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A histone-like protein of *Helicobacter pylori* protects DNA from stress damage and aids host colonization

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Abstract

Genomic DNA in a bacterial cell is folded into a compact structure called a nucleoid, and nucleoid-associated proteins are responsible for proper assembly of active higher-order genome structures. The human gastric pathogen *Helicobacter pylori* express a nucleoid-associated protein encoded by the *hup* gene, which is the homolog to the *Escherichia coli* histone-like protein HU. An *H. pylori hup* mutant strain (X47 *hup*:cat) showed a defect in stationary phase survival. The X47 *hup*:cat mutant was more sensitive to the DNA damaging agent mitomycin C, and displayed a decreased frequency of DNA recombination, indicating Hup plays a significant role in facilitating DNA recombinational repair. The X47 *hup*:cat mutant was also sensitive to both oxidative and acid stress, conditions that *H. pylori* commonly encounters in the host. The *hup* mutant cells survived significantly (7-fold) less upon exposure to macrophages than the wild type strain. In a mouse infection model, the *hup* mutant strain displayed a greatly reduced ability to colonize host stomachs. The geometric means of colonization number for the wild type and *hup* mutant were 6×10^5 and 1.5×10^4 CFU/g stomachs, respectively. Complementation of the *hup* strain by chromosomal insertion of a functional *hup* gene restored oxidative stress resistance, DNA transformation frequency, and mouse colonization ability to the wild type level. We directly demonstrated that the purified His-tagged *H. pylori* Hup protein can protect (in vitro) an *H. pylori*-derived DNA fragment from oxidative damage.

Keywords

Helicobacter pylori; Histone-like protein; Oxidative stress; DNA protection; recombinational repair; Macrophage killing; Mouse colonization

1. Introduction

Helicobacter pylori infects the stomachs of approximately 50% of humans and results in a series of human gastric diseases including gastritis, peptic ulcers, and gastric cancer [1-4]. During the process of colonizing the host, *H. pylori* induces a strong inflammatory response mediated by a variety of host immune cells, leading to generation of a number of reactive oxygen species (ROS) [5-8]. *H. pylori* has excellent capacity and redundant mechanisms to directly detoxify ROS and to repair damaged macromolecules so that it can survive and

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colonize persistently in the harsh conditions within the gastric mucosa [9]. The most severe human gastric diseases are due to the persistent nature of the bacterium.

Studies in recent years have indicated that DNA recombinational repair plays a significant role in *H. pylori*'s persistent colonization of the host [10-14]. In an attempt to identify additional components of the recombinational repair system in *H. pylori*, we screened for mitomycin C-sensitive clones from a random transposon mutagenesis library. In one of the mitomycin C-sensitive strains the transposon was shown to be inserted at the *hup* locus, suggesting that the *hup* gene is involved in DNA protection, recombination and/or repair processes. The *hup* gene encodes a protein that is homologous to the histone-like protein HU of *Escherichia coli*.

Genomic DNA in a bacterial cell is folded into a compact structure called a nucleoid, and the proper assembly of active higher-order genome structures requires accessory proteins, termed nucleoid-associated proteins (NAPs). In *E. coli*, several nucleoid-associated proteins, such as HU, IHF, H-NS, Fis, and Lrp, have been discovered and shown to play roles in DNA organization and protection [15, 16]. These proteins are sometimes referred to as histone-like because they have roles in nucleoid compaction comparable to eukaryotic histones. These proteins are not only involved in DNA supercoiling and compaction, but also modulate DNA functions such as replication, recombination, repair, and transcription [17]. Dps (DNA protection during starvation) is sometimes also referred to as a nucleoid-associated protein. Each bacterial species harbors a specific set of NAPs, with only HU-like proteins being ubiquitous among bacteria. The functions and DNA binding properties of HU-like proteins may vary depending on the specific NAP content and the host conditions [18].

Among the nucleoid-associated proteins, *H. pylori* harbors a HU-like protein Hup and a Dps homolog. The basic properties and substrate DNA binding specificity of *H. pylori* Hup protein were studied previously [19]. In this study, we first examined whether Hup is involved in DNA recombinational repair. Then we investigated the physiological roles of *H. pylori* Hup in DNA protection, particularly focusing on the role in protecting DNA from oxidative damage. Furthermore, the contribution of Hup for bacterial survival within the host stomach was examined herein with a mouse infection model.

2. Materials and Methods

2.1. *H. pylori* culture conditions

H. pylori was cultured on Brucella agar (Difco) plates supplemented with 10% defibrinated sheep blood or 5% fetal bovine serum (called BA plates). Chloramphenicol (50 μg/ml) or kanamycin (40 μg/ml) was added to the medium for culturing mutants. Cultures of *H. pylori* were grown microaerobically at 37°C in an incubator under continuously controlled levels of oxygen (4% partial pressure O_2, 5% CO_2, and the balance was N_2).

2.2. Construction of *H. pylori hup* mutant

A 1.04 kb fragment containing the *H. pylori hup* gene and the flanking regions was amplified by polymerase chain reaction (PCR) from genomic DNA of strain 26695 using the primer pair hupF1 (5'-AAAGTGTATTACGCCACGC-3') and hupR1 (5'-AAAGGGATTTTAATGGCTTG-3'). The PCR product was cloned into pGEM-T vector to generate pGEM-*hup*. Subsequently, a chloramphenicol acetyl transferase (cat) cassette was inserted at the unique BbsI site within the *hup* sequence of pGEM-*hup*. The disrupted *hup* gene was then introduced into *H. pylori* wild type strains by natural transformation *via* allelic exchange and chloramphenicol-resistant colonies were isolated (*hup*::cat). The

disruption of the gene in the genome of the mutant strain was confirmed by PCR showing an increase in the expected size of the PCR product and by direct sequencing of the PCR fragment.

2.3. Construction of *H. pylori hup* complementation strain

The complemented *hup*$^+$ strain was constructed by inserting a wild-type copy of the *hup* gene in the *rdxA* locus of the *hup*::cat chromosome. Disruption of *rdxA* results in metronidazole resistance that will be used for selection in DNA transformation. PCR products corresponding to the 3' end of the *rdxA* gene (266 bp), 523 bp of the full-length *hup* gene and the 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. The final PCR product (1019 bp) was used to transform the *hup::cat* strain by selecting for metronidazole (16 μg/ml) resistant colonies. Through homologous DNA recombination, an intact *hup* gene was inserted at the *rdxA* locus of the *hup::cat* strain.

2.4. Assessment for 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 ~10^8 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 incubation in a microaerobic atmosphere (4% partial pressure O_2) at 37°C, colonies are enumerated.

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).

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. 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) 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 (4% partial pressure O_2) at 37°C.

2.7. Assessment for sensitivity to low pH condition

H. pylori strains were grown on BA plates to late log-phase, and the cells were suspended in the buffer (20 mM Tris-HCl, 150 mM NaCl) with different pH levels (pH 7.0, pH 5.0, or pH 3.0) at a concentration of ~10^8 cells/ml. 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). The percentage of cell survival in pH 5.0 or pH 3.0 relative to that in pH 7.0 was calculated.

2.8. Macrophage killing assay

The survival of *H. pylori* cells within macrophages was investigated following the methods published [14, 20, 21] with minor modifications. Briefly, the macrophage RAW264.7 cells were seeded in 24-well plates in the culture medium (0.5 ml) and incubated at 37°C, 5% CO_2 for 4 days (cell density is about 10^5 cells per well). The medium was replaced by fresh medium to remove the non-adherent cells. *H. pylori* cells were added at a ratio of 20 CFU bacteria per macrophage. Phagocytosis was synchronized by centrifugation at 600 × g for 5 min and then allowed to proceed for 1 h. Extracellular bacteria were removed by washing and incubation in the medium supplemented with gentamicin (100 mg/ml) for 1 h at 37°C, 5% CO_2. After three washes to remove the antibiotics the cells were further incubated in fresh medium for 2 h. After removing the medium, the macrophage cells were lysed with ice-cold PBS with 0.1% saponin for 5 min. Appropriate dilutions of the supernatant were plated on BA plates and incubated at 37°C, 5% CO_2, 2% O_2 for 4 days to count the number of surviving bacteria. The number of surviving bacteria (CFU/ml) is compared with the number of viable bacteria initially added.

2.9. Mouse colonization

Mouse colonization assays were performed essentially as described previously [10, 12]. Briefly, the wild type X47 or isogenic *hup* 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/6NCr 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 [22] to avoid O_2 exposure. Stomach homogenate dilutions (dilutions conducted in sealed tubes containing argon-sparged buffer) were plated on BA plates supplemented with bacitracin (100 μg/ml), vancomycin (10 μg/ml) and amphotericin B (10 μg/ml). The plates were rapidly transported into an incubator containing sustained 4% partial pressure O_2. After incubation for 5 to 7 days *H. pylori* colonies were counted and the data expressed as CFU per gram of stomach.

2.10. Overexpression and purification of *H. pylori* Hup protein

A DNA fragment containing *H. pylori hup* gene was amplified by PCR and cloned into pET-21a vector to generate pET-Hup-6His. *E. coli* BL21 Origami cells harboring pET-Hup-6His were grown at 37°C to an OD at 600 nm of 0.5 in 500 ml of LB medium with ampicillin (100 μg/ml) and kanamycin (40 μg/ml). Expression of the Hup protein was induced by adding 0.5 mM IPTG to the medium followed by further incubation for 3 h; cells were then harvested by centrifugation (5,000×g, 15min, 4°C). All subsequent steps were performed at 4°C. Cells were washed with 200ml of 20mM Na_2HPO_4 (pH7.4), 500mM NaCl, 5mM imidazole (buffer A) and re-suspended in 5 ml of the same buffer. Cells were lysed by two passages through a cold French pressure cell at 18,000 lb/in^2. Cell debris was removed by centrifugation at 20,000 × g. The supernatant was applied to a nickel-nitrilotriacetic acid (Ni-NTA) affinity column (Qiagen), and buffer A was used to wash the

resin until the A_{280} reached the baseline. Proteins were washed with buffer B (buffer A with 30mM imidazole) until the A_{280} reached the baseline, and finally eluted with buffer C (buffer A with 250mM imidazole). Extracts of *E. coli* BL21 Origami containing the vector only did not result in retrievable proteins from this purification (Ni-affinity) procedure. Fractions were analyzed by gel electrophoresis, and the Hup-positive fractions were pooled. Protein concentrations were determined with a bicinchoninic acid protein assay kit (Pierce, Rockford, Ill.).

2.11. DNA protection assay

A linear DNA fragment (800 bp long) was PCR amplified from *H. pylori* genomic DNA. 0.2 μM DNA was incubated with 2 μM purified *H. pylori* Hup protein, or BSA as a control. Then the samples were treated with 20 mM $FeSO_4$ plus 5 mM H_2O_2 or control buffer for 5 minutes. The samples were analyzed on an ethidium bromide stained 1% agarose gel.

3. Results and discussion

3.1. Construction of an *H. pylori hup* mutant and its effect on cell growth

Using a random transposon mutagenesis library of *H. pylori* [23], we identified a dozen genes that confer resistance to mitomycin C (data not shown). In one of the MMC-sensitive strains, the transposon was found to be inserted within the HP0835 locus. In the published *H. pylori* genome sequence [24], HP0835 was annotated as a *hup* gene homolog. The gene product of *H. pylori hup* displays 34% and 36% amino acid identity to *E. coli* HupA and HupB, respectively. In some bacteria including *E. coli*, HU is a heterodimer composed of two highly homologous subunits (HupA and HupB), whereas in many other bacteria including *H. pylori* HU is present as a homodimer [19, 25].

To study the physiological role of *H. pylori* Hup, we constructed a *hup* mutant by inserting a chloramphenicol resistance cassette (CAT) at the restriction site BbsI within the *hup* gene. The *hup* mutant strains can be easily obtained if transformants are screened at a low oxygen (2% partial pressure) condition. The *hup* mutants were constructed in several different strains such as 26695, 43504, and X47. As X47 is a well-adapted strain for mouse colonization assay, most of the in vitro and all the in vivo assays reported herein were done with X47 and its isogenic *hup* mutant.

Under the normal in vitro growth condition (4% O_2, 5% CO_2), the *H. pylori hup* mutant grew similarly to the wild type strain. During the exponential growth phase (before reaching 24 hours), a similar growth rate was observed for the WT and mutant strain (Fig. 1). Upon entering stationary phase, however, the survival of the *hup* mutant decreased much faster than the wild type strain. As shown in Fig.1, the *hup* mutant lost viability completely after 3 days, while the majority of the wild type cells were still viable. Thus, *H. pylori* Hup protein appears to play a significant role in protecting its genomic DNA during stationary phase when toxic metabolites are accumulated. A similar role of a histone-like protein Hlp in *Mycobacterium smegmatis* was reported; Hlp is essential for cell viability while cells are at a dormant state (long-term stationary phase) [26]. A stationary phase survival defect was also observed for *E. coli* HU-like IHF mutant strain [27]. Upon entering stationary phase, a *Salmonella enterica* mutant lacking IHF displayed down-regulated expression of classic stationary phase genes; and this down-regulation was not caused by a reduction in the level of the RpoS sigma factor [28]. *H. pylori* Hup protein may play a similar role in up-regulating some genes responsible for stationary phase survival.

3.2. *H. pylori* Hup is involved in DNA recombinational repair

Random transposon insertion in the *hup* gene resulted in sensitivity to mitomycin C. In this study, we further examined the sensitivity of the X47 *hup::cat* mutant to MMC, compared to the wild type. The result shown in Fig.2 confirmed that the *hup* mutant is much more sensitive to MMC than the wild type strain. For example, after treatment with 200 ng/ml MMC for 20 min, the viability of the wild type cells is 5 orders of magnitude greater than that of the *hup* mutant.

Mitomycin C causes predominantly DNA intra-strand cross-links, leading ultimately to DNA double strand breaks. Repair of DNA double strand breaks usually requires homologous DNA recombination. Histone-like protein HU from other bacteria was shown to bind preferentially to DNA recombination intermediates [29, 30]. Based on the DNA binding specificity, Ghosh and Grove [30] proposed an in vivo role of the deinococcal HU in stabilizing homologous recombination intermediates rather than to function as an architectural element. Similarly, the most preferable binding substrate of *H. pylori* Hup protein was shown to be four-way junction DNA, the recombination intermediate [19]. Thus, *H. pylori* Hup protein is likely involved in DNA recombinational repair process. To test this hypothesis, we examined whether loss of Hup function had an adverse effect on DNA recombination by determining the frequency of DNA transformation.

As described previously [10-12], 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 [31]. 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 1. Using rpoB3 as donor DNA, the X47 *hup::cat* mutant had a transformation frequency of 2.7×10^{-5}, which was 15-fold lower than that for the wild type strain X47 (4.16×10^{-4}). Using *acnB*:Kan as donor DNA, wild type *H. pylori* X47 had a transformation frequency of 1.09×10^{-5}. In contrast, the transformation frequency for the X47 *hup::cat* mutant was 1.1×10^{-6}, which is 10-fold lower than that of the WT strain. For both types of DNA donor (rpoB3 and *acnB*:Kan), the mutant strain data (transformation frequency) are significantly lower than those of the WT strain, according to Student t-test ($P<0.01$). These results provided evidence that Hup plays a significant role in the DNA recombination process of *H. pylori*. Further investigations are required to understand how Hup protein interacts with other components of the recombinational repair machinery.

3.3. *H. pylori hup* mutant is highly sensitive to oxidative and acid stress conditions

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 immune cells 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 [32]. Further studies showed that mutant cells of *ruvC* [14], *mutS* [33], *mutY* [34], *recN* [11], *addA* (*recB*) [12, 13] or *recRO* [10] were more sensitive to oxidative stress, indicating important roles of DNA recombination and repair in *H. pylori* for the bacterial survival of oxidative damage. To examine the role of the Hup protein in protecting DNA from oxidative damage, we examined the sensitivity of the *hup* mutant 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.3). 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. In contrast, the *hup* mutant 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 8 h time point, the *hup* mutant cells were eliminated (i.e. no viable cells recovered). Previous studies demonstrated that oxidative stress causes damage to *H. pylori* genomic DNA [35, 36]. Thus, *H. pylori* Hup appears to play a significant role in protecting its DNA from oxidative stress-induced damage.

H. pylori survives and colonizes an acidic niche on the gastric surface [37]. 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 DNA from acid-induced damage [38]. Previously, we showed that *H. pylori* DNA recombination proteins RecN and RecRO are involved in repair of acid-induced DNA damage [10, 11]. In this study, we characterized the *hup* mutant for its sensitivity to low pH conditions.

The wild type *H. pylori* or the *hup* mutant cells were treated for 1 h by suspending cells in the buffer at different pH (pH 7.0, pH 5.0, or pH 3.0), and the survival was subsequently determined. As shown in Table 2, treatment at pH 5.0 for 1 h did not have a significant effect on survival of the wild type cells, while the same treatment killed approximately 90% of the *hup* mutant cells. About 40% of the wild type *H. pylori* cells survived the pH 3.0 condition for 1 h, whereas the *hup* mutant cells were almost completely killed (>98% lethality) by the same treatment. Thus, the *H. pylori hup* mutant is more sensitive to acid stress compared to the wild type.

Sensitivity of the *hup* mutant to oxidative stress and acid stress could be interpreted as the Hup protein has an ability to physically protect DNA from stress damage (See section 3.7 below). Alternatively, Hup may regulate expression of other genes involved in stress resistance. In *E. coli*, HU influences the expression of genes involved in anaerobic respiration, the response to osmotic stress, the acid stress, and the response to DNA damage [39-41]. By modeling DNA topology, *E. coli* HU is known to play a role in modulation of transcription profiles which has important impacts on cellular physiology [39, 42]. *S. enterica* HU controls three regulons that coordinate virulence, response to stress and general physiology [43]. Thus, determination of the Hup regulon in *H. pylori* is of interest for future studies.

3.4. *H. pylori hup* mutant is more sensitive to macrophage killing

H. pylori infection induces a strong inflammatory response by the host, with the recruitment of lymphocytes, macrophages, and polymorphonuclear cells; however the bacterium is oftentimes able to resist this immune response and establish a persistent gastric infection. In particular, although *H. pylori* can be efficiently ingested by the different types of phagocytic cells, it is able to survive for prolonged periods within these cells and presumably is able to resist damage by free radicals derived from the phagocytic respiratory burst [8, 21]. The mechanisms known to contribute to survival within macrophages include enzymatic detoxification of reactive oxygen species and DNA repair. For example, absence of the H_2O_2-detoxification enzyme catalase [20] or DNA recombinational repair enzyme RuvC [14] in *H. pylori* resulted in reduction of survival ability within macrophages. Given the sensitivity of an *H. pylori hup* mutant to DNA damaging agents and oxidative stress, we investigated whether the Hup protein contribute to survival of *H. pylori* within macrophages.

A macrophage killing assay was performed using a murine macrophage cell line RAW264.7 (See Materials and Methods). Similar numbers (5×10^8 CFU/ml) of *H. pylori* WT X47 or X47 *hup:cat* mutant cells were inoculated to the macrophage (Fig. 4). After extracellular killing by gentamycin and 2 hours incubation within macrophages, the numbers of surviving cells of the WT and of the *hup* mutant strain were compared. As shown in Fig.4, a mean number of 3.0×10^6 CFU/ml wild type cells survived. In contrast, the same treatment resulted in recovery of a mean number of 4.4×10^5 CFU/ml of the *hup* mutant cells (7 fold less than the WT). Based on statistical analysis (Student t-test), the cell survival differences between the WT and the *hup* mutant strains are significant ($P<0.01$). These results indicate a role for Hup in survival of *H. pylori* within macrophages.

The roles of histone-like proteins in macrophage survival have been recently observed in other bacteria. The histone-like protein Lsr2 knockout strain of *M. smegmatis* survived significantly less well than the wild type strain [44]. The *hupAhupB* double mutant of *S. enterica* was shown to be defective in invasion of epithelial cells and in its ability to survive in macrophages [43]. The observed effects on macrophage survival were ascribed either to direct protection of DNA from oxidative damage by histone-like protein [44] or to HU-mediated global gene regulation in response to stress [43]. Interestingly, expression of a mutant version of HU protein can convert the commensal *E. coli* K-12 to an invasive form with characteristics of host cell invasion, phagosomal disruption, and intracellular replication, suggesting the role of HU in modulating bacterial survival within host cells [45].

3.5. *H. pylori hup* mutant displays an attenuated ability to colonize mouse stomachs

As the *H. pylori hup* mutant showed sensitivity to conditions that *H. pylori* encounters in its physiological niche, namely (oxidative and acid) stress and to macrophage killing, we sought to determine the effect of the Hup on *H. pylori* colonization in the host. We performed an assay using a mouse infection model as described previously [11, 12, 33]. The wild type X47 or the isogenic *hup* mutant strain were inoculated into C57BL/6J mice, and the colonization of *H. pylori* cells in the mouse stomachs was examined 3 weeks after inoculation (Fig.5). *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. In contrast, 9 of 12 mice that were inoculated with the *hup* strain were found to harbor *H. pylori*. The geometric mean of the colonization number for the *hup* strain in the 12 mice was 1.5×10^4 CFU/g stomach. According to Wilcoxin rank test analysis, the range of colonization values of the *hup* strain is significantly lower than that of the wild type at the 99% confidence level ($P<0.01$). These results indicate that *H. pylori* Hup plays a significant role in bacterial survival/colonization in the host.

3.6. Complementation of the *hup* strain

It is unlikely that disruption of the *hup* gene has a polar effect on the downstream genes, as the *hup* gene alone forms a transcriptional unit within the *H. pylori* genome [24, 46]. To ensure that the phenotypes (both in vitro and in vivo) observed for the *hup* strain were completely attributed to inactivation of *hup*, we introduced a functional copy of the *hup* gene back into the *hup* strain. The strain X47 *hup::cat-hup*$^+$ contains a mutated *hup* gene at the original locus and an intact *hup* gene at the *rdxA* locus (See Materials and Methods). The growth characteristics of the complemented strain were similar to the wild type (data not shown).

The sensitivity of the complemented strain to stress conditions was shown to be similar to that of the wild type. For example, both the wild type and the complemented strain can survive exposure to air for 10 h, while the *hup* mutant strain was completely killed by this treatment (Fig. 3). The complementation of *hup* function restored the acid sensitivity to the

wild type level (Table 2). The complementation of *hup* function also restored the transformation frequency to the wild type level, as determined by using rpoB3 fragment as DNA donor (Table 1).

The complemented strain was also examined for the mouse colonization ability. *H. pylori* bacteria were recovered from all 6 mice that had been inoculated with the *hup* complementation strain, with a geometric mean number of 5.2×10^5 CFU/g stomach (Fig.5). According to Wilcoxin rank test analysis, the range of colonization values of the *hup* complementation strain is not significantly different from that of the wild type, but significantly ($P<0.01$) higher than that of the *hup* mutant strain. This indicates that the complementation of the *hup* strain restored its ability to colonize mouse stomachs.

3.7. *H. pylori* Hup protein protects DNA against hydroxyl radical damage

All the phenotypes above observed for the *H. pylori hup* mutant strain, such as stationary phase survival defect, sensitivity to oxidative stress, and lower survival within macrophages, pointed to a role for the Hup in protecting bacterial DNA from oxidative damage. To further support this notion, we sought to demonstrate its biochemical activity using the purified *H. pylori* Hup protein. *H. pylori* Hup is a small protein with a molecular weight of 10 kDa. Chen et al. [19] showed that *H. pylori* Hup protein exists as a dimer in solution and exhibits greater thermal stability compared with other bacterial HU homologues. Substrate specificity for *H. pylori* Hup protein binding was mainly investigated in that study [19]. Here, we focus on the function of the Hup in protecting bacterial DNA from oxidative damages.

A His-tagged *H. pylori* Hup protein was overexpressed in *E. coli* and purified using Ni-NTA affinity column (not shown). The ability of the Hup protein to protect DNA against damage by hydroxyl radicals was studied. A linear 800-bp DNA fragment was PCR amplified by using *H. pylori* genomic DNA as template. The DNA was incubated in vitro with H_2O_2 and Fe(II) in the presence or absence of the purified *H. pylori* Hup protein or a BSA control protein. As shown in Fig.6, the DNA was completely destroyed by treatment with 20 mM $FeSO_4$ plus 5 mM H_2O_2 for 5 min (lane 1 vs. lane 2). Notably, DNA was substantially protected from degradation by pretreatment with the Hup protein (lane 4). As a control, pretreatment with an equivalent concentration of BSA did not result in any protection (lane 6).

Eukaryotic histones are known to protect DNA against hydroxyl radical-induced DNA strand breaks by binding DNA and organizing it into higher order chromatin structures [47-49]. A prokaryotic histone-like protein, *Mycobacterium tuberculosis* Lsr2 was also shown to be able to protect DNA against reactive oxygen intermediates (ROI) [44]. Electron microscopy and DNA binding studies suggested that Lsr2 shields DNA from ROI by binding bacterial DNA and physically protecting it [44]. Here we showed that *H. pylori* Hup protein has the capability to physically protect DNA against hydroxyl radical damage, in addition to its potential role in DNA recombinational repair and gene regulation. This is in agreement with the oxygen sensitivity of the *hup* mutant observed in vitro. This also helps explain the decreased survival of the *hup* mutant within macrophages and its attenuated ability in colonizing host stomachs.

Another group of bacterial proteins that can protect DNA from oxidative damage is Dps family proteins [50, 51]. Dps homologues are expressed in many bacterial species and accumulate to high levels under conditions of oxidative stress or nutritional stress. Dps appears to protect DNA through the dual mechanisms of iron sequestration (preventing Fenton mediated ROI generation) and in DNA binding (creating a protective physical barrier) [50, 52]. Histone-like proteins share a number of physical properties with Dps,

including small size, high isoelectric point, and an ability to bind DNA with little specificity. In cintrast to Dps, histone-like proteins do not bind and sequester iron. *H. pylori* has a Dps homolog named NapA (neutrophil activating protein). *H. pylori* NapA has been well documented to play roles in human neutrophil recruitment and in stimulating host cell production of ROI [53-55]. On the other hand, as a nucleoid-associated protein (NAP), *H. pylori* NapA plays a separate role in protecting its DNA from oxidative stress damage [56, 57]. In a bacterium harboring multiple NAPs, these NAPs usually have overlapping and complementary functions. For example, deletion of HU from the *E. coli* genome is not lethal unless IHF and H-NS are deleted as well [58]. In contrast, the disruption of HU is lethal for organisms in which it is the only NAP available [59-61]. For *H. pylori*, we can easily obtain *hup* single mutant strains which show a normal growth at the exponential growth phase in vitro, but we failed in attempts to knock out both *hup* and *napA* at the same time (i.e. to create a double mutant, our preliminary result), suggesting partially complementary functions of the two proteins.

There is keen interest in host DNA damage and the etiology of carcinogenesis due to chronic inflammation caused by persistent pathogens [62]. Indeed, increased DNA damage of human host cells was shown to be related to *H. pylori* infection saturating host cell repair capacity [63]. At the same time, *H. pylori* seems capable of protecting its own DNA from damage. We propose that Hup and NapA play a critical role in this protection. Interestingly, a recent proteomic analysis identified Hup and NapA as two of only three identified *H. pylori* proteins that showed a greater expression level among strains associated with gastric cancer, suggesting their involvement in gastric carcinogenesis [64].

In conclusion, the results from this study provided evidence that the histone-like protein Hup in *H. pylori* facilitates DNA recombinational repair, and has the capability to physically protect DNA against hydroxyl radical damage. It plays an important role in protecting *H. pylori* DNA from damage elicited by oxidative stress and acid stress, conditions that the bacterium commonly encounters in the host. Furthermore, this protein contributes to *H. pylori* survival in the host stomach, which could be the combined effect due to its roles in DNA protection (physically), recombinational repair, and modulation of gene expression in response to stress. Further investigations are required to determine the roles of Hup in global gene regulation, particularly in possible regulation of virulence genes.

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Highlights

A nucleoid associated histone-like protein is identified in the gastric pathogen Helicobacter pylori

It protects DNA from acid and oxidative stress

It permits the pathogen to be more virulent in a mouse model.

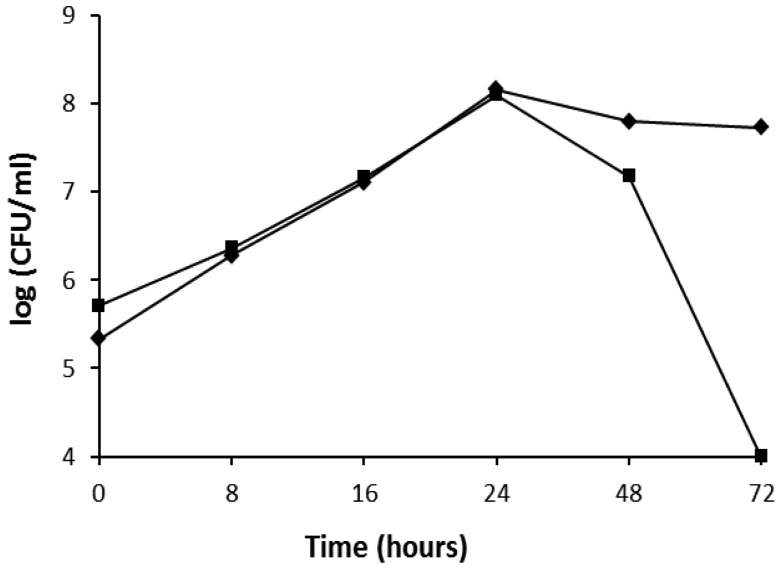

Fig. 1. Growth curve of *H. pylori* cells

H. pylori X47 wild type (diamond) and its isogenic *hup* mutant (square) cells were grown microaerobically (4% O_2, 5% CO_2, and balanced with N_2) at 37°C. At the indicated time points, the number of live cells was determined by serial dilution and plate counting for colony forming units (CFU). The data are from a representative experiment.

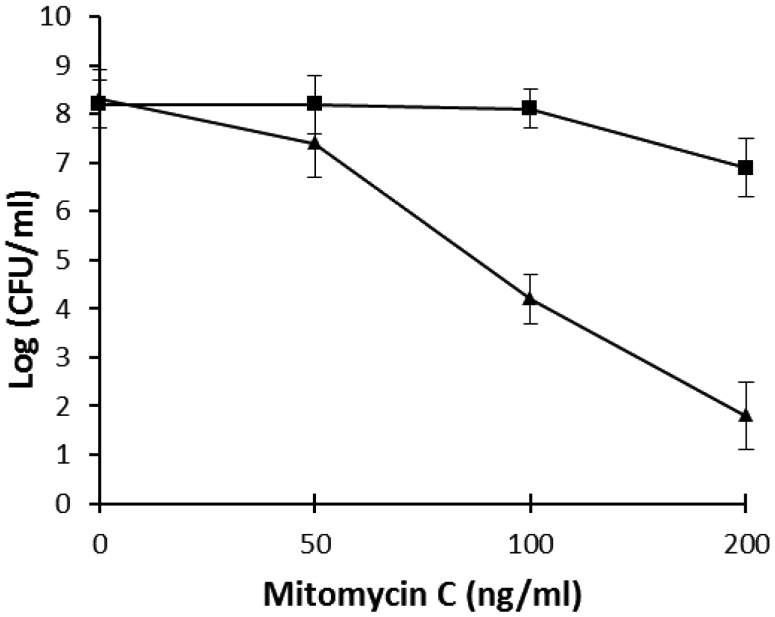

Fig. 2. Sensitivity of *H. pylori* X47 wild type (square) and its isogenic *hup* mutant (triangle) to mitomycin C

H. pylori cell suspensions in PBS were treated with different concentrations (as indicated in the × axis) of mitomycin C for 20 min. The surviving CFU were determined after 4 days incubation under a microaerobic condition. The data are the means of three experiments with standard deviation as indicated.

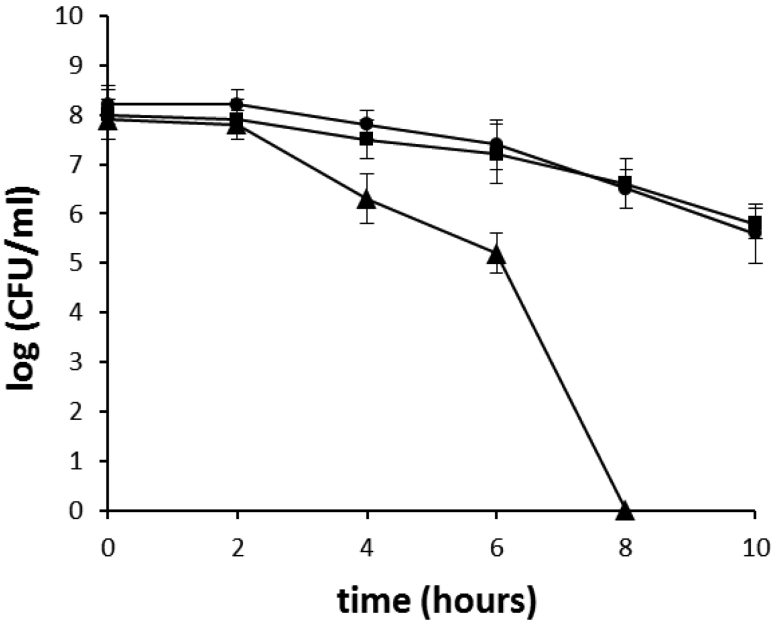

Fig. 3. Survival of *H. pylori* cells upon exposure to air

H. pylori cell suspensions in PBS were incubated at 37°C under normal atmospheric conditions (21% partial pressure O_2). Samples were removed at the times indicated in the x-axis and were used for plate count determinations upon incubation in a 5% oxygen environment. The data are the means of three experiments with standard deviation as indicated. Symbols: square, wild type; triangle, *hup::cat*; circle, *hup* complementation strain. 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.

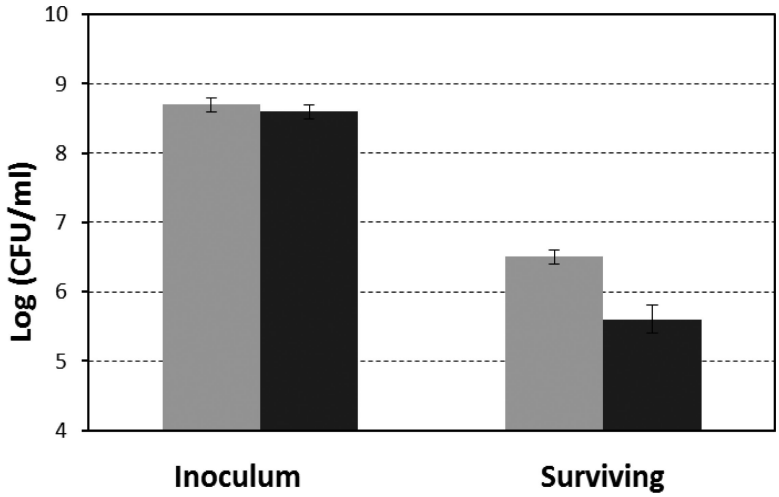

Fig. 4. Survival of *H. pylori* cells in macrophage RAW264.7 cells determined with the gentamycin killing assay

Similar numbers of the WT X47 (gray bars) or X47 *hup:cat* mutant cells (black bars) were inoculated to the macrophages. After extracellular killing by gentamycin and 2 hours incubation within macrophages, the numbers of surviving cells of the WT and the mutant strain were determined. Data are means from three independent determinations with standard deviations.

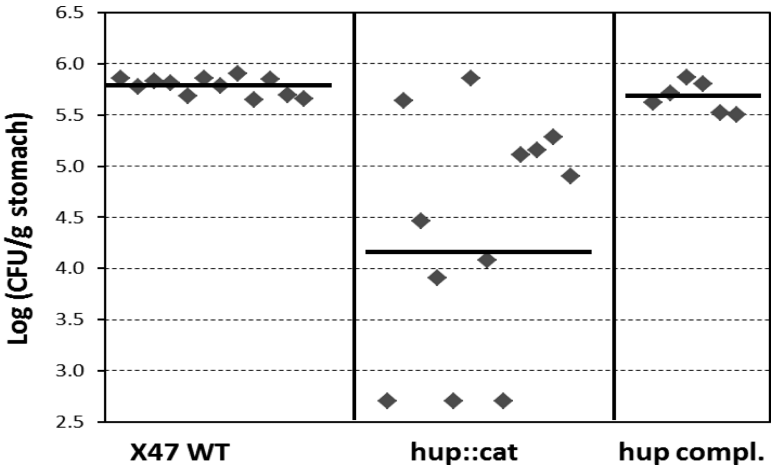

Fig. 5. Mouse colonization results of *H. pylori* strains

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. 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 detection limit of the assay is 500 CFU/g stomach, corresponding to $\log_{10}$ (CFU/g) =2.7.

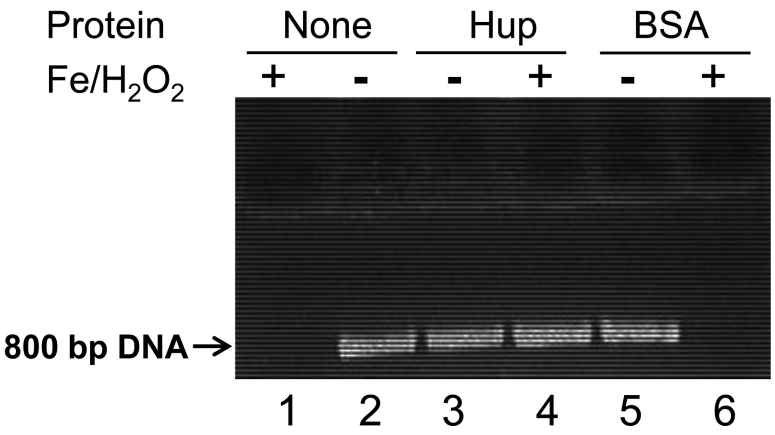

Fig. 6. DNA protection activity of *H. pylori* Hup protein

Linear DNA (~0.2 μM, 800 bp PCR fragment of *H. pylori* genomic DNA) was used alone (lane 1, 2) or after incubation with either 2 μM of purified Hup (lane 3, 4) or BSA (lane 5, 6). Both proteins were suspended in identical imidazole buffers. Samples were treated for 5 min with either 20 mM $FeSO_4$ plus 5 mM H_2O_2 (lane 1, 4, 6) or left untreated (lane 2, 3, 5). The samples were analyzed on an ethidium bromide stained 1% agarose gel

Table 1

Transformation frequency with different types of donor DNA

	Donor DNA	
***H. pylori* strains**	**rpoB3 (330 bp)**	**acnB:Kan (2.5 kb)**
X47 WT	41630 ± 6190	1090 ± 166
X47 *hup::cat*	2710 ± 693	110 ± 13
X47 *hup::cat-hup*$^{+}$	48750 ± 8738	ND

The transformation frequencies are presented as the number of transformants (resistant colonies) per 10^8 recipient cells. Data are means ± standard errors from three independent determinations. ND, not determined.

Table 2

Acid sensitivity of *H. pylori* strains.

	% survival at [a]:		
Strains	**pH 7.0**	**pH 5.0**	**pH 3.0**
X47 WT	100	94 ± 7	39 ± 6
X47 *hup::cat*	100	8.0 ± 3.7	1.6 ± 1.2
X47 *hup::cat-hup*$^{+}$	100	95 ± 8	41 ± 4

[a]The values for pH less than 7.0 are the percentages of cell survival after treatment for 1 h at pH 5.0 or pH 3.0 relative to the survival at pH 7.0. The data are the means ± standard errors from three independent determinations.