

***Helicobacter pylori* AddAB helicase-nuclease and RecA promote recombination-related DNA repair and survival during stomach colonization**

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Summary

***Helicobacter pylori* colonization of the human stomach is characterized by profound disease-causing inflammation. Bacterial proteins that detoxify reactive oxygen species or recognize damaged DNA adducts promote infection, suggesting that *H. pylori* requires DNA damage repair for successful *in vivo* colonization. The molecular mechanisms of repair remain unknown. We identified homologues of the AddAB class of helicase-nuclease enzymes, related to the *Escherichia coli* RecBCD enzyme, which, with RecA, is required for repair of DNA breaks and homologous recombination. *H. pylori* mutants lacking *addA* or *addB* genes lack detectable ATP-dependent nuclease activity, and the cloned *H. pylori* *addAB* genes restore both nuclease and helicase activities to an *E. coli* *recBCD* deletion mutant. *H. pylori* *addAB* and *recA* mutants have a reduced capacity for stomach colonization. These mutants are sensitive to DNA damaging agents and have reduced frequencies of apparent gene conversion between homologous genes encoding outer membrane proteins. Our results reveal requirements for double-strand break repair and recombination during both acute and chronic phases of *H. pylori* stomach infection.**

Introduction

The Gram (–) bacterium *Helicobacter pylori* chronically infects the stomach of half of the world's human population, causing inflammation in the stomach that can lead to peptic ulcer disease and gastric cancers (Kusters *et al.*, 2006). The host immune system wards off these and other bacteria by exposing them to DNA damaging agents. Bacteria overcome this damage in part by repairing their damaged DNA using homologous recombination. Homologous recombination involves three steps. First, a presynaptic step processes the damaged DNA to produce single-stranded DNA coated with RecA protein. The second, synaptic step involves homology searching and strand exchange promoted by RecA to produce a joint DNA molecule between the damaged DNA and intact DNA. A third, postsynaptic step results in resolution of the joint molecule or priming of new DNA synthesis.

In *Escherichia coli* the presynaptic step can be catalysed by two distinct sets of proteins, the heterotrimeric RecBCD complex and the RecFOR proteins, which convert a DNA lesion into a RecA-coated filament (Amundsen and Smith, 2003). It has been proposed that the choice of repair complex depends on whether a double-strand (ds) break (by RecBCD) or a single-strand (ss) break or gap (by RecFOR) must be repaired. Curiously, *H. pylori* and other bacteria in the epsilon branch of Proteobacteria with sequenced genomes were thought to lack many or all components of these complexes owing to a failure to identify homologues in their genomes (Rocha *et al.*, 2005). In *H. pylori*, the only convincing, annotated homologues of presynaptic proteins are RecJ and RecR. This is in spite of the facts that *H. pylori* contains RecA (Schmitt *et al.*, 1995; Thompson and Blaser, 1995) and postsynaptic proteins (Tomb *et al.*, 1997; Loughlin *et al.*, 2003), has a population genetic structure indicative of a high amount of recombination between strains (Suerbaum *et al.*, 1998; Falush *et al.*, 2003), and uses gene conversion to vary expression of surface proteins during the course of infection (Solnick *et al.*, 2004).

Recombination and DNA repair initiated at DNA ds breaks in *E. coli* requires the RecBCD enzyme, a heterotrimer composed of one copy of the products of the *recB*,

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recC and *recD* genes (Taylor and Smith, 1995). The enzyme is an ATP-dependent ds and ss exonuclease, a ss endonuclease, an ATPase, and a highly processive helicase. RecBCD binds tightly to ds DNA ends and initiates unwinding using the fast RecD helicase and the slower RecB helicase (Taylor and Smith, 2003). Although the single nuclease domain resides in RecB (Yu *et al.*, 1998), all three subunits and ATP are required for substantial nuclease activity, because DNA hydrolysis occurs during ATP-dependent DNA unwinding (Amundsen *et al.*, 1986). When the enzyme interacts with a Chi site (5'-GCTGGTGG-3') during unwinding, a 3'-terminated ss DNA end is produced, onto which RecBCD loads RecA protein (Smith *et al.*, 1981; Ponticelli *et al.*, 1985; Anderson and Kowalczykowski, 1997). This presynaptic filament is the substrate for subsequent steps in recombination or repair with an intact duplex.

Activities similar to those of RecBCD enzyme are also found in the two subunit AddAB bacterial enzyme, most extensively studied from *Bacillus subtilis*. Like RecBCD, AddAB has nuclease and helicase activities, both of which are ATP-dependent (Kooistra *et al.*, 1988) and can produce the 3'-terminated ss DNA end required for presynaptic filament formation and recombination (Chedin and Kowalczykowski, 2002). Both the AddA and AddB proteins have nuclease domains, while apparently active helicase motifs are found only in AddA (Kooistra *et al.*, 1997; Yeeles and Dillingham, 2007). Thus, although the structure of AddAB differs from that of RecBCD, both enzymes contain ATP-dependent nuclease and helicase activities.

Here we demonstrate that *H. pylori* and, by homology, other epsilon Proteobacteria do have ATP-dependent nuclease and helicase activities, which, as in most Gram (+) bacteria and some Gram (-) bacteria (Rocha *et al.*, 2005), are encoded by *addA* and *addB* genes. We show that AddAB and RecA are required for efficient colonization of the stomach of mice, RecA more than AddAB. We also demonstrate that AddAB and RecA promote a gene conversion-like event that modulates surface expression of a bacterial adhesin. These results suggest that recombination plays multiple roles during infection: recombination-related repair of DNA damage encountered during infection as well as remodelling of the bacterial surface that may allow evasion of adaptive immune responses or altered bacterial tropism.

Results

Identification of *H. pylori* addAB Homologues

No genes in either of two sequenced *H. pylori* genomes available on the NCBI website (<http://www.ncbi.nlm.nih.gov/>) were annotated as either *recB* or *addA*. Two BLAST

searches against *H. pylori* using the RecB sequence of *E. coli* K12 or the AddA sequence of *B. subtilis* identified a group of three proteins with significant *E*-values (from 8×10^{-7} to 4×10^{-16}) in *H. pylori* strains J99 and 26695. Both AddA and RecB consist of a highly conserved helicase domain and a highly conserved nuclease domain. The helicase domain is also found in a group of related helicases including Rep, UvrD and PcrA. In neither *H. pylori* strain J99 nor strain 26695 did the highest scoring alignments to RecB or AddA include their nuclease domains, suggesting that these *H. pylori* proteins might not be homologues of AddA or RecB but instead might be related helicases.

To identify more likely *H. pylori* AddA or RecB proteins, the group of three high-scoring helicases from each strain was searched for conserved domains against the NCBI Conserved Domain Database. For both strains J99 and 26695 only one protein showed a significant alignment to the RecB profile (COG1074); these alignments included both the helicase and nuclease domains of RecB. These two proteins (HP1553 from strain 26695 and jhp1446 from strain J99) were not those with the maximum scoring BLAST results but were 93% identical to each other. The J99 protein was previously annotated as PcrA. Each of the two protein sequences is annotated with a RecB domain corresponding to essentially the full length of the protein sequence. To classify these proteins as AddA or RecB they were BLASTed against the TIGR profile database (<http://tigrblast.tigr.org/web-hmm/>), which identified them as AddA, rather than RecB, sequences.

No significant hits were obtained in a BLAST search using the *B. subtilis* AddB sequence against *Helicobacter* species. This is likely because AddB proteins consist of a large, poorly conserved RecC-like 'inactivated helicase' domain and a short, well-conserved, RecB-like nuclease domain. Therefore, the highly conserved AddB nuclease motif 'GRIDRID' was used to identify the *H. pylori* AddB homologues. One perfect match to this sequence was identified in both strains 26695 and J99 (proteins HP1089 and jhp0336 respectively). These proteins are 94% identical and include an 'inactivated superfamily I helicase' domain. Both were described as hypothetical proteins, without an assigned function. Alignment of these proteins to *B. subtilis* AddB showed conservation of the nuclease domain (Fig. 1). Interestingly, the HP0275 protein from strain 26695, which was annotated as *addB*, lacks the inactivated helicase domain annotation and does not show significant alignment to the conserved nuclease sequence.

Using the criterion of reciprocal best hit by BLAST with the *H. pylori* 26695 AddAB sequences, we identified highly related proteins in all of the sequenced epsilon Proteobacteria, suggesting that these bacteria all contain AddAB. As shown in Fig. 1, the nuclease domains of the

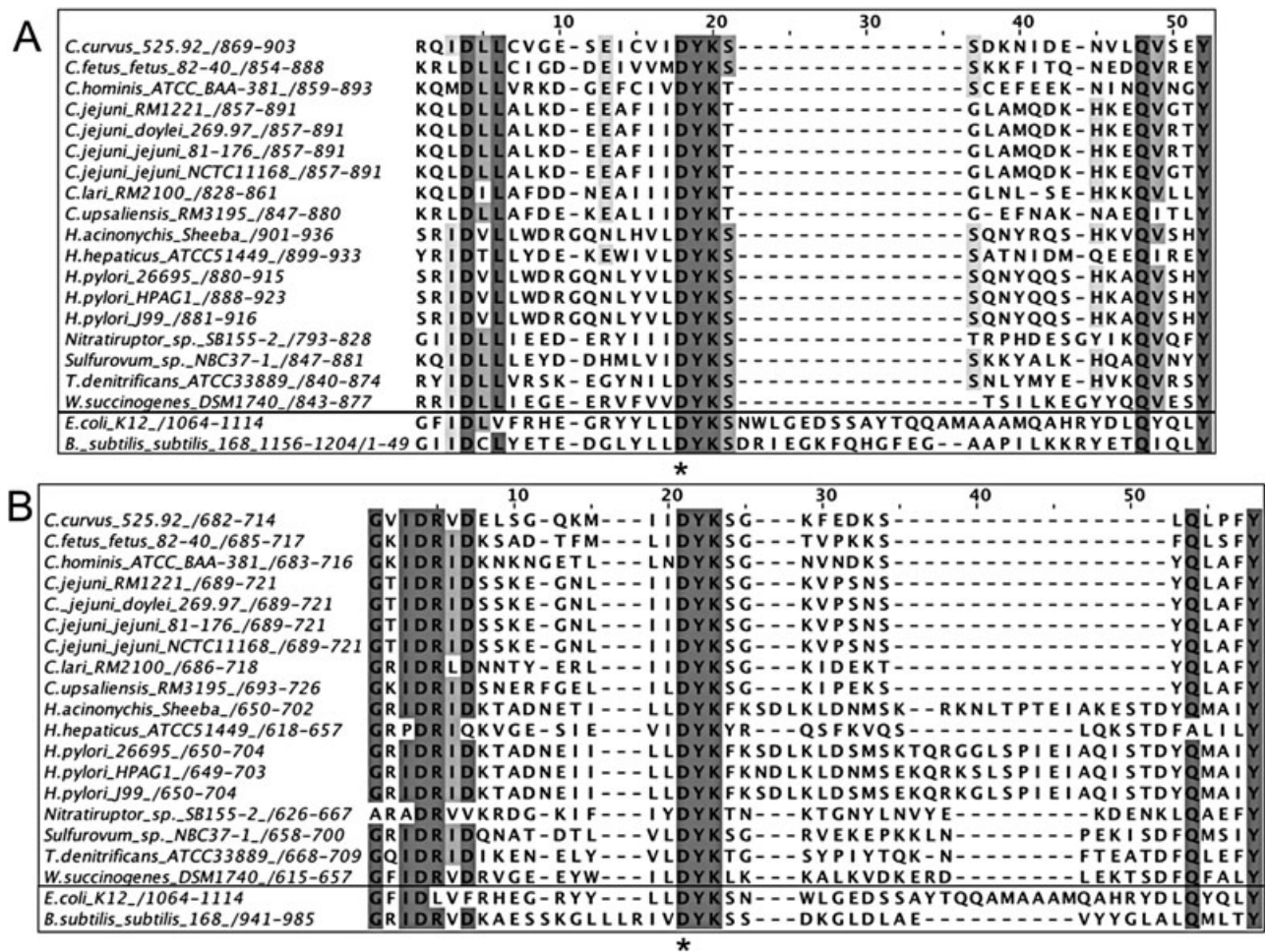


Fig. 1. Epsilon Proteobacteria AddA and AddB homologues with conserved nuclease domains. Nuclease domains of AddA (A) and AddB (B) proteins from epsilon Proteobacteria with fully sequenced genomes show highly conserved residues indicated by shading. Sites where mutations in *B. subtilis* *addA* and *addB* abolish nuclease activity (Kooistra *et al.*, 1997) are indicated by an asterisk. Species names, and first and last residue numbers in the displayed alignment are shown on the left. Alignment to the nuclease domains of *E. coli* RecB and *B. subtilis* AddA (A) and *E. coli* RecB and *B. subtilis* AddB (B) are shown for comparison (bottom of each alignment). The corresponding locus tags and GI numbers for the epsilon Proteobacterial AddA proteins are (top to bottom) CCV52592_0910 (154174808), CFF8240_0386 (118474774), CHAB381_1391 (154148488), CJE1654 (57238504), JJD26997_1828 (153951355), CJJ81176_1474 (121612796), Cj1481c (15792796), CLA0743 (57240914), CUP1844 (57242577), Hac_0287 (109946901), HH1643 (32267142), HP1553 (15646160), HPAG1_1502 (108563927), jhp1446 (15612511), NIS_0366 (152990115), SUN_0187 (152991783), Tmden_0090 (78776291), WS1252 (34557616). The corresponding locus tags and GI numbers for the epsilon Proteobacterial AddB proteins are (top to bottom) Ccur5_02000280 (145956769), CFF8240_0385 (118475773), CHAB381_1392 (154149234), CJE1655 (57238505), Cjejd_02001773 (145960086), CJJ81176_1475 (121613506), Cj1482c (15792797), CLA0742 (57240913), CUP1843 (57242576), Hac_0793 (109947353), HH0025 (32265524), HP1089 (15645703), HPAG1_0358 (108562783), jhp0336 (15611404), NIS_0365 (152990114), SUN_0185 (152991781), Tmden_0088 (78776289), WS0562 (34556981).

epsilon Proteobacteria homologues of both AddA and AddB are highly conserved. Unlike *B. subtilis* AddB (Kooistra and Venema, 1991), *H. pylori* AddB does not contain a detectable Walker A box, which is often involved in ATP hydrolysis. This motif is found in AddB of some, but not all, firmicute species. Many Gram (+) and essentially all Gram (-) AddB sequences lack this motif. While *addA* and *addB* are adjacent in the chromosome in most bacteria, including other epsilon Proteobacteria, this is not the case in *H. pylori*. Both genes that we identified as *addA* and *addB* are considered core genes that are not strain variable, as

they were observed in 56 *H. pylori* clinical isolates from around the world (Gressmann *et al.*, 2005).

H. pylori AddAB has ATP-dependent nuclease and helicase activity

The defining characteristic of AddAB and RecBCD enzymes is ATP-dependent DNA exonuclease activity; this nuclease is apparently active only during DNA unwinding, which requires ATP hydrolysis. Thus, we measured this activity in wild-type, mutant, and complemented

Table 1. *H. pylori* *addA* and *addB* mutants lack ATP-dependent ds DNA exonuclease activity.

Relevant genotype ^a	Allele at <i>rdxA</i> ^b at <i>rdxA</i> ^b	ATP-dependent ds DNA exonuclease (units per mg extract protein)	
		Mean ^c	Range
WT	–	5.3 ± 0.9 (6)	3.4–6.8
$\Delta addA::cat$	–	0.009 ± 0.0007 (6)	0.009–0.010
$\Delta addA::cat$	<i>addA</i> ⁺	6.0 (2)	5.7–6.3
$\Delta addB::cat$	–	0.02 ± 0.017 (6)	0.010–0.040
$\Delta addB::cat$	<i>addB</i> ⁺	7.1 (2)	7.0–7.2
$\Delta recA::cat$	–	6.1 ± 0.25 (3)	5.9–6.4
$\Delta ruvC::cat$	–	4.8 ± 0.4 (3)	4.3–5.1

a. These strains are derivatives of *H. pylori* strain NSH57 with the indicated deletion allele on the chromosome.

b. The indicated allele is at the *rdxA* chromosomal locus. '–' indicates wild-type *rdxA*.

c. The values are the means and standard deviations of the number of extracts assayed in parentheses. For those experiments where two extracts were assayed only the mean is indicated.

H. pylori NSH57 strains; the latter have the *addA*⁺ or *addB*⁺ gene inserted into the chromosomal *rdxA* locus, often used for this purpose (Smeets *et al.*, 2000). As shown in Table 1, cytosolic extracts from wild-type bacteria showed detectable ATP-dependent nuclease activity with ds DNA substrate under conditions optimized for the *H. pylori* enzyme (Fig. S1). Replacement of either the *addA* or *addB* gene with an antibiotic resistance cassette to create deletion (null) alleles abolished activity. ATP-dependent nuclease activity could be restored in these strains by complementation with *addA*⁺ or *addB*⁺. As a further control, we showed that disruption of two other recombination genes, *recA* and *ruvC*, had no effect on ATP-dependent nuclease activity.

To determine if *addA* and *addB* are the structural genes sufficient to confer ATP-dependent nuclease activity, we expressed these proteins in an *E. coli* strain deleted for *recBCD*. Cytosolic extracts of this strain without the *addA* and *addB* genes showed a very low level of ATP-dependent nuclease activity that was not enhanced by introduction of the vector control (Table 2). Introduction of an *addAB* coexpression construct (pETDuet-1 *addA addB*) resulted in a > 40-fold increase in ATP-dependent nuclease activity. The noninducible *recBCD*⁺ control plasmid (pMR3) resulted in a 10-fold increase in activity.

For an intracellular measure of *H. pylori* AddAB nuclease activity in these *E. coli* cells, we examined the ability of the *addAB* genes to restrict T4 phage infection. The T4 gene 2 protein blocks RecBCD-dependent degradation of the phage DNA upon infection, perhaps by binding to the DNA ends in the virion (Oliver and Goldberg, 1977). A T4 gene 2 mutant can productively infect an *E. coli* strain lacking RecBCD nuclease activity (Amundsen *et al.*, 1990) but shows a six-log reduction in plating

efficiency in *recBCD*⁺ *E. coli* (Table 2). Similarly, expression of *addAB* in the *recBCD* deletion strain efficiently restricted T4 2[–] infection. Thus, the *H. pylori* *addAB* genes confer nuclease activity both in cell-free extracts and in intact cells.

In addition to being an ATP-dependent ds DNA exonuclease, RecBCD enzyme is a highly processive DNA helicase (Taylor and Smith, 1980). We tested extracts of *E. coli* *recBCD* mutant cells expressing *H. pylori* *addAB* or *E. coli* *recBCD* for unwinding activity using linearized, 5' end-labelled pBR322 DNA as substrate. These extracts unwound linear DNA, but extracts with the vector (pETDuet-1) lacking the *addA* and *addB* genes did not (Fig. 2). Unwinding activity by AddAB was ATP-dependent but slightly weaker than that by RecBCD. These results confirm that the *H. pylori* *addA* and *addB* genes encode an ATP-dependent helicase-nuclease similar to RecBCD enzyme of *E. coli*. In the absence of ATP the effective Mg²⁺ concentration is elevated and ATP-independent nucleases present in the extract degraded some of the substrate to oligonucleotides (Fig. 2).

addAB and *recA* mutants are hypersensitive to DNA damaging agents

Mutants lacking *E. coli* RecBCD or *B. subtilis* AddAB show increased sensitivity to several antibiotics that damage DNA (Alonso *et al.*, 1993), as do *recA* mutants of several species including *H. pylori* (Schmitt *et al.*, 1995; Thompson and Blaser, 1995). As expected, *H. pylori* *addA* and *addB* mutant strains also showed heightened sensitivity to the alkylating agent mitomycin C and the DNA gyrase inhibitor ciprofloxacin (Table 3), both of which lead to DNA ds breaks (Iyer and Szybalski, 1963; Wolfson and Hooper, 1985; Sioud and Forterre, 1989). The sensitivity

Table 2. Cloned *H. pylori* *addA* and *addB* genes express ATP-dependent exonuclease in *E. coli* cells.

Plasmid	ATP-dependent ds DNA exonuclease (units/mg extract protein) ^a	Efficiency of plating ^b	
		T4	T4 2 [–]
pBR322	18 ± 4 (4)	1.0	1.0
pMR3 (<i>recBCD</i>)	140 ± 11 (6)	0.94	8.3 × 10 ^{–7}
pETDuet-1	24 ± 5 (6)	0.97	0.91 ± 0.04
pSA405 (<i>addA addB</i>)	880 ± 12 (6)	0.91	6.2 × 10 ^{–6}

a. Extracts were prepared from transformants of *E. coli* strain V2831 ($\Delta recBCD2731$ <kan>) with pBR322 or pMR3 or transformants of *E. coli* strain V3060 ($\Delta recBCD2731$ <kan> DE3) with pETDuet-1 or its derivatives. Cells containing pETDuet-1 were harvested 3 h after induction with 1 mM IPTG. Data are the mean ± SEM from the indicated number of extracts (n) from separate cultures.

b. Phage titre on *E. coli* strain V3060 ($\Delta recBCD2731$ <kan> DE3) with the indicated plasmid divided by the phage titre on strain V3060 with pBR322. Data are the mean from three separate experiments.

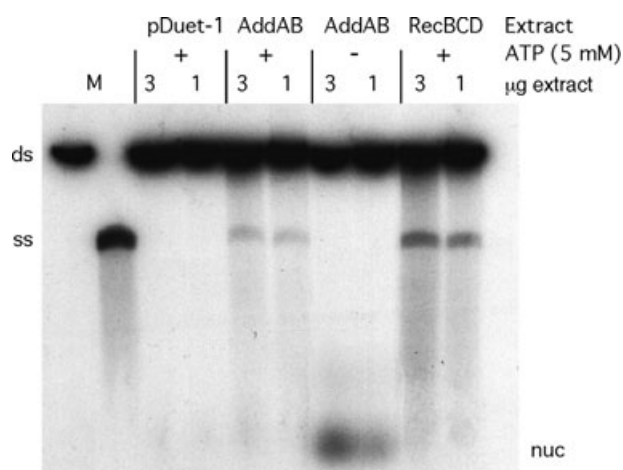


Fig. 2. AddAB promotes ATP-dependent DNA unwinding. Extracts were prepared from strain V3060 ($\Delta recBCD2731$ DE3) carrying vector pETDuet-1 with or without insertion of the *H. pylori addA* and *addB* genes or pBR322 with *recBCD* (pMR3). The indicated amount of extract protein was incubated with pBR322 linearized and 5' end-labelled with ^{32}P . Reactions contained ATP (5 mM) as indicated. The positions of ds DNA substrate (ds), unwound ss DNA (ss) produced by boiling or enzymatic reaction, and degraded DNA (nuc) are shown.

observed for the *addA* and *addB* mutants was similar to that seen for a *recA* mutant and could be complemented by expression of the corresponding gene. We conclude that *H. pylori* AddAB is required for repair of intracellular ds breaks.

In contrast, when we examined UV sensitivity, *addA* and *addB* mutants were markedly less sensitive than a *recA* mutant (Fig. 3), suggesting that ds break repair does not play a major role in repair of this damage in *H. pylori*. The modest UV sensitivity of the *addA* and *addB* mutants could be complemented by the corresponding genes. This complementation was particularly evident at the 4 kJ m⁻² exposure, where the complemented strains were slightly more resistant than wild type. This enhanced resistance may result from a higher-than-wild-type level of expression when the genes are at the *rdxA* locus, which shows

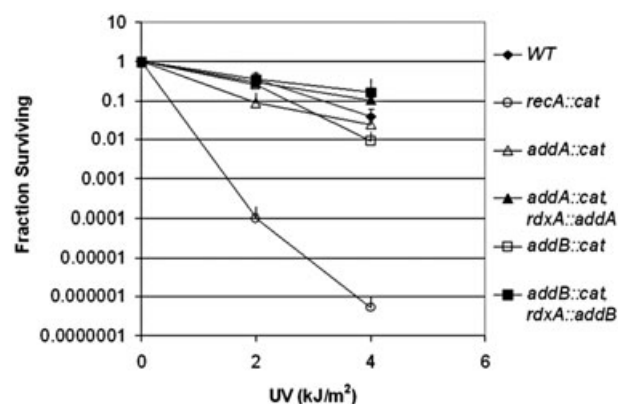


Fig. 3. AddAB confers modest protection against UV damage. The indicated bacterial strains (wild-type, mutant, and complemented mutant strains) were exposed to UV irradiation, and the fraction of bacteria surviving was determined. The data plotted represent averages of three separate experiments, and bars indicate 1 standard deviation.

constitutive high-level expression of several proteins (D.M. Pinto-Santini, L.K. Sycuro and N.R. Salama, unpubl. obs.).

We also queried the role that *H. pylori* AddAB might play in homologous recombination during natural transformation with a chromosomal marker. While the *recA* mutant completely lost the ability to undergo natural transformation, as reported previously (Schmitt *et al.*, 1995; Thompson and Blaser, 1995), there was no measurable difference in transformation efficiency for either the *addA* or *addB* mutant (N.R. Salama unpublished data). Thus, while *H. pylori* AddAB does appear to play an important role in repair of certain types of DNA damage, it does not appear to participate in homologous recombination during natural transformation.

AddAB enzyme and RecA protein are required for optimal stomach colonization

Helicobacter pylori proteins that neutralize reactive oxygen species, such as superoxide dismutase (Seyler *et al.*, 2001) and catalase (Harris *et al.*, 2003), promote stomach colonization. Similarly endonuclease III, a protein involved in recognition and processing of oxidized DNA, promotes stomach colonization (O'Rourke *et al.*, 2003), suggesting that *H. pylori* DNA experiences oxidative damage during infection. A possible role for recombination-based repair during infection was suggested by the observation that a mutant lacking a Holliday junction resolvase homologue RuvC had persistence defects during stomach colonization (Loughlin *et al.*, 2003). Therefore, we investigated the role that AddAB and RecA, proteins whose homologues promote the early

Table 3. Antibiotic sensitivity of recombination mutants.

Genotype ^a	Minimal inhibitory concentration	
	Mitomycin C (ng ml ⁻¹)	Ciprofloxacin (µg ml ⁻¹)
WT	6.0	0.5
$\Delta recA::cat$	1.8	0.1
$\Delta addA::cat$	1.8	0.1
$\Delta addB::cat$	1.8	0.1
$\Delta addA::cat, rdxA::addA$	6.0	0.5
$\Delta addB::cat, rdxA::addB$	6.0	0.5

a. NSH57 strain background.

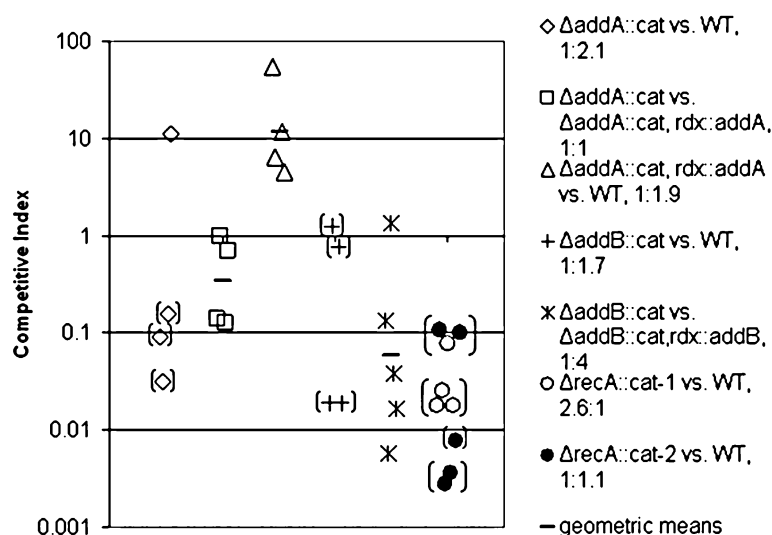


Fig. 4. AddAB and RecA promote stomach colonization. Mice were orally infected with a 1:1 mixture of the indicated strains. The actual ratio in the inoculum of mutant to wild-type or complemented mutants is indicated. After 1 week the bacteria colonizing the stomach were harvested and the competitive index determined (ratio of mutant to wild type in the output corrected for the input ratio) as described in *Experimental Procedures*. Each data point is from one mouse, and the geometric means are indicated by horizontal bars. Infections with two independent $\Delta recA::cat$ clones are indicated by open and filled circles. Bracketed data points represented an upper limit on the competitive index with 95% confidence because of failure to isolate mutant clones from the stomach as described in the *Experimental Procedures*. Competitions of mutant clones with wild type were repeated with similar results (data not shown). A colonization defect is indicated by a competitive index of less than one.

steps of recombination-based repair, might play in stomach colonization.

We first performed competition experiments by oral infection with 1:1 mixtures of mutant and either wild-type or complemented mutant in the NSH57 strain background. We allowed the infection to continue for 1 week and then harvested the bacteria from the stomachs. Plating on selective and nonselective media allowed enumeration of mutant and total bacteria. A competitive index was computed for each animal as the ratio of mutant to wild-type (or complemented mutant) bacteria recovered after 1 week, adjusted for the ratio of strains in the inocula. While there is considerable mouse-to-mouse variation in the assay, the average competitive index of either *addA* or *addB* mutants in competition with either wild-type or the complemented mutant was below 1, indicating a colonization defect for the mutants (Fig. 4). Curiously, when the *addA* complemented strain was competed with wild type, the competitive index was above 1. As described above, expression of *addA* from the *rdxA* locus may result in a higher expression level that is protective under some circumstances. We were unable to complement the *recA* mutant clone because of the requirement of RecA for natural transformation. However, two independently generated *recA* mutant clones both yielded even lower competitive indices than *addA* or *addB* mutants (Fig. 4). While we recovered at least some mutant bacteria in five of eight *addA* mutant and five of nine *addB* mutant competitions with wild-type or comple-

mented strains, we never recovered *recA* mutant bacteria in competition experiments. All three mutants (*recA*, *addA* and *addB*) showed comparable growth to wild type during *in vitro* culture (Fig. S2).

In order to gain further insight into the infection potential of our *H. pylori* strains we infected groups of five animals with decreasing titres of individual strains to determine the dose required for detectable infection of 50% of the animals (ID_{50}) (Table S4). In this experiment the ID_{50} was 2.3×10^4 bacteria for the wild-type strain, 2.0×10^7 for the *addA* mutant strain and greater than 2.4×10^{10} for the *recA* mutant strain. We recovered bacteria from only a single animal at a high-infecting dose of *recA* mutant bacteria. These results mirror the data from the competition experiments showing significantly attenuated colonization by strains lacking AddAB activity and essentially no colonization by strains lacking RecA.

Recombination proteins promote apparent gene conversion at the *babA* locus

As mentioned above, previous work demonstrated that mutants lacking RuvC, a protein required for resolving recombination intermediates, also have partially attenuated stomach persistence (Loughlin *et al.*, 2003). Interestingly, further studies suggested that RuvC function and, by inference, recombination facilitate bacterial immune evasion by altering the adaptive immune response (Robinson *et al.*, 2005). The mechanisms by which the immune

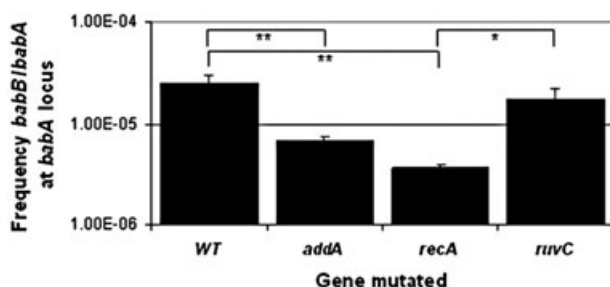


Fig. 5. AddA and RecA promote *babA* to *babB* gene conversion. Quantitative PCR was used to determine the number of copies of *babA* and *babB* at the *babA* locus for genomic DNA prepared from each of three cultures started from individual colonies as described in *Experimental Procedures*. The geometric mean of the frequency of *babB/babA* at the *babA* locus is reported and the bars indicate 1 standard deviation. The difference in means between groups was considered significant (one-way analysis of variance, $P = 0.0002$). Significant P -values for pairwise comparisons between groups are indicated ** $P < 0.001$, * $P < 0.01$.

system becomes redirected remain obscure. We previously showed that *H. pylori* can abolish *babA*-dependent adhesion by a gene conversion-like event between *babA* and a related locus, *babB*, and that this event is selected during infection of some hosts (Solnick *et al.*, 2004). We investigated whether this gene conversion event requires recombination protein activity. To do this, we created null alleles of *recA*, *addA* and *ruvC* in the J166 strain background where we had previously measured spontaneous *babA* to *babB* gene conversion during *in vitro* culture (Solnick *et al.*, 2004). Real-time polymerase chain reaction (PCR) was used to quantify the frequency of *babB* at the *babA* locus in bacterial populations expanded from single colonies *in vitro*. The wild-type J166 strains showed a frequency of 3×10^{-5} , corresponding to an approximate rate of gene conversion of 3×10^{-6} per cell division (Lea and Coulson, 1949). As shown in Fig. 5, loss of AddA or RecA significantly reduced the frequency of the *babA* to *babB* gene conversion. In contrast loss of RuvC only slightly reduced the frequency of gene conversion, and this difference was not statistically significant. The limit of detection of our assay for the frequency of convertants is approximately 4×10^{-7} . These results suggest that this gene conversion event frequently occurs by a RecA-dependent mechanism that may involve a ds break, as it is AddAB-dependent.

Discussion

We report here that the DNA ds break repair enzyme AddAB and the homologous recombination-promoting protein RecA are required for high-level infection by *H. pylori*. AddAB is a functional homologue of the RecBCD enzyme of *E. coli*, *Salmonella enterica* serovar

Typhimurium and *Neisseria gonorrhoea*. The *E. coli* enzyme is crucial for repair of DNA breaks and genetic recombination involving linear DNA (Smith, 2001), and the *recC* gene appears to be under positive selection in uropathogenic strains of *E. coli* (Chen *et al.*, 2006). *N. gonorrhoeae* *recB*, *recC* and *recD* mutants are more sensitive than wild-type strains to hydrogen peroxide (Stohl and Seifert, 2006). We discuss below the properties of *H. pylori* AddAB enzyme and its role in colonization of the stomach of mice and compare its role with that of RecA protein, which is also required for DNA repair as well as homologous recombination.

We identified the *addA* and *addB* genes using BLAST searches that started with AddA and RecB. These polypeptides contain both the canonical seven helicase motifs (Bork and Koonin, 1993) and a RecB nuclease domain (Yu *et al.*, 1998). Although the helicase motifs are highly conserved among a large group of helicases with highly divergent cellular functions, the nuclease domain appears to be unique to RecB-related polypeptides. This dual criterion in our searches may account for our finding the AddA polypeptide of *H. pylori*, whereas previous searches were not successful. The AddB polypeptide also contains a closely related nuclease domain and a large region with only scant similarity to helicases. These properties allowed us to identify *H. pylori* AddB. Identification of the *H. pylori* *addA* and *addB* genes allowed us to discover further that AddAB proteins are in fact well conserved among all the sequenced epsilon Proteobacteria in spite of the fact they have not been annotated in most sequencing projects (Rocha *et al.*, 2005).

Although the *addA* and *addB* genes of many groups of bacteria are adjacent and appear to form an operon, the *H. pylori* *addA* and *addB* genes are not; they are separated by approximately 500 kb. We suppose that the AddA and AddB polypeptides act together in a complex, as do the RecBCD polypeptides and AddAB polypeptides of other bacteria investigated (Kooistra and Venema, 1991; Taylor and Smith, 1995). As noted below, the phenotypes of *H. pylori* *addA* and *addB* mutants are indistinguishable, as expected if the polypeptides act in a complex. If so, the control of the unlinked *addA* and *addB* genes to maintain the proper stoichiometry of the two polypeptides remains an interesting question.

By assaying extracts of *H. pylori*, we detected an ATP-dependent nuclease (Table 1 and Fig. S1), the defining characteristic of the RecBCD class of enzymes, also called exonuclease V. The ATP dependence of the nuclease activity is a consequence of DNA degradation occurring only during unwinding, which requires the energy of ATP hydrolysis. ATP-dependent nuclease activity was undetectable in *addA* and *addB* mutants; as expected, this loss was complemented by insertions of the corresponding genes at a distant locus (Table 1). These genes also con-

ferred ATP-dependent nuclease and ATP-dependent DNA unwinding activity to an *E. coli* *recBCD* deletion mutant (Table 2 and Fig. 2), indicating that they are the structural genes for this enzyme. Activity was detected both in extracts and in intact cells, which blocked the growth of a phage T4 mutant lacking a protein that protects linear DNA from nuclease digestion. Expression of these genes in *E. coli* provides a way to make large amounts of *H. pylori* AddAB enzyme for further analysis.

Our phenotypic analyses suggest that, like the *E. coli* RecBCD enzyme, *H. pylori* AddAB functions to repair DNA damage that results in ds breaks; both *addA* and *addB* mutants were highly sensitive *in vitro* to the alkylating agent mitomycin C and the topoisomerase inhibitor ciprofloxacin but only slightly sensitive to UV-induced damage (Table 3 and Fig. 3). While *addA* and *addB* mutants grow well *in vitro* in the absence of overt DNA damage (Fig. S2), these strains have a lower colonization potential in both competition experiments and during single-strain infections (Fig. 4, supplementary Table S4 and data in *Results*). This may result from DNA damage specifically encountered in the host environment.

Previous studies of enteric pathogens revealed a role for recombinational repair proteins but apparently not recombination *per se* during infection. *S. enterica* serovar Typhimurium *recBC* mutants are severely attenuated for infection and killing, but suppressors that restore recombinational repair by activating the RecFOR pathway of homologous recombination do not suppress the *in vivo* defects (Buchmeier *et al.*, 1993; Cano *et al.*, 2002). Moreover, *recA* mutants, which essentially lack homologous recombination, show a milder phenotype than *recBC* *Salmonella* mutants, and loss of RecA does not impact colonization by the *E. coli* extracellular pathogens EHEC or UPEC (Fuchs *et al.*, 1999). Based on double mutant analyses, the essential *in vivo* function of RecBCD enzyme during *Salmonella* infection appears to be restoration of stalled replication forks (Schapiro *et al.*, 2003), not recombination. In contrast, our results with *H. pylori*, reported here, indicate a direct role for homologous recombination in stomach infection.

Our results show that two enzymes, AddAB and RecA, which process damaged DNA, enhance the ability of *H. pylori* strains to colonize the stomach (Fig. 4 and lower ID₅₀). In contrast to the *Salmonella* results, loss of *H. pylori* RecA causes even more severe attenuation of stomach colonization than loss of AddAB. This result suggests that *H. pylori* experiences a different spectrum of DNA damage during infection than that encountered by *Salmonella*, and that this spectrum includes ds breaks. Furthermore, recombination functions are essential either to repair DNA damage or for other recombination protein-mediated processes early in *H. pylori* infection.

Unlike *Salmonella*, *H. pylori* primarily remains extracellular where it is not exposed to phagosome-specific host defences, although recent studies suggest that a small subpopulation of *H. pylori* do reside in an intracellular niche primarily in epithelial cells (Amieva *et al.*, 2002; Aspholm *et al.*, 2006; Necchi *et al.*, 2007). The different cellular and tissue tropisms of these bacteria may account for the different recombination-protein requirements for successful infection of *Salmonella* and *H. pylori*. Several lines of evidence suggest that even in its extracellular niche, *H. pylori* is exposed to oxidative damage soon after infection. Recent work using the mouse model showed *H. pylori*-dependent infiltration of neutrophils and macrophages 1 and 2 days post infection which then decreased to low levels at 3 and 10 days post infection, suggesting a rapid innate immune response to infection that is then downregulated (Algood *et al.*, 2007). Because neutrophil infiltration is a hallmark of human *H. pylori* infection, the interaction of *H. pylori* and cultured neutrophils has been studied in some detail. Interestingly, while *H. pylori* is readily taken up by neutrophils and is a potent activator of the phagosome NADPH oxidase, active flavocytochrome *b₅₅₈* complex does not assemble on the *H. pylori*-containing phagosome and instead is redirected to the plasma membrane, leading to extracellular superoxide accumulation (Allen *et al.*, 2005). *H. pylori* also induces both macrophages (Chaturvedi *et al.*, 2004) and epithelial cells (Xu *et al.*, 2004) to produce extracellular hydrogen peroxide by stimulating polyamine oxidase. Finally, isolated gastric pit cells express the phagosome NADPH oxidase components at the plasma membrane and show measurable constitutive extracellular superoxide production that is further induced after exposure to *H. pylori*-derived lipopolysaccharide (Teshima *et al.*, 1999). These innate immune responses likely contribute to host cellular damage that may benefit *H. pylori* but also necessitates bacterial mechanisms to combat oxidative damage to its DNA, proteins and lipids. AddAB appears to play such a role for *H. pylori*.

Loss of several *H. pylori* proteins shown or annotated to recognize DNA damage causes lower colonization loads or decreased persistence of *H. pylori* strains in the mouse model, suggesting that the bacteria experience DNA damage stresses during infection. These proteins include HP0585, a homologue of *E. coli* endonuclease III, which repairs oxidized pyrimidine residues (O'Rourke *et al.*, 2003), MutS2, which in *H. pylori* recognizes and binds 8-oxoguanine (Wang *et al.*, 2005), two DNA glycosylases (Baldwin *et al.*, 2007), and a putative RecN homologue (Wang and Maier, 2008). In *E. coli* and *B. subtilis*, RecN promotes RecBCD (AddAB)-dependent ds break repair under some stress conditions and is recruited to large damage foci in the absence of ds break repair (Meddows *et al.*, 2005; Sanchez *et al.*, 2006). Thus, *H. pylori* RecN

may interact with AddAB to promote ds break repair during stomach colonization.

Our *in vitro* data suggest that *H. pylori* AddAB, like *E. coli* RecBCD, functions specifically at ds breaks. Mutants with loss of AddAB or RecA show equivalent sensitivity to chemicals leading to ds breaks, but the *recA* mutant is much more sensitive to UV exposure than *addAB* mutants (Table 3, Fig. 3). These results suggest that an additional RecA-dependent pathway operates in *H. pylori* to repair damage induced by UV. A likely candidate is an analogue of the *E. coli* RecFOR pathway. A *recR* homologue has been annotated in the *H. pylori* genome (Tomb *et al.*, 1997). The more severe stomach colonization phenotype of *recA* mutants than of *addAB* mutants (Fig. 4 and Table S4) may result from an additional requirement for the RecFOR DNA repair pathway during infection. The role of RecA in competence for natural transformation may also contribute to the more severe attenuation of this mutant. Unlike other bacteria, such as *Rhizobium*, *Bacillus* and *Neisseria* (Haijema *et al.*, 1996; Mehr and Seifert, 1998; Zuniga-Castillo *et al.*, 2004), *H. pylori* AddAB does not appear to contribute to competence, while RecA is absolutely required (Schmitt *et al.*, 1995). Two studies have suggested that competence contributes to stomach colonization, even at early time points (Kavermann *et al.*, 2003; Baldwin *et al.*, 2007). Natural transformation is thought to contribute to genetic diversification of the *H. pylori* population later in infection by allowing new alleles to spread through the population via recombination (Suerbaum and Josenhans, 2007). Multiple mutant analyses involving *addAB*, *recR* and *com* (DNA transformation competence) genes should begin to address the relative importance of these pathways during infection.

RecA and AddAB may also contribute to long-term adaptation to the host environment. We discovered a role for RecA and AddAB in promoting gene conversion between two outer membrane protein (OMP) genes, *babA* and *babB* (Fig. 5). *H. pylori* genomes encode a large number of OMPs (60), some of which have been annotated as porins, adhesins or outer membrane transporters. Subsets of OMPs have been grouped into paralogous families suggested to be at least partially redundant (Alm *et al.*, 2000). The largest family of OMPs is the Hops which include the Lewis B blood group antigen-binding adhesin BabA and two highly related Hops BabB and BabC (Alm *et al.*, 2000; Hennig *et al.*, 2006). Gene conversion via conserved 5'- and 3'-terminal sequences in *babB* or *babC* can eliminate *babA* adhesin-gene expression. Loss of *babA* expression has been observed in a majority of clones from the infecting bacterial population after the initial colonization event (between 4 and 8 weeks post infection) during experimental monkey infection (Solnick *et al.*, 2004), and BabA can be

encoded by one or more of the three *bab* loci (Solnick *et al.*, 2004; Colbeck *et al.*, 2006; Hennig *et al.*, 2006). Loss of BabA protein from the cell surface may result in an altered immune response or, alternatively, modify bacterial tropism by changing host receptor-binding interactions. In *Neisseria*, pilin antigenic variation is mediated by a RecA-dependent gene conversion event that also requires the RecFOR complex (Mehr and Seifert, 1998) or the RecBCD complex (Hill *et al.*, 2007), depending on the strain background. While an engineered gene conversion event in *H. pylori* is RecA-dependent (Pride and Blaser, 2002), the requirement for RecA and other recombination proteins for *bab* locus conversion had not been examined. We find that the *babA* to *babB* gene conversion event significantly depends on RecA and AddAB (Fig. 5). If AddAB, like RecBCD, depends on a DNA end for activity, this result suggests that the mechanism of this conversion involves a ds break.

Colonization by *H. pylori* of its host for decades is required for development of disease. The data presented here suggest that recombinational repair proteins, including AddAB and RecA, play multiple roles during infection. The *in vivo* phenotypes we report here after 1 week of infection likely result from the requirement for these proteins to combat DNA damage stress induced soon after infection. Interestingly, while *recA* mutants have a very severe phenotype, *addAB* mutants can still colonize. This outcome will allow study of genetic diversification during long-term colonization to further dissect additional roles of recombinational repair proteins during infection. Recombinational repair has been suggested as a target that could enhance the efficacy of other antibiotics that lead to intracellular oxidative stress (Kohanski *et al.*, 2007). Our work suggests that for *H. pylori* and perhaps other bacteria AddAB could be a promising direct target for a novel antimicrobial drug, as this class of enzymes is widely distributed in prokaryotes but not in eukaryotes.

Experimental procedures

Bacterial strains and growth

Escherichia coli strains (Table S1) were grown in media containing Difco tryptone and yeast extract (Luria-Bertani), Terrific Broth (Fisher) or Difco tryptone (TB). These media, phage suspension medium (SM) and top agar have been described (Cheng and Smith, 1989). *H. pylori* strains (Table S1) were grown on solid horse blood agar (HB) plates containing 4% Columbia agar base (Oxoid), 5% defibrinated horse blood (HemoStat Laboratories), 0.2% β -cyclodextrin (Sigma), vancomycin (Sigma; 10 μ g ml⁻¹), cefsulodin (Sigma; 5 μ g ml⁻¹), polymyxin B (Sigma; 2.5 U ml⁻¹), trimethoprim (Sigma; 5 μ g ml⁻¹) and amphotericin B (Sigma; 8 μ g ml⁻¹) at 37°C either under a microaerobic atmosphere generated using a CampyGen sachet (Oxoid) in a gas pack jar or in an

incubator equilibrated with 14% CO₂ and 86% air. For liquid culture, *H. pylori* was grown in Brucella broth (Difco) containing 10% fetal bovine serum (BB10; Invitrogen) with shaking in a gas pack jar with a CampyGen sachet. For antibiotic resistance marker selection, bacterial media were additionally supplemented with ampicillin (100 µg ml⁻¹), kanamycin (50 µg ml⁻¹), chloramphenicol (Cm; 15 µg ml⁻¹) or metronidazole (Mtz; 36 µg ml⁻¹). When culturing bacteria from mouse stomachs, Bacitracin (Bac; 200 µg ml⁻¹) was added to eliminate normal mouse microbiota contamination.

DNA manipulations

DNA manipulations, such as restriction digestion, PCR and agarose gel electrophoresis, were performed according to standard procedures (Ausubel *et al.*, 1997). Genomic DNA was prepared from *H. pylori* by Wizard genomic DNA preparation kits (Promega). Primers used for PCR and sequencing are in Table S2, and plasmids used in this study are in Table S3.

Generation of *H. pylori* isogenic knockout mutants

In the NSH57 strain background, null alleles of *addA*, *addB*, *recA* and *ruvC* were constructed using a vector-free allelic replacement strategy to generate alleles in which a chloramphenicol acetyl transferase resistance cassette replaced 80–90% of the coding sequence of the gene while preserving the start and stop codons (Chalker *et al.*, 2001; Salama *et al.*, 2004). The primers used for this procedure are in Table S2. The resistance cassette contains its own promoter but lacks a transcriptional terminator and in all cases was inserted in the same direction of transcription as that of the native gene. After natural transformation (Wang *et al.*, 1993) with the appropriate PCR product and selection on Cm-containing media, four to eight clones were evaluated by PCR to confirm replacement of the wild-type allele with the null allele; urease activity and flagella-based motility were also confirmed. Single clones were used for infection experiments and phenotypic characterization. J166 deletion mutants of *recA*, *addA* and *ruvC* were constructed by allelic exchange using methods similar to those previously described (Akopyants *et al.*, 1998). Briefly, a nonpolar kanamycin resistance (*aphA*) cassette (Menard *et al.*, 1993) and ~1 kb upstream and downstream 'arms' for each gene were PCR amplified using primers (Table S2) with compatible 5' restriction sites. All three fragments were restricted with the appropriate enzymes, ligated with pBluescript SK(-) (Stratagene, La Jolla, CA, USA) plasmid DNA which was previously digested with XhoI/NotI, and transformed into One Shot TOP10 competent *E. coli* (Invitrogen, Carlsbad, CA, USA) with kanamycin selection. Each shuttle plasmid was verified by sequencing and transferred into *H. pylori* J166 using natural transformation with kanamycin selection, thereby deleting most of the coding region of each gene.

Generation of *E. coli* expression constructs and *H. pylori* complementation constructs

The *E. coli* expression construct pJF30 containing both AddA and AddB in pETDuet-1 (Novagen) was made by separately

amplifying *addA* (HP1553) and *addB* (HP1089) from *H. pylori* strain 26695 using primers AddA-C1 (Sall), AddA-N1 (NcoI), AddB N1 (NdeI), and AddB C1 (AvrII). The reaction conditions included six cycles with 54°C annealing temperature followed by 24 cycles with 62°C annealing temperature and used High Fidelity Taq and Supermix (Invitrogen). Each gene was separately cloned into pETDuet-1 after digestion of the PCR product and the vector with the indicated restriction enzymes using standard procedures to generate pJF25 (*addA*) and pJF22 (*addB*). pJF30 was made by subcloning *addB* from pJF22 into pJF25 using NdeI and AvrII. Vectors for complementation were made by subcloning each gene individually into pRdxA and introduced into *H. pylori* NSH57 by natural transformation and selection on Mtz-containing media (Smeets *et al.*, 2000). The *addA* complementing vector pJF29 was made by subcloning *addA* from pJF31 using XbaI and Sall. The *addB* complementing vector pJF27 was made by subcloning *addB* from pJF22 using AvrII and XbaI. All inserted genes contained the expected nucleotide sequences, except for *addB* in pJF22, pJF30, pJF31 and pJF27, which contained a single point mutation [T2311 → C2311] changing serine 771 to proline (S771P). This residue is not part of any conserved domain of AddB, and the S771P-containing clone fully complemented drug sensitivity and animal infectivity in *H. pylori* (Table 3 and Fig. 4). For assaying enzymatic activities, this mutation was repaired using QuikChange (Stratagene) on the pJF30 template to generate pSA405.

Preparation of cell-free extracts and enzymatic assays

Extracts were prepared as described by Tomizawa and Ogawa (1972). For *H. pylori* extracts bacteria were harvested from 24 to 48 h plate-grown cultures. For *E. coli* extracts bacteria were harvested 3 h after addition of 1 mM IPTG to induce expression of AddA and AddB. The ds exonuclease activity was assayed as ATP-dependent solubilization of uniformly [³H]-labelled T7 DNA (specific activity 2 × 10⁴ cpm µg⁻¹; Eichler and Lehman, 1977). Each assay included two or three protein concentrations that gave a linear relationship between acid-solubilized DNA and protein assayed. Assays of haploid *H. pylori* extracts contained 10 mM MgCl₂ and 1 mM ATP (see Fig. S1). Assays of *H. pylori* AddAB in *E. coli* extracts contained 50 µM ATP (Table 2).

The substrate for DNA unwinding was plasmid pBR322 digested with HindIII (New England Biolabs), treated with shrimp alkaline phosphatase (US Biochemicals), and labelled at the 5' ends with [^γ-³²P] ATP (GE Biosciences). Unincorporated nucleotides were separated from the DNA substrate by passage through an SR200 minicolumn (GE Biosciences).

DNA unwinding assay mixtures contained 4.0 nM DNA substrate in 15 µl of buffer containing 25 mM Tris acetate (pH 7.5), 2 mM magnesium acetate, 5 mM ATP, 1 mM DTT and 1 µM single-stranded DNA binding protein (Promega). Reactions were for 2 min at 37°C with the amount of extract protein indicated in Fig. 2. Reactions were terminated by the addition of 5 µl of buffer containing 0.1 M EDTA, 2.5% SDS, 0.125% bromophenol blue, 0.125% xylene cyanol and 10% ficoll. Reaction products were separated on a 0.7% agarose gel (22 cm long) in Tris acetate electrophoresis buffer (Ausubel *et al.*, 1997) at 100 V for 2 h and visualized by autoradiography.

Efficiency of plaque formation by phage T4 and T4 2⁻

Escherichia coli strain V3060 (chromosomal $\Delta recBCD$) containing plasmids bearing the *E. coli* *recBCD* or *H. pylori* *addAB* genes was grown in TB broth containing ampicillin ($100 \mu\text{g ml}^{-1}$) to about 2×10^8 cells ml^{-1} . Phage T4 or T4 2⁻ in SM ($50 \mu\text{l}$) were added to 0.1 ml of bacteria and incubated for 15 min at 37°C. Top agar (2.5 ml) was added and the mixture poured onto a TB agar plate. Plates were incubated at 37°C overnight, after which plaques were enumerated.

Antibiotic resistance testing

Bacteria taken from fresh plates (incubated for 18–36 h) were grown in liquid culture to an optical density at 600 nm (OD_{600}) between 0.1 and 1 and examined for spiral shape and motility. Fivefold serial dilutions were spotted onto plates containing increasing concentrations of mitomycin C or ciprofloxacin (Sigma). The minimal inhibitory concentration was determined when drug prevented growth of at least two fivefold serial dilutions.

UV sensitivity

Bacteria were grown as described for antibiotic resistance testing. Dilutions were plated in duplicate, each on one half of a plate, and exposed to UV using a UV Stratalinker 2400 (Stratagene) on the energy setting. After UV exposure, plates were incubated for 3–4 days until single colonies could be counted. The per cent survival was calculated in comparison with plates that were mock UV treated. The data presented are the average from three experiments.

Mouse infections

Female C57BL/6 mice 24–28 days old were obtained from Charles River Laboratories and certified free of endogenous *Helicobacter* infection by the vendor. The mice were housed in sterilized microisolator cages with irradiated PMI 5053 rodent chow, autoclaved corn cob bedding, and acidified, reverse-osmosis purified water provided ad libitum. All studies were done under practices and procedures of Animal Biosafety Level 2. The facility is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International, and all activities were approved by the FHCRC Institutional Animal Care and Use Committee.

For competition experiments each indicated null mutant strain and the parental wild-type strain or complemented mutant were grown from frozen stock in liquid culture to mid-to-late logarithmic growth phase. The wild-type and mutant bacteria were combined to give approximately 5.0×10^8 bacteria of each in 2.5 ml, and 0.5 ml inoculated by oral gavage into each of five mice. After inoculation, a portion of the inoculum was plated on HB-Bac plates and HB-Cm plates to determine the number of wild-type and mutant bacteria, respectively, in the inocula. After 1 week the mice were euthanized by inhalation of CO_2 . The glandular stomach was removed and cut along the greater curvature, and any food was removed. The stomach was then cut in half longitudinally, and one-half stomach placed in 0.5 ml of BB10 broth

for homogenization with disposable pellet pestles (Fisher Scientific). Dilutions of the homogenate were plated on HB-Bac to enumerate total bacteria and on HB-Cm plates to enumerate mutant bacteria. If no mutant bacteria were recovered we set the number of colonies on the lowest dilution plated to three. From the Poisson distribution we estimate with 95% confidence that a plate containing zero colonies corresponds to three or less colony-forming units (cfu). The average bacterial colonization load for wild type in these experiments was 1.3×10^5 cfu g^{-1} and the range was 3.3×10^3 – 9.6×10^5 cfu g^{-1} . The competitive index (CI) was computed as follows: $\text{CI} = (\text{output mutant cfu/output wild-type cfu})/(\text{input mutant cfu/input wild-type cfu})$.

To determine the dose for 50% infection (ID_{50}), the indicated strains were grown in liquid culture to mid-to-late logarithmic growth phase and concentrated to give 5×10^9 – 5×10^{10} bacteria per millilitre. Serial 10-fold dilutions were prepared and inoculated into each of five mice. After 7–10 days mice were euthanized and processed as described above; bacteria were enumerated on HB-Bac plates. The number of infected mice at each titre was determined (Table S4), and the ID_{50} was calculated using the method of Reed and Muench (1938).

babA gene conversion assay

Real-time quantitative PCR was used to determine the frequency of apparent gene conversion of *babA* to *babB*, using methods similar to those described (Solnick *et al.*, 2004). Briefly, genomic DNA was prepared from individual colonies of wild-type *H. pylori* J166, and from isogenic mutants with deletions of *recA*, *addA* or *ruvC*. Each 20 μl reaction contained 10 μl of iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA), 5 μl of primer pair (1 μM each) contain a primer (Table S2) upstream of the *babA* locus (F14) and a primer in either *babA* (160R) or *babB* (178R), and 5 μl of DNA template (0.0375 ng μl^{-1} for *babA*; 5 ng μl^{-1} for *babB*). Amplification was carried out in a Bio-Rad iCycler (3 min at 95°C; 45 cycles of 30 s at 95°C, 30 s at 58°C, 1 min at 72°C) and the cycle threshold (Ct) was determined. Standard curves were constructed for *babA* and *babB* using plasmids (Table S3) in which *babA* (pJ150) or *babB* (pJ151) was amplified and cloned into pGEM-T Easy (Promega, Madison, WI, USA) using TA cloning. Template for amplification of the *babA* and *babB* fragments was chromosomal DNA from wild-type *H. pylori* J166 (*babA*) or *H. pylori* J166 passaged through rhesus macaques (*babB*), in which the *babA* gene was converted by *babB*. Primers for amplification of *babA* and *babB* (Table S2) were therefore the same, as the upstream primer (HP0898F) was not in *babA/babB* and the downstream primer (AN5954) was in a region in which *babA* and *babB* are identical.

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References

- Akopyants, N.S., Clifton, S.W., Kersulyte, D., Crabtree, J.E., Youree, B.E., Reece, C.A., *et al.* (1998) Analyses of the *cag* pathogenicity island of *Helicobacter pylori*. *Mol Microbiol* **28**: 37–53.
- Algood, H.M., Gallo-Romero, J., Wilson, K.T., Peek, R.M., Jr, and Cover, T.L. (2007) Host response to *Helicobacter pylori* infection before initiation of the adaptive immune response. *FEMS Immunol Med Microbiol* **51**: 577–586.
- Allen, L.A., Beecher, B.R., Lynch, J.T., Rohner, O.V., and Wittine, L.M. (2005) *Helicobacter pylori* disrupts NADPH oxidase targeting in human neutrophils to induce extracellular superoxide release. *J Immunol* **174**: 3658–3667.
- Alm, R.A., Bina, J., Andrews, B.M., Doig, P., Hancock, R.E., and Trust, T.J. (2000) Comparative genomics of *Helicobacter pylori*: analysis of the outer membrane protein families. *Infect Immun* **68**: 4155–4168.
- Alonso, J.C., Stiege, A.C., and Luder, G. (1993) Genetic recombination in *Bacillus subtilis* 168: effect of *recN*, *recF*, *recH* and *addAB* mutations on DNA repair and recombination. *Mol Gen Genet* **239**: 129–136.
- Amieva, M.R., Salama, N.R., Tompkins, L.S., and Falkow, S. (2002) *Helicobacter pylori* enter and survive within multivesicular vacuoles of epithelial cells. *Cell Microbiol* **4**: 677–690.
- Amundsen, S.K., and Smith, G.R. (2003) Interchangeable parts of the *Escherichia coli* recombination machinery. *Cell* **112**: 741–744.
- Amundsen, S.K., Taylor, A.F., Chaudhury, A.M., and Smith, G.R. (1986) *recD*: the gene for an essential third subunit of exonuclease V. *Proc Natl Acad Sci USA* **83**: 5558–5562.
- Amundsen, S.K., Neiman, A.M., Thibodeaux, S.M., and Smith, G.R. (1990) Genetic dissection of the biochemical activities of RecBCD enzyme. *Genetics* **126**: 25–40.
- Anderson, D.G., and Kowalczykowski, S.C. (1997) The recombination hot spot Chi is a regulatory element that switches the polarity of DNA degradation by the RecBCD enzyme. *Genes Dev* **11**: 571–581.
- Aspholm, M., Olfat, F.O., Norden, J., Sonden, B., Lundberg, C., Sjostrom, R., *et al.* (2006) SabA is the *H. pylori* hemagglutinin and is polymorphic in binding to sialylated glycans. *PLoS Pathog* **2**: e110.
- Ausubel, F., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1997) *Short Protocols in Molecular Biology*. New York: John Wiley & Sons.
- Baldwin, D.N., Shepherd, B., Kraemer, P., Hall, M.K., Sycuro, L.K., Pinto-Santini, D.M., and Salama, N.R. (2007) Identification of *Helicobacter pylori* genes that contribute to stomach colonization. *Infect Immun* **75**: 1005–1016.
- Bork, P., and Koonin, E.V. (1993) An expanding family of helicases within the 'DEAD/H' superfamily. *Nucleic Acids Res* **21**: 751–752.
- Buchmeier, N.A., Lipps, C.J., So, M.Y., and Heffron, F. (1993) Recombination-deficient mutants of *Salmonella typhimurium* are avirulent and sensitive to the oxidative burst of macrophages. *Mol Microbiol* **7**: 933–936.
- Cano, D.A., Pucciarelli, M.G., Garcia-del Portillo, F., and Casadesus, J. (2002) Role of the RecBCD recombination pathway in *Salmonella* virulence. *J Bacteriol* **184**: 592–595.
- Chalker, A.F., Minehart, H.W., Hughes, N.J., Koretke, K.K., Lonetto, M.A., Brinkman, K.K., *et al.* (2001) Systematic identification of selective essential genes in *Helicobacter pylori* by genome prioritization and allelic replacement mutagenesis. *J Bacteriol* **183**: 1259–1268.
- Chaturvedi, R., Cheng, Y., Asim, M., Bussiere, F.I., Xu, H., Gobert, A.P., *et al.* (2004) Induction of polyamine oxidase 1 by *Helicobacter pylori* causes macrophage apoptosis by hydrogen peroxide release and mitochondrial membrane depolarization. *J Biol Chem* **279**: 40161–40173.
- Chedin, F., and Kowalczykowski, S.C. (2002) A novel family of regulated helicases/nucleases from Gram-positive bacteria: insights into the initiation of DNA recombination. *Mol Microbiol* **43**: 823–834.
- Chen, S.L., Hung, C.S., Xu, J., Reigstad, C.S., Magrini, V., Sabo, A., *et al.* (2006) Identification of genes subject to positive selection in uropathogenic strains of *Escherichia coli*: a comparative genomics approach. *Proc Natl Acad Sci USA* **103**: 5977–5982.
- Cheng, K.C., and Smith, G.R. (1989) Distribution of Chi-stimulated recombinational exchanges and heteroduplex endpoints in phage lambda. *Genetics* **123**: 5–17.
- Colbeck, J.C., Hansen, L.M., Fong, J.M., and Solnick, J.V. (2006) Genotypic profile of the outer membrane proteins BabA and BabB in clinical isolates of *Helicobacter pylori*. *Infect Immun* **74**: 4375–4378.
- Eichler, D.C., and Lehman, I.R. (1977) On the role of ATP in phosphodiester bond hydrolysis catalyzed by the recBC deoxyribonuclease of *Escherichia coli*. *J Biol Chem* **252**: 499–503.
- Falush, D., Wirth, T., Linz, B., Pritchard, J.K., Stephens, M., Kidd, M., *et al.* (2003) Traces of human migrations in *Helicobacter pylori* populations. *Science* **299**: 1582–1585.
- Fuchs, S., Muhldorfer, I., Donohue-Rolfe, A., Kerenyi, M., Emody, L., Alexiev, R., *et al.* (1999) Influence of RecA on *in vivo* virulence and Shiga toxin 2 production in *Escherichia coli* pathogens. *Microb Pathog* **27**: 13–23.
- Gressmann, H., Linz, B., Ghai, R., Pleissner, K.P., Schlappbach, R., Yamaoka, Y., *et al.* (2005) Gain and loss of multiple genes during the evolution of *Helicobacter pylori*. *PLoS Genet* **1**: e43.
- Haijema, B.J., Noback, M., Hesseling, A., Kooistra, J., Venema, G., and Meima, R. (1996) Replacement of the lysine residue in the consensus ATP-binding sequence of the AddA subunit of AddAB drastically affects chromosomal recombination in transformation and transduction of *Bacillus subtilis*. *Mol Microbiol* **21**: 989–999.
- Harris, A.G., Wilson, J.E., Danon, S.J., Dixon, M.F., Donegan, K., and Hazell, S.L. (2003) Catalase (KatA) and KatA-associated protein (KapA) are essential to persistent colonization in the *Helicobacter pylori* SS1 mouse model. *Microbiology* **149**: 665–672.
- Hennig, E.E., Allen, J.M., and Cover, T.L. (2006) Multiple chromosomal loci for the *babA* gene in *Helicobacter pylori*. *Infect Immun* **74**: 3046–3051.
- Hill, S.A., Woodward, T., Reger, A., Baker, R., and Dinse, T. (2007) Role for the RecBCD recombination pathway for *pilE* gene variation in repair-proficient *Neisseria gonorrhoeae*. *J Bacteriol* **189**: 7983–7990.
- Iyer, V.N., and Szybalski, W. (1963) A molecular mechanism

- of mitomycin action: linking of complementary DNA strands. *Proc Natl Acad Sci USA* **50**: 355–362.
- Kavermann, H., Burns, B.P., Angermüller, K., Odenbreit, S., Fischer, W., Melchers, K., and Haas, R. (2003) Identification and characterization of *Helicobacter pylori* genes essential for gastric colonization. *J Exp Med* **197**: 813–822.
- Kohanski, M.A., Dwyer, D.J., Hayete, B., Lawrence, C.A., and Collins, J.J. (2007) A common mechanism of cellular death induced by bactericidal antibiotics. *Cell* **130**: 797–810.
- Kooistra, J., and Venema, G. (1991) Cloning, sequencing, and expression of *Bacillus subtilis* genes involved in ATP-dependent nuclease synthesis. *J Bacteriol* **173**: 3644–3655.
- Kooistra, J., Vosman, B., and Venema, G. (1988) Cloning and characterization of a *Bacillus subtilis* transcription unit involved in ATP-dependent DNase synthesis. *J Bacteriol* **170**: 4791–4797.
- Kooistra, J., Haijema, B.J., Hesselting-Meinders, A., and Venema, G. (1997) A conserved helicase motif of the AddA subunit of the *Bacillus subtilis* ATP-dependent nuclease (AddAB) is essential for DNA repair and recombination. *Mol Microbiol* **23**: 137–149.
- Kusters, J.G., van Vliet, A.H., and Kuipers, E.J. (2006) Pathogenesis of *Helicobacter pylori* infection. *Clin Microbiol Rev* **19**: 449–490.
- Lea, D.E., and Coulson, C.A. (1949) The distribution of the numbers of mutants in bacterial populations. *J Genet* **49**: 264–285.
- Loughlin, M.F., Barnard, F.M., Jenkins, D., Sharples, G.J., and Jenks, P.J. (2003) *Helicobacter pylori* mutants defective in RuvC Holliday junction resolvase display reduced macrophage survival and spontaneous clearance from the murine gastric mucosa. *Infect Immun* **71**: 2022–2031.
- Meddows, T.R., Savory, A.P., Grove, J.I., Moore, T., and Lloyd, R.G. (2005) RecN protein and transcription factor DksA combine to promote faithful recombinational repair of DNA double-strand breaks. *Mol Microbiol* **57**: 97–110.
- Mehr, I.J., and Seifert, H.S. (1998) Differential roles of homologous recombination pathways in *Neisseria gonorrhoeae* pilin antigenic variation, DNA transformation and DNA repair. *Mol Microbiol* **30**: 697–710.
- Menard, R., Sansonetti, P.J., and Parsot, C. (1993) Nonpolar mutagenesis of the *ipa* genes defines IpaB, IpaC, and IpaD as effectors of *Shigella flexneri* entry into epithelial cells. *J Bacteriol* **175**: 5899–5906.
- Necchi, V., Candusso, M.E., Tava, F., Luinetti, O., Ventura, U., Fiocca, R., et al. (2007) Intracellular, intercellular, and stromal invasion of gastric mucosa, preneoplastic lesions, and cancer by *Helicobacter pylori*. *Gastroenterology* **132**: 1009–1023.
- O'Rourke, E.J., Chevalier, C., Pinto, A.V., Thiberge, J.M., Ielpi, L., Labigne, A., and Radicella, J.P. (2003) Pathogen DNA as target for host-generated oxidative stress: role for repair of bacterial DNA damage in *Helicobacter pylori* colonization. *Proc Natl Acad Sci USA* **100**: 2789–2794.
- Oliver, D.B., and Goldberg, E.B. (1977) Protection of parental T4 DNA from a restriction exonuclease by the product of gene 2. *J Mol Biol* **116**: 877–881.
- Ponticelli, A.S., Schultz, D.W., Taylor, A.F., and Smith, G.R. (1985) Chi-dependent DNA strand cleavage by RecBC enzyme. *Cell* **41**: 145–151.
- Pride, D.T., and Blaser, M.J. (2002) Concerted evolution between duplicated genetic elements in *Helicobacter pylori*. *J Mol Biol* **316**: 629–642.
- Reed, L.J., and Muench, H. (1938) A simple method of estimating fifty per cent endpoints. *Am J Hygiene* **27**: 493–497.
- Robinson, K., Loughlin, M.F., Potter, R., and Jenks, P.J. (2005) Host adaptation and immune modulation are mediated by homologous recombination in *Helicobacter pylori*. *J Infect Dis* **191**: 579–587.
- Rocha, E.P., Cornet, E., and Michel, B. (2005) Comparative and evolutionary analysis of the bacterial homologous recombination systems. *PLoS Genet* **1**: e15.
- Salama, N.R., Shepherd, B., and Falkow, S. (2004) Global transposon mutagenesis and essential gene analysis of *Helicobacter pylori*. *J Bacteriol* **186**: 7926–7935.
- Sanchez, H., Kidane, D., Castillo Cozar, M., Graumann, P.L., and Alonso, J.C. (2006) Recruitment of *Bacillus subtilis* RecN to DNA double-strand breaks in the absence of DNA end processing. *J Bacteriol* **188**: 353–360.
- Schapiro, J.M., Libby, S.J., and Fang, F.C. (2003) Inhibition of bacterial DNA replication by zinc mobilization during nitrosative stress. *Proc Natl Acad Sci USA* **100**: 8496–8501.
- Schmitt, W., Odenbreit, S., Heuermann, D., and Haas, R. (1995) Cloning of the *Helicobacter pylori* *recA* gene and functional characterization of its product. *Mol Gen Genet* **248**: 563–572.
- Seyler, R.W., Jr, Olson, J.W., and Maier, R.J. (2001) Superoxide dismutase-deficient mutants of *Helicobacter pylori* are hypersensitive to oxidative stress and defective in host colonization. *Infect Immun* **69**: 4034–4040.
- Sioud, M., and Forterre, P. (1989) Ciprofloxacin and etoposide (VP16) produce a similar pattern of DNA cleavage in a plasmid of an archaebacterium. *Biochemistry* **28**: 3638–3641.
- Smeets, L.C., Bijlsma, J.J., Boomkens, S.Y., Vandenbroucke-Grauls, C.M., and Kusters, J.G. (2000) *comH*, a novel gene essential for natural transformation of *Helicobacter pylori*. *J Bacteriol* **182**: 3948–3954.
- Smith, G.R. (2001) Homologous recombination near and far from DNA breaks: alternative roles and contrasting views. *Annu Rev Genet* **35**: 243–274.
- Smith, G.R., Kunes, S.M., Schultz, D.W., Taylor, A., and Triman, K.L. (1981) Structure of Chi hotspots of generalized recombination. *Cell* **24**: 429–436.
- Solnick, J.V., Hansen, L.M., Salama, N.R., Boonjakuakul, J.K., and Syvanen, M. (2004) Modification of *Helicobacter pylori* outer membrane protein expression during experimental infection of rhesus macaques. *Proc Natl Acad Sci USA* **101**: 2106–2111.
- Stohl, E.A., and Seifert, H.S. (2006) *Neisseria gonorrhoeae* DNA recombination and repair enzymes protect against oxidative damage caused by hydrogen peroxide. *J Bacteriol* **188**: 7645–7651.
- Suerbaum, S., and Josenhans, C. (2007) *Helicobacter pylori* evolution and phenotypic diversification in a changing host. *Nat Rev Microbiol* **5**: 441–452.
- Suerbaum, S., Smith, J.M., Bapumia, K., Morelli, G., Smith,

- N.H., Kunstmann, E., *et al.* (1998) Free recombination within *Helicobacter pylori*. *Proc Natl Acad Sci USA* **95**: 12619–12624.
- Taylor, A., and Smith, G.R. (1980) Unwinding and rewinding of DNA by the RecBC enzyme. *Cell* **22**: 447–457.
- Taylor, A.F., and Smith, G.R. (1995) Monomeric RecBCD enzyme binds and unwinds DNA. *J Biol Chem* **270**: 24451–24458.
- Taylor, A.F., and Smith, G.R. (2003) RecBCD enzyme is a DNA helicase with fast and slow motors of opposite polarity. *Nature* **423**: 889–893.
- Teshima, S., Tsunawaki, S., and Rokutan, K. (1999) *Helicobacter pylori* lipopolysaccharide enhances the expression of NADPH oxidase components in cultured guinea pig gastric mucosal cells. *FEBS Lett* **452**: 243–246.
- Thompson, S.A., and Blaser, M.J. (1995) Isolation of the *Helicobacter pylori* *recA* gene and involvement of the *recA* region in resistance to low pH. *Infect Immun* **63**: 2185–2193.
- Tomb, J.F., White, O., Kerlavage, A.R., Clayton, R.A., Sutton, G.G., Fleischmann, R.D., *et al.* (1997) The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature* **388**: 539–547. [Published erratum appears in: *Nature*, 1997, **389**: 412.]
- Tomizawa, J., and Ogawa, H. (1972) Structural genes of ATP-dependent deoxyribonuclease of *Escherichia coli*. *Nature: New Biology* **239**: 14–16.
- Wang, G., and Maier, R.J. (2008) Critical role of RecN in recombinational DNA repair and survival of *Helicobacter pylori*. *Infect Immun* **76**: 153–160.
- Wang, G., Alamuri, P., Humayun, M.Z., Taylor, D.E., and Maier, R.J. (2005) The *Helicobacter pylori* MutS protein confers protection from oxidative DNA damage. *Mol Microbiol* **58**: 166–176.
- Wang, Y., Roos, K.P., and Taylor, D.E. (1993) Transformation of *Helicobacter pylori* by chromosomal metronidazole resistance and by a plasmid with a selectable chloramphenicol resistance marker. *J Gen Microbiol* **139**: 2485–2493.
- Wolfson, J.S., and Hooper, D.C. (1985) The fluoroquinolones: structures, mechanisms of action and resistance, and spectra of activity *in vitro*. *Antimicrob Agents Chemother* **28**: 581–586.
- Xu, H., Chaturvedi, R., Cheng, Y., Bussiere, F.I., Asim, M., Yao, M.D., *et al.* (2004) Spermine oxidation induced by *Helicobacter pylori* results in apoptosis and DNA damage: implications for gastric carcinogenesis. *Cancer Res* **64**: 8521–8525.
- Yeeles, J.T., and Dillingham, M.S. (2007) A dual-nuclease mechanism for DNA break processing by AddAB-type helicase-nucleases. *J Mol Biol* **371**: 66–78.
- Yu, M., Souaya, J., and Julin, D.A. (1998) The 30-kDa C-terminal domain of the RecB protein is critical for the nuclease activity, but not the helicase activity, of the RecBCD enzyme from *Escherichia coli*. *Proc Natl Acad Sci USA* **95**: 981–986.
- Zuniga-Castillo, J., Romero, D., and Martinez-Salazar, J.M. (2004) The recombination genes *addAB* are not restricted to gram-positive bacteria: genetic analysis of the recombination initiation enzymes RecF and AddAB in *Rhizobium etli*. *J Bacteriol* **186**: 7905–7913.

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