Critical Role of RecN in Recombinational DNA Repair and Survival of *Helicobacter pylori*[▽]

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Homologous recombination is one of the key mechanisms responsible for the repair of DNA double-strand breaks. Recombinational repair normally requires a battery of proteins, each with specific DNA recognition, strand transfer, resolution, or other functions. *Helicobacter pylori* lacks many of the proteins normally involved in the early stage (presynapsis) of recombinational repair, but it has a RecN homologue with an unclear function. A *recN* mutant strain of *H. pylori* was shown to be much more sensitive than its parent to mitomycin C, an agent predominantly causing DNA double-strand breaks. The *recN* strain was unable to survive exposure to either air or acid as well as the parent strain, and air exposure resulted in no viable *recN* cells recovered after 8 h. In oxidative stress conditions (i.e., air exposure), a *recN* strain accumulated significantly more damaged (multiply fragmented) DNA than the parent strain. To assess the DNA recombination abilities of strains, their transformation abilities were compared by separately monitoring transformation using *H. pylori* DNA fragments containing either a site-specific mutation (conferring rifampin resistance) or a large insertion (kanamycin resistance cassette). The transformation frequencies using the two types of DNA donor were 10- and 50-fold lower, respectively, for the *recN* strain than for the wild type, indicating that RecN plays an important role in facilitating DNA recombination. In two separate mouse colonization experiments, the *recN* strain colonized most of the stomachs, but the average number of recovered cells was 10-fold less for the mutant than for the parent strain (a statistically significant difference). Complementation of the *recN* strain by chromosomal insertion of a functional *recN* gene restored both the recombination frequency and mouse colonization ability to the wild-type levels. Thus, *H. pylori* RecN, as a component of DNA recombinational repair, plays a significant role in *H. pylori* survival in vivo.

DNA repair is a fundamental process in all free-living organisms and is used by pathogenic bacteria as one of the defense mechanisms that allow them to survive in their hosts. Well-known pathways of DNA repair include postreplication mismatch repair (MutSLH) (30), base excision repair (glycosylases) (29), and nucleotide excision repair (UvrABCD) (35). In addition, homologous recombination is one of the key mechanisms responsible for the repair of DNA double-strand breaks (DSBs) (5). DSBs occur as a result of a variety of physical or chemical insults that modify the DNA. Damage at a single-strand site, if not repaired immediately, leads to a DSB. More commonly, if a replication fork meets damaged bases that cannot be replicated, the fork can collapse, leading to a DSB. In *Escherichia coli*, 20 to 50% of replication forks require recombinational repair to overcome damage (4).

Helicobacter pylori, a pathogenic bacterium infecting over 50% of humans, is the etiological agent of gastritis, peptic ulcers, and gastric cancer (7). In physiological conditions (i.e., in vivo), *H. pylori* is thought to frequently suffer both oxidative and acid stress, leading to DNA damage (31, 50). Several DNA repair pathways in *H. pylori* have been found to be important for its survival and pathogenesis. For example, *H. pylori* mutants that cannot repair oxidized pyrimidines due to loss of the DNA glycosylase EndoIII (*nth* gene product) function are

more sensitive to the mutagenic and lethal effects of activated macrophages, and they show an attenuated colonization capacity (31). The *H. pylori* MutY glycosylase was shown to function in avoidance of the mutagenic effect of 8-oxo-guanine (8-oxoG) by removing adenine from an 8-oxoG · A mispair, and the loss of MutY leads to attenuation of the colonization ability (8, 18, 26). However, genes encoding some other DNA glycosylases (e.g., *tag*, *alkA*, and *mutM* found in other bacteria) are not present in the *H. pylori* genome. *H. pylori* encodes homologues of all four members of the nucleotide excision repair pathway (UvrA, UvrB, UvrC, and UvrD), all of which are well conserved in bacteria. Notably, *H. pylori* does not have the postreplication mismatch repair system, which most likely contributes to the high mutation frequencies observed (1). Instead, *H. pylori* MutS (a member of the MutS2 family) has been shown to play a role in maintaining genome integrity by suppressing homologous and homeologous DNA recombination (33) and by repairing oxidative DNA damage (8-oxoG) (49).

Recombinational DNA repair requires a large number of proteins that act at various stages of the process (5, 24). The most crucial step in DNA recombination is DNA strand exchange catalyzed by RecA (synapsis). With the involvement of RuvAB helicases, the RecA-promoted strand transfer produces a branched DNA molecule termed the Holliday junction, which is subsequently resolved by RuvC resolvase (postsynapsis). The processes of synapsis and postsynapsis appear to be highly conserved in organisms ranging from bacteria to humans. However, there are many differences between species in the initial step of recombinational repair (presynapsis),

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which involves recognition of the DNA damage site, generation of 3' single-stranded DNA ends that can be used for annealing with the homologous sequence, and recruitment of RecA. In *E. coli*, there are two separate pathways, RecBCD and RecFOR, for initiation of DNA recombination (24). Several other genes (*recJ*, *recQ*, and *recN*) are also required for recombination, although their functions are unclear (11, 41). Recently, RecN, RecO, and RecF were found to be localized to distinct foci on the DNA in *Bacillus subtilis* cells after induction of DSBs (22). These proteins form active repair centers at DSBs and recruit RecA, initiating homologous recombination. RecN was shown to play an important role in repairing DSBs, probably coordinating alignment of the broken segments with intact duplexes to facilitate recombination (27). The roles of RecN, as well as other components of DNA recombinational repair, in protection against oxidative damage in *Neisseria gonorrhoeae* have recently been demonstrated (42, 43).

A limited number of genes that are involved in recombinational repair are present in the *H. pylori* genome. *H. pylori* RecA is critical in DNA recombination and repair (13, 47). Genes for RuvABC proteins are present in *H. pylori*; thus, *H. pylori* may be able to restore Holliday junctions in a way similar to the way observed for *E. coli*. A *ruvC* mutant of *H. pylori* was sensitive to oxidative stress conditions and to DNA-damaging agents and was unable to establish a long-term infection in a mouse model (25). Notably, many genes coding for the components of DNA recombinational repair that are involved in the presynapsis stage, such as RecBCD, RecF, RecO, and RecQ, are missing in the *H. pylori* genome. Thus, *H. pylori* likely has a unique mechanism for DNA recombinational repair, particularly for the presynapsis stage.

In this study, we characterized a *recN* mutant of *H. pylori*. We showed that this *H. pylori recN* mutant is much more sensitive to various types of DNA-damaging agents than the wild type, indicating that RecN plays an important role in DNA repair in *H. pylori*. Loss of RecN function results in a significant decrease in the DNA recombination frequency, suggesting that the DNA repair performed by RecN involves homologous recombination. We demonstrated that under oxidative stress conditions *H. pylori recN* mutant cells accumulate much more fragmented DNA than wild-type cells, providing direct evidence that RecN functions in DNA recombinational repair. Finally, an *H. pylori recN* mutant clearly displays an attenuated ability to colonize mouse stomachs, highlighting the importance of recombinational DNA repair in survival of *H. pylori* within its host.

MATERIALS AND METHODS

***H. pylori* culture conditions.** *H. pylori* was cultured on brucella agar (Difco) plates supplemented with 10% defibrinated sheep blood or 5% fetal bovine serum (BA plates). Chloramphenicol (50 µg/ml) or kanamycin (40 µg/ml) was added to the medium used for mutants. Cultures of *H. pylori* were grown microaerobically at 37°C in an incubator using continuously controlled levels of oxygen (5% [partial pressure] O₂, 5% CO₂, 90% N₂).

Construction of *H. pylori recN* mutant. A 1.76-kb fragment containing the *H. pylori recN* gene was amplified by PCR from genomic DNA of strain 26695 using primers *recNF* (5'-ACGACCACCAAGCTCTTAC-3') and *recNR* (5'-TAGACGCGTTCAAGCGTTT-3'). The PCR product was cloned into the pGEM-T vector to generate pGEM-*recN*. Subsequently, a chloramphenicol acetyltransferase cassette was inserted into the *recN* sequence of pGEM-*recN* at the Eco47III sites. The disrupted *recN* gene was then introduced into *H. pylori*

wild-type strains by natural transformation via allelic exchange, and chloramphenicol-resistant colonies were isolated. Disruption of the gene in the genome of the mutant strain was confirmed by PCR that showed that there was an increase in the expected size of the PCR product and by direct sequencing of the PCR fragment.

Construction of *H. pylori recN* complementation strain. A 2.76-kb fragment covering the HP1394-*recN* sequence and the upstream promoter sequence was amplified by PCR from genomic DNA of strain 26695 using primers *recNCF* (5'-TTGACAGAAACAACCAAGC-3') and *recNR* (5'-TAGACGCGTTCAAGCGTTT-3'). The PCR product was cloned into the pGEM-T vector to generate pGEM-*recN*⁺. Subsequently, a kanamycin resistance cassette (Kan) was inserted behind the *recN* gene in the plasmid, yielding pGEM-*recN*⁺-Kan. The insert portion of this recombinant plasmid was then excised and ligated into plasmid pEU39, yielding pEU-*recN*⁺-Kan. Plasmid pEU39 (from TIGR) contains a 2.04-kb fragment of *H. pylori* genomic DNA covering the HP0405 open reading frame. Previously, our laboratory showed that disruption of HP0405 in *H. pylori* resulted in no obvious phenotype. In plasmid pEU-*recN*⁺-Kan, HP0405 was disrupted into two pieces flanking *recN*⁺-Kan. When this plasmid was used to transform *H. pylori* X47 *recN*:Cm (with selection for Cm^r Kan^r), the intact *recN* gene (together with HP1394 and its promoter) and the Kan cassette were inserted into the genome at the HP0405 locus. This produced a merodiploid strain, X47 *recN*:Cm-*recN*⁺-Kan, which contains the original interrupted *recN* gene and an intact copy of *recN* at an unrelated site.

Assessment of the susceptibility to mitomycin C. *H. pylori* strains were grown on BA plates to late log phase, and the cells were suspended in phosphate-buffered saline (PBS) at a concentration of ~10⁸ cells/ml. The cell suspensions were treated with 0, 50, 100, or 200 ng/ml mitomycin C for 20 min. The samples were serially diluted and spread on BA plates. After 4 days of incubation in a microaerobic atmosphere (5% [partial pressure] O₂) at 37°C, colonies were counted.

Air survival assay. *H. pylori* strains were grown on BA plates to late log phase, and the cells were suspended in PBS at a concentration of ~10⁸ cells/ml. The cell suspensions were incubated at 37°C under normal atmospheric conditions (21% O₂) with moderate shaking. Samples were then removed at various time points (2, 4, 6, 8, and 10 h), serially diluted, and spread onto BA plates. Colony counts were recorded after 4 days of incubation in a microaerobic atmosphere (5% [partial pressure] O₂) at 37°C.

Assessment of sensitivity to low-pH conditions. *H. pylori* strains were grown on BA plates to late log phase, and the cells were suspended in buffer (20 mM Tris-HCl, 150 mM NaCl) at different pH values (pH 7.0, 5.0, or 3.0) at a concentration of ~10⁸ cells/ml. The cell suspensions were incubated under microaerobic conditions (5% O₂) at 37°C for 1 h. The samples were serially diluted and plated to determine CFU counts (after 4 days of incubation under microaerobic growth conditions). The percentage of cell survival at pH 5.0 or 3.0 relative to that in pH 7.0 was calculated.

DNA transformation assay to assess recombination frequency. The donor DNA used in this study included (i) a 330-bp PCR fragment of the *H. pylori rpoB* gene containing a site-specific mutation (at the center of the fragment) conferring rifampin resistance, (ii) a linear DNA fragment containing a kanamycin resistance cassette (1.4 kb) flanked by *H. pylori acnB* gene sequences (about 550 bp on each side of the Kan cassette), and (iii) shuttle plasmid pHPI.

H. pylori strains were grown on BA plates to late log phase, and the cells were suspended in PBS at a concentration of ~10⁸ cells/ml (recipient cells for transformation). Thirty microliters of the suspension was mixed with 100 ng of donor DNA and spotted onto a BA plate. After incubation for 18 h under microaerobic conditions at 37°C, the transformation mixture was harvested and suspended in 1 ml PBS. Portions (100 µl) of the suspension (or appropriate dilution) were plated onto either BA plates or BA plates containing a selective antibiotic (20 µg/ml rifampin or 40 µg/ml kanamycin, depending on the donor DNA used). The plates were incubated for 4 days under microaerobic conditions at 37°C, and the numbers of colonies were counted. The transformation frequency was determined by dividing the number of resistant colonies by the total number of CFU. In a normalized DNA transformation assay, the frequency of transformation was expressed as the number of transformants per 10⁸ recipient cells. As negative controls, *H. pylori* strains with no DNA added were tested under the same assay conditions; no antibiotic-resistant colonies were observed.

DNA fragmentation analysis by electrophoresis. The wild-type or *recN* mutant cells were grown on BA plates under microaerobic conditions (5% O₂) to mid-log phase (for about 1 day). To elicit DNA damage, the plates were exposed to atmospheric conditions (air, 21% O₂) for 6 h. The cells on the plates were either directly used for DNA fragmentation analysis or incubated further under the microaerobic conditions for 4 h, followed by DNA fragmentation analysis.

FIG. 1. Organization of the *recN* gene and surrounding genes in the *H. pylori* genome and DNA constructs for generating the *recN* mutant. HP1394 encodes a hypothetical protein, and HP1392 encodes a fibronectin/fibrinogen binding protein. The gray bar in the N-terminal portion of *recN* represents the SMC domain encompassing the internal ATP/GTP binding site. A fragment containing the *recN* gene (1,760 bp) was PCR amplified using the *recNF/recNR* primer pair and cloned into pGEM-T to obtain pGEM-*recN*. A chloramphenicol resistance cassette (CAT) (800 bp) was then inserted within the *recN* fragment at the *Eco47III* sites, and plasmid pGEM-*recN*:CAT was used for transformation of wild-type *H. pylori* to create the *recN* mutant.

The cells grown continuously under the microaerobic conditions (i.e., without exposure to air) were used as controls.

Analysis of DNA fragmentation was performed as described by Zirkle and Krieg (53), with the following minor modifications. Cells were suspended in PBS to an optical density at 600 nm of 0.5. Then 500 μ l of the sample was centrifuged for 1 min at 15,000 $\times$ g, the pellet was washed in Tris-EDTA buffer (50 mM Tris HCl, 5 mM EDTA; pH 8) at 4°C, and the final pellet was suspended in 10 μ l of Tris-EDTA buffer. The resulting suspension was then added to 50 μ l of 1% low-melting-point agarose at 37°C. The agarose and cells were mixed thoroughly, and 60- μ l blocks were made by pipetting the mixture onto Parafilm. After solidification, the blocks were placed in a lysing solution (0.25 mM EDTA, 0.5% Sarkosyl, 0.5 mg/ml proteinase K) and incubated at 55°C for 1 h, followed by overnight incubation at room temperature. The next day, the blocks were washed three times (10 min each) in cold Tris-EDTA buffer. Agarose plugs were then submerged in a 0.8% agarose gel. Samples were subjected to gel electrophoresis for 7 h at 30 V. Gels were then stained for 30 min with ethidium bromide (0.5 μ g/ml), destained in H₂O, and then visualized under UV light.

Mouse colonization. Mouse colonization assays were performed essentially as described previously (49, 51). Briefly, wild-type strain X47 or X47 *recN* mutant cells were harvested after 48 h of growth on BA plates (37°C, 5% oxygen) and suspended in PBS to an optical density at 600 nm of 1.7. The headspace in each tube was sparged with argon gas to minimize oxygen exposure, and the tube was tightly sealed. The bacterial suspensions were administered to C57BL/6J mice (3×10^8 *H. pylori* cells/mouse) twice, using the oral deliveries made 2 days apart. Three weeks after the first inoculation, the mice were sacrificed, and the stomachs were removed, weighed, and homogenized in argon-sparged PBS (40) to avoid O₂ exposure. Stomach homogenate dilutions were plated on BA plates supplemented with bacitracin (100 μ g/ml), vancomycin (10 μ g/ml), and amphotericin B (10 μ g/ml), and the plates were rapidly placed in an incubator containing O₂ at a sustained partial pressure of 5%. After incubation for 5 to 7 days, the fresh *H. pylori* colonies were enumerated and the data were expressed as CFU per gram of stomach.

RESULTS

Bioinformatic analysis of *H. pylori* RecN homolog and construction of a *recN* mutant. In the published *H. pylori* genome sequence (48), HP1393 was annotated as a *recN* gene homolog. Upstream of the *recN* gene is an open reading frame (HP1394) coding for a hypothetical protein, and downstream is a gene (HP1392) encoding a fibronectin/fibrinogen binding protein that could be involved in biosynthesis and degradation of cell surface polysaccharides and lipopolysaccharides (Fig. 1). To study the physiological role of RecN, we constructed a *recN* mutant by inserting a chloramphenicol resistance cassette at

FIG. 2. Sensitivity of *H. pylori* wild-type strain X47 (◆) and its isogenic *recN* mutant (■) to mitomycin C. *H. pylori* cell suspensions in PBS were treated with different concentrations of mitomycin C (indicated on the x axis) for 20 min. The numbers of surviving CFU were determined after 4 days of incubation under microaerobic conditions. The data are the means $\pm$ standard deviations of three experiments.

the *Eco47III* restriction sites in the *recN* gene. This cassette replaced the 15-bp fragment between the two *Eco47III* sites (Fig. 1). Correct insertion of the cassette within the *recN* gene in the *H. pylori* genome was confirmed by PCR, which showed the expected increase in the size of the PCR product (data not shown). *recN* mutants were constructed for several different strains, such as 26695, J99, and X47. As X47 is a strain well adapted for the mouse colonization assay, most of the in vitro assays and all of the in vivo assays reported here were done with X47 and its isogenic *recN* mutant.

The gene product of *H. pylori* *recN* (1,575 bp) displays 22% amino acid identity (42% similarity) with the homolog in *E. coli*. *E. coli* RecN is strongly induced by DNA damage (SOS response) and has been implicated in repair of DSBs (12, 32). In the N-terminal portion of the RecN sequence there is a conserved domain related to the SMC (structural maintenance of chromosome) family proteins that are involved in chromosome scaffolding and partitioning (15). An ATP/GTP binding site motif is present in the SMC domain of the RecN protein.

***H. pylori* *recN* mutant is highly sensitive to DNA-damaging agents.** To investigate the role of RecN in DNA repair, we examined the sensitivity of the *recN* mutant to various types of DNA-damaging agents. Mitomycin C causes predominantly DNA strand cross-links, leading ultimately to DNA DSBs, which require DNA recombination for repair. We determined the mitomycin C sensitivities of the wild-type X47 strain and the isogenic *recN* mutant. The results (Fig. 2) indicated that the *recN* mutant is much more sensitive to mitomycin C than the wild-type strain. For example, after treatment with 100 ng/ml mitomycin C for 20 min, the viability of the wild type was 5 orders of magnitude greater than that of the *recN* mutant. The same experiment was also performed with *H. pylori* strains 26695 and J99 and their isogenic *recN* mutant strains. The results were similar to those obtained for the X47 strain and its *recN* mutant (data not shown).

Oxidative stress is a major stress condition that *H. pylori* encounters in its physiological niche. To examine the sensitivity of *H. pylori* strains to oxidative stress, we performed an air survival assay, and the results (i.e., survival curves) are shown in Fig. 3. After exposure of approximately 10^8 cells to air for

FIG. 3. Survival of *H. pylori* wild-type strain X47 (◆) and its isogenic *recN* mutant (■) upon exposure to air. *H. pylori* cell suspensions in PBS were incubated at 37°C under normal atmospheric conditions (21% [partial pressure] oxygen). Samples were removed at the times indicated on the x axis and were used for plate counting in a 5% oxygen environment. The data are the means $\pm$ standard deviations of three experiments. Based on a statistical analysis (Student *t* test), the differences in cell survival between the wild type and the *recN* strain are significant ($P < 0.01$) for all the time points except the 2-h time point.

10 h, about 10^6 viable cells of the wild-type strain could be recovered. In contrast, all of the *recN* mutant cells were killed (no viable cells were recovered) by exposure to air for 8 h. As an additional control for the assay, cell suspensions in PBS were incubated under microaerobic conditions (5% O₂, 5% CO₂, and 90% N₂, with occasional shaking). Under these conditions, no significant difference in cell survival between the wild-type and *recN* strains was observed at any time point (not shown). The results clearly showed that the *recN* mutant is significantly more sensitive to atmospheric oxygen than the wild type.

Low pH is another stress condition that *H. pylori* encounters in its physiological niche. The sensitivity of the *recN* mutant to low-pH conditions was characterized. The wild-type *H. pylori* and *recN* mutant cells were treated for 1 h by suspending them in buffer at different pH values (pH 7.0, 5.0, and 3.0), and the survival was determined. As shown in Table 1, treatment at pH 5.0 for 1 h did not have a significant effect on the survival of the wild-type cells, while the same treatment killed approximately one-half of the *recN* mutant cells. About 40% of the wild-type *H. pylori* cells survived the pH 3.0 treatment for 1 h, whereas all of the *recN* mutant cells were killed by the same treatment.

***H. pylori recN* mutant has a reduced frequency of DNA recombination.** The results obtained for the sensitivity to DNA-damaging agents described above strongly suggest that

TABLE 1. Acid sensitivity of *H. pylori* wild-type strain X47 and its isogenic *recN* mutant

Strain	% Survival at ^a :		
	pH 7.0	pH 5.0	pH 3.0
X47	100	96 $\pm$ 6	41 $\pm$ 4
X47 <i>recN</i> :Cm	100	53 $\pm$ 7	0

^a The values for pH 5.0 and 3.0 are the percentages of cell survival after treatment for 1 h at pH 5.0 or 3.0 relative to the survival at pH 7.0. The data are the means $\pm$ standard errors from three independent determinations.

TABLE 2. Transformation frequencies with different types of donor DNA

<i>H. pylori</i> strain	Transformation frequency with the following donor DNA ^a :		
	<i>rpoB3</i> (330 bp)	<i>acnB</i> :Kan (2.5 kb)	Plasmid pHP1
X47	46,800 $\pm$ 2,300	630 $\pm$ 70	240 $\pm$ 31
X47 <i>recN</i> :Cm	4,340 $\pm$ 580	12 $\pm$ 1	210 $\pm$ 27
X47 <i>recN</i> :Cm- <i>recN</i> ⁺ -Kan	43,700 $\pm$ 2,100	ND ^b	ND

^a The transformation frequencies are expressed as the number of transformants (resistant colonies) per 10^8 recipient cells. The data are the means $\pm$ standard errors from three independent determinations.

^b ND, not determined (as the complemented strain had a Kan^r marker, the transformation frequency with *acnB*:Kan or pHP1 could not be determined).

RecN is involved in *H. pylori* DNA repair. To investigate if this repair function is due to a DNA recombination process, we tested whether loss of RecN function had an adverse effect on DNA recombination. The frequency of DNA recombination can be experimentally determined by DNA transformation. Natural transformation of *H. pylori* involves uptake of DNA into the cell, followed by recombination into the genome at the site of the homologous sequence (allelic exchange). Assuming that the wild-type *H. pylori* strain and its isogenic *recN* mutant strain are equally competent for DNA uptake, the frequency of natural transformation is an indicator of DNA recombination frequency.

We used two different types of DNA to examine DNA transformation of *H. pylori*. A specific A-to-G mutation in the *H. pylori rpoB* gene (*rpoB3* allele) confers rifampin resistance (52). A 330-bp PCR fragment containing this specific mutation at the center of the fragment was used to transform *H. pylori* strains by using rifampin resistance as a selective marker. The other type of DNA used for transformation was the sequence of the *H. pylori acnB* gene (a 1.1-kb housekeeping gene), in which a 1.4-kb kanamycin resistance cassette (Kan) was inserted at the center (*acnB*:Kan). A plasmid that did not contain any *H. pylori* gene sequence (pHP1) was used as a control, as transformation with this plasmid did not involve DNA recombination (the plasmid remained extrachromosomal).

The results of the transformation analysis are shown in Table 2. First, we validated the use of the small DNA fragment of the *rpoB3* allele for transformation to distinguish it from spontaneous mutations that lead to rifampin resistance. The spontaneous Rif^r mutation frequency in *H. pylori* is 10^{-8} (52), whereas the transformation frequency using the *rpoB3* fragment was 10^{-4} . Under the transformation assay conditions used in this study, no single spontaneous Rif^r colony (i.e., no DNA added) was observed. Compared to the transformation frequency for the wild-type X47 strain (4.68×10^{-4}), the transformation frequency for the *recN* mutant was 10-fold lower (4.34×10^{-5}). Using *acnB*:Kan as donor DNA, wild-type *H. pylori* had a transformation frequency of 630×10^{-8} . In contrast, the transformation frequency for the *recN* mutant was only 12×10^{-8} , which is 50-fold lower than that of the wild type. For both types of DNA donor (*rpoB3* and *acnB*:Kan), the *recN* strain transformation frequencies were significantly lower than those of the wild type, according to a Student *t* test ($P < 0.01$). In the control experiments using plasmid pHP1 as the

FIG. 4. Agarose gel electrophoresis showing genomic DNA fragmentation in cells of *H. pylori* X47 (WT) and its isogenic *recN* mutant. Lanes 1 and 2, cells not exposed to air; lanes 3 and 4, cells exposed to air for 6 h; lanes 5 and 6, cells exposed to air for 6 h and then incubated under microaerobic conditions for 4 h. Fragmented DNA was visualized as smears on the agarose gel. The sizes of DNA standards are indicated on the right. The experiments were repeated three times with similar results.

donor DNA, there was no significant difference in the transformation frequency between the wild type and the *recN* mutant. The transformation assay was also performed once with *H. pylori* strain 26695 and its isogenic *recN* strain; the results were similar to those obtained with the X47 strain and its *recN* mutant (data not shown). These results indicate that RecN plays a significant role in the DNA recombination process in *H. pylori*.

***H. pylori* *recN* mutant cells under oxidative stress contain more fragmented DNA.** To further investigate the role of RecN in DNA repair, we examined the level of fragmentation of genomic DNA in the *recN* mutant cells compared to that in the wild-type cells (Fig. 4). *H. pylori* cells were grown on BA plates under the microaerobic conditions (5% O₂) to mid-log phase (for about 1 day). When the genomic DNA was examined at this time point, both the wild-type and *recN* mutant strains appeared to contain intact genomic DNA (Fig. 4, lanes 1 and 2). This indicated that there is no significant DNA damage in *H. pylori* cells in vitro under optimal or near-optimal growth conditions. Next, we subjected cells to oxidative stress by exposing them to atmospheric conditions (21% O₂) for 6 h. Based on the results shown in Fig. 3, this treatment should have resulted in losses of viability of about 1 order of magnitude for the wild-type strain and 6 orders of magnitude for the *recN* mutant. After this treatment, the cells were either directly analyzed by the DNA fragmentation assay or incubated further under the microaerobic conditions for 4 h (to allow DNA repair), which was followed by a DNA fragmentation analysis. In each case, the wild-type *H. pylori* cells contained a small amount of fragmented DNA (Fig. 4, lanes 3 and 5). In contrast, the *recN* mutant cells contained significantly larger amounts of fragmented DNA; this was shown by large smears (size range, 1 to 10 kb) on the agarose gel (lanes 4 and 6). Using densitometric scanning, the intensity of DNA fragments whose sizes

FIG. 5. Mouse colonization assays with *H. pylori* X47 and its isogenic *recN* mutant. The mice were inoculated with *H. pylori* two times (2 days apart) using a dose of 1.5×10^8 viable cells. The colonization of *H. pylori* in mouse stomachs was examined 3 weeks after the first inoculation. The data are presented as scatter plots of the number of CFU per gram of stomach as determined by plate counting. Each symbol indicates the CFU count for one stomach. The baseline (2.7 log₁₀ CFU/g) is the detection limit of the assay, which represents a value less than 500 CFU/g of stomach. Panels A and B show the results for two independent experiments. WT, wild type.

ranged from 1 to 10 kb in the *recN* cells was determined to be ninefold higher than the intensity in the wild-type cells.

***H. pylori* *recN* mutant displays an attenuated ability to colonize the mouse stomach.** To determine whether loss of RecN activity has a significant effect on *H. pylori* colonization of the host, the relative abilities of the wild-type X47 and X47:*recN* mutant strains to colonize the mouse stomach were compared (Fig. 5). In one experiment, each strain was injected into nine C57BL/6J mice, and the colonization of *H. pylori* cells in the mouse stomachs was examined 3 weeks after inoculation (Fig. 5A). *H. pylori* was recovered from all nine mice that had been inoculated with the wild-type strain, and the average level was 1.06×10^6 CFU/g stomach. In contrast, the average number of *H. pylori* cells recovered from the mice that were inoculated with the *recN* mutant strain was 1.1×10^5 CFU/g stomach (Fig. 5A). According to a Wilcoxon rank test analysis, the lower colonization values for the mutant strain were significantly less than the colonization values for the wild type at the 99% confidence level ($P < 0.01$). The entire mouse colonization assay was performed an additional time, and a similar difference between the wild type and the mutant strain was observed

TABLE 3. Assessment of mouse colonization at 3 or 5 weeks after inoculation with *H. pylori* wild-type strain X47, the *recN* mutant, or the complemented strain

<i>H. pylori</i> strain	No. of <i>H. pylori</i> cells (10 ⁵ CFU/g of stomach) at ^a :	
	3 wk	5 wk
X47	11.3 ± 4.3	10.4 ± 4.1
X47 <i>recN</i> :Cm	1.78 ± 0.8	1.44 ± 0.7
X47 <i>recN</i> :Cm- <i>recN</i> ⁺ -Kan	10.2 ± 3.5	9.80 ± 3.7

^a The data are the mean numbers of *H. pylori* cells per gram of mouse stomach ± standard errors for groups of six mice.

(Fig. 5B). These results suggest that RecN plays a significant role in survival and colonization in the host.

Complementation of the *recN* strain. To ensure that the phenotypes (both in vitro and in vivo) observed for the *recN* strain were completely attributed to inactivation of *recN*, we introduced a functional copy of the *recN* gene back into the *recN* strain. Strain X47 *recN*:Cm-*recN*⁺-Kan contains a mutated *recN* gene at the original locus and an intact *recN* gene at an unrelated site (see Materials and Methods). The sensitivity of this strain to DNA-damaging agents was shown to be similar to that of the wild type. For example, both the wild type and the complemented strain could survive treatment with 200 ng/ml mitomycin C for 20 min or exposure to air for 10 h (data not shown), while all cells of the *recN* mutant strain were killed by these treatments (Fig. 2 and 3). Complementation of the *recN* function also restored the transformation frequency to the wild-type level, as determined by using the *rpoB3* fragment as the DNA donor (Table 2).

The complemented strain was also examined to determine its mouse colonization ability. In an additional mouse colonization experiment, groups of mice (12 animals for each strain) were inoculated with wild-type strain X47, with the *recN* mutant, or with the complemented strain. Six mice from each group were sacrificed after 3 weeks, and the other six mice were sacrificed after 5 weeks. The viable counts of the strains recovered from the mouse stomachs at each time point were determined (Table 3). Consistent with the previous results (Fig. 5), the average level of the wild-type strain was about 1×10^6 CFU per g of stomach, and similar levels were detected at 3 and 5 weeks postinoculation. The average levels of the *recN* mutant strain at both time points (3 and 5 weeks postinoculation) were significantly lower than those of the wild type. In contrast, the average levels of the complemented strain at both time points were similar to those of the wild type (Table 3). This indicates that complementation of the *recN* strain restored its ability to colonize mouse stomachs.

DISCUSSION

In its physiological environment, *H. pylori* induces strong host inflammatory responses that involve recruitment of neutrophils, lymphocytes, and macrophages; these cells release free radicals that damage DNA. Although *H. pylori* is equipped with a variety of enzymes that detoxify reactive oxygen species, these enzymes are not sufficient to avoid damage to the genomic DNA. Indeed, the DNA of the pathogen was shown to be a target for host-generated oxidative stress based on

studies of *H. pylori nth* strains that are unable to repair oxidized pyrimidines (31). Thus, a highly competent DNA repair system is especially important for survival of a pathogen like *H. pylori* in its host. However, published *H. pylori* genome sequences have revealed that a limited number of genes are involved in DNA repair. Interestingly, some major components of the DNA recombination machinery, such as RecA and RuvC, have been shown to be important for *H. pylori* survival (25, 47).

DNA DSBs pose a major threat to the structural and functional integrity of the genome. Homologous DNA recombination is a key mechanism used to repair DNA DSBs and to prevent replication fork collapse. Repair of DSBs in eukaryotic cells involves the orchestrated recruitment of various repair proteins to the sites of DSBs (5). Two members of this repair complex belong to the SMC family of proteins, which are key players in a variety of chromosome dynamics, from chromosome condensation and cohesion to transcriptional repression and DNA repair (16). SMC family proteins have a structural characteristic, an extensive coiled-coil domain located between globular domains at the N and C termini, that brings together Walker A and B motifs associated with ATP binding (15). Bacterial RecN protein is related to this family. *E. coli* RecN is strongly induced during the SOS response and was shown to be involved in RecA-mediated recombinational repair of DSBs (27). In *B. subtilis*, RecN was shown to be recruited to DSBs at an early time point during repair (21, 22, 37). In vitro, RecN was shown to bind and protect 3' single-stranded DNA ends in the presence of ATP (36).

In this study, we showed that an *H. pylori recN* mutant is much more sensitive to mitomycin C (Fig. 2), an agent that predominantly causes DNA DSBs, indicating that RecN plays an important role in DSB repair in *H. pylori*. Under physiological conditions, DSBs often result from exposure of cells to oxidative agents (6). The *H. pylori recN* mutant is highly sensitive to exposure to atmospheric oxygen (Fig. 3), as well as to other forms of oxidative stress (such as hydrogen peroxide and paraquat [data not shown]). The results indicate that RecN confers protection against oxidative damage in *H. pylori*. Similar roles of RecN in protection against oxidative damage have recently been demonstrated for *N. gonorrhoeae* (42, 43). Our results also showed that an *H. pylori recN* mutant is much more sensitive to low pH than the wild-type strain, suggesting that RecN is also involved in repair of acid-induced DNA damage. This could be relevant to the organisms's physiological condition, as *H. pylori* appears to colonize an acidic niche on the gastric surface (39).

Loss of *H. pylori* RecN function results in a significant decrease in the DNA recombination frequency, suggesting that RecN is a critical factor in DNA recombinational repair. Similarly, mutant strains in which the major components of DNA recombination machinery were disrupted (*recA*, *ruvC*) had greatly decreased DNA recombination frequencies (13, 25). In contrast, loss of UvrD or MutS in *H. pylori* resulted in an increase in DNA recombination frequency in *H. pylori* (19, 33). Suppression of DNA recombination by UvrD or MutS may play a role in maintaining genome integrity. Recombinational repair could be mutagenic due to homeologous recombination or could cause rearrangement due to recombination with direct repeat sequences (see below). In addition, recombinational repair systems are much more complex and require

more energy to operate than nucleotide excision repair (NER) and base excision repair (BER) systems. Thus, UvrD, a component of an NER system, and MutS, a likely component of a BER (8-oxoG glycosylase) system (49), both suppress DNA recombination. Both NER and BER systems would be expected to continuously function in low-stress conditions. Under severe-stress conditions when large amounts of DSBs are formed, RecN perhaps recognizes DSBs and recruits RecA, facilitating recombinational repair.

H. pylori displays exceptional genetic variability and intraspecies diversity (45). Allelic diversity is obvious as almost each (unrelated) isolate of *H. pylori* has a unique sequence when a fragment of only several hundred base pairs of either housekeeping or virulence genes is compared among strains (10, 20, 46). A key mechanism for generating diversity is thought to be DNA recombination (23). In vivo, there appears to be continuous DNA damage from oxidative and acid stress in *H. pylori* cells. Some of this damage could result in DNA DSBs and replication fork collapse that require repair via DNA recombination processes. We propose that the requirement for recombinational DNA repair could be a driving force contributing to the genetic diversity of *H. pylori*. *H. pylori* is naturally competent for uptake of exogenous DNA, and a special apparatus homologous to type IV secretion systems (*comB* locus) has a dedicated role in DNA uptake (17). Unlike several other bacterial species, *H. pylori* does not seem to require specific DNA sequences for uptake of related DNA (38). Thus, small DNA segments could be taken up from neighboring cells of the same strain (homologous recombination) or from cocolonizing strains (homeologous recombination) and then used to repair damaged genes by recombination. Using mathematical modeling approaches with sequence data from 24 pairs of sequential *H. pylori* isolates, Falush et al. (9) estimated that the mean size of imported fragments was only 417 bp, which is much shorter than the sizes observed for other bacteria. In this study, we used a 330-bp DNA fragment (*rpoB3*) to assess the transformation frequency. The wild-type frequency of DNA recombination was 4 orders of magnitude higher than the spontaneous Rif^r mutation frequency (10^{-4} versus 10^{-8}). Therefore, DNA recombination provides a mechanism to generate new genotypes much more rapidly than mutation alone can provide (2, 14).

In normal laboratory growth conditions, an *H. pylori* *recN* mutant does not show a growth defect, but its survival is greatly reduced under oxidative stress conditions. The latter conditions most likely closely resemble the in vivo stress conditions, at least in later stages of infection. Consistent with this idea, very little fragmented DNA was observed in either strain (wild type or *recN* mutant) when cells were cultured under normal microaerobic conditions. However, after oxidative stress treatment, a significantly higher proportion of the DNA was fragmented in the *recN* mutant cells than in the wild-type cells (Fig. 4). This strain comparison provided evidence that RecN functions in DNA recombinational repair. For the DNA fragmentation assays, we tested two oxidative stress exposure conditions: (i) exposure to air for 6 h, followed by a DNA fragmentation assay; and (ii) further incubation (after exposure to air for 6 h) under microaerobic conditions for 4 h (allowing time for DNA repair), followed by DNA fragmentation analysis. The same results were obtained for the two

conditions, indicating that recombinational repair was functional during the exposure to air.

Repair of damaged DNA is known to be important for bacterial survival and pathogenesis (3, 28, 44). For *H. pylori*, it was documented that loss of DNA repair functions, such as EndoIII, RuvC, MutS, or MutY, resulted in a reduced ability to colonize the host stomach (8, 25, 31, 49). Our data demonstrated that *H. pylori* cells with *recN* disrupted were less able to colonize hosts than wild-type cells (Fig. 5). These results suggested that there were significant DSBs in the *H. pylori* DNA in the host environment and that the *recN* mutant strain was unable to efficiently repair them. Another interpretation is that DNA recombination may provide an alternative mechanism for gene regulation (e.g., immune modulation) that is essential for the long-term survival of *H. pylori* within the stomach (25). For example, while the wild-type *H. pylori* strain elicited a T helper 2 response in mice and established a persistent infection, a *ruvC* mutant elicited a T helper 1-mediated immune response that was more effective in eradicating *H. pylori* (34). Thus, an *H. pylori* *ruvC* mutant displayed decreased colonization ability in a relatively short time period (within 3 weeks) after infection, and this strain was completely eliminated from the murine gastric mucosa over a longer period (5 weeks after inoculation) (25). In contrast to RuvC, which is a major component of the DNA recombination machinery, RecN is a protein specific for repair of DSBs by linking DSB recognition and DNA recombination initiation. In our mouse colonization assay, the *recN* mutant cells were not completely eradicated 5 weeks after infection, although the colonization level of this strain was significantly lower than that of the wild-type strain (Table 3). We therefore propose that the attenuated ability of the *recN* mutant to colonize mouse stomachs was mainly due to its failure to repair DSBs through a DNA recombinational repair pathway.

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