicin resistance cassette in either *HP0645* (A) or *HP1186* (B) was introduced into wild-type (wt) or *mutS* X47-2AL strains.

(C and D) Chromosomal DNA from X47-2AL strains carrying a gentamicin-resistance cassette in either *HP0645* (C) or *HP1186* (D) was introduced into wild-type (wt) or *mutS* X47-2AL strains. *$p < 0.001$; **$p < 0.01$ between wt and *mutS2* using the Mann-Whitney nonparametric test.

All the recombination experiments described above require the integration into the chromosome of a large piece of heterologous DNA corresponding to the cassette coding for the antibiotic resistance used as a marker. HpMutS could act as a barrier toward the incorporation of such nonhomologous regions into the bacterial chromosome. To determine whether MutS also inhibits the recombination into the chromosome of a sequence differing only in one or two nucleotides, we isolated DNA from a rifampicin-resistant X47-2AL derivative strain and used it to transform rifampicin-sensitive X47-2AL or its *mutS2* mutant. As it was the case for the incorporation by HR of a cassette, the inactivation of HpMutS2 resulted in a large increase of rifampicin-resistant clones (25-fold), confirming the general hyperrecombination phenotype of *mutS2* strains (Figure 3C).

When a nonintegrating plasmid conferring chloramphenicol resistance was tested as donor DNA, no difference in the yield of antibiotic-resistant colonies was observed between X47-2AL and X47-2AL *mutS2* (data not shown), showing that the mutant strain is not defective in its transformation proficiency. Taken together, these results show that *mutS2* disruption in *H. pylori* results in an increased frequency of HR, both between perfectly matched DNA molecules and between molecules with divergent sequences.

HpMutS Has ATPase Activity and Binds to Recombination Intermediate DNA Structures

To explore the biochemical properties of HpMutS2, we cloned the *mutS2* gene from X47-2AL strain in frame with a histidine tag at its C terminus and expressed it in *E. coli*. After purification, we obtained a near homogenous protein of apparent molecular weight closely matching the predicted MW of 86.7 kDa (Figure 4A). The presence of a putative ATP binding domain suggested that this protein could have an ATPase activity. Indeed, MutS2 has an ATP hydrolyzing activity (Figures 4C and 4D). Since other MutS proteins family members have their ATPase activities stimulated by specific DNA substrates (Parker and Marinus, 1992; Snowden et al., 2004; Su and Modrich, 1986), we analyzed the capacity of

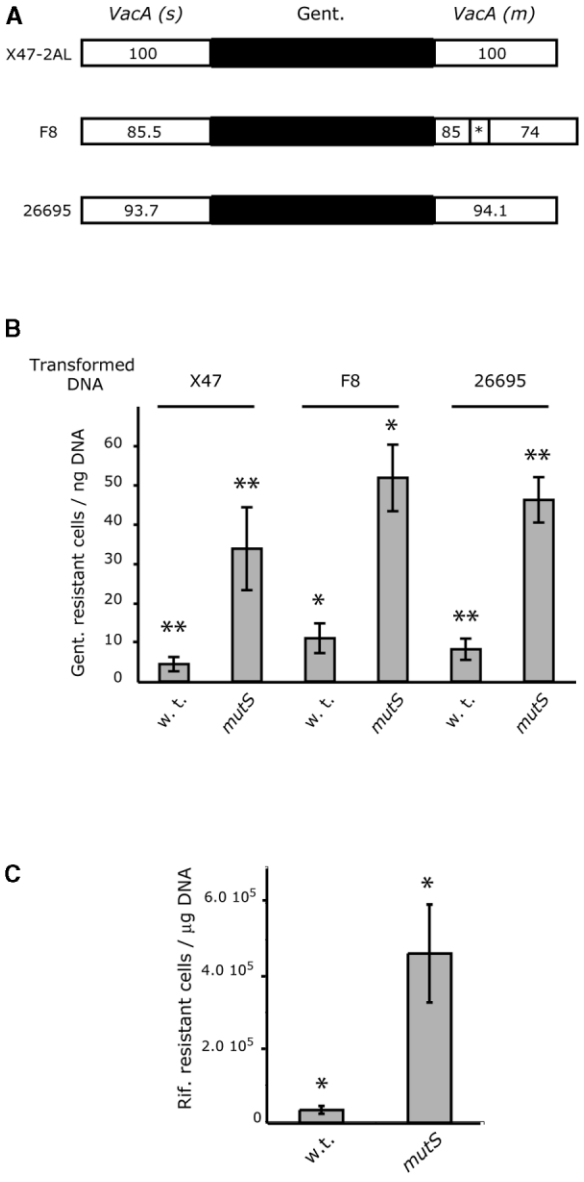

Figure 3. The Degree of Heterology Does Not Affect the Hyperrecombination Phenotype of *H. pylori mutS2* Strains

(A) Schematic representation of the DNA constructions used for transformation of *H. pylori* strains. Numbers in the boxes represent the percentage of identity between the source strains and X47-2AL. The box with the asterisk corresponds to a 57 bp insertion.

(B) Integration efficiency of the constructs shown in (A) into either wild-type (wt) or *mutS* X47-2AL strains.

(C) Chromosomal DNA from a rifampicin-resistant X47-2AL strain was introduced into wild-type (wt) or *mutS* X47-2AL strains. *$p < 0.001$; **$p < 0.01$ between wt and *mutS2* using the Mann-Whitney nonparametric test.

various DNA substrates mimicking either MMR or HR substrates (Figure 4B) to stimulate an HpMutS2 ATPase activity. The HpMutS2 ATP hydrolyzing activity is stimulated by the presence of four-way junction (FWJ) or fork DNA structures (Figure 4C). The incubation in the presence of a single-stranded, homoduplex or heteroduplex double-stranded DNA resulted in a weak activation when compared to the FWJ or the fork. To rule out the possibility of a contaminant ATPase in our protein preparation, a parallel purification was carried out for a MutS2 in which the conserved glycine 338 of the nucleotide binding domain was mutated to arginine (Figure 4A). No ATPase activity was detectable for the mutated protein in the absence or in the presence of FWJ DNA (Figure 4D).

The ATPase experiments suggested the possibility that HpMutS could specifically bind to DNA structures mimicking recombination intermediates, rather than mismatches or homoduplex DNA. The capacity of HpMutS2 to bind DNA was tested by filter retention experiments where the protein was incubated in the presence of end-labeled double-stranded or FWJ DNA. The titration curves presented in Figure 4E show that HpMutS2 binds more efficiently a FWJ-structured DNA than homoduplex DNA. To compare the relative affinities of HpMutS for different DNA structures, filter retention experiments where the labeled FWJ is competed by the various DNAs shown in Figure 4B were performed. As expected, addition of unlabeled FWJ competes away the binding of HpMutS2. A Scatchard analysis (Scatchard, 1949) of the data yielded an apparent K_D of 58 nM. Homoduplex DNA, duplex DNA harboring a G/T mismatch, or single-stranded DNA inhibit weakly the binding of HpMutS to the FWJ probe, while the fork structure shows an inhibition comparable to that of the FWJ (Figure 4F). While these experiments were carried out at 130 mM NaCl, similar results were obtained at 50 mM NaCl (data not shown). These results suggest a selective affinity of HpMutS for single- to double-strand transitions, likely to be found during recombination or other DNA repair events.

HpMutS Blocks In Vitro DNA Strand Transfer

To determine whether HpMutS2 inhibits HR in vitro, we studied its effect on RecA-promoted strand exchange reactions between linear double-stranded DNA (dsl) and circular single-stranded DNA (ssc) (Figure 5A). In the absence of HpMutS2, after 90 min of incubation almost all the dsl has disappeared, and substantial levels of the final strand exchange product, a nicked circular duplex (nc), are present. When HpMutS2 was added, disappearance of nc was observed (Figures 5B and 5C), indicating an inhibitory effect of the protein on the strand exchange reaction. Moreover, identical results were obtained when the strand exchange experiment was performed between DNAs displaying 8% heterology (data not shown). This is in agreement with our in vivo results showing that inactivation of *HpmutS2* has indistinguishable effects on the integration of homologous and heterologous sequences (Figure 3).

Discussion

Here we provide evidence of a significant phenotype associated with a protein member of the prokaryote MutS2 family by showing that the HpMutS2 is inhibitory to HR. In addition, we demonstrate that HpMutS2, unlike the MutS-I proteins, does not participate in MMR. A previous study on three isolates of *H. pylori* showed that the spontaneous frequency of rifampicin-resistant mutants was not enhanced by the disruption of the

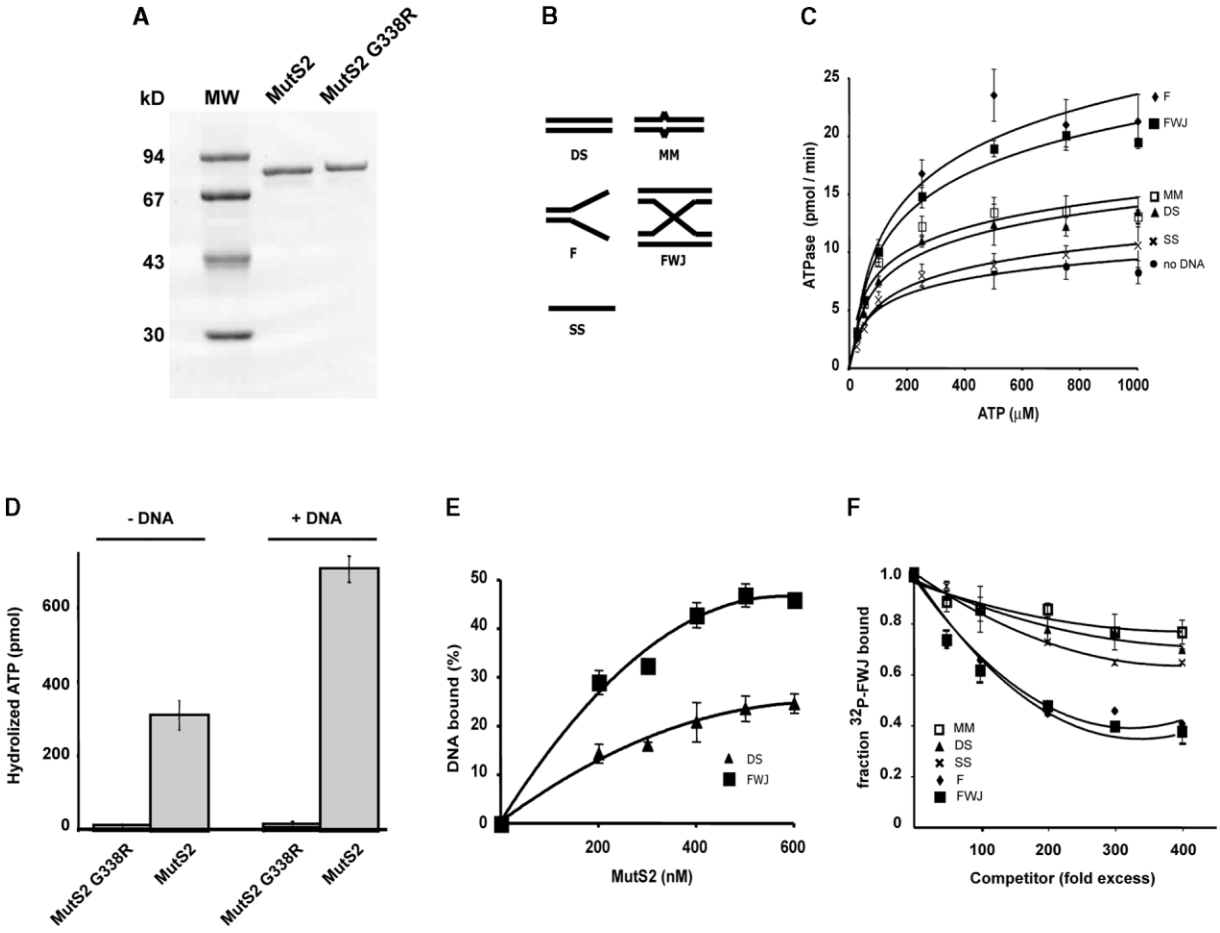

Figure 4. ATPase and DNA Binding Activities of HpMutS

(A) The HpMutS2 and HpMutS2 G338R overexpressed in *E. coli* were purified to near homogeneity as judged by analysis on 10% SDS-polyacrylamide gel electrophoresis and staining with Coomassie blue.

(B) Schematic representation of the DNA substrates used for binding or stimulation experiments: DS, double-stranded homoduplex DNA; MM, as DS but with a G/T mismatch; SS, single-stranded DNA; F, fork structure; FWJ, four-way junction.

(C) Stimulation of the HpMutS ATPase activity by DNA. Purified HpMutS (25 nM) was incubated with [γ-^{32}P]ATP and the indicated concentration of unlabeled ATP without DNA (●), with 100 nM of FWJ (■), F (◆), DS (▲), MM (□), or SS (X) for 35 min.

(D) ATPase activity of wild-type or G338R MutS2 proteins. Incubations were carried out as in (C) with 200 μM ATP in the absence (−DNA) or presence (+DNA) of 100 nM FWJ DNA.

(E) Filter binding analysis of HpMutS2 binding to FWJ (■) and DS (▲) DNAs. Increasing concentrations of HpMutS2 were incubated with 10 fmol of the corresponding end-labeled oligo. Protein-DNA complexes retained on filter were quantified with a PhosphorImager. Error bars indicate the standard deviation of at least three independent experiments.

(F) Competition analysis of HpMutS2 bound to a FWJ structure. HpMutS2 (400 nM) and 10 fmol end-labeled FWJ were incubated with increasing amounts of unlabeled competitor DNA: FWJ (■), F (◆), DS (▲), MM (□), or SS (X) in a 20 μl reaction. Quantification of the products was as in (E).

HpmutS gene (Bjorkholm et al., 2001). However, MMR-defective, and in particular *mutS*, strains are often found among clinical isolates of bacterial pathogens (LeClerc et al., 1996), and because for the *H. pylori* strains there were no other phenotypes to test, it could not be ruled out that the parental strains' *mutS* gene was already nonfunctional. In the case of two of the strains used in this work, X47-AL and 26695, the *mutS2* product displays enzymatic and DNA binding activities, and the inactivation of the gene leads to a recombination phenotype but not to a hypermutator one, thus confirming that the MutS2 proteins are not involved in MMR. Moreover, *HpmutS2* disruption does not increase the frequency of inactivation of *rdxA* (Table 1), ruling out a specifc role of MutS2 in the avoidance of frameshifts. In *B. subtilis*, where both *mutS1* and *mutS2* are present, disruption of the latter did not affect the spontaneous mutation frequency (Rossolillo and Albertini, 2001). The lack of a hypermutator phenotype in *H. pylori mutS2* strains is not surprising since HpMutS2, as the other MutS2 proteins, lacks the N-terminal domain responsible for mismatch recognition in the MutS-I proteins (Malik and Henikoff, 2000; Sixma, 2001). Indeed the DNA binding experiments shown in this work demonstrate that HpMutS2 does not discriminate between mismatch-containing DNA and homoduplex DNA. Similarly, MutS2 from *Pyrococcus furiosus* (Vijayvargia and Biswas, 2002) or *Thermus thermophilus* (Fukui et al., 2004) has affinity

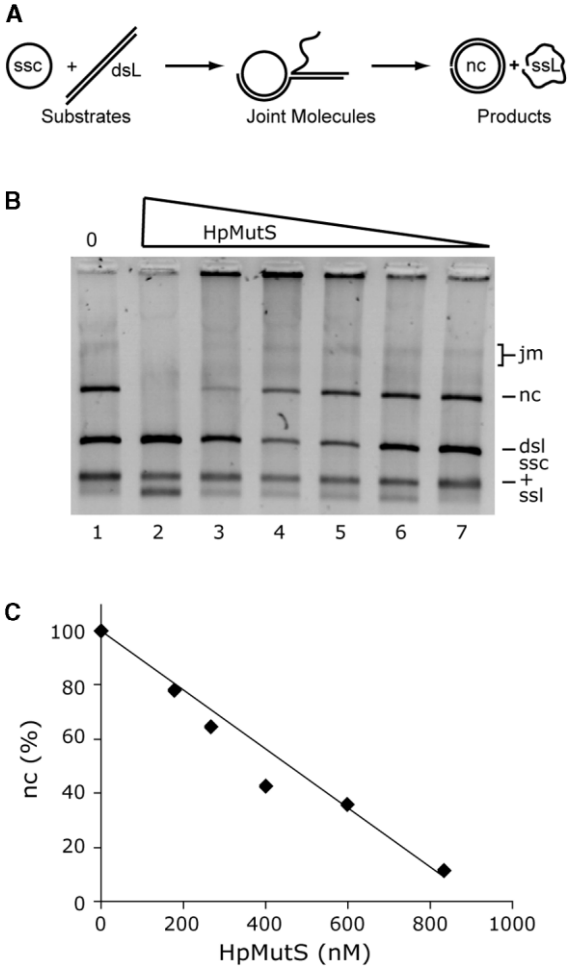

Figure 5. HpMutS Inhibits Strand Exchange Catalyzed by RecA

(A) Scheme of DNA strand exchange reaction.

(B) Increasing amounts of HpMutS were added at the initiation time of the strand exchange reaction (lanes 1–8 correspond to 0, 834, 601, 401, 267, 178, and 119 nM HpMutS, respectively). Abbreviations: dsl, double-stranded linear DNA; jm, joint molecules; nc, nicked circular double-stranded DNA; ssl, single-stranded linear DNA; ssc, single-stranded circular DNA.

(C) Quantification of the reactions shown in (B).

for double-stranded DNA but no detectable mismatch-specific binding activity.

Consistent with the lack of mismatch discrimination, the degree of inhibition of incorporation of exogenous DNA by a functional HpMutS2 is independent of the level of heterology. This observation strongly suggests that HpMutS2 acts differently than the MutS-I proteins whose inhibitory action on recombination depends on the presence of heterologies between the DNAs (Rayssiguier et al., 1989). Moreover, our in vitro experiments show that HpMutS suppresses RecA-catalyzed strand exchange reactions independently of the presence of heterology between the sequences. In the case of *E. coli* MutS, the protein does not inhibit recombination between completely homologous sequences (Worth et al., 1994). Taken together, these results imply that HpMutS acts by a different mechanism than the MutS1 proteins to inhibit recombination. It remains to be analyzed what the role of the MutS2 ATPase activity could be and whether other factors are necessary to complete HpMutS2 action in vivo.

The experiments measuring the recombination of exogenous DNA into the MutS2-proficient *H. pylori* chromosome yielded the surprising result that the efficiency of incorporation of the foreign DNA was in all cases independent of the level of heterology of the incorporated DNA with respect to the chromosomal sequences. Indeed, the yield of recombinant bacteria was essentially the same whether the DNA used was isogenic or up to 26% divergent with respect to the recipient chromosomal DNA. This suggests that in *H. pylori*, once the strand exchange reaction is initiated, probably by pairing of relative short homologous sequences, the presence of nonhomologous sequences is not a barrier for the completion of the recombination event.

The conserved and widespread presence of *mutSII* genes leads to the obvious question of their role in evolution. The presence of MutS2 proteins in some naturally competent species such as *H. pylori* or *B. subtilis* could regulate the incorporation of foreign DNA. Likewise, in *H. pylori* it could be one of the unknown factors proposed to regulate genome plasticity (Aras et al., 2003). Interestingly, although in yeast MSH4 and MSH5 facilitate interhomolog crossover during meiosis, *msh5* mutants display an increased frequency of gene conversion (Hollingsworth et al., 1995), an event similar to the allele exchange measured here for *H. pylori*. It is also possible that MSH4 and MSH5 are only very distant relatives of prokaryotic MutS2 proteins and have simply conserved the ATPase domain and a structure-dependent DNA binding capacity (Snowden et al., 2004) to evolve to very different functions.

In conclusion, our findings on the genetic and biochemical characteristics of HpMutS2 support the idea that the bacterial MutS2 proteins, so far of unknown function, modulate HR. In the case of bacterial species that, as *H. pylori*, show a high level of genetic diversity, MutS2 could control the plasticity of their genomes by regulating both the integration of exogenous DNA and the reshuffling of sequences in their chromosomes. The levels of MutS2 activity could have important consequences in the appearance of phenotypic variants and new antibiotic resistances.

Experimental Procedures

H. pylori Strains

Parental *H. pylori* strains used were X47-2AL (Londono-Arcila et al., 2002), 26695 (Tomb et al., 1997), F8 (Ng et al., 1999), ADM1, J99, and N6 (Ferrero et al., 1992). To generate the corresponding *mutS* derivatives, pILL570-HP0621-Km was constructed by replacing 1689 bp (between positions 300 and 1986) of *ORF HP0621* from strain 26695 with a nonpolar kanamycin (Km) resistance cassette (Skouloubris et al., 1998). For disruption of *HP0645* and *HP1186*, similar plasmids were constructed but with a gentamicin (Gm) resistance cassette. Plasmids were introduced into *H. pylori* strains by natural transformation, colonies were selected on either 20 μg/ml Km or 5 μg/ml Gm, and allelic replacement was verified by PCR. Both independent mutants in *HP0621* or pools of mutants were used according to the experiments.

H. pylori Rates of Spontaneous Mutation

Independent cultures were grown for 24 hr on plates with Blood Agar Base Number 2 (Oxoid) supplemented with 10% horse blood

(BAB). Appropriate dilutions were plated either on BAB to determine the number of viable cells or on BAB with either 20 μg/ml rifampicin or 8 μg/ml metronidazole to score for mutants. The mutation rates and their standard deviations were calculated by the method of the median (Lea and Coulson, 1949).

Recombination Substrates

Genomic DNA from *H. pylori* strains was prepared using the Qiamp (Qiagen) protocol. For the plasmid constructs, the hypervariable *s* and *m* regions of the *VacA* gene (HP0887) from three *H. pylori* strains were PCR amplified. The primers 3′ for the *s* region and 5′ for the *m* region also contained sequences homologous to the 5′ and 3′ ends of a Gm resistance cassette, respectively. The amplification products s and m were utilized as primers for the Gm cassette amplification obtaining the construction VacAs-Gm-VacA*m*. These products were cloned into plasmid pILL570 and introduced into *E. coli* MC 1061 strain (Invitrogen).

Recombination Assay

Chromosomal DNA from *H pylori* or plasmids containing the different constructions of VacAs-Gm-VacAm were introduced into X47-2AL or X47-2AL *mutS* by natural transformation. For each experiment, ten independent transformations were carried out using 1 μg of DNA per in 10 μl patches (approximately 5×10^6 cells) on BAB plates. Appropriate dilutions were plated on BAB with 5 μg/ml Gm or 20 μg/ml rifampicin.

Cloning and Purification of the Recombinant HpMutS Protein

ORF *HP0621* was amplified from *H. pylori* X47-2AL genomic DNA. A 3′-terminal sequence coding for a six-histidine tag was added. The amplification product was cloned into pET-3b (Novagen). For the G338R substitution, site-directed mutagenesis was performed using the QuikChange system (Stratagene) as indicated by the manufacturer. *E. coli* Rosetta cells (Novagen) transformed with pET-Hp*mutS2* or pET-Hp*mutS2*-G338R were grown at 37°C to an A_{600} of 0.6. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 0.5 mM. After incubation for 3 hr at 37°C, cells were harvested, resuspended in buffer A (20 mM phosphate [pH 7.4], 500 mM NaCl, and 1 mM DTT), and disrupted by sonication. After centrifugation at 100,000 × g for 20 min, the soluble fraction was applied on a HiTrap chelating column (Amersham Biosciences) equilibrated with buffer A plus 20 mM imidazole. An elution gradient of 20–500 mM imidazole in buffer A was applied. Fractions containing HpMutS2 were pooled and loaded on a HiTrap Heparin column (Amershan Biosciences) equilibrated with buffer B (20 mM phosphate buffer, 100 mM NaCl, and 1 mM 2-mercaptoethanol). An elution gradient of 200–1000 mM NaCl was applied in buffer B. Fractions containing HpMutS2 were then loaded into a Ressource S (Amersham Bioscience) 1 ml column and eluted with a 0.1–0.5 M NaCl gradient in buffer A. Purification of the protein was monitored on SDS-polyacrylamide gels.

Filter Retention Assay

Synthetic DNA substrates used to determine the affinity of HpMutS for DNA were constructed by annealing combinations of oligonucleotides with sequences as those described by Harmon and Kowalczykowski (1998). For the mismatch-containing substrate, a G/T mismatch was introduced into the dsDNA oligonucleotide. To construct the various DNA substrates shown in Figure 4B, equimolar concentrations of oligonucleotides were mixed in annealing buffer (20 mM Tris-HCl [pH 8], 10 mM MgCl, and 50 mM NaCl), heated to 100°C for 6 min, and then allowed to cool to room temperature. Labeled substrates were obtained by end-labeling one of the oligonucleotides with [γ-^{32}P]ATP and polynucleotide kinase. Reactions in 25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 5% v/v glycerol, 1 mM DTT, and 50 μg/ml bovine serum albumin (buffer C) were initiated by the addition of 10 fmol ^{32}P-labeled DNA and the indicated concentrations of HpMutS2 protein. After incubation at 4°C for 30 min, the reaction mixtures were loaded with a slot blot apparatus onto a nitrocellulose Hybond-C membrane (Amersham) previously treated with 0.4 M KOH to avoid DNA binding (Wong and Lohman, 1993). Membranes were then washed and air-dried. MutS2-bound DNA was determined by quantifying the radioactivity bound to the nitrocellulose, using a Storm Phosphorimager (Amersham). For competition assays, unlabeled competitor DNA was added at the start of the reaction.

ATP Hydrolysis Assay

Assays were performed in 20 μl buffer C, with the indicated amounts of ATP, and 13.3 nCi [γ-^{32}P]ATP. Reactions were started by the addition of 25 nM HpMutS, incubated at 37°C, and stopped with 2 μl 0.5 M EDTA (pH 8). ATP hydrolysis was measured as described (Iggo and Lane, 1989). When DNA was used as cofactor, DNA structures were added at 100 nM to the reaction.

DNA Strand Exchange Reaction

M13CEF3 ssDNA (4.5 μM nucleotides) was incubated in 30 mM Tris HCl (pH 7.6), 9 mM $MgCl_2$, 1.8 mM DTT, 1.1 mM ATP, 7.2 mM phosphocreatine, and 9 U/ml phosphocreatine kinase for 3 min at 37°C before the addition of 1.5 μM RecA protein and 0.26 μM SSB. The reaction was kept at 37°C for 10 min. Variable amounts of MutS protein were then added followed by the introduction of PacI-linearized M13CEF3 dsDNA (4.5 μM nucleotides). After 90 min incubation, the reaction mixture was deproteinized and analyzed by electrophoresis in 0.8% agarose gel in TAE buffer. The gels were stained with SybrGold (Molecular Probes) and quantified by ImageQuant software.

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