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Role of a MutY DNA Glycosylase in Combating Oxidative DNA damage in *Helicobacter pylori*

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Abstract

MutY is an adenine glycosylase that has the ability to efficiently remove adenines from adenine/7,8-dihydro-8-oxoguanine (8-oxo-G) or adenine/guanine mismatches, and plays an important role in oxidative DNA damage repair. The human gastric pathogen *Helicobacter pylori* has a homolog of the MutY enzyme. To investigate the physiological roles of MutY in *H. pylori*, we constructed and characterized a *mutY* mutant. *H. pylori mutY* mutants incubated at 5% O₂ have a 325 fold higher spontaneous mutation rate than its parent. The mutation rate is further increased by exposing the mutant to atmospheric levels of oxygen, an effect that is not seen in an *E. coli mutY* mutant. Most of the mutations that occurred in *H. pylori mutY* mutants, as examined by *rpoB* sequence changes that confer rifampicin resistance, are GC to TA transversions. The *H. pylori* enzyme has the ability to complement an *E. coli mutY* mutant, restoring its mutation frequency to the wild-type level. Pure *H. pylori* MutY has the ability to remove adenines from A/8-oxo-G mismatches, but strikingly no ability to cleave A/G mismatches. This is surprising because *E. coli* MutY can more rapidly turnover A/G than A/8-oxo-G. Thus, *H. pylori* MutY is an adenine glycosylase involved in the repair of oxidative DNA damage with a specificity for detecting 8-oxo-G. In addition, *H. pylori mutY* mutants are only 30% as efficient as wild-type in colonizing the stomach of mice, indicating that *H. pylori* MutY plays a significant role in oxidative DNA damage repair *in vivo*.

1. Introduction

Helicobacter pylori is common gastric pathogen that infects more than half of the world's population [1]. It inhabits the gastric mucosa of the human stomach and causes various diseases including gastritis, peptic ulcers, gastric cancer, and MALT lymphoma [2]. *H. pylori* is a microaerophilic bacterium and therefore cannot survive atmospheric levels of oxygen. It has been shown previously that *H. pylori* infections cause a strong inflammatory response from the host, including the production of reactive oxygen species (ROS) [3]. *H. pylori* has a variety of enzymes for combating oxidative stress including ROS detoxification enzymes such as catalase, superoxide dismutase, and alkyl hydroperoxide reductase [4-6]. In addition, *H. pylori* is equipped with DNA repair machinery as a second line of defense against oxidative stress [7,8].

One of the deleterious effects of oxidative stress is the damage on biological molecules such as DNA. The oxidation of guanine can occur while it is incorporated in the DNA or while it is in the nucleotide pool as dGTP forming the stable oxidized DNA product 7,8-dihydro-8-oxoguanine (8-oxo-G) [9]. This oxidized form of guanine has the ability to base pair with either adenine or cytosine [10]. If 8-oxo-G is paired with adenine and DNA replication occurs, the

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daughter strand will have a GC to TA transversion mutation. *E. coli* protects itself from the damaging effects of 8-oxo-G using the GO system comprised of three proteins, MutT, MutY, and MutM [11]. MutT is a hydrolase that converts 8-oxo-dGTP to 8-oxodGMP and pyrophosphate [12]. This prevents the oxidized dGTP from being incorporated into the DNA. MutM is a formamidopyrimidine-DNA glycosylase that excises 8-oxo-G when paired with cytosine [13]. MutY is an adenine glycosylase that excises adenines paired with 8-oxo-G or guanine [14]. Mutants in these genes display a mutator phenotype indicated by an increased spontaneous mutation frequency. The *mutT* mutant has the highest frequency, followed by the *mutY* mutant, then the *mutM* mutant [9]. *H. pylori* has homologs to MutT and MutY, but not to MutM. It also has a homolog to MutS, which is normally a part of the post-replication mismatch repair system along with MutH and MutL [15]. *H. pylori* lacks homologs to MutH and MutL. *H. pylori* MutS is not functional in conventional DNA mismatch repair, but instead it confers protection from oxidative DNA damage [7]. It has been shown that this MutS homolog has the ability to bind 8-oxo-G in DNA as well as Holliday junctions. Lack of some important DNA repair enzymes in *H. pylori* may be part of the reason that it has such extraordinary genetic diversity between strains [16].

MutY homologs are present in eukaryotes such as humans, mice, and yeast, as well as in the prokaryotes *E. coli*, *B. subtilis*, *P. aeruginosa*, *D. radiodurans*, *N. meningitidis*, and *N. gonorrhoeae* [17-22]. The functions and mechanisms of *E. coli* MutY have been well studied [13,14,23,25,26,28-31].

To examine the physiological role of the MutY homolog in *H. pylori*, we characterized an *H. pylori* *mutY* mutant and determined the biochemical function of purified *H. pylori* MutY (HpMutY). Here we show that HpMutY behaves in a similar manner as MutY from *E. coli* but with some striking differences. HpMutY has the ability to excise adenine paired with 8-oxo-G, but not adenine paired with guanine. *H. pylori* *mutY* mutants showed a dramatically increased spontaneous mutation frequency and this is further increased by exposure to oxygen. *H. pylori* *mutY* mutants are deficient in mouse colonization compared to wild-type, and HpMutY appears to be much more specific for 8-oxo-G detection than other MutY enzymes previously studied. It plays a critical role in oxidative DNA damage defense.

2. Materials and Methods

2.1 Bacterial strains and creation of mutants

H. pylori was cultured on Brucella agar (BA) with either 10% defibrinated sheep blood or 5% bovine serum. Cultures were grown in 5% CO₂/5% O₂ incubators at 37° C. The *H. pylori* strains used were SS1 and X47-2L. All strains are listed in Table 1. *E. coli* strains were grown on LB agar plus necessary antibiotics. Mutants were created by PCR amplifying the *mutY* gene plus several hundred base pairs upstream and downstream of the coding region and subsequently ligating this product into the pGEM-T vector. The *mutY* gene was then cut at the unique restriction site BamHI and a kanamycin resistance cassette was ligated into the vector. The disrupted *mutY* gene was introduced into *H. pylori* by natural transformation via allelic exchange and colonies were selected for by growth on the respective antibiotic. Insertions were confirmed by PCR.

2.2 Oxidative stress sensitivity assays

Assays were done similar to the procedure described by Wang [7]. Briefly, *H. pylori* cell suspensions were spread plated onto BA plates. A sterile filter paper disc was then placed in the center of each plate. To this disc 10 µl of each of the following agents was added (in separate experiments): 1M H₂O₂, 0.2M cumene hydroperoxide, 0.2M t-butyl hydroperoxide, 20mM

paraquat. Plates were then incubated for 48 hours at 5% O₂. Zones were measured from the edge of the disc to where growth began.

2.3 Determination of spontaneous mutation rate

H. pylori was grown on plates as described above for 1.5 days. A cell suspension in PBS was made (10⁹ cells/ml) and then diluted 10⁻³ before spread plating onto 10 plates to give 10 parallel cultures. The assay for a particular strain was repeated three times to obtain 30 independent cultures. For an oxidative stress condition the same original cell suspension was poured into an empty petri dish and exposed to atmospheric oxygen levels with shaking at 37° C for 4 hours. The cell suspension was then diluted and spread plated. These independent parallel cultures were allowed to grow for 2 days. Cells were then harvested from these plates and made into cell suspensions (O.D.₆₀₀ = 0.5 for wt and 0.3 for mutant). These suspensions were serially diluted and plated on nonselective plates to determine the total viable cell number and also plated without dilution on plates containing rifampicin (20µg/ml) to determine the number of rifampicin resistant cells. The mutation rate was calculated according to the methods described (36). The most likely number of mutations per culture (*m*) was first calculated from the distribution of numbers of resistant mutants in the independent cultures by using Drake formula ($r/m - \ln(m) = 0$), where *r* is the median number of resistant mutants from a particular strain. Then the mutation rate (μ) per cell division was calculated as $\mu = m/Nt$, where *Nt* is the total cell number per culture.

2.4 Determination of mutation specificity

Single colonies were picked from rifampicin containing plates of the spontaneous mutation rate experiment. Only one colony from each plate was taken to ensure that the mutation would represent an independent event. A 330 bp fragment of the *rpoB* gene, where rifampicin resistance causing mutations are known to occur, was PCR amplified from each of the colonies [32]. These PCR products were sequenced by the DNA sequencing core at the University of Michigan. The mutant sequences were aligned with a wild-type sequence to identify the mutation that had occurred.

2.5 E. coli complementation

E. coli strains PR8 (Su-*lacZ* X74 *galU galK* Sm^r) and *mutY* mutant strain PR70 (similar to PR8 but *micA68::Tn10kan*) were obtained from A-Lien Lu at the University of Maryland, Baltimore [33]. The mutant strain makes a truncated MutY that lacks the C-terminal domain. This strain has been shown to have a mutator phenotype [34]. The *mutY* mutant was complemented by the addition of the same pGEV-HpMutY plasmid used for the protein overexpression and purification experiment. Due to leaky expression from the vector no addition of IPTG was needed to achieve complementation. As a control the empty pGEV1 vector was used. Complementation phenotype was assessed by measuring spontaneous mutation frequency on rifampicin plates by the same method as Lina Li [26]. Data was analyzed and expressed in the same manner as was done for the *H. pylori* mutational rate in section 2.3.

2.6 Protein Expression and Purification

The *H. pylori mutY* sequence was amplified by PCR, purified, cut with NheI and XhoI and ligated into the pGEV1 vector which was cut with the same enzymes (vector obtained from A-Lien Lu, University of Maryland, Baltimore). This plasmid has been used previously to solve solubility problems with MutY proteins [26]. pGEV1 creates an N-terminal fusion with streptococcal protein G (GB1 domain) and a C-terminal fusion with a 6-His tag [35]. The size of the HpMutY fusion protein is 45 kDa. This fusion was to increase the solubility of HpMutY. For expression, pGEV-HpMutY was transformed into *E. coli* BL21 Rosetta pLys. Cells were grown to an O.D.₆₀₀ of 0.5 before being induced with IPTG (final conc. 500mM). After

induction, cells were grown for 2 hours before being harvested, washed, and frozen at -80° C. Protein was purified using a Ni-NTA column following the instructions provided by Qiagen. Protein was dialyzed to remove imidazole. Cell extracts and purified protein are shown in Figure 2.

2.7 Glycosylase Assay

This assay was done similar to the procedure from Pope [19]. Briefly, DNA substrates for glycosylase assays were created by 5' end labeling 37 base oligonucleotides with ³²P ATP and T4 polynucleotide kinase. Labeled strands were annealed to unlabeled complementary strands by heating to 90° C for 5 minutes and then cooling to room temperature over several hours. DNA was cleaned by precipitating with ammonium sulfate and 100% ethanol, then washing with 70% ethanol, before being resuspended in glycosylase assay buffer (20mM Tris-HCl pH 7.6, 1mM DTT, 1mM EDTA, 50mM NaCl, 50µg/ml BSA, 3% glycerol). Reactions were conducted by mixing 500 ng HpMutY protein, DNA substrates to approximately 200,000 cpm of radiation, and assay buffer to a total of 10 µl. Reactions are incubated at 37° C for 30-40 minutes before the addition of 2µl of 0.5 M NaOH and heating at 90° C for 5 minutes to cleave apurinic sites. 2 µl of 0.5 M HCl were added to balance the pH. 10µl of denaturing DNA dye was added followed by heating at 90° C for 5 minutes. Samples were loaded onto a 17.5% acrylamide denaturing DNA gel and electrophoresed for 1.5 hrs at 250 V. Images are obtained by phosphorimaging.

2.8 Mouse colonization

Wild-type and *mutY* mutant X47-2L *H. pylori* cultures were grown as described above for 36 hrs before being suspended in PBS to an O.D.₆₀₀ of 1.7 (X47-2L was used in place of SS1 because of its increased colonization efficiency). These suspensions were administered to C57BL/6 J mice orally (150µl/per mouse). This is 1.5× 10⁸ viable cells. This inoculation was done twice, 48 hours apart. After 3 weeks, mice were sacrificed and their stomachs were removed, homogenized in PBS and the suspensions were diluted and plated on BA plates supplemented with bacitracin (50µg/ml), vancomycin (10µg/ml), and amphotericin B (10µg/ml). Colonies were counted after 5 days of incubation at 37° C at 5% O₂[7].

3. Results

3.1 Bioinformatic analysis of *H. pylori* *mutY* and construction of *mutY* mutant

In *H. pylori*, a *mutY* gene homolog is located between the genes for lactate permease (*lctP*) and 2-oxoglutarate/malate translocator (*SODiT1*) as shown in figure 1A. *mutY* may be in an operon with *SODiT1* since the stop codon of *SODiT1* directly precedes the start codon of *mutY*. To study the physiological role of MutY, we constructed a *mutY* mutant by inserting a kanamycin resistance cassette at the unique restriction site BamHI within the *mutY* gene (figure 1A). The correct insertion of the cassette within the *mutY* gene in the genome was confirmed by PCR showing the increase in the expected size of the PCR product (data not shown). Since *lctP* is transcribed on the opposite strand, it is very unlikely that there would be downstream polar effects from disrupting *mutY*.

MutY from *H. pylori* has 34% amino acid identity with the homolog in *E. coli* as shown by the alignment in figure 1B. The majority of the identical and similar residues shared between these two MutY enzymes are in the N-terminal domain (figure 1B), which has been shown to be the catalytic domain of the protein. The *H. pylori* protein is slightly smaller, containing 328 amino acids versus 350 amino acids. *H. pylori* MutY also has the Glu37 and Asp 138 previously shown to make up the catalytic site of the *E. coli* enzyme (highlighted in figure 1B) [23]. Like in *E. coli*, *H. pylori* MutY has an endonuclease III domain, a helix-hairpin-helix domain, and

an iron sulfur cluster. This could indicate that the enzymes from the two organisms have similar functions.

3.2 Sensitivity to oxidative stress agents

Disc assays using the oxidative stress agents 1M H₂O₂, 0.2M cumene hydroperoxide, 0.2M t-butyl hydroperoxide, and 20mM paraquat were conducted. These disc assays showed no differences between wild-type *H. pylori* SS1 and the *mutY* mutant (data not shown).

3.3 Spontaneous vs. induced mutation rate

Mutation rate was determined by screening for rifampicin resistance. The spontaneous mutation rate of wild-type SS1 (incubated at 5% O₂) was low (1.6×10^{-9}). Disruption of *mutY* in *H. pylori* resulted in a large increase in spontaneous mutation rate (325 fold increase) (Table 2). This effect could be further amplified 763 fold by exposing the cells to atmospheric oxygen levels for 4 hours. Oxygen exposure had no significant effect on the mutational rate of wild-type cells (1.75 fold increase). It has been shown previously in *E. coli* that growing *mutY* mutants in aerobic or anaerobic conditions did not affect the spontaneous mutation frequency [11].

3.4 Specificity of mutations

Specificity of rifampicin resistance conferring mutations was determined by sequencing a 330 bp region of the *rpoB* gene (RNA polymerase beta subunit) known to confer rifampicin resistance [32]. Rifampicin resistance occurs because of changes in the rifampicin-binding site of RNA polymerase, so that rifampicin cannot bind/inactivates the RNA polymerase. Fifty-six Rif^r mutants were isolated for *rpoB* sequence analysis. Most of these mutants were taken from plates under normal growth conditions (i.e. 5% O₂) and five were taken from the plates with 4 hours air exposure. There were no significant differences between the mutation specificity between the two conditions. Of the 56 Rif^r mutants sequenced, 49 showed GC to TA transversions, 1 showed AT to TA transversion, and 6 showed no visible mutations in the sequenced region (Table 3). The mutants showing no visible mutations most likely had mutations outside of the 330 bp sequenced area. Forty-seven of the 49 GC to TA transversion mutants had the exact same mutation in the same base pair. In the sequenced *rpoB* gene region there are four GC to TA transversions that have been previously shown to confer rifampicin resistance [32]. This indicates a special mutational hot spot for conferring rifampicin resistance in *H. pylori mutY* background. This same base pair has been shown to commonly mutate in *E. coli mutY* mutants [33] and *H. pylori mutS* mutants [7], but not in such a high percentage as in the *H. pylori mutY* mutant.

3.5 *E. coli* complementation

To demonstrate that MutY from *H. pylori* has a similar function to the homologous MutY protein of *E. coli*, a complementation experiment was done to determine if HpMutY could reduce the spontaneous mutation rate of an *E. coli mutY* mutant. The *E. coli mutY* mutant PR70 makes a truncated MutY, lacking the C-terminal domain; it has catalytic activity but lacks the specificity for the detection of 8-oxo-G. This strain has been shown previously to display a mutator phenotype [34]. HpMutY was expressed from the same plasmid that was used for protein expression and purification (pGEV-HpMutY). Spontaneous rifampicin resistance was used to assess the phenotype of the organisms. The *mutY* mutant (PR70) has a spontaneous mutation rate 11 fold higher than that of wild-type (PR8). Expression of HpMutY in *E. coli* was able to return the spontaneous mutation frequency of the *E. coli mutY* mutant (PR70) back to wild-type (PR8) levels as seen in Table 4. Introduction of the plasmid vector alone had no effect on the mutation rate.

3.6 Glycosylase Assay

To examine the enzyme activity of HpMutY, we overexpressed (in *E. coli*) HpMutY fused with streptococcal protein G at the N-terminus and a 6-His tag at the C-terminus. By using a Ni-NTA column, this fusion protein (45 kDa) was purified to near homogeneity (Figure 2). The purified HpMutY enzyme was tested for its ability to cleave mismatched DNA substrates. HpMutY cleaved adenine from an A/8-oxo-G pair but surprisingly not from an A/G pair (Figure 3). As expected, HpMutY has no activity towards either C/8-oxo-G mismatches or to the normal base pair C/G. It was also demonstrated that HpMutY does not have the ability to remove 8-oxo-G from 8-oxo-G/A and 8-oxo-G/C mismatches (data not shown).

3.7 Mouse Colonization

To determine if MutY is important for colonization of a mouse stomach, 1.5×10^8 cells were injected into the stomachs of nine C57BL/6 J mice twice, 48 hours apart. The stomachs were taken 3 weeks later and homogenized and plated to determine the cfu/g of stomach. Wild-type *H. pylori* X47 showed an average of 8.58×10^5 cfu/g of stomach whereas the *mutY* mutant had an average of 2.55×10^5 cfu/g of stomach. This indicates that the mutant can only colonize approximately 30% as well as wild-type. The nine colonization numbers for each strain were converted to log₁₀ and plotted as shown in figure 4. According to a Wilcoxon rank test, the colonization efficiency of the *mutY* mutant is significantly ($P < 0.01$) lower than that of wild-type.

4. Discussion

To examine the physiological roles of *H. pylori* MutY in oxidative DNA damage repair, we characterized a *mutY* gene disruption mutant. The *H. pylori mutY* mutant showed a dramatic increase in spontaneous mutation rate compared to wild-type. The 325 fold increase under non-stress conditions is quite high compared to that of either the *E. coli mutY* mutant tested (11 fold increase) or the *H. pylori mutS* mutant, which showed no increase in mutation rate at low oxygen levels. This may reflect a greater role for *H. pylori* MutY in mutation avoidance. When the *H. pylori mutY* mutant is exposed to atmospheric levels of oxygen for 4 hours the mutation rate increase was 763 fold more than wild-type, and was more than two times the low oxygen concentration rate for the same strain. The *H. pylori mutS* mutant showed a 10 fold increase when exposed to the same atmospheric oxygen stress [7]. It has been shown previously in *E. coli* that growing *mutY* mutants in aerobic or anaerobic conditions did not affect the spontaneous mutation frequency [11]. The observation that oxygen levels affect the mutation frequency in *H. pylori* may reflect the microaerophilic nature of the organism. A similar induced mutation rate was seen in the *H. pylori nth* (endonuclease III) mutant after it was exposed to macrophages [8]. The increased oxygen stress on the cells causes increased formation of endogenous ROS which in turn could create more 8-oxo-G.

H. pylori 26695 wild-type has been previously shown to have 67% transition mutations and 33% transversion mutations conferring Rif resistance [7]. *E. coli* has been similarly shown to have a distribution of 49% transitions and 51% transversions [33]. GC to TA transversions are the characteristic mutations caused by 8-oxo-G and 98% of the mutations in the *H. pylori mutY* mutant are GC to TA transversions. Strikingly, 47 of the 49 identified GC to TA transversions are all at the same position (His540). This residue is one of the mutation hotspots for conferring rifampicin resistance. The mutation in this particular residue was seen 2 out of 15 times in a *H. pylori mutS* mutant and 7 out of 35 times in an *E. coli mutYmutM* mutant [33]. It is not clear why this particular mutation is so predominant in the *H. pylori mutY* mutant.

Biochemical analysis of the purified HpMutY resulted in the surprising finding that HpMutY has no ability to cleave A/G mismatches. The *E. coli* protein can actually process the A/G

substrate faster than the A/8-oxo-G substrate, even though it has a greater affinity for the latter. This is due to the fact that *E. coli* MutY releases from an A/G pair more rapidly after cleavage of adenine than it does from an A/8-oxo-G pair [28,29]. This DNA release is the rate limiting step of the reaction. It is thought that the longer retention time of the A/8-oxo-G substrate may serve some protective role to prevent premature cleavage of the 8-oxo-G by MutM which could cause a double strand break if the AP site had not been fixed yet. It is thought that A/8-oxo-G is the major physiological substrate for MutY enzymes and that A/G is not as commonly occurring within the cell [9]. The lack of similarity between the C-terminal domains of *E. coli* MutY and *H. pylori* MutY may help to explain HpMutY's inability to cleave A/G mismatches. The C-terminal domain has been shown to confer specificity for the detection of 8-oxo-G [26,30]. It remains to be determined if the altered C-terminus of HpMutY is responsible for its lack of activity towards A/G.

The attraction of HpMutY to A/8-oxo-G mismatches over A/G mismatches is similar to what has been seen for *H. pylori* MutS. In DNA binding experiments, *H. pylori* MutS showed a high binding affinity for A/8-oxo-G but a low binding affinity for A/G [7]. This similarity may indicate that these enzymes work together in *H. pylori* to combat the effects of oxidative DNA damage. *H. pylori* MutS may be involved in taking the place of the absent MutM. MutM is responsible for removing 8-oxo-G from C/8-oxo-G mismatches. After MutY removes the adenine opposite the 8-oxo-G, there would be an apurinic site left that would be filled with a cytosine by a DNA polymerase [9]. This creates MutM's substrate. Without a functional MutM enzyme or another mechanism of removing 8-oxo-G incorporated in the DNA the cell would keep repeating a futile process. If this 8-oxo-G is not removed and another round of replication occurs the A/8-oxo-G pair would be formed once again. This futile cycle could be another reason for the very high genetic diversity of *H. pylori*, but it seems unlikely that the cell would waste its energy making MutY if there was no other enzyme available to act on the C/8-oxo-G substrate that is created after MutY's activities.

Although it has been shown that the substrate specificities for *E. coli* MutY and *H. pylori* MutY are not exactly the same, we have shown that HpMutY has the ability to complement the *E. coli* *mutY* mutant (PR70) and return its spontaneous mutation rate to near wild-type levels (table 4). This would indicate that the two enzymes share a similar role in the cell, which is removing adenines from A/8-oxo-G mismatches.

H. pylori infection in the host causes a strong inflammatory response, leading to the production of ROS. Although *H. pylori* is equipped with a variety of ROS-detoxifying enzymes, it may still suffer continuous oxidative DNA damage *in vivo*. In this study, the *H. pylori* *mutY* mutant was shown to only be able to colonize the mouse stomach 30% as well as wild-type, indicating a role of MutY in DNA damage repair *in vivo*. The extremely high mutation rate in *mutY* mutant cells may cause them to acquire many more deleterious mutations compared to wild-type. It is possible the colonization deficiency observed at three weeks may be further amplified by allowing the mice to live longer than that after inoculation. In the *H. pylori* endonuclease III mutants the colonization proficiency continued to decrease over a two month period, most likely due to the build up of mutations [8]. The *H. pylori* *mutS* mutant was also shown to have a deficiency in its colonization ability [7]. These results taken together provide evidence that these DNA repair enzymes (MutY, MutS, and Endo III) are needed to protect the genome of *H. pylori* from oxidative DNA damage *in vivo*.

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A

B

H.p	--METLHNALLKWYEEFGRKGLPFRNLKGINAPYEVYIS	EVMSQQTQINTVIERFYSPFL	58
E.c	MQASQFSAQVLDWYDKYGRKTLPWQIDK---	TPYKVWLS	56
	* * *	* * * * *	
H.p	EAFPTLKDLANAQLEEVLLLWRGLGYYSRAKNLKKSAEICVKEHHSQLPNDY	QSLKLPG	118
E.c	ARFPTVTDLANAPLDEVHLWTGLGYARARNLHKAQQVATLHGGKFPETFE	EEVAALPG	116
	***	*****	
H.p	IGAYTANAILCF	GFREKRACV	177
E.c	VGRSTAGAILSL	SLGKHFPIL	176
	* * *	* * * * *	
	N-term / C-term		
H.p	---HNQALIDL	GAL	229
E.c	VERFNQAMMDLGAM	ICTRSPKPKCSLCPLONGCIAA	236
	***	*****	
H.p	GVVIQNNQIALEKIEQKLYLGMHHFPDLKENLECKLPFLGAIKHSHTKFKLHLNLYSAAI		289
E.c	LLQHEDEVLLAQRPPSGLWGGLYCFPQFADEESLRQWLAQRQIAADNLTQLTAFRHTFSH		296
	* * *		
H.p	KDLKNPVRFYSLKDLETLPISMTLKILNFLKQKNLFGG		328
E.c	FHLDIVPMW-----	LPVSSFTGCMDEGNALWYNLAQPPSVGLAAPVERLLQQLRTGAPV	350
	*	* * * *	

Figure 1.

Genome region and sequence alignment. A) genome region surrounding *mutY* in *H. pylori* strain 26695. *lctP* is lactate permease and *SODiT1* is 2-oxoglutarate/malate translocator (authentic frameshift). Location of Kanamycin cassette insertion is indicated. B) Sequence alignment comparing *H. pylori* MutY to *E. coli* MutY. The catalytic residues in the active site are highlighted black. Conserved residues are marked with "*". Endonuclease III domain is underlined. Helix-Hairpin-Helix domain is highlighted grey with black lettering. Iron Sulfur domain is highlighted grey with white lettering. Division between N-terminal and C-terminal domains is marked with a "/".

Figure 2. Overexpression and purification of HpMutY. Lane M is a marker, lane 1 is BL21 Rosetta pLys (pGEV-HpMutY) whole cell extract prior to induction with IPTG, lane 2 is the same cells 2 hours after induction, lane 3 is purified HpMutY. The size of HpMutY fusion protein is 45 kDa.

Figure 3.

Glycosylase activities of HpMutY with different mismatched substrates. Oligonucleotide substrates (labeled with 200,000 cpm ^{32}P) were mixed with 500 ng HpmutY and incubated at 37° C for 30 minutes. Control lane lacks enzyme. The first base in the pair is in the strand that is labeled. Substrates are 37 mers and products are 18mers. GO = 8-oxo-G

Figure 4.

Mouse colonization of X47 wild-type and *mutY*::Kan X47. Nine mice were inoculated twice (2 days apart) with 1.5×10^8 cells. Stomachs were homogenized 3 weeks after initial inoculation. Each point represents the cfu count for one stomach, expressed per gram stomach tissue. The baseline $2.7 \log_{10}$ cfu/g is the limit of detection for the assay. The value for wild-type and mutant are significantly different ($P = < 0.01$) based on Wilcoxon rank test.

Table 1**Strains and Primers**

Strains	Description
SS1	<i>H. pylori</i> wt used for mutation frequency
<i>mutY</i> ::Kan SS1	<i>H. pylori</i> mutant used for mutation frequency
X47-2L	<i>H. pylori</i> wt used for mouse colonization
<i>mutY</i> ::Kan X47	<i>H. pylori</i> mutant used for mouse colonization
PR8	<i>E. coli</i> wt
PR70	<i>E. coli mutY</i> mutant
PR70 pGEV-HpMutY	<i>E. coli mutY</i> mutant complemented with <i>H. pylori</i> MutY
PR70 pGEV1	<i>E. coli mutY</i> mutant with empty vector (negative control)
BL21 Rosetta pLys pGEV-HpMutY	<i>E. coli</i> overexpressing HpMutY
Primers	Name and Description
GGTTTGGCTTTCACCTTTTCTCG	mutYF- creation of <i>H. pylori mutY</i> mutant
CACCATTTATTACGGGAGCGG	mutYB- creation of <i>H. pylori mutY</i> mutant
CGCGCGGGCTAGCGAACTTTACACAAC	GmutYF- creation of HpMutY overexpression plasmid
GTGGAGCCTCGAGACCCCAAATAAAATTTT	GmutYR- creation of HpMutY overexpression plasmid
TTTGATTGCTCATGCCCAT	rpoBP1- amplification of <i>H. pylori rpoB</i>
CACAACCTTTTATAAGGGGC	rpoBP2- amplification of <i>H. pylori rpoB</i>
ATTTCCTTCAGCAGATAG/8-oxo-G/AACCATACTGATTCACAT	8-oxo-G strand for Glycosylase assay
ATTTCCTTCAGCAGATAGGAACCATACTGATTCACAT	G strand for Glycosylase assay
ATGTGAATCAGTATGGTTCCTATCTGCTGAAGGAAAT	C strand for Glycosylase assay
ATGTGAATCAGTATGGTACTATCTGCTGAAGGAAAT	A strand for Glycosylase assay

Table 2
Mutation rates of *H. pylori* and *mutY* mutant strains

Strain	Mutation rate per cell division ^a	Fold Increase ^b
SS1 (wild-type)	1.6×10^{-9}	1
SS1 with air stress	2.8×10^{-9}	1.75
<i>mutY</i> ::Kan SS1	5.2×10^{-7}	325
<i>mutY</i> ::Kan SS1 with air stress	12.2×10^{-7}	763

^a Mutation rate was determined with rifampicin resistance and calculated with Drake formula as described in Materials and Methods.

^b fold increase relative to wild-type.

Table 3Mutation Specificity of *mutY*::Kan SS1 strain

Base Change	Mutations conferring Rif ^{Ra}	Occurrence in <i>mutY</i> ::Kan strain ^b
	Amino Acid Change ^a	
AT → GC	Q527R	0
	D530G	0
	H540R	0
GC → AT	D530N	0
	H540Y	0
	S526L	0
	L525P	0
AT → TA	D530V	1
	I586P	0
	I586N	0
GC → TA	L525I	0
	Q527K	1
	H540N	47
	D530Y	1
Unknown	Unknown	6

^a All the possible mutations in *rpoB* gene known to confer rifampicin resistance in *H. pylori* (ref 32).

^b number of times this particular mutation was seen out of the 56 total sequenced spontaneous mutants

Table 4
Complementation of *E. coli mutY* mutant with HpMutY

Strain	Mutation rate per cell division ^a	Fold Increase ^b
PR8 (wild-type)	3.3×10^{-8}	1
PR70	3.7×10^{-7}	11.2
PR70 (pGEV-HpMutY)	5.2×10^{-8}	1.6
PR70 (pGEV1)	3.0×10^{-7}	9.1

^a Mutation rate was determined with rifampicin resistance and calculated with Drake formula as described in Material and Methods.

^b fold increase relative to wild-type