Genetic variability and DNA repair: base excision repair activities in *Helicobacter pylori*.

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Abstract

One of the remarkable characteristics of *Helicobacter pylori* is the high genetic diversity it displays. Based on the genome sequencing results, the absence of certain DNA repair activities has been postulated to be one of the causes for the genetic variability of this pathogen. We explored the possible base excision repair (BER) pathways present in *H. pylori.* We analyzed the activities corresponding to the enzymes participating in the first two steps of the pathway, the DNA glycosylases, specific for each kind of base damage, and the endonuclease that cleaves the resulting abasic (AP) site. We review here the data on the repair of alkylating DNA damage and oxidized pyrimidines and present results on studies carried out on bacterial extracts and cloned genes for the other BER activities. The combined approaches allowed the identification of a 3-methyl adenine DNA glycosylase, an endonuclease III, an uracil glycosylase, an adenine DNA glycosylase specific for 8-oxoguanine / adenine base pairs and an AP endonuclease activity. We also discuss the possible role of the host in the bacterial genetic variability and the potential appearance of new alleles that could influence *H. pylori* persistence.

Key words: base excision repair, DNA glycosylase, genetic diversity, H. pylori.

Introduction

Because it has been proposed that the genotype of the bacteria could have an impact on the pathogenicity and persistence of a *Helicobacter pylori* infection (1, 2), the intra-species genetic variability of this infective agent has been the focus of many investigations. The study of natural isolates suggests that the genetic diversity of *H. pylori* exceeds that recorded in virtually all other bacterial species studied (3). The differences detected can be interpreted as being due both to recombination events or point mutations (4). However, point mutations are at the source of most genetic changes, that can later on be transferred between genomes through recombination events. Point mutations are the consequences of either replication errors or the final product of DNA modifications induced by genotoxics of endogenous or exogenous origin. All cells have DNA repair systems that allow them to avoid the lethal or mutagenic effects of DNA lesions (5). From the genomic sequences of *H. pylori*, it has been suggested that the absence of some of the DNA repair pathways could lead to the genetic variability described for this species (6). For the replication errors, most organisms have a mismatch repair (MMR) system. In the case of *H. pylori*, the evidence gathered so far points to the absence of such a system. Indeed, no homolog for MutL, an essential component of MMR in the organisms studied, can be detected at the sequence level (4, 6). Furthermore, mutants in the other supposed component of this pathway, the gene *mutS*, do not display a hypermutator phenotype (7). However, the analysis of the number and the kind of sequence changes found between strains suggests that replications errors by themselves cannot account for the genetic diversity characteristic of this pathogen. Furthermore, the repair of bulky DNA lesions seems to be functional since a complete set of genes homologous to the nucleotide excision repair genes is present in the *H. pylori* genome. This lead to the

study of other mutation sources, mainly those produced by base modifications. Base modifications or losses can be spontaneous or induced by genotoxic agents, both endogenous or environmental. Base excision repair is the most specialized DNA repair system involved in the removal and repair of modified bases (Figure 1). The repair is initiated by the recognition of the damaged base by a DNA glycosylase that excises the base leaving an abasic (AP) site. This initial step is performed by specific proteins capable of recognizing and excising specific lesions (8). The abasic site, which can also be regarded as a lesion itself, is then cleaved by an AP endonuclease to yield a nick with a 3'-OH and a 5' deoxyribose phosphate (dRP) residue that is subsequently removed by a dRPase activity (9). The resulting gap is a substrate for a repair DNA polymerase that can incorporate a nucleotide and the resulting nick is sealed by a DNA ligase. The DNA glycosylases involved in the repair of oxidative damage possess also an AP lyase activity that will cleave the DNA strand 3' of the AP site, and are therefore classified as bifunctional (10). In these cases the AP endonuclease will eliminate the resulting open aldehyde from the 3' extremity. The unveiling of the complete genomic sequence of *H. pylori* allowed the search by homology of the BER components in this organism. Several genes were identified as candidates for participating in the BER. Sequences with homology to the enzymes carrying on the common steps of BER were present. Indeed orthologs of the AP endonuclease Exo III or Xth (*HP1526*), the DNA polymerase Pol I (*hp1470*), and DNA ligase (*hp615*) were easily identified. However, only a few open reading frames were annotated as coding for the DNA glycosylases involved in the recognition of the different kinds of base lesions. Putative genes for Udg (uracil DNA glycosylase; *hp1347*), MutY (A/8-oxoG adenine DNA glycosylase *hp0142*) and surprisingly for two Nth (Endonuclease III), a DNA glycosylase that excises oxidised pyrimidines, (*hp585*

and *hp602*) were proposed. However, no functional tests were done to confirm the predicted activities of the genes. It is worth noting that no candidates for DNA glycosylases recognizing alkylated bases or oxidized purines, which are the most common base adducts, were reported. This a surprising observation since most organisms studied do have DNA glycosylases that remove 3-methyl-adenine, a toxic lesion that blocks DNA replication (11), and 8-oxoguanine, a common product of guanine oxidation that induces G to T transversions (12). Here we review recent data on some of the genes potentially involved in BER and we provide results on some of the remaining BER activities. We also discuss the potential relevance of DNA repair and the pathogen-host interaction in some of the unique characteristics of *H. pylori* infection.

Materials and Methods

Bacterial strains and culture media. Bacterial strains used in this study were *E. coli* PR180 (CC104 *mutY*) (13), *E. coli* JM109 (New England Biolabs), *H. pylori* ADM1 (14), *H. pylori* Gomez (clinical isolate, this work) and *H. pylori* X47-2AL *nucT* (O'Rourke et al., in preparation), a nuclease defective derivative of X47-2AL (15). *E. coli* cultures were done in LB agar containing 200 □g/ml of ampicillin when required. Selection for rifampicin resistant colonies was done on LB agar plates containing 100 □g/ml. *H. pylori* was cultured in liquid BHI medium (Oxoid) containing 0.2% cyclodextrine. Complementation assays of the mutator phenotype of strain PR180 were done as described (16).

Cell extracts. Exponentially growing cells from *E. coli* or *H. pylori* X47 *nucT* were harvested by centrifugation, washed twice and resuspended in lysis buffer (20 mM Tris Hcl pH 8, 1 mM EDTA, 250 mM NaCl, 0.8 □g/ml antipain, 0.8 □g/ml leupeptine, 0.8 □g/ml aprotinin) containing 0.1 mg/ml lysozyme. After incubation for 30 minutes at 4°C and sonication the cell lysates were centrifuged at 45000 rpm for one hour and the supernatant recovered.

Plasmid constructions. ORFs *hp0142* and *hp1347* were amplified by PCR from strain ADM1 using primers deduced from the published genome sequence of strain 26695 (6). The primers for *hp0142* were 5'-GAAGATCTCTGGAAACTTTACACAACGC and 5'-GGAATTCACCCCCAAATAAATTTTTTT. For *hp1347* the primers were 5'-GAAGATCTATGAAGCTTTTTTGACTAC and 5'-GGAATTCTAAACTAAAATCCTTGCGATAAAC. The products of amplification were cloned into the *Bgl*II / *Eco*RI sites of pGEX-4T1 (Amersham Biosciences). The

resulting plasmids were named pGEX-HP0142 and pGEX-HP1347 and expressed HP0142 and HP-1347 repectively, fused to glutathione-S transferase (GST).

Overproduction and purification of the GST fused proteins. For the purification of GST-HP0142, *E. coli* PR180 carrying pGEX-HP0142 was were used to inoculate 500 ml of LB medium supplemented with ampicillin and were incubated at 37°C with shacking until the A_{600} reached 1,5. IPTG was added to 50 µM, and growth was continued overnight at 18°C with gentle shaking. Cells were harvested by centrifugation and HpMutY was purified by GST affinity chromatography. The eluted protein was dialyzed against 20 mM Tris-HCl pH 8, 5 mM □-mercaptoetanol and 10% glycerol. The same protocol was followed for the purification of GST-HP1347 from strain JM109 harbouring plasmid pGEX-HP1347.

DNA substrates and enzymatic activity assays. The 34-mer oligodeoxyribonucleotides used in this study have the following sequence: 5'-GGCTTCATCGTTGTC**[X]**CAGACCTGGTGGATACCG-3' with **X** being uracil (U) (Oligo Express, France), 8-oxoG (kind gift of Jean Cadet, CEA Grenoble) or, tetrahydrofurane (THF) (Eurogentec). The complementary strand with each of the four bases opposite the lesion in the duplex, were purchased from Oligo Express (France). For the cleavage of lesion-containing DNA duplexes, the oligonucleotide carrying the modified base or the adenine opposite 8-oxoG was ^{32}P-labelled at the 5'-end using T4 polynucleotide kinase (New England Biolabs) and annealed to its complementary strand. In a standard reaction (10 □l final volume), 50 fmoles of labelled duplex were incubated in the reaction buffer (25 mM Tris-HCl pH 7.6, 2 mM Na_2EDTA, 50 mM NaCl) with the indicated protein fraction at 37°C. Reactions were, unless otherwise stated, stopped by addition of 0.1 N NaOH. After addition of 6 □l of

formamide dye the products were separated by 7 M urea 20% PAGE. Gels were analyzed with a Storm phosporimager (Molecular Dynamics).

***H. pylori* library construction and screening for an 8-oxoG specific DNA glycosylase**

Genomic DNA from the Gomez strain was purified and partially digested with EcoRI. An expression library was constructed on Lambda ZAPII (Invitrogen). The representation of different full length genes was tested by PCR. The rationale and methodology of the screening were as previously described (17). Briefly, the *E. coli* strain BH1190 was transformed by electroporation with the plasmidic form of Lambda ZAPII and plated on papillation plates (18). After 5 days the reversion of the β-galactosidase activity was analyzed by direct observation. The clones with 1 to 3 papillae were harvested, grown in LB and frozen. The spontaneous mutation frequency of each clone was independently measured by the appearance of rifampicin resistant colonies. From those clones with a mutation frequency lower than 10^{-7}, the plasmids were purified and sequenced.

Results and Discussion

Base excision repair of modified bases is initiated by DNA glycosylases specific for each kind of damage or by the second enzyme of the pathway, the AP endonuclease, in the event that an AP site is the original lesion. In the following paragraphs we will review and analyze the BER enzymes and activities present (or absent) in *H. pylori*, drawing the parallel with the well characterized *Escherichia coli* pathway.

Uracil removal. Uracil arises in DNA through the erroneous incorporation by DNA polymerases or by deamination of cytosine (5). In *E. coli,* two specific DNA glycosylases are responsible for the removal of this base from DNA: Ung and Mug. They belong to the UDG families 1 and 2 respectively (19), the latter one having a specificity for G:U mismatches, likely to be generated by deamination. When we tested *H. pylori* cell extracts for their uracil DNA glycosylase activity using a 34-mer duplex substrate harbouring a single uracil residue, a cleavage product was observed, indicating that *H. pylori* possesses an UDG activity (Figure 2A, lane 2). Because the cleavage product was only obtained when the reactions were stopped with NaOH (compare lanes 2 and 3), we can infer that the activity present in the extracts is monofunctional. To assess to which UDG family the activity detected in *H. pylori* extracts belongs to, its susceptibility to inhibition by the phage PBS1/2 UGI protein (20) was tested. The present of the phage peptide completely inhibits the UDG activity present in the *H. pylori* extract (Figure 2, lane 4). This result, together with the fact that the activity was observed on a C:U mismatch, suggests that the main UDG activity of *H. pylori* belongs to the UDG family 1. Consistently, analysis of the genomic sequences can only reveal one potential ORF coding for a UDG, that, by

sequence comparison, codes for a protein of the UDG family 1 (40% identity with *E. coli* Ung). In order to confirm the assignment of *hp1347* to the gene coding for the UDG, the ORF was cloned in an *E. coli* expression vector, and the product was purified as a GST fusion. After purification, the enzymatic activity of the GST-HP1347 protein was tested on a uracil containing DNA substrate. As shown in Figure 2B, *hp1347* product possesses a UDG activity. Therefore, *hp1347* corresponds indeed to *Hpung.*

Repair of oxidized pyrimidines. During the course of infection, *H. pylori* is exposed to an oxidative stress induced in the host by the infection and subsequent inflammation. Such a stress can lead to DNA damage in the bacterial chromosome (16). Among oxidized pyrimidines formed, some, such as thymine glycol (Tg), can be toxic by blocking either RNA or DNA polymerases while others such as oxidized cytosines, are mutagenic (21). To avoid these effects from oxidized pyrimidines in *E. coli,* two DNA glycosylases are able to initiate their repair: Nth (or Endo III) and Nei (or Endo VIII) (22, 23). The initial analysis of the *H. pylori* genome presented two candidates for coding Nth orthologs (*hp0585* and *hp0602*), but none for Nei. The cloning of *hp0602* and characterization of its product showed that this gene does not code for an Endo III (14). In contrast, the *hp0585* sequence presents all the characteristics of a canonical endonuclease III. The work described in O'Rourke et al. (16) confirms the original annotation for *hp0585*. Its cloning and expression in *E. coli* showed that the product of *hp0585* can complement the mutator phenotype of a double *nth nei* mutant and the purified HP0585 protein showed a DNA glycosylase activity on DNA harbouring an oxidized thymine. We concluded that the protein coded by *hp0585* is indeed HpNth. As shown by their hypersensitivity to the exposure

to menadione or to activated macrophages, Endo III-deficient *H. pylori* strains fail to repair lethal oxidative DNA lesions, a phenotype that requires a double *nth nei* inactivation in *E. coli.* Interestingly, the inactivation of the *Hpnth* gene leads to a markedly reduced capacity of infection translated by a gradual decrease in the bacterial load of the stomachs of the infected mice. In laboratory culture conditions there are no differences in the growth rates of the mutant and wild type strains. This suggests that lethal base modifications, induced by the oxidative stress triggered during the infection, accumulate in the pathogen genome. Competitive infection experiments were performed in which mixtures of a mouse-adapted wild type strain and its *Hpnth* derivative were used to inoculate mice. Even when added in a 10-fold excess, the mutant strain is lost 15 days post-infection, while the parental strain colonizes normally the stomachs. These results suggest that not only lethal oxidized pyrimidines are formed during the infection, but also mutagenic lesions are produced that, by their deleterious effects, can be counter-selected in a genetic background selected for its capacity to colonize the mouse. However, mutation of a non-adapted genetic background, what is a more realistic situation during new host colonization or in-host adaptation to environmental changes, could be beneficial to the pathogen population (24). It has been proposed that *H. pylori* uses DNA mutation as a strategy to adapt to changing environments rather than homeostasis based on the regulation of gene expression at the level of transcription. Our results show that the host could act as a mutagen and we propose that it could thereby benefit *H. pylori* adaptation and long term persistence. Taken together these experiments show the importance of the *H. pylori* Nth to protect the bacterial DNA, challenged by the host response during the infection process. They also suggest that the host, by damaging the

bacterial DNA, might actually contribute to its persistence by increasing the pathogen mutation rate.

Avoidance of 8-oxoguanine-induced mutagenesis. Guanines are specially susceptible to oxidation. The main oxidation product is 8-oxoguanine (8-oxoG) which by its adenine mispairing capacity (25) leads to G to T transversions. *E. coli* has evolved a sophisticated BER strategy to avoid mutations from this oxidized purine present in DNA (26). The DNA glycosylase Fpg removes 8-oxoG paired with C, while the MutY DNA glycosylase removes adenine opposite 8-oxoG resulting from mispairing with unrepaired 8-oxoG during replication. *E. coli* mutants defective in Fpg or MutY exhibit higher-than-wild-type spontaneous mutation frequencies, about 5- to 10-fold and 10- to 50-fold respectively. Moreover the double *fpg mutY* mutant displays a synergistic effect, with a spontaneous mutation frequency between 200- and 800-fold higher than the wild type parental strain. More recent data showed that Nei can also act as a partial backup for the repair of 8-oxoG in *E. coli* (27). In *H. pylori,* an ortholog of MutY (*hp0142*) has been detected by sequence analysis. Surprisingly, no homolgous sequences to the *fpg* gene were found. Together with the lack of a Nei homolog, this result would suggest a lack of 8-oxoG specific DNA glycosylase in *H. pylori*. To test the possibility of the existence of a novel kind of 8-oxoG DNA glycosylase, cell extracts from *H. pylori* were assayed for their capacity to excise 8-oxoG from DNA. Using standard conditions for the known 8-oxoG DNA glycosylases, no cleavage activity was detected on duplex oligonucleotides harbouring an 8-oxoG residue (data not shown). A negative result was also obtained in assays were a reaction intermediate for the bifunctional DNA glycosylases is trapped by a reducing agent such as borohydride (data not shown). Consistently with

the sequence analysis, these data point to a lack of 8-oxoG DNA glycosylase activity in *H. pylori.* However, if an enzyme carrying out the 8-oxoG repair exists in *H. pylori* and is of a new kind, we cannot rule out the possibility that the failure to detect its activity is the consequence of inadequate assay conditions.

To minimize the *in vitro* suboptimal conditions and in order to corroborate the presence or absence of an 8-oxoG DNA glycosylase in *H. pylori* we performed a genomic complementation assay. We constructed a *H. pylori* expression library in Lambda ZapII and we performed a screening by mean of a LacZ based analysis. We rescued 98 clones with a number of reversions lower than that of the *mutY*, *fpg* deficient *E. coli* BH1190 strain. The complementation of the mutator phenotype was confirmed by measuring the median rifR reversion frequency of the clones. By this quantitative method, three clones showed a mutation frequency between the wild type and the *mutY*, *fpg* mutator strain. The sequencing of these clones showed that one encoded for the thiorredoxin reductase and two of them for the predicted MutY ORF (*hp0142*). Even when these results do not confirm the absence of an 8-oxoG DNA glycosylase activity in *H. pylori*, they suggest that if this pathogen possesses an activity able to remove oxidized purines from DNA, this enzyme acts by a mechanism different from that described for *E. coli* activities and/or it strictly depends on species specific partners.

Although an ortholog of MutY was identified at the sequence level, assays on extracts using a duplex 34-mer carrying an A:8oxoG base pair failed to detect an adenine DNA glycosylase activity (data not shown). However, the cloning of *hp0142* and its expression as a GST-fusion in a *E. coli mutY* strain (PR180) allowed to establish that the purified GST-HP0142 fusion possesses an adenine DNA glycosylase activity on A:8-oxoG base pairs (Figure3). Moreover, the expression of

the same fusion partially complemented the spontaneous mutator phenotype of strain PR180 (Table 1). The complementation was also confirmed by the screening of the *H. pylori* library. Taken together these results indicate that *hp0142* codes for HpMutY.

Repair of alkylated bases. The cloning of *hp0602* and the characterisation of its product showed that this ORF, originally annotated as an Endo III ortholog codes for Mag III, a 3-methyl adenine DNA glycosylase of a novel family (14). This enzyme belongs to the large family Endo III-like glycosylases by its sequence (28), and its expression is induced by exposure of the cells to alkylating agents. In *E. coli*, there are two 3-methyl adenine DNA glycosylases. The first one, AlkA, which also belongs to the Endo III super family, is inducible and has a broad substrate specificity. The other 3-methyl adenine DNA glycosylase, Tag, is constitutive, and is not structurally related to the Endo III family and has a very narrow substrate specificity. Surprisingly, among the different alkylated substrates tested, Mag III, although related structurally to AlkA, has only activity on 3-methyl adenine. No activity was detected on 7-methyl guanine, a supposedly mutagenic lesion that is efficiently removed by AlkA. Therefore, in spite of being inducible and structurally related to AlkA, Mag III, almost exclusively removes the toxic 3-methyl adenine bases from DNA as it is the case for Tag. Consistently with the absence of other predicted alkylation-modifications specific enzymes, the *H. pylori magIII* mutant strains are hypersensitive to MMS. It is worth noting that in *E. coli* AlkA and Tag can substitute for each other in the protection against the lethal consequences of alkylated DNA.

Repair of abasic sites. AP sites can result from either the spontaneous loss of bases or as a consequence of the removal of a modified by a DNA glycosylase (9). In

either case they are the substrate for an AP endonuclease that can cleave the 5'-phosphate bond to yield a 3'-OH terminus that is an effective primer for DNA polymerases. *E. coli* harbours two AP endonucleases, Endonuclease IV (Endo IV) and Exonuclease III (Exo III) that are coded by *nfo* and *xth,* respectively. Exo III accounts for >99% of the AP endonuclease activity in extracts of wild-type *E. coli.* Unlike Endo IV, Exo III activity is Mg^{2+} dependent (9). *H. pylori* genome sequencing revealed the presence of an ORF potentially coding for an Exo III like protein. To confirm the presence of such an enzyme, we analysed the AP endonucleases activities present in *H. pylori* extracts both in the presence or absence of Mg^{2+}. Figure 4 shows that in the presence of 1 mM Mg^{2+}, *H. pylori* extracts display an endonuclease activity capable of cleaving the AP analog THF. However, when the reactions were carried out in the presence of the Mg^{2+} chelator EDTA, no cleavage was observed (compare lanes 3 and 4). Taken together, these results and the sequence analysis suggest that *H. pylori* has an Exo III-like enzyme but not an Endo IV-like. Considering the high level of homology with other bacterial Exo III it is likely that *hp1526* corresponds to *Hpxth,* coding for HpExo III.

Conclusions

The sequence analysis of *H. pylori* genome showed an unprecedented level of intra-species allelic variants. Results reported by Bjorkholm *et al* show an increased mutation rate in *H. pylori* compared to other monoploids species when measured *in vitro*. The data available suggests the absence of a MMR system. However the the level and type of mutations observed can not be explained by the only absence of a complete MMR system. The results reviewed and presented here show an incomplete set of BER activities in *H. pylori*. The most striking difference with respect

to most organisms is given by the apparent absence of an 8-oxoG DNA glycosylase. Considering the mutagenic consequences of this oxidised base, it is tempting to speculate that such an absence can be in part responsible for the high mutation rates observed in *H. pylori.* However, the presence of MutY seems to question this hypothesis. A similar situation is encountered in the fission yeast *Schizosaccharomyces pombe.* As mentioned above, we cannot rule out that *H. pylori* has evolved a different biochemical strategy to eliminate 8-oxoG from its DNA. In the case of alkylated bases it is also interesting to note that *H. pylori*, through Mag III, can eliminate the toxic 3-methyl adenine but has no apparent activity that would excise other mutagenic alkylateed bases such as 7-methyl guanine. Another remarkable feature of the *H. pylori* BER pathway when compared to *E. coli*, is the lack of redundancy in the enzymatic activities involved. Although this limited redundancy in *H. pylori* gene content can be extended to other gene families (29) in the case of DNA repair genes it suggests a high potential for genetic variation, specially if, as it has been shown (16), the stress induced in the host by the infection process can trigger a higher mutation frequency on the pathogen. This could be used by the bacterial population to increase the chances of adapting to new environments (i.e. a new host) or to select variants more resistant to therapeutic treatments.

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Table 1. Complementation of the mutator phenotype of *E. coli mutY* (PR180) by HpMutY.

Strain	Rifampicin resistant colonies per 10^8 cells	
	Experiment 1	Experiment 2
Wild type	1.2	2.3
PR180 pGEX	11.3	10.4
PR180 pGEX-HpMutY	7.5	6.8

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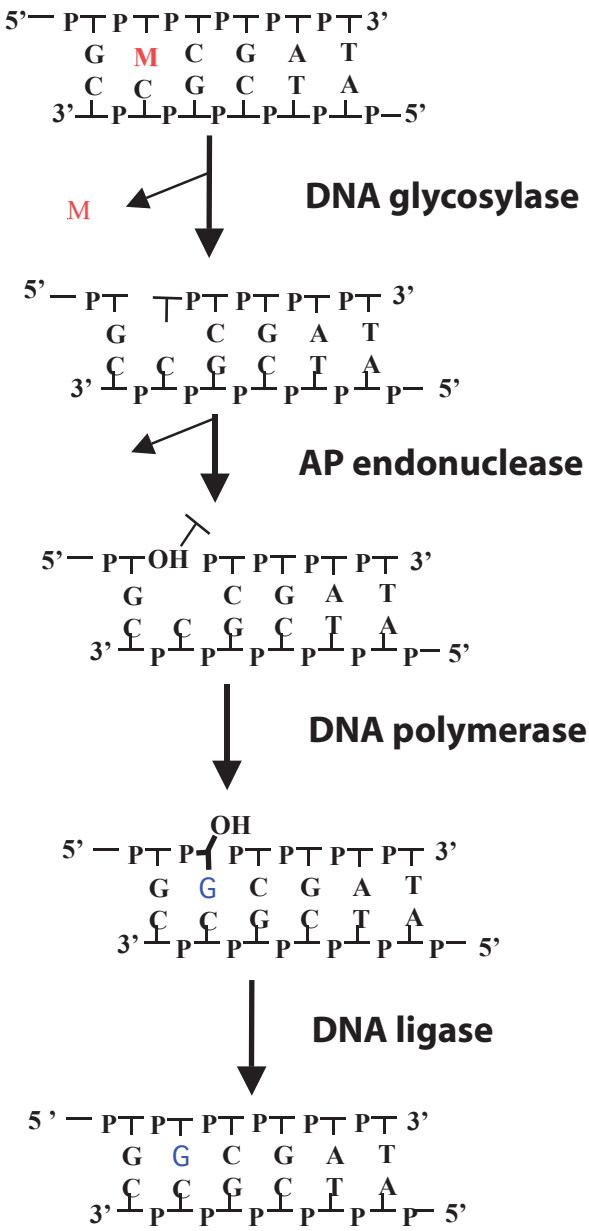
Figure legends

Figure 1. Schematic representation of the base excision repair pathway. For details refer to the text.

Figure 2. A. Uracil DNA glycosylase activities in *H. pylori* X47 *nucT* cell free extracts. Radioactively labelled 34-mer duplexes containing an uracil residue were incubated at 37°C for 45 minutes in the presence of 0.5 □g of total protein (lanes 2, 3 and 4), one unit of UDG inhibitor (lane 4) or 10 ng of Ung from *E. coli* (lane 5). Reactions were stopped by the addition of NaOH (except on lane 3) to cleave the abasic sites left and the products (P) separated from the substrate (S) by denaturing gel electrophoresis. **B.** Uracil DNA glycosylase activity of the GST-HP1347 fusion protein. Reactions were carried out as in A except that incubations were for 15 minutes and contained: 10 ng of GST (lane 6); 10 ng of GST-HP1347 (lane 7) or 10 ng of Ung from *E. coli.*

Figure 3. Adenine DNA glycosylase activity of HP0142. A duplex 34-mer harbouring an A:8-oxoG base pair was incubated for 30 minutes at 37°C with 25 ng of the proteins indicated. After stopping the reactions with NaOH, the products (P) were separated from the substrate (S) by denaturing PAGE.

Figure 4. AP endonuclease activity in *H. pylori* cell extracts. A duplex 34-mer carrying an abasic site analog (tetrahydrofurane) was incubated for 30 minutes at 37°C with 10 ng of human APE1 or 80 ng of protein extract from *H. pylori* X47 *nucT* with or without EDTA as indicated. The reaction products were analyzed by denaturing PAGE.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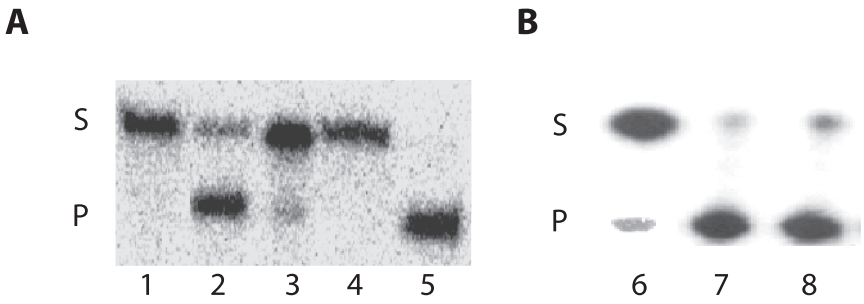
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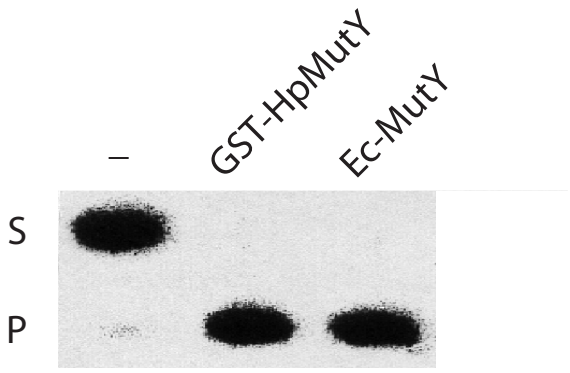
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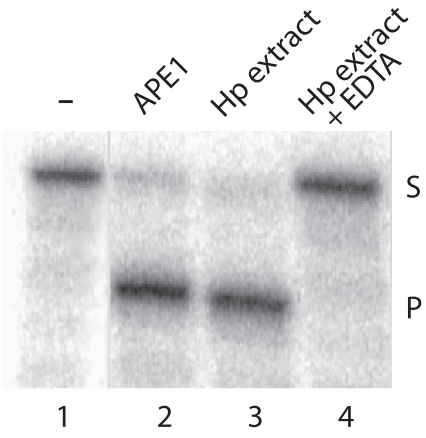
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APE1
Hp extract
Hp extract
+ EDTA
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4