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 a RecA-coated filament. 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,
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 helicas 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 helicas.

To identify more likely H. pylori AddA or RecB proteins, the group of three high-scoring helicas 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
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 chromosomes of 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...
Recombination proteins promote H. pylori infection

H. pylori 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>
<thead>
<tr>
<th>Relevant genotypea</th>
<th>Allele at rdxA at rdxb</th>
<th>ATP-dependent ds DNA exonuclease (units per mg extract protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>–</td>
<td>5.3 ± 0.9 (6)</td>
</tr>
<tr>
<td>ΔaddA::cat</td>
<td>–</td>
<td>0.009 ± 0.0007 (6)</td>
</tr>
<tr>
<td>ΔaddB::cat</td>
<td>–</td>
<td>0.02 ± 0.017 (6)</td>
</tr>
<tr>
<td>ΔaddB::cat addB+</td>
<td>7.1 (2)</td>
<td>7.0–7.2</td>
</tr>
<tr>
<td>ΔrecA::cat</td>
<td>–</td>
<td>6.1 ± 0.25 (3)</td>
</tr>
<tr>
<td>ΔruvC::cat</td>
<td>–</td>
<td>4.8 ± 0.4 (3)</td>
</tr>
</tbody>
</table>

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.

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

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>ATP-dependent ds DNA exonuclease (units/mg extract protein)a</th>
<th>Efficiency of platingb</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBR322</td>
<td>18 ± 4 (4)</td>
<td>1.0</td>
</tr>
<tr>
<td>PMR3 (recBCD)</td>
<td>140 ± 11 (6)</td>
<td>0.94 8.3 × 10⁻⁷</td>
</tr>
<tr>
<td>pETDuet-1</td>
<td>24 ± 5 (6)</td>
<td>0.97 0.91 ± 0.04</td>
</tr>
<tr>
<td>pSA405 (addA addB)</td>
<td>880 ± 12 (6)</td>
<td>0.91 6.2 × 10⁻⁶</td>
</tr>
</tbody>
</table>

a. Extracts were prepared from transformants of E. coli strain V2831 (ΔrecBCD2731 <kan>) with pBR322 or PMR3 or transformants of E. coli strain V3060 (ΔrecBCD2731 <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 (ΔrecBCD2731 <kan> DE3) with the indicated plasmid divided by the phage titre on strain V3060 with pBR322. Data are the mean from three separate experiments.

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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 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

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**Table 3.** Antibiotic sensitivity of recombination mutants.

<table>
<thead>
<tr>
<th>Genotype*</th>
<th>Mitomycin C (µg ml⁻¹)</th>
<th>Ciprofloxacin (µg ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>6.0</td>
<td>0.5</td>
</tr>
<tr>
<td>ΔrecA::cat</td>
<td>1.8</td>
<td>0.1</td>
</tr>
<tr>
<td>ΔaddA::cat</td>
<td>1.8</td>
<td>0.1</td>
</tr>
<tr>
<td>ΔaddB::cat</td>
<td>1.8</td>
<td>0.1</td>
</tr>
<tr>
<td>ΔaddA::cat, rdxA::addA</td>
<td>6.0</td>
<td>0.5</td>
</tr>
<tr>
<td>ΔaddB::cat rdxA::addB</td>
<td>6.0</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* a. NSH57 strain background.
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, 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 complemented 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 (ID50) (Table S4). In this experiment the ID50 was $2.3 \times 10^4$ bacteria for the wild-type strain, $2.0 \times 10^7$ for the addA mutant strain and greater than $2.4 \times 10^{10}$ 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...
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 **\( P < 0.001 \), *\( P < 0.01 \).

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 (Hajjema 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 μg ml⁻¹), cefsulodin (Sigma; 5 μg ml⁻¹), polymyxin B (Sigma; 2.5 U ml⁻¹), trimethoprim (Sigma; 5 μg ml⁻¹) and amphotericin B (Sigma; 8 μg 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 (Mt; 36 μg ml⁻¹). When culturing bacteria from mouse stomachs, Bactracin (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 (Ncol), AddB-N1 (Ndel), 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 Ndel 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 Xbal and Sall. The *addB* complementing vector pJF27 was made by subcloning *addB* from pJF22 using AvrII and Xbal. 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 (Strategene) 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 [³²P]-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 *ΔrecBCD*) containing plasmids bearing the *E. coli recBCD* or *H. pylori addAB* genes was grown in TB broth containing ampicillin (100 µg ml−1) to about 2 × 10⁶ cells ml−1. Phage T4 or T4 2− in SM (50 µl) 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 plaque testing**

Bacteria taken from fresh plates (incubated for 18–36 h) were grown in liquid culture to an optical density at 600 nm (OD₆₀₀) between 0.1 and 1 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 five-fold 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 were grown from frozen stock in liquid culture to mid-to-late logarithmic growth phase and concentrated to give 5 × 10⁹ cells ml⁻¹. Phage T4 or T4 2− in SM (100 µl) were added to 0.1 ml of bacteria and incubated for 15 min at 37°C. Phage T4 or T4 2− in SM (50 µl) 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.

**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 l⁻¹ for *babA*; 5 ng l⁻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 T4 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 (ANS954) was in a region in which *babA* and *babB* are identical.

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**Supplementary material**

This material is available as part of the online article from: [http://www.blackwell-synergy.com/doi/abs/10.1111/j.1365-2958.2008.06336.x](http://www.blackwell-synergy.com/doi/abs/10.1111/j.1365-2958.2008.06336.x)

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