Characterization of a \textit{Helicobacter hepaticus} putA Mutant Strain in Host Colonization and Oxidative Stress\textsuperscript{V,†} \\
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\textit{Helicobacter hepaticus} is a gram-negative, spiral-shaped microaerophilic bacterium associated with chronic intestinal infection leading to hepatitis and colonic and hepatic carcinomas in susceptible strains of mice. In the closely related human pathogen \textit{Helicobacter pylori}, \textit{l}-proline is a preferred respiratory substrate and is found at significantly high levels in the gastric juice of infected patients. A previous study of the proline catabolic PutA flavoenzymes from \textit{H. pylori} and \textit{H. hepaticus} revealed that \textit{Helicobacter} PutA generates reactive \textit{oxygen species} during \textit{proline} oxidation by transferring electrons from reduced flavin to molecular oxygen. We further explored the preference for proline as a respiratory substrate and the potential impact of proline metabolism on the redox environment in \textit{Helicobacter} species during host infection by disrupting the \textit{putA} gene in \textit{H. hepaticus}. The resulting \textit{putA} knockout mutant strain was characterized by oxidative stress analysis and mouse infection studies. The \textit{putA} mutant strain of \textit{H. hepaticus} exhibited increased proline levels and resistance to oxidative stress relative to that of the wild-type strain, consistent with proline’s role as an antioxidant. The significant increase in stress resistance was attributed to higher proline content, as no upregulation of antioxidant genes was observed for the \textit{putA} mutant strain. The wild-type and \textit{putA} mutant \textit{H. hepaticus} strains displayed similar levels of infection in mice, but in mice challenged with the \textit{putA} mutant strain, significantly reduced inflammation was observed, suggesting a role for proline metabolism in \textit{H. hepaticus} pathogenicity in vivo.

\textit{Helicobacter hepaticus} is a gram-negative, spiral-shaped microaerophilic bacterium. The organism was first isolated from the liver tissues of mice that had a high prevalence for hepatitis and liver tumors. Subsequently, \textit{H. hepaticus} was found to be the causative agent of hepatic and intestinal inflammation in mice, leading to hepatic and intestinal tumorigenesis (11–14). Although it is frequently isolated from the liver and associated with hepatitis, like the other enterohepatic \textit{Helicobacter} species (EHS), \textit{H. hepaticus} colonizes primarily the lower bowel, where it is associated with persistent typhlocolitis (13, 17, 20, 28, 43, 55, 56). About 59\% of mice bred for research purposes have been shown to carry \textit{H. hepaticus} alone or in combination with other EHS (49). The potential impact of \textit{Helicobacter} infection of laboratory mice on biomedical research is therefore a major concern. \textit{H. hepaticus} is also the prototype EHS, which makes it an excellent candidate for understanding the molecular basis of intestinal and hepatic infections associated with EHS in general.

\textit{H. hepaticus} is closely related to the gastric human pathogen \textit{Helicobacter pylori}, which is responsible for acute gastric inflammation that can then progress from superficial gastritis to peptic ulceration and gastric cancer. \textit{H. pylori} is classified by the World Health Organization as a group 1 carcinogen (8, 31–33). One of the important features in the pathogenesis of \textit{Helicobacter} infection is its ability to establish persistent colonization within the gastrointestinal mucosa. The initial stage of \textit{H. pylori} colonization is associated with considerable neutrophil exudation along the gastric mucosa (38). The reactive \textit{oxygen species} (ROS) produced during the neutrophil response are thought to play an important role in the oxidative damage to the host mucosa (42). To combat this oxidative stress, \textit{Helicobacter} uses a number of different antioxidant systems (52).

The focus of this study was to investigate the role of proline metabolism in oxidative stress and infection by \textit{H. hepaticus}. Previous studies have implicated proline as having an important role in \textit{Helicobacter} species’ ability to colonize and persist at the host mucosal surfaces. Nagata et al. showed that the gastric juice of patients infected with \textit{H. pylori} had 10-fold higher levels of the amino acid proline than that of noninfected individuals (40). They also showed that the concentration of \textit{l}-proline was higher than all the other amino acids in cultured \textit{H. pylori} cells and that \textit{l}-proline was a preferred respiratory substrate, along with \textit{l}-serine (40). These observations suggested that in the gut environment, proline serves as a key energy source for this gastrointestinal pathogen. Using signature-tagged mutagenesis, it has been shown that the high-affinity proline-specific transporter gene \textit{putP} is one of the 47 genes absolutely essential for gastric colonization by \textit{H. pylori} (22). There is also evidence that the human serum prolidase enzyme is upregulated during the course of \textit{H. pylori} infection (2). Prolidase is an iminodipeptidase that releases proline or hydroxyproline from the carboxy terminus of a glycine-proline dipeptide, thereby increasing serum proline levels (51). Taken

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together, these observations suggest an important role for proline metabolism in *H. pylori* colonization and pathogenesis.

Proline oxidation in gram-negative bacteria is catalyzed by the bifunctional PutA flavoenzyme, which has two catalytic domains in a single polypeptide (19, 25). PutA from *E. coli* (PutA<sub>Ec</sub>) and *H. pylori* (PutA<sub>Hp</sub>) share about 64% amino acid sequence identity (26). The first step in proline oxidation is performed by the flavin adenine dinucleotide-dependent PRODH reaction (Fig. 1, reaction scheme), which couples the 2e− oxidation of proline to the reduction of the electron chain transport system of the cytoplasmic membrane. The product of the PRODH reaction, α1-pyrroline-5-carboxylate (PSC), is subsequently hydrolyzed to glutamate-γ-semialdehyde (GSA), which is then oxidized to glutamate by the NAD+−dependent PSCDH domain of PutA (4). Glutamate that is formed by the oxidation of proline eventually enters the tricarboxylic acid cycle via α-ketoglutarate. In a previous study, PutA<sub>Ec</sub> and PutA<sub>Hp</sub> were shown to transfer electrons to molecular oxygen during catalytic turnover with proline, forming superoxide anion and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (Fig. 1) (26). This oxygen reactivity during turnover with proline is essentially absent in the PutA protein from *Escherichia coli*. The impact of *Helicobacter* PutA reactivity with oxygen was assessed by oxidative stress studies with an *E. coli* model system that demonstrated that the enzyme activities of PutA<sub>Ec</sub> and PutA<sub>Hp</sub> are toxic to *E. coli* (26). Thus, it seemed plausible that proline metabolism in *Helicobacter* species may lead to changes in the redox environment by the formation of superoxide anion by PutA and the subsequent decrease in the levels of proline, an imino acid that has been shown to act as a potent antioxidant in yeast, plants, and mammalian cells (6, 7, 27, 46).

To better understand the role of proline metabolism in disease associated with *Helicobacter* species infection, a putA mutant strain of *H. hepaticus* was made by cassette mutagenesis of the *putA* gene. The *putA* gene knockout strain was compared to the wild-type *H. hepaticus* strain to assess proline metabolism in oxidative stress and infection. The present study suggests that proline metabolism in *H. hepaticus* can modify the redox environment in vitro and that it affects pathogenicity in vivo.

**MATERIALS AND METHODS**

Chemicals, bacterial strains, and culture conditions. All chemicals and buffers, unless otherwise noted, were purchased from Fisher Scientific and Sigma-Aldrich, Inc. Restriction endonucleases and T4 DNA ligases were purchased from Fermentas and Promega, respectively. Cloning and genetic manipulations were performed with *H. hepaticus* strain ATCC 51449<sup>T</sup> and *Escherichia coli* DH5α (BRL). *H. hepaticus* was grown on brucella agar plates (Difco) supplemented with 10% defibrinated sheep blood (BA) without any antibiotics. The *H. hepaticus putA* mutant strain was grown on BA plates supplemented with kanamycin (30 μg/ml). Both the wild-type and the *putA* mutant strains were grown in a microaerobic environment (5% CO<sub>2</sub> and 1% O<sub>2</sub>) at 37°C. *E. coli* was grown aerobically at 37°C in Luria-Bertani medium containing ampicillin (100 μg/ml) or chloramphenicol (34 μg/ml). For analysis of the *H. hepaticus* population in fecal and tissue matter, samples were plated on BA medium containing amphotericin B (10 μg/ml), vancomycin (10 μg/ml), and cefoperazone (20 μg/ml). The sequences of all the primers used in this study are provided in Table S1 in the supplemental material.

Construction of the *H. hepaticus* putA mutant strain by insertional mutagenesis. The *putA* gene was amplified using *H. hepaticus* ATCC 51449<sup>T</sup> genomic DNA as a template and the *putA*-specific primers (see Table S1 in the supplemental material). The PCR fragment was ligated into a pGEM-T vector (Promega) according to recommendations of the manufacturer to generate the construct pGEM-T:*putA*. Subsequently, a kanamycin resistance cassette (1.1 kb) was inserted into the *putA* gene (3.505 kb) using unique AflII and XhoI sites that were incorporated within the *putA* gene by QuikChange (Stratagene) site-directed mutagenesis at bp 745 (AflII primer) and 2728 (XhoI primer), respectively. The resulting construct containing the kanamycin insertion (pGEM-T:*putA::Kan) was introduced into *H. hepaticus* ATCC 51449<sup>T</sup> by electroporation (2.5-kV pulse; Gene Pulser; Bio-Rad). Transformants were then plated on kanamycin-BA plates to isolate the *putA* mutant strain. Insertion of the *H. hepaticus* gene into the *putA* gene of *H. hepaticus* was confirmed by PCR using the *putA*-specific primers described above. PCR conditions were 30 s at 95°C, 1 min at 52°C, and 4 min at 72°C for 30 cycles.

Growth of the wild-type and *putA* knockout mutant strains. The impact of disrupting the *putA* gene and proline catabolism on cell growth, proline content, and antioxidant gene expression was assessed by comparing the wild-type and the *putA* mutant *H. hepaticus* strains grown on BA plates under microaerobic conditions. The *H. hepaticus* wild-type and *putA* mutant strains were grown onBA plates and diluted in brucella broth medium to ~1.0 × 10<sup>9</sup> cells/ml, assuming that an optical density at 600 nm (OD<sub>600</sub>) of 1.0 is ~1.6 × 10<sup>9</sup> cells/ml, as previously described (37). Cells (3.0 × 10<sup>9</sup>) were then plated on fresh BA plates and grown for 0, 24, 48, 96, 144, and 192 h. The number of cells at 0 h was 3.0 × 10<sup>9</sup> cells/ml. Cell numbers from 24 to 192 h were monitored by resuspending cells from the BA plates at different time points in brucella broth medium to a final volume of 1 ml and estimating the number of cells, assuming that an OD<sub>600</sub> of 1.0 is ~1.6 × 10<sup>9</sup> cells/ml (37). The cell suspensions were used for determining proline content and for analysis of PutA, superoxide dismutase (SOD), and catalase expression as described below. Measurements at 0 h were performed with cell suspensions before the cells were plated on BA plates.

**Quantitation of proline in the wild-type and *putA* mutant strains.** The cell suspensions as described above were used for determining proline content. The wild-type and *putA* mutant strains grown on BA plates under microaerobic conditions were harvested by centrifugation, resuspended in 0.5 ml of sterile water, and lysed by boiling for 10 min. The amount of proline was quantitated by PRODH activity assays using the *E. coli* PRODH domain construct, EcPutA<sub>86-630</sub>, which was purified as previously described (59). Briefly, proline:dichlorophenolindophenol oxidoreductase assays were performed in 50 mM Tris buffer (pH 7.5) as previously described, except for the use of EcPutA<sub>86-630</sub> (100 μM) and 100 μl of cell extract (60). A standard curve of EcPutA<sub>86-630</sub> PRODH activity from 0 to 0.5 mM proline in the assay was used to determine the amount of proline in the total cell lysates.

**Western blotting analysis.** Antiserum directed against purified recombinant PutA<sub>Ec</sub> was prepared by ProteinTech Inc. Recombinant PutA<sub>Ec</sub> was purified as previously described (26). Cells (~3 × 10<sup>10</sup> were lysed by gentle sonication for 1 min (15-s pulse on, 30-s pulse off) in ice-cold phosphate-buffered saline (PBS) buffer containing 0.2% Triton, unless otherwise indicated, and the lysates were centrifuged for 10 min at 16,000 × g at 4°C. Total protein concentration in the solubilized portion of the cell lysates was determined by using bichinonic acid (Pierce) and bovine serum albumin as a standard. Samples of 20 μg or 50 μg of total protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted onto a polyvinylidene difluoride immunoblotting membrane described (27). The polypeptide band corresponding to the 44-kDa protein was visualized by using a monoclonal anti-Flag–fluorescein isothiocyanate conjugate (Sigma-Aldrich) and were visualized by using a LI-COR Odyssey imager as previously described.

![FIG. 1. Generation of H<sub>2</sub>O<sub>2</sub> by PutA in *H. hepaticus*. Shown are the reactions catalyzed by the PRODH and the PSC dehydrogenase (PSCDH) domains of PutA. During the oxidation of proline, the PRODH domain of PutA can catalyze the transfer of electrons from reduced flavin adenine dinucleotide to molecular oxygen, leading to H<sub>2</sub>O<sub>2</sub> formation. The intermediates in the overall conversion of proline to glutamate are PSC and glutamate-γ-semialdehyde (GSA).](http://iai.asm.org/content/2/5/3038/F1.large.jpg)
described (27). PutA enzymes from other bacterial sources such as E. coli were not recognized by the PutA_{Hhe} antiserum.

**Oxidative stress studies.** Oxidative stress was induced by using three different cytotoxic agents and was evaluated by disk assays (54). Prolinemediated stress was also studied by the same method using 1 M proline. Sterile filter paper disks, 7.5 mm in diameter, containing 10 μl of 1 M hydrogen peroxide (H₂O₂), 0.2 M tert-butyl hydroperoxide (tBHP), and 50 mM paraquat were placed on BA plates (100 by 15 mm) that had been previously streaked for confluent growth with either the putA mutant or wild-type cells. The plates were then placed in a 2% O₂ incubator. Following a 48-h incubation period, the clear zones or inhibition zones surrounding the disks were measured. The zone of inhibition represents the distance from the edge of the disk to where growth begins. No growth inhibition was seen with disks containing only H₂O₂. The mean zone inhibition diameter for the wild-type and the putA mutant H. hepaticus strains with each oxidant was obtained from three separate experiments (six samples per experiment). The statistical significance of the data was assessed using Student’s t test (two-tailed). Differences observed between the parent and the mutant strains were considered statistically significant at a P value of <0.05.

**RNA isolation and RT-PCR.** Expression of the putA, the neutrophil-activating protein (napA), and the proline transporter (putP) genes in the wild-type and the putA mutant H. hepaticus strains was analyzed using cells grown on BA plates under microaerobic conditions. Expression profiles of SOD (sodF), catalase (katA), alkyl hydroperoxide reductase TsaA (tacA), and thiol peroxidase (tpx) were assessed in the wild-type and putA mutant H. hepaticus strains, using ~3 × 10⁸ cells harvested at 0, 24, 48, 96, 144, and 192 h of growth on BA plates, as described above. For RNA isolation, cells were lysed in 50 mM Tris-HCl (pH 8.0) with 1 mM EDTA and 50 mM NaCl supplemented with 1.5% sodium dodecyl sulfate (SDS) and 50°C. Total RNA was extracted and purified using TRIzol (Invitrogen) and then dissolved in diethylpyrocarbonate-treated water. The concentration of the isolated RNA was then determined at 260 nm. The collected RNA was reverse transcribed to synthesize total cDNA in the presence of 1 μM of random hexamers in a final volume of 20 μl. All reagents were from Invitrogen. Reactions were carried out in an MJ mini-thermal cycler unit (Bio-Rad) for 1 cycle at 95°C for 3 min and 40 cycles each of 95°C for 30 s and 65°C for 30 s.

**Quantitative analysis of liver and cecum.** Because H. hepaticus grows as a continuously spreading colony, counting bacterial colonies is not possible. Instead, real-time PCR was used to estimate H. hepaticus populations in different tissues as previously described (13, 15, 37, 58). DNA was extracted from 25 mg of homogenized liver and cecum tissue, using a DNeasy tissue kit (Qiagen). Real-time PCR (Cycler Thermal Cycler) was performed using 6-carboxyfluorescein-labeled H. hepaticus-specific cdT primers (see Table S1 in the supplemental material), which were designed using the PLEXor primer design tool from Promega. A BLAST search against known genomes was used to verify that the cdT primers and the cdB gene probe (coding for subunit B of the cytotoxic distending toxin) were highly specific for H. hepaticus. Real-time PCR analysis was performed by using a 25-μl mixture containing 12.5 μl of Supermix (Promega), 200 nM concentrations each of the forward and reverse primers, and 5 μl of tissue DNA (equivalent to 1.0 mg of tissue). Conditions for real-time PCR were 1 cycle at 95°C for 3 min and 40 cycles each of 95°C for 30 s and 65°C for 30 s. Real-time PCR generated an amplified product of 110 bp. A standard curve (not shown) was generated from H. hepaticus genomic DNA samples ranging from 10⁵ to 10⁹ fg, from which the number of genome copies was estimated for each sample as described previously (37).

**Histopathological studies.** Tissue specimens consisting of the median lobe of the liver, the cecum, the proximal colon, and the spleen were fixed overnight in 10% buffered formalin, processed, and embedded in paraffin. Sections (5 μm thick) were stained with hematoxylin and eosin (H&E) and examined microscopically. The histopathological scores of tissues were quantified from the infected and putA mutant H. hepaticus-infected cecal and hepatic lesions were analyzed by the nonparametric Mann-Whitney test, using SPSS statistical software, with statistical significance set at a P value of <0.05.

### Results

**Disruption of the putA gene.** The putA gene in H. hepaticus was disrupted by the insertion of a kanamycin cassette. Insertion of the cassette was confirmed by a comparison of the ~4.6-kb PCR product for the putA mutant with the ~3.5-kb product for the wild-type strain. Disruption of the gene was also confirmed at the transcript and protein levels by RT-PCR and by Western blotting analysis, respectively (Fig. 2). RT-PCR targeting the putA cDNA did not give any product for the putA mutant strain, whereas significant product was observed with the wild-type strain. Figure 2 shows an ~3.5-kb band from wild-type H. hepaticus, indicating expression of the full-length putA gene. Additional smaller-sized bands were also observed with the wild-type H. hepaticus strain and were most likely due to off-target priming events within the cDNA of putA. Western blotting analysis was performed using antibodies raised against purified full-length H. hepaticus PutA. Immunoreactive bands of the expected size (133.4 kDa) were not observed for the mutant strain, but a 133-kDa band corresponding to PutA_{Hhe} was detected with the wild-type strain. To establish that there were no polar effects, the expression levels of the neighboring upstream putP and downstream napA genes were tested in both the wild-type and the mutant strains by RT-PCR. The putP gene encodes a high-affinity proline transporter, while the napA gene encodes the neutrophil-activating protein. Both strains showed similar levels of expression of the putP and napA genes, indicating that these genes were not disrupted by
the kanamycin cassette mutagenesis of the putA gene (data not shown).

Increased oxidative stress and proline toxicity resistance of the putA mutant strain. We characterized the in vitro oxidative stress response for the wild-type and the putA mutant strains by performing paper disk assays with hydrogen peroxide (1 M), \( t \)BH (0.2 M), and paraquat (0.05 M). The putA mutant strain exhibited a zone-of-inhibition diameter with \( H_2O_2 \) that was 2-fold smaller than that of the wild-type strain (Table 1). With \( t \)BH and paraquat, the putA mutant strain had a 3-fold smaller zone-of-inhibition diameter than the wild-type strain (Table 1).

Thus, the mutant strain exhibited significantly higher resistance to oxidative stress than the parental wild-type strain. Because PutA has been shown to produce ROS in vitro, we performed paper disk assays with hydrogen peroxide (1 M), \( t \)BH (0.2 M), and paraquat (0.05 M). The putA mutant strain exhibited a zone-of-inhibition diameter with \( H_2O_2 \) that was 2-fold smaller than that of the wild-type strain (Table 1). With \( t \)BH and paraquat, the putA mutant strain had a 3-fold smaller zone-of-inhibition diameter than the wild-type strain (Table 1).

Thus, the mutant strain exhibited significantly higher resistance to oxidative stress than the parental wild-type strain. Because PutA has been shown to produce ROS in vitro, we determined whether the addition of proline also leads to stress and cell death. After a wide range of proline concentrations was tested, 1 M proline was used on the filter disks for these experiments. The wild-type strain showed a high sensitivity to oxidative stress. In contrast, proline did not have any effect on the putA mutant strain, as no inhibition zone was observed. These observations agree with our previous results in which the overexpression of PutAHh in E. coli proved to be toxic to the cells when it was supplemented with proline (5 mM) (26). Here, it is shown that the enzymatic action of PutAHh is also toxic to H. hepaticus when proline levels are elevated.

Increased levels of proline in the putA mutant strain. Cells were grown on BA plates for up to 192 h. No significant differences were observed between the growth rates of the wild-type and those of the putA mutant strains (Fig. 3A). Proline content in the cells was measured at different time points during growth. The mutant strain had higher levels of intracellular proline than the wild-type strain under normal growth conditions (Fig. 3B). The proline levels were about 2-fold higher in the mutant strain than in the wild-type strain throughout the 192-h growth period. This result is in agreement with the mutant strain’s lack of ability to catabolize proline in the absence of PutA. As proline has been suggested to be an antioxidant with radical scavenging properties (1), the higher levels of proline in the putA mutant strain may explain the increased stress resistance observed with the mutants, along with the lack of PutA-generated ROS.

Antioxidant gene expression levels. To better understand the reason for the increased resistance to oxidative stress observed with the putA mutant strain, mRNA levels of two main antioxidant genes, sodF and katA, were determined for the wild-type and for the putA mutant strains. Under normal growth conditions, the transcript levels of sodF and katA were significantly higher in the wild-type strain than in the putA

![FIG. 2. RT-PCR and Western blotting analyses confirm the disruption of the putA gene in H. hepaticus. (A) RT-PCR performed with putA gene-specific primers. Lanes: 1, kb ladder; 2, wild-type H. hepaticus strain; 3, empty; 4 to 7, different colonies screened for putA deletion. (B) Western blotting analysis of protein extracts from the wild-type and putA H. hepaticus strains. Two different amounts of total protein were loaded for each cell lysate sample. Lanes: 1 and 2, putA mutant cell lysates (20 \( \mu \)g and 50 \( \mu \)g, respectively); 3 and 4, wild-type cell lysates (50 \( \mu \)g and 20 \( \mu \)g, respectively); 5 and 6, purified recombinant PutAHh (20 \( \mu \)g and 50 \( \mu \)g, respectively).](http://iai.asm.org/)

**TABLE 1. Disk oxidative stress assays**

<table>
<thead>
<tr>
<th>H. hepaticus strain</th>
<th>Mean inhibition zone diam (mm) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Water ( H_2O_2 ) ( 1 ) M</td>
</tr>
<tr>
<td>Wild type</td>
<td>None</td>
</tr>
<tr>
<td>putA mutant</td>
<td>None</td>
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* Zones of inhibition were measured from filter paper disks treated with 10 \( \mu \)l of the indicated compounds. Water was used as the control, which did not result in any zones of growth inhibition. Results are the means ± standard deviations (SD) from three separate experiments. The inhibition zones for the putA mutant strain with \( H_2O_2 \), paraquat, and \( t \)BH are significantly lower than those for the parental wild-type strain at the 95% level of confidence (\( P < 0.05 \)), based on Student’s \( t \) test.

![FIG. 3. (A) Growth curves for the wild-type (solid curve) and the putA mutant (dashed curve) H. hepaticus strains incubated on BA plates for 0, 24, 48, 96, 144, and 192 h. Cells were resuspended in brucella medium for OD measurements at each time point. (B) Proline content for the wild-type and putA mutant H. hepaticus strains at 0, 24, 48, 96, 144, and 192 h of growth on BA plates. Approximately \( 3 \times 10^8 \) cells were used for determining proline content at each time point.](http://iai.asm.org/)
mutant strain over the entire 192-h growth period (Fig. 4A). Transcript levels of the \( \text{tsaA} \) (alkyl hydroperoxide reductase) and \( \text{tpx} \) (thiol peroxidase) genes were also checked, but no significant differences were observed between the expression of the wild-type and that of the \( \text{putA} \) mutant strains. The expression of PutA was confirmed in wild-type \( H. \text{hepaticus} \) during the same growth period used for monitoring SOD and catalase expression. Western analysis shows that PutA protein levels appear constant from 0 to 192 h (Fig. 4B), consistent with the correlation between PutA expression and higher SOD and catalase levels in the wild-type \( H. \text{hepaticus} \) strain. Thus, SOD and catalase expression may be required to protect cells from ROS generated by PutA during turnover with proline.

**Analysis of \( H. \text{hepaticus} \) colonization in liver, cecum, and feces in mice.** A/J mice were inoculated with \( H. \text{hepaticus} \) wild-type and \( \text{putA} \) mutant strains. A/J mice inoculated with sterile PBS served as negative controls. Twenty-one days after inoculation, liver and cecum homogenates, as well as fecal suspensions, from each mouse were collected and plated on BA plates. No growth was observed for plates with homogenates from the control mice. Growth was observed with homogenates from both the \( \text{putA} \) mutant and the wild-type strains, indicating colonization of \( H. \text{hepaticus} \) in the liver and cecum. Real-time PCR of the \( H. \text{hepaticus} \) \( \text{cdtB} \) gene was also used to quantify the colonization of the wild-type and mutant strains. A standard plot was used to estimate the genome copy numbers based on quantitation of the \( \text{cdtB} \) gene. Similar genome copy numbers were observed in the livers and cecum from mice inoculated with either the wild-type or the \( \text{putA} \) mutant strains (data not shown). There was no PCR product detected in the tissues obtained from the negative-control mice. The average genome copy among all samples obtained from mice inoculated with either the \( H. \text{hepaticus} \) wild type or the mutant strain were in the range of \( 10^2 \) (liver) to \( 10^3 \) (cecum). Thus, there was no significant difference between the genome copy number of the wild type and that of the \( \text{putA} \) mutant strain. This strongly suggests that PutA expression is not important for colonization of \( H. \text{hepaticus} \) in the liver or the cecum.

**Histopathology results.** The pathological changes associated with \( H. \text{hepaticus} \) infection in mice are very well documented (37, 39). The histopathologies of the liver, colon, spleen, and cecum were examined in the control mice (10 animals), the mice inoculated with wild-type \( H. \text{hepaticus} \) (10 animals), and the mice inoculated with the \( \text{putA} \) mutant strain (9 animals). No significant gross changes were seen in the livers, intestines, and other visceral organs of the control mice, the mice infected with the \( H. \text{hepaticus} \) wild type, and the \( \text{putA} \) strains at necropsy. In the liver, as in every vascularized tissue, cell death (necrosis) evokes an inflammatory reaction which is also the hallmark of \( H. \text{hepaticus} \) infection. Figure 5 summarizes the histopathology scoring results of liver samples from mice in each group. The control mice did not show any evidence of liver infection, as no portal or lobular inflammation or biliary lesions were present. All the mice that were inoculated with the wild-type \( H. \text{hepaticus} \) strain clearly showed portal inflammation. Mild inflammation was observed in 8/10 animals, and moderate inflammation was seen in 2/10 mice. In contrast, significantly less inflammation (\( P \) value, 0.003) was found in mice challenged with the \( \text{putA} \) mutant strain than in mice infected with wild-type \( H. \text{hepaticus} \). Only 4/9 mice infected with the \( \text{putA} \) mutant strain exhibited mild portal inflammation, and none of the mice was scored for moderate inflammation. Mild to moderate lobular inflammation or hepatocellular necrosis was present in the 10 mice inoculated with the wild-type \( H. \text{hepaticus} \) strain, while only 2/9 mice inoculated with the \( \text{putA} \) mutant strain exhibited mild lobular inflammation. Biliary lesions and oval cell changes were also seen in 8/10 mice inoculated with the wild-type strain, whereas only 1/9 mice inoculated with the \( \text{putA} \) mutant strain had similar changes. The differences observed between the lobular inflammation (\( P \) value, 0.007) and biliary lesions/oval changes (\( P \) value, 0.007) in mice infected with the wild-type strain and those of mice infected with the \( \text{putA} \) mutant \( H. \text{hepaticus} \) strain are considered statistically significant according to the Mann-Whitney test. Inflammation was not observed in the cecum, colon, or spleen from any of the mice.
from oxidative stress is through maintaining reduced glutathione (27, 44). High levels of proline appear to support cell proliferation (40, 50). These interesting observations led us to characterize the PutA proteins from *H. pylori* and from *H. hepaticus*. We found that similar to human PRODH, PutA from *Helicobacter* spp. generates superoxide anion during turnover with proline and molecular oxygen (26). The oxidase activities of PutA<sub>Hh</sub> and PutA<sub>Hp</sub> were considered unusual, since PutA homologs from other gram-negative bacteria exhibited very minimal oxidase activity. The oxidase activities of PutA<sub>Hh</sub> and PutA<sub>Hp</sub> proved to be toxic to *E. coli* and severely inhibited cell survival (26). Thus, in addition to proline’s role as an energy substrate, it seemed plausible that proline metabolism may have a dual effect on the redox environment in *H. hepaticus*, with PutA activity generating ROS and proline accumulation providing protection against oxidative stress. Therefore, it was of interest to test the pathophysiological roles of PutA and proline metabolism in *H. hepaticus*.

To explore the impact of proline metabolism on *H. hepaticus* physiology and infection, a putA mutant strain of *H. hepaticus* was generated. The mutant strain was found to accumulate more proline and was significantly more resistant to oxidative stress than the wild-type strain. The ability of the mutant strain to survive high concentrations of oxidants indicates that the absence of PutA causes a change in the redox environment which is favorable for cell survival. The main reason for the increased resistance to oxidative stress exhibited by the putA mutant strain may be due to the hydroxy radical-scavenging properties of proline (27). Alternatively, expression levels of the antioxidant enzymes may be higher in the putA mutant strain. We found the opposite trend, however, as the wild-type strain exhibited higher expression levels of SOD and catalase than the putA mutant strain. Thus, proline appears to serve as a cytoprotectant in *H. hepaticus*, a role which has not been demonstrated previously in this pathogen.

High levels of proline were also shown to be toxic to the *H. hepaticus* wild-type strain but had no lethal effect on the putA mutant strain. These results provide evidence that PutA activity causes proline toxicity and cell death in the *H. hepaticus* wild-type strain. The toxic effects of PutA may be mediated by ROS formation or by production of the intermediate P5C, which has been shown to induce apoptosis in mammalian cells (35). As reported in a previous study, *Helicobacter* most likely resides in ecological niches that have elevated proline levels (40); therefore, it must balance the beneficial properties of proline metabolism with the harmful prooxidant effects of PutA activity. The unexpected increase in the sodF and katA gene expression in the wild-type cells relative to that in the putA mutant strain under normal growth conditions suggests that *H. hepaticus* uses SOD and catalase to combat the adverse effects of PutA activity. Because earlier studies with *H. pylori* have shown that proline is an important respiratory substrate, *H. hepaticus* may use SOD and catalase to control the toxic side effects of PutA activity while benefiting from the energetic and growth features of proline metabolism.

Analysis of tissue samples showed no significant differences between colonization of the liver and cecum of mice inoculated with the *H. hepaticus* wild type and that of mice inoculated with the putA mutant strain, indicating that the disruption of the putA gene does not affect the colonization ability of *H. hepaticus*. Histopathology examination of the liver tissue from mice infected with the putA mutant strain, however, exhibited significantly less inflammation than liver samples from mice inoculated with the wild-type strain. This suggests that although PutA may not be critical for colonization of the host, PutA and proline metabolism help to promote the pathogenesis of *H. hepaticus*. Inflammation observed with the liver samples is thought to be due primarily to neutrophil activation and ROS.

**DISCUSSION**

To combat oxidative stress and persist in the gastric mucosa successfully, *Helicobacter* species are endowed with a very effective antioxidant system (23, 47, 52, 53). *Helicobacter* spp. are also capable of producing ROS for signaling and regulating the redox environment at the site of infection (52, 53). Proline metabolism has been associated with a number of different ecological niches, with proline having multifaceted roles in abiotic stress protection and energy utilization (16, 24, 27, 50).

In humans, the proline oxidative pathway has been shown to play an important role in cellular redox homeostasis. The up-regulation of human PRODH by p53 produces ROS, which leads to apoptosis and is thought to be an important cancer-preventing mechanism (10, 18, 29, 30, 35, 36, 41, 45). Paradoxically, proline biosynthesis has been shown to be upregulated in cells exposed to oxidative stress and in certain cancer cell lines (27, 44). High levels of proline appear to support cell proliferation and enable proline to function as an antioxidant (27, 44). One of the mechanisms by which proline protects cells from oxidative stress is through maintaining reduced glutathione levels in the cell (27).

The gut of patients infected with *H. pylori* has been shown to have high levels of proline, and in separate studies, proline supplementation was observed to be critical for *H. pylori* proliferation (40, 50). These interesting observations led us to characterize the PutA proteins from *H. pylori* and from *H. hepaticus*. We found that similar to human PRODH, PutA from *Helicobacter* species generates superoxide anion during turnover with proline and molecular oxygen (26). The oxidase activities of PutA<sub>Hh</sub> and PutA<sub>Hp</sub> were considered unusual, leading to apoptosis and is thought to be an important cancer-preventing mechanism (10, 18, 29, 30, 35, 36, 41, 45). The oxidase activities of PutA<sub>Hh</sub> and PutA<sub>Hp</sub> were considered unusual, since PutA homologs from other gram-negative bacteria exhibited very minimal oxidase activity. The oxidase activities of PutA<sub>Hh</sub> and PutA<sub>Hp</sub> proved to be toxic to *E. coli* and severely inhibited cell survival (26). Thus, in addition to proline’s role as an energy substrate, it seemed plausible that proline metabolism may have a dual effect on the redox environment in *H. hepaticus*, with PutA activity generating ROS and proline accumulation providing protection against oxidative stress. Therefore, it was of interest to test the pathophysiological roles of PutA and proline metabolism in *H. hepaticus*.

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**FIG. 6.** Representative photomicrographs of liver sections taken on day 21 postinoculation from mice inoculated with the wild-type *H. hepaticus* or with the putA mutant *H. hepaticus* strain and from tissue from sham-inoculated control mice (hematoxylin-eosin stain; magnification, ×20). (A) Wild-type *H. hepaticus* strain-inoculated mouse tissue displays mild hepatocellular necrosis with inflammatory cell infiltration (arrow). (B) Wild-type *H. hepaticus* strain-inoculated mouse tissue exhibits mild biliary hyperplasia (arrow). (C) Mild portal inflammation and no evidence for tissue necrosis are seen in the liver of a mouse inoculated with the *H. hepaticus* putA mutant strain. (D) Control mouse liver shows an absence of hepatic damage or inflammation.
formation around the site of H. hepaticus infection (53). Apparently, the lack of PutA activity and proline utilization diminishes ROS-induced tissue damage and perhaps host neutrophil activation. The accumulation of proline in the H. hepaticus putA mutant may result in lower ROS levels, thereby minimizing or slowing down the inflammatory response. This would be consistent with the increased oxidative stress resistance of the putA mutant strain. Conversely, PutA activity in the wild-type strain would help accelerate or exacerbate inflammation and the pathogenesis of H. hepaticus infection.

Due to the dual effects of proline as a ROS scavenger and as a prooxidant via PutA, proline metabolism is well positioned to modulate inflammatory responses and the carcinogenesis of H. hepaticus infection. Future work will focus on discovering whether proline metabolism invokes certain signaling pathways involved in oxidative stress response and inflammation. For example, virulence factors such as catalase and NapA, an iron-binding protein, are upregulated in H. pylori by oxidative stress to help protect H. pylori and enable chronic inflammation to persist (5, 9, 21). The higher levels of catalase expression observed with the wild-type H. hepaticus strain than with the putA mutant strain suggest that proline oxidation via PutA generates redox signals in the cell that help maintain catalase expression and perhaps virulence. Additional experiments need to be performed to determine whether disrupting the putA gene attenuates the expression of other putative virulence factors. Proline transport will also be explored to distinguish between intracellular and extracellular pools of proline. Proline uptake has been shown to play a crucial role in the survival of the human pathogen Staphylococcus aureus, as deletion of the putP gene encoding the high-affinity proline transporter causes marked attenuation in several animal models of infection (3, 48, 57).

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