ROLE OF A MUTY DNA GLYCOSYLASE IN COMBATING OXIDATIVE DNA DAMAGE IN HELICOBACTER PYLORI

by

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(Under the direction of Robert Maier)

ABSTRACT

MutY is an adenine glycosylase that has the ability to 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 gastric pathogen *Helicobacter pylori* has a homolog of the MutY enzyme. To investigate the physiological roles of MutY in *H. pylori*, I characterized a *mutY* mutant and also purified the *H. pylori* MutY enzyme. The mutant has an elevated spontaneous mutation frequency when incubated at 5% O_2. This effect can be amplified by exposure to atmospheric oxygen levels. Most of the mutations sequenced are GC to TA transversions. 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. The *H. pylori mutY* gene was able to complement an *E. coli mutY* mutant. *H. pylori mutY* mutants are only 30% as efficient as wild-type in colonizing the stomachs of mice.

INDEX WORDS: *Helicobacter pylori*, *mutY*, DNA damage, oxidative stress

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Helicobacter pylori

Helicobacter pylori was originally isolated by Barry Marshall and Robin Warren in 1982 [37]. It had been previously seen in the mucosal layer of gastric biopsy samples but had never been successfully isolated. The organism was characterized and originally named *Campylobacter pyloridis*. In the few years following its isolation, it became evident that *H. pylori* infection was strongly associated with inflammation of the gastric mucosa [6]. *H. pylori* is a microaerophile, incapable of growing at atmospheric levels of oxygen. It also has complex nutritional requirements that are not completely understood. It is commonly grown on complex media supplemented with either blood or serum.

H. pylori isolated from gastric specimens has a spiral shape, but when it is grown on solid media the cells appears more rod-shaped [23,24]. After prolonged culturing on solid media or in liquid culture, the cells take on a coccoid morphology [8]. These are rod-shaped cells folded over on themselves. These coccoid cells are metabolically active but cannot be cultured. *H. pylori* is gram negative and is classified with the epsilon proteobacteria. It is motile by four to six unipolar sheathed flagella [23].

There are currently two strains of *H. pylori* whose genomes have been sequenced. Strain 26695 was sequenced by The Institute for Genomic Research in 1997 [61]. It has a 1.6 Mb genome with 1630 annotated genes. Its G + C percentage is 39%.

The genome for strain J99 was later sequenced by Astra and Genome Therapeutics. *H. pylori* shows very high diversity between strains. On average it also has a higher mutational frequency and a higher rate of recombination than most organisms [59].

H. pylori has been found in humans from all parts of the world. It has also been isolated from other primates. People in developing countries have been shown to be more likely to carry *H. pylori* with 70 to 90% of the population being infected. This is somewhat less in developed countries with 20 to 50% of the population carrying *H. pylori* [60]. It has been shown that most people acquire *H. pylori* during childhood. There are no substantial reservoirs for *H. pylori* except for the stomachs of primates. *H. pylori* is believed to be spread from person to person through fecal-oral transmission and oral-oral transmission [41]. In the past, before improvements in disinfecting techniques, it was possible to transmit *H. pylori* with a contaminated endoscope [1].

H. pylori infections can cause a variety of disease states. Everyone infected with *H. pylori* develops chronic gastric inflammation, but this is very often asymptomatic [6]. One of the common illnesses caused by *H. pylori* infection is peptic ulcers. This is the most common cause of gastric and duodenal ulcers in the human population [45]. The infection was classified as a class I human carcinogen by International Agency for Research on Cancer in 1994 [30]. *H. pylori* is known to cause gastric adenocarcinoma and gastric lymphoma of the mucosa-associated lymphoid tissue (MALT lymphoma) [58]. *H. pylori* infections are easily curable. They are commonly treated with a triple therapy consisting of bismuth subsalicylate, metronizadole, and tetracycline. This treatment usually lasts for two weeks [18].

H. pylori has several virulence factors that help to make it a highly successful gastric pathogen. The enzyme urease is critical to the survival of *H. pylori* in the harsh environment of the stomach. It has been shown that urease mutants are unable to colonize the stomachs of mice [62]. This enzyme takes urea and breaks it down into carbon dioxide and ammonia. This helps to create a pH neutral microenvironment around the cell to help it resist the low pH of the stomach. *H. pylori* also has a vacuolating cytotoxin (VacA) that induces the formation of acid vacuoles in host cells; this in turn causes damage to the gastric mucosa [51]. *H. pylori* also possesses the cytotoxin-associated gene (CagA) located on a 40kb pathogenicity island. This pathogenicity island contains a type IV secretion system that is responsible for injecting CagA into host cells. This enzyme is believed to disrupt host cell signaling pathways and therefore contribute to disease [9]. *H. pylori* also has oxidative stress resistance genes that contribute to virulence. These will be discussed in the next section.

Oxidative stress resistance in *H. pylori*

H. pylori infection induces the recruitment and activation of host immune cells such as macrophages and neutrophils. These cells produce reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, and hydroxyl radicals [16]. Inflammation also induces nitric oxide synthase (iNOS) which produces large amounts of nitric oxide (NO) over time [43]. Reaction of NO with oxygen radicals leads to the formation of nitrogen oxides and peroxynitrate, which are reactive nitrogen species (RNS). Production of ROS and RNS can have damaging effects on both the host and pathogens biological molecules. Superoxide and hydrogen peroxide can interact with the Fe-S

clusters of enzymes [20]. This can cause inactivation of the enzymes as well as release of free iron. Hydrogen peroxide can interact with free iron through Fenton reactions and produce hydroxyl radicals, which are potent oxidizers of biological molecules [26]. The oxidative stress response over time caused by infection with *H. pylori* is believed to play a role in the formation of gastric cancer [14].

H. pylori has several enzymes dedicated to combating this oxidative stress. They fall into the categories of: ROS detoxifying enzymes, oxidative DNA damage repair enzymes, and oxidative protein damage repair enzymes. The enzyme superoxide dismutase (SOD) is responsible for the breakdown of superoxide molecules. The reaction of SOD with superoxide takes two molecules of superoxide and two protons and creates hydrogen peroxide and oxygen. *H. pylori* has one SOD enzyme (encoded by *sodB*) whereas *E. coli* has three SOD enzymes. *H. pylori sodB* mutants show an increased sensitivity to oxygen. These mutants also show an increased mutation frequency, indicating the role of SOD in protecting against oxidative DNA damage. Compared to

E. coli, H. pylori makes a large amount of SOD but with a low specific activity [42]. SOD also plays a role in controlling of the levels of peroxynitrate. This occurs because peroxynitrate is formed from nitric oxide and superoxide. The enzyme catalase is responsible for the breakdown of hydrogen peroxide. This enzyme can take two molecules of hydrogen peroxide and create two water molecules and one oxygen molecule. The gene for catalase in *H. pylori* is *katA*. *H. pylori katA* mutants are more sensitive to killing by hydrogen peroxide, professional phagocytes, and macrophage phagosomes [5,27,54]. *katA* mutants are also deficient in long term colonization of mice,

indicating that this enzyme is important for persistence in the host [28]. *H. pylori* has another protein that is associated with catalase that is known as KapA (KatA-associated protein). Disruption of this gene does not affect catalase activity but does result in increased sensitivity to hydrogen peroxide [27]. This gene appears to be specific to *H. pylori* and has no known homologs. *E. coli* has two catalases, one cytoplasmic and one periplasmic, whereas *H. pylori* only has one catalase which is located in both the cytoplasm and periplasm. *H. pylori* has a group of peroxiredoxins that are responsible for the breakdown of hydrogen peroxide, peroxynitrate, and organic hydroperoxides. The enzyme AhpC has the ability to breakdown hydrogen peroxide, t-butyl hydroperoxide, and linoleic acid hydroperoxide [4]. In *H. pylori ahpC* mutants were only able to grow at very low oxygen conditions. These mutants were also shown to be unable to colonize the stomach of a mouse [46]. *H. pylori* has two thiol-peroxidases, Tpx and Bcp. Mutants in either of these genes in *H. pylori* show increased sensitivity to oxidative stress agents as well as reduced colonization ability in the mouse model [47,66].

H. pylori has homologs to the DNA repair proteins Nth, MutS, MutT, and MutY. Nth encodes endonuclease III, which is responsible for removing oxidized pyrimidines such as thymine glycol. *H. pylori nth* mutants show increased sensitivity to oxidizing agents and are deficient in colonizing mouse stomachs when compared to wild-type [49]. It is strange that *H. pylori* would have a homolog to *mutS* because it has no homologs to *mutL* and *mutH*. These three enzymes together normally form what is known as the methyl-directed mismatch repair system. MutS in *H. pylori* does not function in conventional mismatch repair. It has been shown that *mutS* mutants in *H. pylori* have increased sensitivity to oxidative stress agents. They showed an increased mutation

frequency when exposed to oxidative stress conditions and the predominance of G/C to T/A transversions, which are characteristic of 8-oxo-G. These mutants contained three times the amount of 8-oxo-G as wild-type cells. Purified *H. pylori* MutS showed the ability to bind double stranded DNA containing 8-oxo-G [65]. MutT and MutY have not yet been studied in *H. pylori*. The study presented in this thesis will focus on the role of MutY in *H. pylori*.

For repair of oxidized proteins, *H. pylori* has methionine sulfoxide reductase (Msr). Methionine is one of the most oxidation sensitive amino acids. It can be oxidized to form methionine sulfoxide, rendering many methionine-containing proteins inactive. Methionine sulfoxide in proteins or as a free amino acid is the substrate for Msr; the repair enzyme can restore the methionine back to normal [2,64].

Oxidative DNA damage

One of the biological molecules in the cell that can be easily damaged by oxidation is DNA. This oxidative damage can occur through exogenous or endogenous sources. Reactive oxygen species (ROS) can be produced endogenously as a common byproduct of aerobic respiration. The electron transport chain in aerobic respiration is leaky in nature and often allows the release of free electrons [13]. These free electrons can react with oxygen, which normally has two unpaired electrons, to form superoxide (O_2^-). Superoxide can be further reduced by reacting with additional electrons to form hydroxyl radicals and hydrogen peroxide. ROS can also be formed by ionizing and UV radiation. Another common source of ROS that pathogens encounter is the oxidative burst from host immune cells. This commonly occurs when cells are engulfed by

macrophages. Superoxide is generated by NADPH oxidase, which catalyzes a one electron reduction of oxygen using NADPH as the electron donor [50].

These ROS can react with DNA causing harmful lesions and modified bases that have detrimental effects on the cell. There are more than 20 different types of damaged bases that can occur after oxidative stress. The most commonly damaged purine is guanine, which is converted to 7,8-dihydro-8-oxoguanine (more commonly known as 8-oxoguanine or 8-oxo-G). The most commonly damaged pyrimidine is thymine, which is converted into thymine glycol [57]. 8-oxo-G can be created in several ways through reactions with ROS such as, hydroxyl radical reactions, one-electron oxidation reactions, and singlet oxygen oxidation. The hydroxyl radical reaction to the formation of 8-oxo-G through the 8-dihydroxy-7,8-dihydroguanyl radical. This intermediate arises either through addition of an OH group at C8 or through hydration of the guanine radical cation. This intermediate can either go through an oxidation or reduction reaction, depending on the reducing features and the availability of oxygen in the surrounding environment. If oxidation occurs, then 8-oxo-G is formed. If a one electron reduction occurs, then 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Fapy) is formed. When single nucleosides are the target, the predominant product is Fapy. When isolated DNA is the target the predominant product is 8-oxo-G. This may be due to the fact that DNA is a more reducing environment, likely caused by bound transition metals, than the free nucleoside. The formation of 8-oxo-G through one-electron reactions is a minor process. This reaction proceeds through the formation of the guanine radical cation followed by conversion to the 8-dihydroxy-7,8-dihydroguanyl radical.

Guanine can be ionized by ionizing radiation, UV light, type I photosensitizers, and peroxyl and oxyl radicals. Guanine is the only base that has the ability to react with singlet oxygen. This reaction is a Diels-Alder [4+2] cycloaddition across the 4,8-bond of the imidazole ring. This leads to the formation of 4,8-endoperoxide, which is further converted to 8-hydroperoxyl-2'-deoxyguanosine before finally forming 8-oxo-G [10].

The presence of 8-oxo-G in DNA is harmful to cells because of its altered base pairing properties. Where guanine normally pairs with cytosine, 8-oxo-G has the ability to pair with adenine. This can occur due to a conformational switch in the position of the guanine. Guanine is normally in the anti conformation when base pairing with cytosine. Once converted to 8-oxo-G, it is sterically more feasible for it to adopt the syn conformation. This makes it less likely to bind with cytosine and increasing more likely to bind with adenine. The polymerase which is incorporating the nucleotides also has an effect on which base is paired with 8-oxo-G. A replicating polymerase has been shown to be more likely to incorporate the incorrect adenine across from 8-oxo-G whereas a repair polymerase is more likely to incorporate the correct base which is cytosine [56]. The A/8-oxo-G mismatch that is created causes a G/C to T/A transversion mutation that is characteristic of 8-oxo-G DNA damage.

E. coli has a group of enzymes dedicated to the prevention of damage caused by 8-oxo-G. It is known as the GO system and is comprised of three enzymes: MutT, MutM, and MutY [40]. MutT is a hydrolase that converts 8-oxo-dGTP to 8-oxodGMP and pyrophosphate [35]. 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 [12]. MutY is an adenine glycosylase that excises adenines paired

with 8-oxo-G [3]. 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 [19].

MutY in *E. coli*

The *mutY* gene locus was originally discovered in *E. coli* as a locus whose disruption generates a large quantity of GC to TA transversions within a cell [44]. This was accomplished using a *lacZ* mutant that would only revert to wild-type if certain transversion mutations occurred. It was shown early on that MutY could act as an adenine glycosylase which was capable of repairing A/G mismatches [3]. G/A to G/C repairs were shown to be possible *in vitro* by mixing purified MutY, a 5' apurinic/apyrimidinic-site endonuclease, DNA polymerase I, and DNA ligase [3]. Several years later it was discovered that MutY had glycosylase activity on A/8-oxoG mismatches. It was also shown that overexpressing MutM in a *mutY* mutant background caused the mutational frequency to return to wild-type levels [39]. This provided early evidence for a relationship between these two enzymes in an oxidative DNA damage repair system. These two enzymes along with MutT came to be known as the GO system, whose job it was to protect the cell from the damaging effects of 8-oxo-G [40]. MutY served as an adenine glycosylase, removing adenines from A/8-oxo-G mismatches. MutM was an 8-oxo-G DNA glycosylase that could remove 8-oxo-G from 8-oxo-G/C basepairs [12]. MutT is a hydrolase that converts 8-oxo-dGTP to 8-oxodGMP and pyrophosphate [35]. This prevented any oxidized dGTP from being incorporated into DNA.

It was originally thought that MutY was a bifunctional enzyme possessing both a glycosylase and apurinic (AP) lyase activity [34]. This AP lyase function was debated throughout the years [25,67,68,69]. It has more recently been determined that this lyase activity is uncoupled from the glycosylase activity, meaning the enzyme is not truly bifunctional. The lyase activity is believed to be due to active site plasticity and slow dissociation of the enzyme from the reaction product [36].

MutY can be trapped in a stable covalent enzyme-DNA Schiff base complex using sodium borohydrate [34,67]. This is characteristic of bifunctional glycosylase enzymes. A Schiff base is carbon double bonded to a nitrogen with the nitrogen being attached to an aryl or alkyl group. This crosslinking was shown to involve lysine residue number 142 [73]. However, this residue and the covalent complex it generates are not necessary for the enzyme's catalytic activity. It was shown that a site directed mutant strain (K142A) had normal glycosylase activity without Schiff base formation [70].

The active site of the MutY enzyme is a deep cleft that binds adenine and contains the two catalytic residues, glutamic acid 37 and aspartic acid 138 [25]. Adenine is flipped out of the helix where it can be cleaved. Once binding has occurred there is a coupled protonation of N7 of adenine and a nucleophilic attack by an activated water molecule on the C1′ of the deoxyribose leading to the cleavage of the glycosyl bond and the formation of an AP site [36].

MutY displays unusual enzyme kinetics. It holds onto its substrate for some time after catalysis has occurred. The adenine cleavage step is very rapid compared to MutY releasing from the DNA substrate; the latter is the rate limiting step [38]. MutY has different affinities for its various substrates. The K_d for an A/8-oxo-G substrate is

0.112 nM versus 5.6 nM for an A/G substrate [31]. This reflects the greater affinity for the 8-oxo-G containing substrate. Even though it has a greater affinity for the A/8-oxo-G substrate, it can turnover the A/G substrate faster. This is due to a longer retention time of the A/8-oxo-G substrate [38]. It is thought that this substrate is retained longer to protect the AP/8-oxo-G from MutM, which could potentially cause a double strand break if it acts before a polymerase has time to fill the AP site.

MutY can be digested with thermolysin to yield two stable fragments, an N-terminal fragment of 25 kDa and a C-terminal fragment of 12 kDa [21]. The N-terminal domain is the catalytic domain of the protein. It contains an endonuclease III domain, a helix-hairpin-helix motif, and a 4Fe-4S cluster. This N-terminal domain functions just as well as the intact protein for the recognition of A/G mismatches, with a K_d of 5.6 nM for intact enzyme versus 6.8 nM for the N-terminal domain. On the other hand, the N-terminal domain shows a deficiency in the recognition of A/8-oxo-G mismatches, with a K_d of 0.112 for intact enzyme and 4.3 for the N-terminal domain [31]. This indicates that the role of the C-terminal domain of MutY is in specific recognition of 8-oxo-G.

The 4Fe-4S cluster of MutY has been shown to be important for DNA binding and catalytic activity but not for protein folding or thermal stability [53]. Through site-directed mutagenesis of individual cysteine residues it was determined that the 4Fe-4S cluster plays a role in substrate recognition and protein stability *in vivo* [22]. It has also been shown that lysine 198 within the iron sulfur cluster motif plays a role in damage recognition and removal [11]. The 4Fe-4S clusters may also play a role in DNA damage detection through changes in their redox state. This could occur through a mechanism called DNA-mediated charge transport. In this system, the MutY enzyme has a different

redox state depending on whether it is bound to DNA or free in solution. When bound to DNA the redox state is +3 and when free it is +2. Two MutY enzymes bound to DNA can exchange electrons down the length of undamaged DNA. These electrons will reduce the enzymes and cause them to release from the DNA. They can then bind DNA again and become oxidized. This electron transfer cannot occur through damaged DNA. This causes MutY to stay attached to and accumulate near DNA damage sites, to enable and facilitate its repair function. Once the damage is repaired, the MutY can be reduced. This will cause it to be released and relocate to another piece of DNA where the process can continue [7,71].

MutY has been shown to be downregulated by oxidative stress. Its activity was reduced by 30% when cells were grown in the presence of 0.5 mM paraquat or 0.1 mM H_2O_2. Activity was doubled under anaerobic conditions compared to aerobic conditions. This increased activity under anaerobic conditions could be further enhanced by mutations in *fur, fnr*, and *arcA* [72]. This indicates that *mutY* is under the positive control of these regulatory genes. This expression is opposite of the control of MutM which is upregulated under conditions of oxidative stress. This may be occurring because under conditions of oxidative stress the oxidation of dGTP in the nucleotide pool will be much higher than that of guanine already incorporated into the DNA. This is because the dGTP is more easily accessible to reactive oxygen species. If MutT cannot keep up with the demand of 8-oxo-dGTP formed, then the MutY enzyme can itself become mutagenic. This scenario is supported by observations of a $MutT^-$ strain where an A/T pair can have an 8-oxo-dGTP incorporated into it during replication to form an A/8-oxo-G mismatch.

This mismatch then is converted to C/8-oxo-G after the actions of MutY and then to C/G after the actions of MutM. The end result of this is an A/T to C/G transversion mutation [63]. Therefore if 8-oxo-dGTP is allowed to be incorporated into the DNA, then MutY becomes mutagenic for the cell. This could be one explanation for why MutY is downregulated under conditions of oxidative stress.

MutY in other organisms

Although MutY has been best characterized in *E. coli*, it has been somewhat characterized in several other organisms. This repair enzyme is important in both prokaryotes and eukaryotes. So far it has been studied in *Deinococcus radiodurans*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Neisseria meningitidis*, *Neisseria gonorroheae*, *Schizosaccharomyces pombe*, mice, and humans.

MutY in *D. radiodurans* has 35% identity to the *E. coli* protein. Heterologous expression of *D. radiondurans* MutY has been shown to be able to functionally complement an *E. coli mutY* mutant. It has been shown to have adenine glycosylase activities on A/G, A/C, and A/8-oxo-G mismatch substrates. The kinetic parameters of this enzyme are similar to that of *E. coli* MutY [32]. MutY in *P. aeruginosa* is 53% identical to MutY in *E. coli*. The *Pseudomonas* enzyme was shown to be able to complement an *E.coli mutY* mutant [48].

A *mutY* mutant is *B. subtilis* was shown to have a high spontaneous mutation frequency. 71% of these mutations were GC to TA transversions mutations, which are characteristic of 8-oxo-G DNA damage. MutY from *Bacillus* was also shown to be able to complement an *E. coli mutY* mutant [55].

Both *N. meningitides* and *N. gonorroheae* contain *mutY* homologs. When *mutY* mutants were made in *Neisseria*, an increase in mutation frequency is observed as well as an increase in the percentage of GC to TA transversions. Purified *Neisseria* MutY shows adenine glycosylase activity towards A/8-oxo-G and A/C mismatches. The A/G substrate was not tested [14].

The *mutY* homolog in the fission yeast *Schizosaccharomyces pombe* is known as SpMYH. It shows 28% identity to the *mutY* homolog in *E. coli*. The yeast homolog can complement the increased mutational frequency phenotype in an *E. coli mutY* mutant. Purifed SpMYH shows adenine glycosylase activity on A/8-oxo-G and A/G substrates. It has less of an affinity for A/C than the *E. coli* enzyme. Like *E. coli*, SpMYH can catalyze A/G substrate turnover three times more rapidly than the A/8-oxo-G substrate [33]. Surprisingly, SpMYH also shows a guanine glycosylase activity towards G/8-oxo-G mismatches that is equally as strong as its adenine glycosylase activity [17].

The murine homolog of *mutY* is known as mMYH. Like *E. coli* MutY, mMYH shows adenine glycosylase activity towards A/8-oxo-G and A/G but the intrinsic rates of adenine removal is diminished 10-fold compared to that of *E. coli*. mMYH can remove 2-hydroxyadenine at the same rate as adenine, where *E. coli* MutY has a much lower rate of 2-hydroxyadenine removal compared to adenine [52].

The human homolog of *mutY* is hMYH. This enzyme is a monofunctional glycosylase with no associated lyase activity [29]. Biallelic mutations in hMYH can cause an autosomal recessive condition of adenomatous colorectal polyposis as well as high colorectal cancer risk. This was the first link shown between an inherited disorder in a DNA glycosylase and human cancer [29].

Helicobacter pylori has a homolog to MutY from *E. coli* with 34% amino acid identity. They share common sequence elements of the endonuclease III domain, helix-hairpin-helix motif, and a 4Fe-4S cluster domain. They share the same residues in the active site which have been proven to be the catalytic residues in the *E. coli* MutY enzyme. To date no experimental work has been published on *mutY* in *H. pylori*. The purpose of this study is to elucidate the role of the *mutY* homolog in *H. pylori* strain SS1. We will determine if it functions in a manner similar to that of *E. coli*. One rationale for studying its role in

H. pylori is the unusual genetic variability of this organism. The mutator phenotype of a *H. pylori mutY* mutant will be determined as well as the catalytic abilities of the purifed *H. pylori* MutY enzyme. The role of *H. pylori mutY* will also be tested in a mouse model, to determine if this gene is important for virulence or colonization.

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CHAPTER 2

ROLE OF A MUTY DNA GLYCOSYLASE IN COMBATING OXIDATIVE DNA DAMAGE IN HELICOBACTER PYLORI[1]

[1] Eutsey, R., G. Wang, and R. J. Maier. Submitted to *DNA Repair*, 6/22/06

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. An *H. pylori mutY* mutant incubated at 5% O_2 have a 343 fold higher spontaneous mutation frequency than its parent. The mutation frequency 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* gene 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 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-oxo-dGMP 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% CO_2/5% O_2 incubators at 37^o C. The *H. pylori* strains used were SS1 and X47-2L. *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: 1M H_2O_2, 0.2 M cumene hydroperoxide, 0.2 M t-butyl hydroperoxide, 20mM paraquat. Plates were then incubated for 48 hours at 5% O_2. 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 (10^9 cells/ml) and then diluted 10^{-3} before spread plating onto 10 plates to give 10 parallel 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^o C for 4 hours. The cell suspension was then diluted and spread plated. These 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 mutational frequency is expressed as a ratio of rifampicin resistant cells to the total number of cells. The assay was done a minimum of 3 times for each strain. The procedure was adapted from G. Wang et al. [7].

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 *micA*68::Tn*10*kan) 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].

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^{o} 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 ^{32}P ATP and T4 polynucleotide kinase. Labeled strands were annealed to unlabeled complementary strands by heating to 90^{o} 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. Labeling specific activity is approximately 5,000 cpm/ pmol DNA. Reactions are incubated at 37^{o} C for 30-40 minutes before the addition of 2l µl of 0.5 M NaOH and heating at 90^{o} C for 5 minutes to cleave apurinic sites. 2 µl of 0.5 M HCl were added to balance the pH. Ten ul of denaturing DNA dye was added followed by heating at 90^{o} 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 *H. pylori* cultures were grown as described above for 36 hrs before being suspended in PBS to an O.D. $_{600}$ of 1.7. These suspensions were administered to C57BL/6 J mice orally (150µl/per mouse). This is 1.5 x 10^8 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^o C at 5% O_2 [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 (*SODiT*1) as shown in figure 1A. *mutY* may be in an operon with *SODiT*1 since the stop codon of *SODiT*1 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 binding domain. 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 H_2O_2, 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 frequency

Mutation frequency was determined by screening for rifampicin resistance. The spontaneous mutation frequency of wild-type SS1 (incubated at 5% O_2) was low (0.4×10^{-8}). Disruption of *mutY* in *H. pylori* resulted in a large increase in spontaneous mutation frequency (343-fold increase) (Table 2). This effect could be further amplified 1125-fold by exposing the cells to atmospheric oxygen levels for 4 hours. Oxygen

exposure had no significant effect on the mutational frequency of wild-type cells (1.25 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% O_2) 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 4). The mutants showing no visible mutations most likely had mutations outside of the 330 bp sequenced area. 47 of the 49 GC to TA transversion mutants had the exact same mutation in the same base pair. This indicates a mutational hot spot for conferring rifampicin resistance. 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 frequency 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 frequency 12 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 3. Introduction of the plasmid vector alone had no effect on the mutation frequency.

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 under argon gas 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 $\log_{10}$ 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 343-fold increase under non-stress conditions is quite high compared to that of either the *E. coli mutY* mutant tested (12-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 1125-fold more

than wild-type, almost four 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]. Most possibly, 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 [7]. *E. coli* has been shown to have a distribution of 49% transitions and 51% transversions [33]. GC to TA transverions 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 3). 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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Table 1. Strains and Primers

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

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

Strain	$Rif^r/10^8$ cells	Fold Increase [a]
SS1 (wild-type)	0.4 ± 0.1	1
SS1 with air stress	0.5 ± 0.3	1.25
mutY::Kan SS1	137 ± 31	343
mutY::Kan SS1 with air stress	450 ± 75	1125

a: fold increase relative to wild-type

Table 3. Complementation of *E. coli mutY* mutant with HpMutY

Strain	$Rif^r/10^8$ cells	Fold Increase [a]
PR8 (wild-type)	7 ± 1	1
PR70	83 ± 9	12
PR70 (pGEV-HpMutY)	13 ± 3	1.9
PR70 (pGEV1)	72 ± 9	10

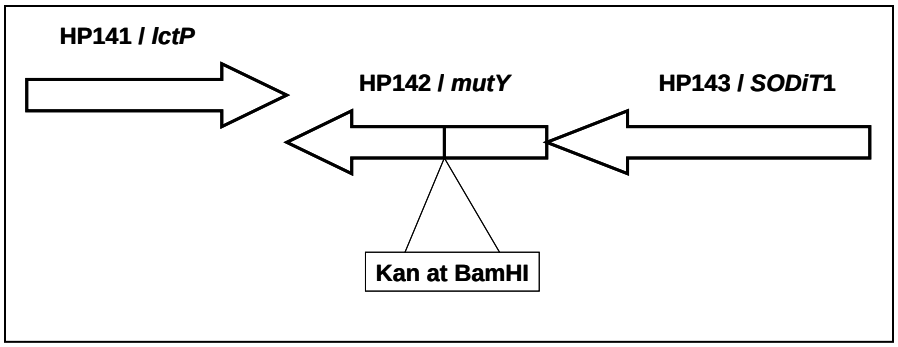
a: fold increase relative to wild-type

Table 4. Mutation Spectrum (*mutY*::Kan SS1)

Base Change	Amino Acid Change	Occurrence [a]
GC → TA	His 540 → Asn	47
GC → TA	Gln 527 → Lys	1
GC → TA	Asp 530 → Tyr	1
AT → TA	Asp 530 → Val	1
Unknown	Unknown	6

a: number of times this particular mutation was seen out of the 56 total sequenced spontaneous mutants

A



B

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H.p  --METLHNALLKWYEEFGRKGLPFRNLKGINAPYEVYISEVMSQQTQINTVIERFYSPFL 58
E.c  MQASQFSAQVLDWYDKYGRKTLPWQIDK---TPYKVWLSEVMLQQTQVATVIP-YFERFM 56
               * **   *** **    *    ** *  **** ****  ***      *

H.p  EAFPTLKDLANAQLEEVLLLWRGLGYYSRAKNLKKSAEICVKEHHSQLPNDYQSLLKLPG  118
E.c  ARFPTVTDLANAPLDEVLHLWTGLGYYARARNLHKAAQQVATLHGGKFPETFEEVAALPG  116
     ***  ***** * *** ** ***** ** ** * *      *    *        ***

H.p  IGAYTANAILCFGFREKRACVDANIKRVLLRLFGLDPNIHAKDLQIKANEFLNLNESFN-  177
E.c  VGRSTAGAILSLSLGKHFPILDGNVKRVLARCYAVSGWPGKKEVENKLWSLSEQVTPAVG  176
      *  ** ***           * * **** *          *    *

                                                  N-term / C-term
H.p  ---HNQALIDLGALICS-PKPKCAICPFNPYCLG—-KNHLEKHTLKKKQE/IIQEER--YL 229
E.c  VERFNQAMMDLGAMICTRSKPKCSLCPLQNGCIAAANNSWALYPGKKPKQ/TLPERTGYFL 236
         ***  **** **   ****  **    *     *       **       *     *

H.p  GVVIQNNQIALEKIEQKLYLGMHHFPDLKENLECKLPFLGAIKHSHTKFKLHLNLYSAAI  289
E.c  LLQHEDEVLLAQRPPSGLWGGLYCFPQFADEESLRQWLAQRQIAADNLTQLTAFRHTFSH  296
                    *  *   **                          *

H.p  KDLKNPVRFYSLKDLETLPISSMTLKILNFLKQKNLFGG 328
E.c  FHLDIVPMW--------LPVSSFTGCMDEGNALWYNLAQPPSVGLAAPVERLLQQLRTGAPV350
       *              ** ** *
```

Figure 1. Genome region and sequence alignment. A) genome region surrounding *mutY* in *H. pylori* strain 26695. *lctP* is lacate permease and *SODiT*1 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 this a "/".

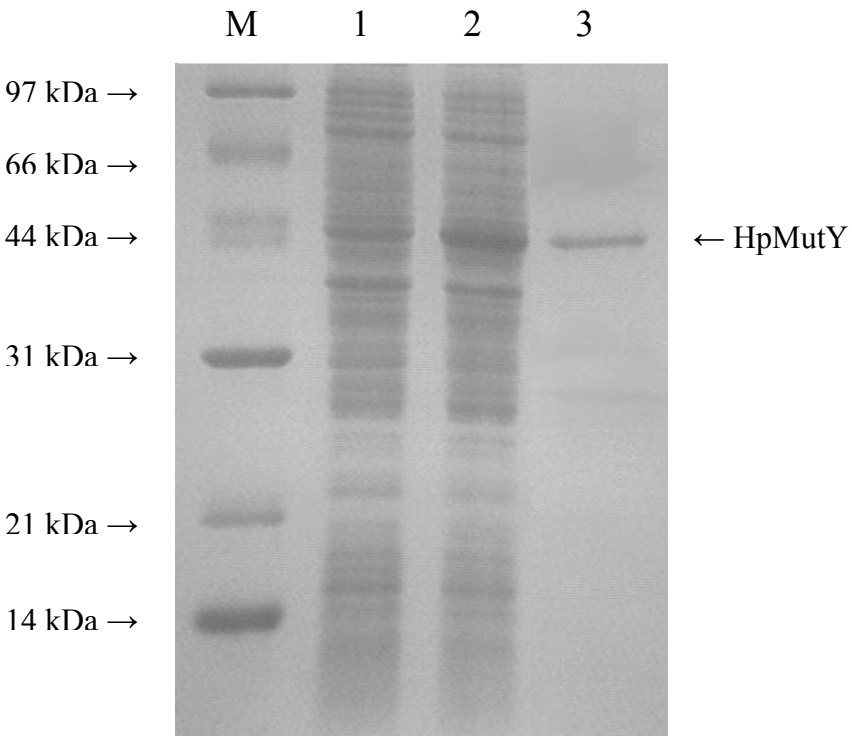

Figure 2. Overexpression and purification of HpMutY. Lane M is a marker, lane 1 is BL21 Rosetta pLys (pGEV-HpMutY) whole cell extract prior to induction with IPTG, lane 2 is the same cells 2 hours after induction, lane 3 is purified HpMutY. The size of HpMutY fusion protein is 45 kDa.

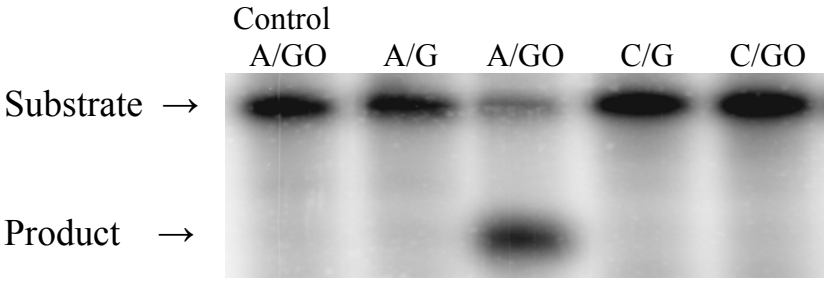

Figure 3. Glycosylase activities of HpMutY with different mismatched substrates. Oligonucleotide substrates (labeled with 200,000 cpm ^{32}P, specific activity is 5,000 cpm/ pmol DNA) were mixed with 500 ng HpmutY and incubated at 37^{o} C for 30 minutes. Control lane lacks enzyme. The first base in the pair is in the strand that is labeled. Substrates are 37 mers and products are 18mers. GO = 8-oxo-G

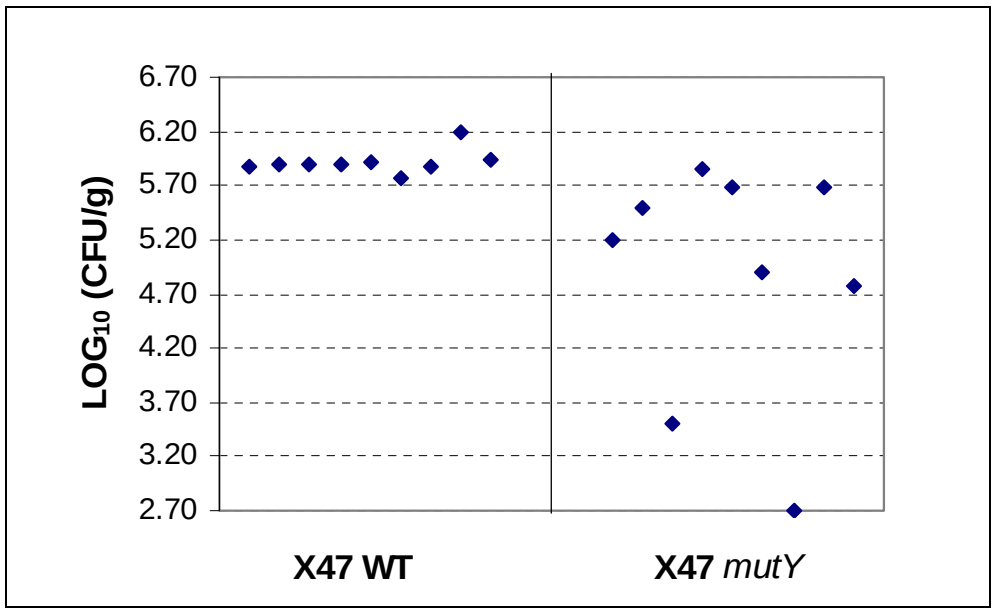

Figure 4. Mouse colonization of X47 wild-type and *mutY*::Kan X47. Nine mice were inoculated twice (2 days apart) with 1.5 x 10^8 cells. Stomachs were homogenized 3 weeks after initial inoculation. Each point represents the cfu count for one stomach, expressed per gram stomach tissue. The baseline 2.7 $\log_{10}$ 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.

CHAPTER 3

CONCLUSIONS

Cells are constantly faced with the threat of oxidative stress from reactive oxygen species (ROS). These ROS can be generated by endogenous sources, such as electron leakage from the electron transport chain during aerobic respiration, or by exogenous sources, such as the oxidative burst of macrophages that a pathogen would face inside a host [2,3]. ROS can damage biological molecules such as DNA. One of the most common results of oxidative DNA damage is the formation of 7,8-dihydro-8-oxoguanine (8-oxo-G) [8]. This damaged base pairs incorrectly and causes mutations in the DNA [7]. Cells have dedicated systems of enzymes to deal with oxidative DNA damage [4]. One of these enzymes is MutY, an adenine glycosylase that has the ability to remove adenines from adenine/7,8-dihydro-8-oxoguanine (8-oxo-G) or adenine/guanine mismatches [1].

I studied the role of the MutY homolog in the gastric pathogen *Helicobacter pylori*. To study the physiological role of this enzyme, I created a gene deletion mutant and also purified the enzyme. *H. pylori mutY* mutants incubated at 5% O_2 have a 343-fold higher spontaneous mutation frequency compared to the wild-type strain. The mutation frequency is further increased by exposing the mutant to atmospheric levels of oxygen. This effect is not seen in an *E. coli mutY* mutant and may reflect *H. pylori's* microaerophilic physiology. 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. These mutations are characteristic of 8-oxo-G DNA damage [7].

The *H. pylori* MutY enzyme when expressed from a plasmid in *E. coli* 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 [5,6]. 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*.

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