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The RecRO pathway of DNA recombinational repair in *Helicobacter pylori* and its role in bacterial survival in the host

Ge Wang, **Leja F. Lo**, and **Robert J. Maier***
Department of Microbiology, University of Georgia, Athens, Georgia 30602

Abstract

Two pathways for DNA recombination, AddAB (RecBCD-like) and RecRO, were identified in *Helicobacter pylori*, a pathogenic bacterium that colonizes human stomachs resulting in a series of gastric diseases. In this study, we examined the physiological roles of *H. pylori* RecRO pathway in DNA recombinational repair. We characterized *H. pylori* single mutants in *recR* and in *recO*, genes in the putative gap repair recombination pathway, and an *addA recO* double mutant that is thus deficient in both pathways that initiate DNA recombinational repair. The *recR* or *recO* single mutants showed the same level of sensitivity to mitomycin C as the parent strain, suggesting that the RecRO pathway is not responsible for the repair of DNA double strand breaks. However, *H. pylori recR* and *recO* mutants are highly sensitive to oxidative stress and separately to acid stress, two major stress conditions that *H. pylori* encounters in its physiological niche. The complementation of the *recR* mutant restored the sensitivity to oxidative and acid stress to the wild type level. By measuring DNA transformation frequencies, the *recR* and *recO* single mutants were shown to have no effect on inter-genomic recombination, whereas the *addA recO* double mutant had a greatly (~12-fold) reduced transformation frequency. On the other hand, the RecRO pathway was shown to play a significant role in intra-genomic recombination with direct repeat sequences. Whereas the *recA* strain had a deletion frequency 35-fold lower than that of background level, inactivation of *recR* resulted in a 4-fold decrease in deletion frequency. In a mouse infection model, the three mutant strains displayed a greatly reduced ability to colonize the host stomachs. The geometric means of colonization number for the wild type, *recR*, *recO*, and *addA recO* strains were 6×10^5, 1.6×10^4, 1.4×10^4 and 4×10^3 CFU/g stomach, respectively. *H. pylori* RecRO-mediated DNA recombinational repair (intra-genomic recombination) is thus involved in repairing DNA damage induced by oxidative and acid stresses and plays an important role in bacterial survival and persistent colonization in the host.

Keywords

Helicobacter pylori; Gastric pathogen; Oxidative stress; DNA repair; RecRO pathway; Intra-genomic recombination

*Corresponding author: Robert J. Maier, Department of Microbiology, 815 Biological Sciences Building, University of Georgia, Athens, GA 30602, rmaier@uga.edu, Tel: 706 542 2323, Fax: 706 542 2674.

1. Introduction

DNA recombinational repair is crucial for maintaining genomic integrity by repairing DNA double strand (ds) breaks and stalled replication forks [1]. DNA recombinational repair requires a large number of proteins that act at various stages of the process [2]. The central step in DNA recombination is the DNA strand exchange (synapsis) mediated by the nucleoprotein filament formed by RecA on single stranded (ss) DNA [3]. In the pre-synapsis stage, complex systems (pathways) are required for DNA damage recognition, processing of DNA substrate and recruiting of RecA. In the well-studied bacterial model system *Escherichia coli*, RecFOR and RecBCD pathways are required to initiate homologous DNA recombination [3,4]. Whereas the RecBCD pathway is responsible for dsDNA break repair, the RecFOR pathway is mainly used for ssDNA gap repair. The RecFOR pathway can also repair dsDNA breaks when the RecBCD pathway is inactivated. The RecF, RecO and RecR proteins act together to promote loading of RecA onto ssDNA [5].

Helicobacter pylori colonizes the stomachs of about half the human population and is implicated in the etiology of gastric ulcers and cancer [6]. In its physiological environment, *H. pylori* frequently suffers both oxidative and acid stress, leading to DNA damage [7–10]. Therefore, DNA recombinational repair plays an important role in *H. pylori*'s survival and persistence in the host [11,12]. It is also well known that *H. pylori* exhibits a high frequency of DNA recombination [13] which plays a critical role in generating genetic diversity by allowing genomic DNA rearrangements and integration of exogenous DNA into the genome through transformation [14].

Although a limited number of genes in the *H. pylori* genome were predicted to be involved in DNA recombinational repair [15–17], recent studies revealed the existence of both pathways for DNA recombinational repair in *H. pylori*. An AddAB class of helicase-nuclease enzymes, related to the *E. coli* RecBCD enzyme, was shown to be functional in *H. pylori* [18,19], and the RecB-like helicase (now termed AddA) was also characterized in other studies [20,21]. Although RecF historically served as a reference for RecFOR pathway, it is absent from genomes of many bacteria including *H. pylori* [4]. By bioinformatics analysis, Marsin et al [20] identified a novel RecO orthologue, suggesting the presence of the RecRO pathway in *H. pylori*. Recent studies in *E. coli* indicated that RecOR in the absence of RecF can perform recombination by loading RecA[22,23]. Whereas the RecO protein can displace ssDNA-binding protein (SSB) and bind to ssDNA, RecR is the key component for loading RecA onto ssDNA [22,24].

In characterizing DNA recombination mutants of *H. pylori*, there were discrepancies in measuring DNA recombination rate from different studies using different DNA substrates [18,20,21,25,26]. While the RecRO is supposed to have a major role in intragenomic recombination at repeat sequences [27], the effect of *H. pylori* RecR and RecO on the intragenomic recombination reported by Marsin et al [20] was marginal (less than 2-fold difference compared to the wild type). Thus, the roles of RecRO pathway in *H. pylori* DNA repair and recombination remain to be clarified, and the relative contributions of the two pathways (RecRO and AddAB) in DNA recombinational repair in *H. pylori* are unknown. More importantly, the in vivo role of *H. pylori* RecRO as a DNA recombinational repair pathway remain to be determined. In this study we further examined the roles of RecR and RecO in *H. pylori* DNA repair and recombination using different types of DNA substrates. Furthermore, we examined the contribution of the RecRO pathway to bacterial survival in vivo by using a mouse infection model.

2. Materials and Methods

2.1. *H. pylori* strains and culture conditions

H. pylori strain 26695, 43504 or X47 was cultured on Brucella agar (Difco) plates supplemented with 10% defibrinated sheep blood or 4% fetal bovine serum (called BA plates). Cultures of *H. pylori* were grown microaerobically at 37°C in an incubator under continuously controlled levels of oxygen (5% partial pressure O_2, 5% CO_2, and the balance was N_2). For assessing the susceptibility to mitomycin C, *H. pylori* strains were grown on BA plates containing different concentration of mitomycin C under microaerobic conditions, and the growth was recorded after 2 days.

2.2. Construction of *H. pylori* isogenic mutants and complementation

To construct the *recR* mutant, a DNA fragment containing the *recR* gene was amplified by polymerase chain reaction (PCR) from genomic DNA using the primer pair listed in Table 1, and the PCR product was cloned into pGEM-T vector. Subsequently, a part of the target gene sequence in the recombinant plasmid was replaced by insertion of a chloramphenicol acetyl transferase (cat) cassette. The disrupted gene was then introduced into *H. pylori* wild type strain by natural transformation *via* allelic exchange and chloramphenicol (50 μg/ml) resistant colonies were isolated. The *recO* mutant was constructed by an overlapping PCR method in which the major portion of the gene was replaced by a kanamycin resistance cassette (aphA). The primers used for this procedure are listed in Table 1. The appropriate PCR product was used to transform the *H. pylori* wild type strain by selecting on kanamycin-containing (40 μg/ml) BA plates that were incubated at low O_2 (1% partial pressure) condition. Each resistance cassette (cat or aphA) contains its own promoter but lacks a transcription terminator and in all cases was inserted in the same direction of transcription as that of the native gene. After transformation, four to eight clones were selected and evaluated by PCR to confirm replacement of the wild type allele with the null allele, and a single clone was selected for use in further experiments. To construct the *recR recO* and *addA recO* double mutants, the PCR fragment of *recO::aphA* was used to transform *recR::cat* or *addA::cat* mutant, respectively.

The complemented *recR*$^+$ strain was constructed by inserting a wild-type copy of the *recR* gene in the *rdxA* locus of the *recR*::cat chromosome. PCR products corresponding to the 3′ end of the *rdxA* gene (266 bp), 810 bp of the full-length *recR* gene (including 186 bp of upstream sequence containing its promoter), and the 5′ end of the *rdxA* gene (256 bp) were amplified in three separate PCRs and then stitched together in subsequent PCR using the primers listed in Table 1. The final PCR product was used to disrupt the *rdxA* locus of the *recR::cat* strain by selecting for metronidazole (16 μg/ml) resistant colonies (96 bp at the center of the *rdxA* gene was deleted and replaced by an intact *recR* gene).

2.3. Oxygen sensitivity (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 ~10^8 cells/ml. The cell suspensions were incubated at 37°C under normal atmospheric conditions (21% O_2, without alteration of CO_2 partial pressure) with moderate shaking. Samples were then removed at various time points (2, 4, 6, 8, and 10 hours), serially diluted, and spread onto BA plates. Colony counts are recorded after 4 days of incubation in a microaerobic atmosphere (5% partial pressure O_2) at 37°C.

2.4. Assessment for sensitivity to low pH condition

H. pylori strains were grown on BA plates to late log-phase, and the cells were suspended at a concentration of ~10^8 cells/ml in the buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.0), or, for acid stress condition, in the same solution pre-adjusted to pH 3.0 by adding appropriate

amount of HCl. The cell suspensions were incubated under a microaerobic condition (4% O_2) at 37°C for 1 hour. The samples were serially diluted and plated for CFU counts (after 4 days incubation under microaerobic growth condition, see text above). The percentage of cell survival in pH 3.0 relative to that in pH 7.0 was calculated.

2.5. DNA transformation assay to assess inter-genomic recombination frequency

The donor DNA used in this study included: (i) a 330 bp PCR fragment of *H. pylori rpoB* gene fragment containing a site-specific mutation (at the center of the fragment) conferring rifampicin 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) pHP1, a *H. pylori-E. coli* shuttle plasmid carrying a Kan cassette [28].

H. pylori strains were grown on BA plates to late log-phase, and the cells were suspended in PBS at a concentration of ~ 10^8/ml (recipient cells for transformation). A 30 μl cell suspension sample was mixed with 100 ng of donor DNA and spotted onto a BA plate. After incubation for 18 hours under microaerobic condition at 37°C, the transformation mixture was harvested and suspended in 1 ml PBS. 100 μl portions of the suspension (or appropriate dilution as needed) were plated onto either BA plates or BA plates containing selective antibiotic (20 μg/ml rifampicin or 40 μg/ml kanamycin, depending on the donor DNA used). The plates were incubated for 4 days under the microaerobic condition at 37°C, and the numbers of colonies were counted. The transformation frequency was determined by the number of resistant colonies divided by the total number of CFU. In a normalized DNA transformation assay, the frequency of transformation is expressed as the number of transformants per 10^8 recipient cells. As negative controls, *H. pylori* strains with no DNA added were tested under this assay condition; no antibiotic-resistant colonies were observed.

2.6. Construction of the intra-genomic recombination substrates (deletion cassettes)

A plasmid (pUre100) was kindly provided by Dr. Martin Blaser. pUre100 contains a deletion cassette (IDS100) and was described and used for determination of the intra-genomic recombination frequency (deletion frequency) resulting from recombination on direct repeat sequences [26,29,30]. The deletion cassette IDS100 contains a chloramphenicol resistance gene (CAT) with flanking identical repeat segments (100 bp long) of the kanamycin resistance gene (aphA). By using a multiplex overlapping PCR method (primers listed in Table 1) and IDS100 as the starting template, we constructed IDS350 in which the length of the identical repeat segments is 350 bp. The complete nucleotide sequence of IDS350 was confirmed by DNA sequencing. Subsequently, the recR::IDS and recA::IDS were constructed by overlapping PCR method (primers listed in Table 1) in which the major portion of the gene was replaced by the deletion cassette (IDS100 or IDS350). The appropriate PCR product was used to transform *H. pylori* wild type strain by selecting Cm-resistant colonies. As controls, the deletion cassettes were inserted into hp405 locus which has no known effect on DNA recombination or any other characteristics.

2.7. Determination of the intra-genomic recombination (deletion) frequency

Strains to be tested were grown on BA plates containing chloramphenicol (50 μg/ml) under normal microaerobic condition (4% O_2) to late log phase. The cells were suspended in PBS at a concentration of 10^8 – 10^9 cells/ml and serial dilutions were made. Appropriate dilutions were plated onto BA plates with or without kanamycin (40 μg/ml), and the plates were incubated for 4 to 5 days followed by counting the number of colonies. The frequencies of intra-genomic recombination (deletion of cat cassette and recovery of intact aphA cassette) were calculated as the number of Kan^R cells divided by the number of total cells. The average frequencies and their standard deviations were calculated from at least five independent experiments.

2.8. Mouse colonization

Mouse colonization assays were performed essentially as described previously [11,21,31]. Briefly, the wild type X47 or isogenic *rec* mutant cells were harvested after 48 h of growth on BA plates (37°C, 4% oxygen) and suspended in PBS to an OD_{600} of 1.7. Headspace in the 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, with each of 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 [32] to avoid O_2 exposure. Stomach homogenate dilutions (dilutions conducted in tubes in argon-sparged buffer) were plated on BA plates supplemented with bacitracin (100 μg/ml), vancomycin (10 μg/ml) and amphotericin B (10 μg/ml), and the plates were rapidly transported into an incubator containing sustained 4% partial pressure O_2. After incubation for 5 to 7 days the fresh *H. pylori* colonies were enumerated and the data expressed as CFU per gram of stomach.

3. Results and Discussion

3.1 Sensitivity of *H. pylori recR* and *recO* mutants to DNA damaging agents

Previously, we characterized *H. pylori recN* and *addA* mutants [11,21]. To focus on the physiological roles of the RecRO pathway herein, we constructed *recR* and *recO* mutants. The mutants were constructed in strain X47, a mouse-colonizing strain, to also determine the effects on mouse colonization ability. In addition, we constructed an *addA recO* double mutant that is deficient in both pathways of DNA recombinational repair. All three mutant strains, X47 *recR::cat*, X47 *recO::aphA*, and X47 *addA::cat recO::aphA*, grew much slower than the wild type X47 under the normal microaerobic conditions (the growth rate is about half that of the WT). In the report of Marsin et al [20], the *recR* and *recO* single mutants (in the strain 26695 background) did not show obvious growth impairment by measuring the colony size after 4 days growth on plates. To determine whether these are strain-dependent difference, we constructed *recR* mutants in both background strains 26695 and X47, and measured the growth curves (data not shown). We observed that both *recR* mutants grew at a slower rate than the wild type in exponential growth phase, but can eventually reach a similar yield to the wild type at stationary phase (after 3–4 days).

In the study of Marsin et al [20], the *recR* and *recO* single mutants showed marked sensitivity to DNA damaging agents metronidazole and UV light, indicating roles of RecR and RecO in DNA repair. On the other hand, the *recR* and *recO* single mutants did not show significant sensitivity to ionizing radiation (IR), suggesting that all the recombinational repair of IR-induced damage is mediated by the AddA (RecB)-dependent pathway. We examined UV sensitivity of our strains; similar results were obtained, i.e. the *recR* and *recO* single mutants are more sensitive to UV light than the wild type (data not shown), confirming the role of *H. pylori* RecRO pathway in repairing UV-induced DNA damage.

Mitomycin C (MMC) causes predominantly DNA intra-strand cross-links, leading ultimately to DNA double strand breaks. In this study, we determined the sensitivity of the mutant strains to MMC by growing *H. pylori* strains on BA plates containing different concentrations of MMC. The wild type *H. pylori* strain can grow on the plates containing 5 ng/ml MMC, but not on 10 ng/ml MMC. The *recN* mutant strain is more sensitive than the wild type, as it tolerated 2.5 ng/ml MMC but could not grow on the plates containing 5 ng/ml MMC [11]. The *addA* mutant strain showed even higher sensitivity with no growth on plates containing MMC at 2.5 ng/ml or higher concentrations [21]. In contrast, the MMC sensitivity of the *recR* or *recO* single mutant was the same as the wild type, and the *addA recO* double mutant showed the same level of sensitivity to MMC as the *addA* single mutant

(data not shown). These results indicate that the RecRO pathway is not responsible for the repair of MMC-induced DNA damage.

3.2. *H. pylori recR* and *recO* mutants are sensitive to oxidative stress and acid stress

Oxidative stress is a major stress condition that *H. pylori* encounters in its physiological niche. *H. pylori* induces strong host inflammatory responses that involve recruitment of neutrophils, lymphocytes and macrophages; these release reactive oxygen species that damage DNA. *H. pylori* DNA 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 [7]. Further studies showed that mutant cells of *ruvC* [12], *recN* [11] or *addA* (*recB*) [21] were more sensitive to oxidative stress, indicating an important role of DNA recombinational repair in *H. pylori* for the bacterial survival of oxidative damage. To examine the role of the RecRO pathway in repairing DNA damage derived from oxidative stress, we examined the sensitivity of the *H. pylori* strains to oxidative stress by an air survival assay. The cell suspensions (~ 5×10^8 cells/ml) were exposed to air, and the numbers of surviving cells were determined at various time points (Fig. 1). The number of wild type cells decreased slowly; and at the 10 h time point, about 5×10^6 cells (~1% of that at the time zero) survived. The *recR* complemented strain behaved like the wild type. In contrast, the *recO*, *recR*, and *addA recO* mutants showed a greater sensitivity to air exposure. Two hours after exposing cells to air, the number of surviving mutant cells started to decrease at a rate much faster than that of the wild type cells. At the 10 h time point, the *recO* mutant cells were completely killed (i.e. no viable cells recovered), and the *recR* mutant cells were completely killed at the 8 h time point. The *addA recO* double mutant is statistically significantly more air-sensitive than the *recO* single mutant and is slightly more sensitive than the *recR* single mutant. The results indicated that both RecRO and AddAB pathways are important for survival of oxidative damage. Similar roles of the RecBCD and the RecFOR pathways for survival of oxidative (H_2O_2) damage were also observed in *E. coli* [3,33] and in *Neisseria gonorrhoeae* [34]. In those bacteria, however, the RecBCD appeared to be the predominant (over the RecFOR) repair pathway for oxidative damage. Our results suggest that the two pathways in *H. pylori* play similarly important roles in repairing oxidative stress-derived DNA damage.

H. pylori appears to colonize an acidic niche on the gastric surface [10]. Therefore, low pH is another stress condition that *H. pylori* encounters in its physiological niche. Despite the existence of sophisticated pH homeostasis systems and acid tolerance mechanisms, bacteria may still suffer DNA damage from acid stress. Indeed, *E. coli* O157:H7 chromosomal DNA was shown to be significantly damaged by acid stress, and the Dps protein plays an important role in protecting acid-induced damage [35]. Notably, the acid tolerance of the *recA* mutant was significantly lower than that of the *dps* mutant and parent strain, suggesting that DNA recombinational repair might play an even more important role in acid tolerance than Dps [35]. Previously, we showed that *H. pylori* RecN is involved in repair of acid-induced DNA damage [11]. In this study, we characterized the *recR*, *recO*, and *addA recO* mutants for their sensitivity to low pH conditions. The cells of wild type *H. pylori* or the *rec* mutants were treated for 1 hour at different pH (pH 7.0 or pH 3.0) conditions, and the survival was determined. As shown in Fig. 2, about 40% of the wild type *H. pylori* cells survived the pH 3.0 condition for 1 hour. In contrast, only 1–2% of the *recR* cells or the *recO* cells survived the same stress condition. The complementation of the *recR* mutant restored the acid sensitivity to the wild type level. The *addA recO* double mutant cells were almost completely killed (>99.9% lethality) by the same treatment. The results clearly indicated that the RecRO pathway plays an important role in repairing DNA damage derived from acid stress.

3.3. Disruption of *H. pylori recR* and *recO* has no effect on inter-genomic recombination

H. pylori is naturally competent for DNA transformation, and has a highly efficient system for recombination of short-fragment involving multiple recombination events within a single locus [25,36]. Natural transformation involves uptake of DNA into the cell followed by recombination into the genome at the site of homologous sequence (inter-genomic recombination). DNA uptake in *H. pylori* is mediated by a composite system involving proteins at the *comB* locus and ComEC [37,38]. Assuming the wild type *H. pylori* and its isogenic *rec* strains are equally competent for DNA uptake, the frequency of natural transformation is an indicator of DNA recombination frequency. Previously, different results regarding the effect of *rec* genes on transformation frequency were reported by different research groups using different *H. pylori* strains and different DNA substrates. For example, Marsin et al [20] concluded that DNA integration into the chromosome following transformation is AddA (RecB)-independent. In contrast, studies from our group [21] and Kulick et al [25] indicated that inactivation of *addA* (*recB*) significantly reduced transformation frequency. In this study, we examined the effect of *recR* and *recO* on DNA transformation. As described previously [11,21], we used two different types of DNA for examining DNA transformation of *H. pylori*. A specific A-to-G mutation in the *H. pylori rpoB* gene (rpoB3 allele) confers rifampicin resistance [39]. A 330-bp PCR fragment containing this specific mutation at the center of the fragment was used to transform *H. pylori* strains by using rifampicin resistance as a selective marker. Another type of DNA used for transformation was the sequence of *H. pylori acnB* gene (a housekeeping gene, 1.1 kb) in which a kanamycin resistance cassette (Kan, 1.4 kb) was inserted at the center (acnB:Kan).

The results for transformation are shown in Table 2. The *recR* and *recO* single mutants had a transformation frequency at a level similar to the wild type strain. According to Student t-test, the differences between the wild type and the mutants were not statistically significant. In contrast, the *addA recO* double mutant had a greatly reduced transformation frequency (2.13×10^{-5} transformants/recipient cell), which was ~12-fold lower than that of the wild type strain (2.56×10^{-4} transformants/recipient cell). According to Student t-test, this difference was highly significant ($P<0.001$). The effect of the *addA recO* double mutation was similar (no significant difference) to that observed for the *addA* single mutation (3.09×10^{-5} transformants/recipient cell). These results indicated that recombination of exogenous DNA into *H. pylori* genome in the process of transformation is dependent on AddA, but not on RecRO-pathway.

3.4. Roles of RecR and RecA on intra-genomic recombination at repetitive sequence

The *E. coli* paradigm of recombinational repair is that the RecBCD pathway acts on double-strand breaks and the RecFOR pathway acts on single-strand DNA (ssDNA) gaps. The transformation assays described above mainly measure the recombination activity involving DNA double strand breaks for which the RecBCD pathway is responsible in the organisms that have been well studied. For testing the role of the RecFOR pathway, an assay is required to measure intra-genomic recombination with repeat sequences [27]. We adopted an assay developed by Aras et al [26] and Kang et al [29,30] to assess the deletion frequency resulting from recombination on direct repeat sequences, as described in Materials and Methods and in Fig. 3A. We made two DNA constructs (deletion cassettes) that contain identical repeat sequences of different length (IDS100 and IDS350).

The deletion cassettes were inserted into the target *rec* gene locus in the *H. pylori* genome. As a control, the deletion cassettes were inserted into hp405 locus which has no known effect on DNA recombination. Using this assay, we measured the deletion frequency of *H. pylori recR* and *recA* mutants (Fig. 3B). For IDS100, the deletion frequency in the wild type

background was approximately 1.7×10^{-5} Kan^R clones/cell. Inactivation of *recR* or *recA* resulted in a 2- or 4-fold decrease in deletion frequency, respectively. According to Student's t-test, the decreases are significant ($P<0.01$). These results indicated that the intra-genomic recombination of 100 bp-long direct repeat sequences in *H. pylori* is partially dependent on RecR and RecA. However, a big portion of the recombination event is RecR- and RecA-independent. This is basically in agreement (with small variance) with the results of Aras et al [26] who reported that the repeat sequences of 100 bp or shorter recombined through a RecA-independent pathway. Currently we are trying to identify such a RecA-independent recombination pathway in *H. pylori*.

For IDS350, the deletion frequency in the wild type background was approximately 1.4×10^{-5} Kan^R clones/cell. In contrast to IDS100, recombination on IDS350 was almost completely dependent on RecA, as the deletion frequency in *recA* strain was only 4×10^{-7} Kan^R clones/cell, 35-fold lower than that of background level. Inactivation of *recR* resulted in a significant ($P<0.01$) 4-fold decrease in deletion frequency, indicating that the intra-genomic recombination of IDS350 is RecR-dependent. The earlier report from Marsin et al [20] also observed that the *H. pylori recO* and *recR* mutants had a reduced recombination frequency compared to the wild type; however the difference was much smaller (less than 2-fold) than what we observed here. The discrepancy from the two studies could be due to the difference of the *H. pylori* strains and the assay systems used. Thus, our results here clearly indicate that RecR plays a significant role in recombination of IDS350. It is interesting to note that the deletion frequencies in the *recR* strain for both IDS100 and IDS350 were higher than those observed for the *recA* strain. Thus, the RecA-dependent IDS recombination (for both IDS100 and IDS350) is only partially dependent on RecR. Most likely, the AddAB pathway is also involved in intra-genomic recombination.

3.5. *H. pylori recR* and *recO* mutants have attenuated ability to colonize mouse stomachs

Repair of damaged DNA is known to be important for *H. pylori* survival and pathogenesis [7,31]. Defects in DNA recombinational repair due to loss of RuvC, RecN, or AddAB function in *H. pylori* resulted in reduced ability to colonize the host stomach [11,12,18,21]. As *H. pylori recR* and *recO* mutants showed sensitivity to both oxidative stress and acid stress, the condition that *H. pylori* encounters in its physiological niche, we sought to determine the effect of RecRO pathway on *H. pylori* colonization in the host. We performed an assay using a mouse infection model as described previously [11,21,31].

The wild type X47 or the isogenic mutant strains of *recR*, *recO*, and *addA recO*, as well as the *recR* complementation strain, were inoculated into 12 C57BL/6J mice, and the colonization of *H. pylori* cells in the mouse stomachs was examined 3 weeks after inoculation (Fig. 4). *H. pylori* was recovered from all 12 mice that had been inoculated with the wild type strain, with a geometric mean number of 6.0×10^5 CFU/g stomach. Similar results were obtained for the *recR* complementation strain (mean of 5.1×10^5 CFU/g). In contrast, 8 of 12 mice that were inoculated with the *recR* or *recO* strain were found to harbor *H. pylori*. The geometric mean of the colonization number for the *recR* or *recO* strain in the 12 mice was 1.6×10^4 or 1.4×10^4 CFU/g stomach, respectively. According to Wilcoxin rank test analysis, the ranges of colonization values of the *recR* or *recO* strain are significantly lower than that of the wild type at the 99% confidence level ($P<0.01$). Therefore, the RecRO pathway plays a significant role in *H. pylori* survival/colonization in the mouse stomachs. Of the 12 mice that were inoculated with the *addA recO* double mutant strain, only 6 mice had detectable *H. pylori* in their stomachs. The geometric mean of the colonization number for the *addA recO* strain in the 12 mice was 4×10^3 CFU/g stomach. Based on the previous data [21], the geometric mean of the colonization number for the *addA* single mutant (14 mice tested) was 1×10^4 CFU/g stomach. According to Wilcoxin rank test analysis, the ranges of colonization values of the *addA recO* double mutant is significantly lower than that of the

addA or *recO* single mutant at the 95% confidence level ($P<0.05$). Thus, the effect of the double mutation on attenuation of mouse colonization is more severe than those observed for *addA* or *recO* single mutation.

3.6. Conclusions

H. pylori RecRO pathway is not responsible for repairing DNA double strand breaks. In accord with this, it is not involved in the integration of exogenous DNA fragments into the genome during the transformation process. Similarly to the RecFOR pathway in *E. coli* and other bacteria, the RecRO pathway in *H. pylori* plays a significant role in intra-genomic recombination involving direct repeat sequences as substrate. *H. pylori recR* and *recO* mutants were shown to be much more sensitive to oxidative stress and acid stress than the wild type strain, indicating that *H. pylori* RecRO pathway is involved in repairing DNA damage induced by these stress conditions. Furthermore, we demonstrated that RecRO-mediated DNA recombinational repair in *H. pylori* plays an important role in bacterial survival and persistent colonization in the host.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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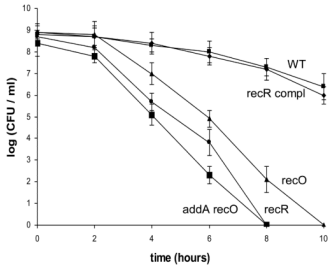

Fig. 1. Survival of *H. pylori* cells upon exposure to air

H. pylori cell suspensions in PBS were incubated at 37°C under normal atmospheric conditions (21% partial pressure O_2, and no alteration of CO_2 partial pressure). Samples were removed at the times indicated in the x axis and were used for plate count determinations in a 5% oxygen environment. The data are the means of three experiments with standard deviation as indicated. Symbols: square, wild type; diamond, *recR* complementation strain; triangle, *recO::aphA*; circle, *recR::cat*; large square, *addA::cat recO::aphA*. Based on statistical analysis (Student t-test), the cell survival differences between the WT and the mutant strains are significant ($P<0.01$) for all the data points except for the 2 h time point.

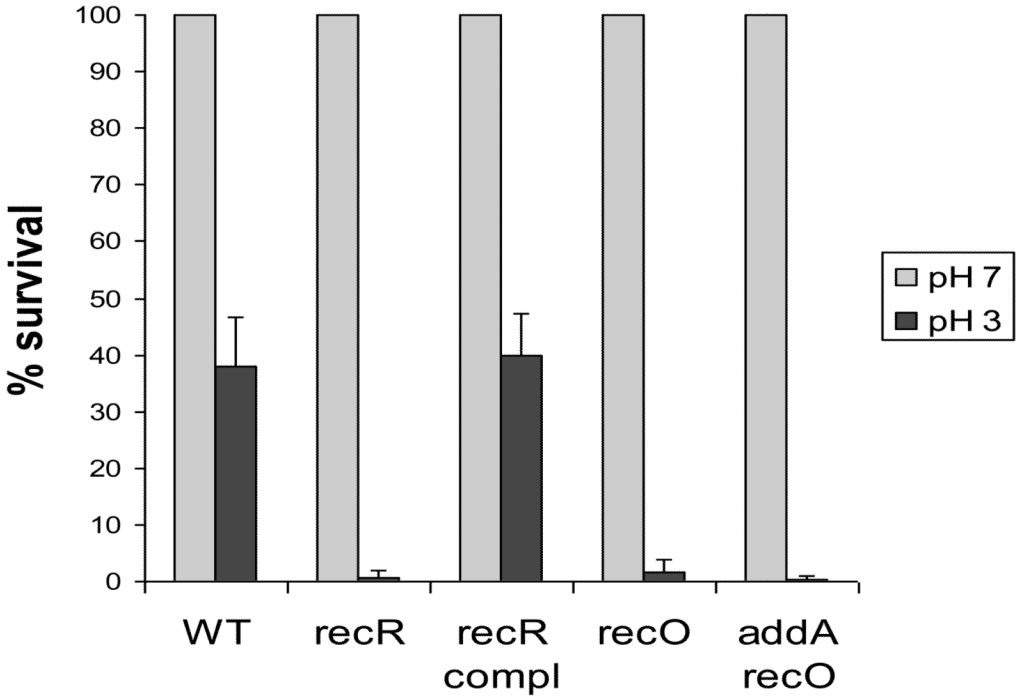

Fig. 2. Acid sensitivity

H. pylori cell suspensions were treated in solutions at different pH levels (pH 7.0 or pH 3.0) under a microaerobic condition (5% O_2) at 37°C for 1 hour, and the numbers of surviving cells were determined. Numbers shown are the percentages of cell survival after treatment in pH 3.0 relative to that in pH 7.0. Data are means ± standard errors from three independent determinations.

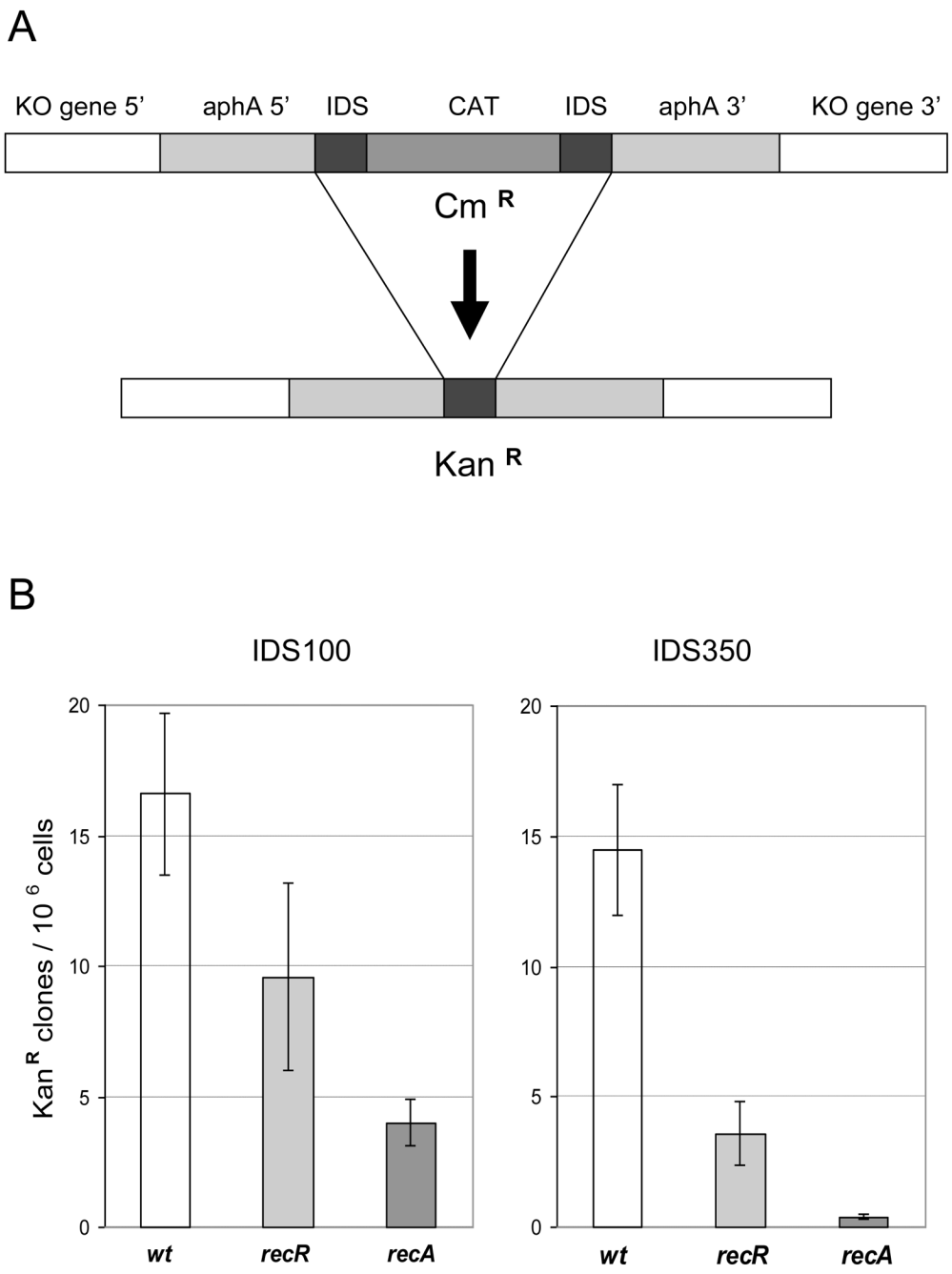

Fig. 3. Intra-genomic recombination frequency in *H. pylori recR* and *recA* mutant strains

(A) Description of the experimental system. A target knockout (KO) gene (recR, recA or hp405 representing WT) on *H. pylori* genome was disrupted by insertion of a deletion cassette (gray bars). The deletion cassette contains the *aphA* gene interrupted by *cat* gene with two flanking identical repeat sequences (IDS, black bars, 100 bp or 350 bp long). Recombination between the two IDS sequences gives rise to a functional aphA gene conferring kanamycin resistance. **(B)** Recombination frequencies determined for different KO genes with IDS100 or IDS350. Recombination frequencies were determined as described in Materials and Methods. Shown data are average frequency and standard deviation of at least 5 independent determinations.

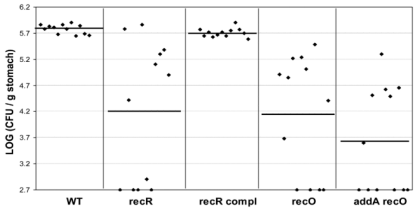

Fig. 4. Mouse colonization results for *H. pylori* rec mutants

The mice were inoculated with *H. pylori* two times (two days apart) with a dose of 1.5×10^8 viable cells administered per animal each time. Colonization of *H. pylori* in mouse stomachs was examined 3 weeks after the first inoculation. Conditions were used during stomach homogenization and homogenate dilution to minimize oxygen exposure (see text). Data are presented as a scatter plot (log scale) of colony forming units per gram of stomach as determined by plate counts. Each point represents the CFU count from one mouse stomach, and the solid horizontal lines represent the geometric means of the colonization numbers for each group. The base line [$\log_{10}$ (CFU/g) =2.7] is the detection limit of the assay, which represents a count below 500 CFU/g stomach.

Table 1

Primers used in this study.

Name	Sequence (5′ – 3′)	Description
PrecR-F	AACGCTTCATTAAGCGCTTT	To construct recR::cat mutant
PrecR-R	ACAAAGGCACTTCATGCAC	
recR-1	GTTGAATCTCCTGGCACGAGTTTGATATTG	For recR complementation
recR-2	ACGGCCACTATCGCATGGTTATAAGCATGC	
rdxA-L	CACAACCAAGTAATCGCATC	
rdxA-1	CTCGTGCCAGGAGATTCAACCACAGCATGC	
rdxA-2	AACCATGCGATAGTGGCCGTGTGGTAACAA	
rdxA-R	AGCGCCATTCTTGCAAGATG	
PrecO-F	CATGCGGAAATGGTAATGGC	To construct recO::aphA mutant
PrecO-R	GCTTGTTCTGTGTTTTCGCC	
PrecO-11	CGAATCACCTAGTATCCGCTATGGCGTTTGCCATAG	
PrecO-22	GATTCAGGAACTGATCGCATTAGAGCGTTCTGTCGC	
KanF-11	CGGATACTAGGTGATTCGAGACGATAAATGCGTCGG	Forward primer for aphA cassette
KanR-22	GCGATCAGTTCCTGAATCGACATCTAAATCTAGGTAC	Reverse primer for aphA cassette
D350P1	TTATCGAGCTGTATGCGGAG	To construct IDS350
D350P2	TCCATACGATACCGGGTACCTGCCATCTTTCACAAAGATG	
D350P3	CTCCGCATACAGCTCGATAA	
D350P4	GGTACCCGGTATCGTATGGA	
Hp405-F	CTCCCTTACTCAACCCTAAC	To construct hp405::IDS100 and hp405::IDS350
Hp405-R	CGTGAAGTGCAAGCTCACTC	
Hp405-11	CGAATCACCTAGTATCCGCCATATCTTTCAAATACGGC	
Hp405-22	GATTCAGGAACTGATCGCATACGCTAATCGCACACGGC	
RecR-F	CCTCATCTATAATGACCACA	To construct recR::IDS100 and recR::IDS350
RecR-R	GAGATGAATTGAGTCGTCAG	
RecR-11	CGAATCACCTAGTATCCGACCCAAATGATACGCCA	
RecR-22	GATTCAGGAACTGATCGCGACTCAGTTTCGCTCTCA	
RecA-F	TTGCGCTCACATCATAAGTG	To construct recA::IDS100 and recA::IDS350
RecA-R	CTTGATTGATCGCTTCAAGC	
RecA-11	CGAATCACCTAGTATCCGGAGCCTGTAGAAATAGAGTC	
RecA-22	GATTCAGGAACTGATCGCCTCTAATGAAGAGATCATGC	

Table 2

Transformation frequency with different types of donor DNA

	Donor DNA	
***H. pylori* strains**	**rpoB3 (330 bp)**	**acnB:Kan (2.5 kb)**
X47 WT	$2.56 \pm 0.38 \times 10^{-4}$	$7.20 \pm 0.84 \times 10^{-6}$
X47 recR::cat	$3.25 \pm 0.76 \times 10^{-4}$	$5.60 \pm 0.32 \times 10^{-6}$
X47 recO::aphA	$3.04 \pm 0.82 \times 10^{-4}$	ND
X47 addA::cat recO::aphA	$2.13 \pm 0.68 \times 10^{-5}$	ND
X47 addA::cat	$3.09 \pm 0.62 \times 10^{-5}$	$2.90 \pm 0.30 \times 10^{-7}$

The transformation frequencies are presented as the number of transformants (resistant colonies) per recipient cell. Data are means ± standard errors from three independent determinations. ND: When a strain contains a Kan^R marker (aphA gene), the transformation frequency with acnB:Kan cannot be determined.