lkyl Hydroperoxide Reductase Is Required for Helicobacter cinaedi Intestinal Colonization and Survival under Oxidative Stress in BALB/c and BALB/c Interleukin-10-/- Mice

Alkyl hydroperoxide reductase is required for *Helicobacter cinaedi* intestinal colonization and survival under oxidative stress in BALB/c and BALB/c IL10$^{-/-}$ mice

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Abstract

Helicobacter cinaedi, a common human intestinal bacterium, has been implicated in various enteric and systemic diseases in normal and immunocompromised patients. Protection against oxidative stress is a crucial component of bacteria-host interactions. Alkyl hydroperoxide reductase C (AhpC) is an enzyme responsible for detoxification of peroxides and is important in protection from peroxide-induced stress. *H. cinaedi* possesses a single *ahpC*, which was investigated with respect to its role in bacterial survival during oxidative stress. The *H. cinaedi ahpC* mutant had diminished resistance to organic hydroperoxide toxicity, but increased hydrogen peroxide resistance compared with the wild type (WT) strain. The mutant also exhibited an oxygen-sensitive phenotype and was more susceptible to killing by macrophages than the WT strain. I*n vivo* experiments in BALB/c and BALB/c IL10$^{-/-}$ mice revealed that the cecal colonizing ability of the *ahpC* mutant was significantly reduced. The mutant also had diminished ability to induce bacterial-specific immune responses *in vivo*, as shown by immunoglobulin (IgG2a and IgG1) serum levels. Collectively, these data suggest that *H. cinaedi ahpC* not only contributes to protecting the organism against oxidative stress but also alters its pathogenic properties *in vivo.*

Introduction

Historically, *Helicobacter cinaedi* was classified under the genus *Campylobacter*; however, it was subsequently classified as a *Helicobacter* sp. based on DNA-DNA hybridization, 16S rRNA analysis, and biochemical properties (36). *H. cinaedi* has been reported by Vandamme P. *et al*. to form a 16S rRNA taxonomic cluster with *H. canis, H. bilis, and Flexispira rappini*, separate from the *H. pylori* cluster (42). *H. cinaedi* is now recognized as an enterohepatic helicobacter colonizing the lower gastrointestinal tract of numerous mammals, including dogs, cats, hamsters, and monkeys (12). Although the epidemiology and pathogenesis of *H. cinaedi* infections are not fully elucidated, it was first isolated from rectal swabs obtained from homosexual men (40). It is also implicated as a cause of gastroenteritis, particularly in immunocompromised individuals, such as HIV-infected or cancer patients, and recently was isolated from a healthy heterosexual male with cellulitis (16). Unlike some other *Helicobacter* spp. and *Campylobacter* spp.-related organisms, which colonize the intestinal tract (36), *H. cinaedi* has been cultured from the blood of patients with sepsis (16, 20, 23) and can cause cellulitis, bacteremia, and gastroenteritis with a high potential for recurrence (38).

In general, innate immunity is programmed to respond immediately when a host is challenged by an infectious pathogen, whereas adaptive immunity, mounted in response to infection, requires time to react and generate a microbe-specific response. One of the primary defense mechanisms of the innate response is macrophage killing, in which activated macrophages produce various reactive oxygen species (ROS), including organic hydroperoxides. These compounds cause damage to DNA, RNA, protein, and lipids of invading microorganisms. In response, bacterial pathogens have

developed both non-enzymatic and enzymatic mechanisms to protect themselves from damage and facilitate successful resistance to macrophage killing. An important example of this microbial defense mechanism is the enzyme alkyl hydroperoxide reductase C (AhpC), which catalyzes the hydrolysis of toxic compounds such as organic hydroperoxide to the corresponding alcohol and water. AhpC is classified as a member of the peroxiredoxin (Prx) family because it contains the CXXC motif, a common feature of Prx-type peroxidases (9). Its peroxidatic cysteine reacts with peroxides to yield the corresponding alcohol and cysteine sulfenic acid (Cys-SOH), which is then reduced by the free thiol of the cysteine residue to form a disulfide bond to complete the catalytic cycle. Reflecting its importance in protecting organisms against oxidative stress, *ahpC* has been identified in a wide variety of eubacteria and archaea. We therefore hypothesized that it contributes to the survival of *H. cinaedi* during infection, and not only plays an important role in colonization, but also in potential virulence. *In vitro* and *in vivo* studies were performed to assess the oxidative stress response of WT *H. cinaedi* and isogenic mutants lacking *ahpC*.

Material and Methods

Bacterial strains and growth conditions. *H. cinaedi* (CCUG18818) and *E. coli* DH5a were used for genetic manipulations. *H. cinaedi* was grown on Tryptic Soy Agar (TSA) supplemented with 10% sheep's blood) or brucella broth (BB) supplemented with 10% fetal calf serum; 25 μg/ml of chloramphenicol was added as appropriate. Plates were grown microaerobically at 37^{o}C in an incubator with 10% CO_2, 10% H_2, and 80% N_2 for

3 to 5 days. *E. coli* was grown in Luria-Bertani (LB) media supplemented with 100 μg/ml of ampicillin, or 30 μg/ml carbenicillin and incubated aerobically at 37^{o}C (13). **Construction of *H. cinaedi ahpC* mutant strain by insertional mutagenesis.** Briefly, the *ahpC* gene was PCR amplified from *H. cinaedi* chromosomal DNA using primers encompassing a *SmaI* restriction site in the middle of the gene. The products were ligated into pGemTeasy Vector (Promega Madison, WI) and transformed into *E. coli* DH5a, generating the plasmid pGemTeasy-*ahpC*. It was digested by *SmaI* and ligated to a chloramphenicol cassette that was cut by *Hinc*II from pUC20CAT. The pGemTeasy-*ahpC*::CAT was transformed into the *H. cinaedi* parental strain by electroporation facilitating a double crossover event at the flanking regions, resulting in inactivation of *ahpC* gene. The chloramphenicol resistant clones were selected, and the presence of the *ahpC* mutation was verified by PCR and sequencing. Mutants were confirmed by Southern blot analysis, as follows. Genomic DNA was digested by *Hind*III, separated on 1% agarose gel, transferred to a membrane, and hybridized with probes (amplified by using NC5; 5' ATATGTTAGTTACAAAACTTGC 3' and NC8; 5' ATTAAAGCTTAATGGAATTTTCT 3'). *Hind*III digestion of *H. cinaedi* genomic DNA produces a 1210-bp positive hybridization band, whereas the integration of pUC20CAT (2.0 kb) into the gene results in one large band: 2010-bp. As shuttle vectors are not available for *H. cinaedi*, an independent *ahpC* mutant was constructed and its phenotype tested to confirm that the altered phenotype arose from *ahpC* inactivation, not from random mutations.

Complementation of the functional *H.cinaedi* AhpC. To generate the complementation plasmid, the *H.cinaedi ahpC* structural gene and its ribosome binding site (618 bp) were amplified by *Pfu* polymerase using primers NC16 (5' TTCTTAAGGAGTTTGATATG 3') and NC17 (5' AAGATTAAAGCTTGTTAGCG 3'). The blunt PCR product was cloned into the SmaI site of pBBR-MCS2 (containing a kanamycin resistant cassette) to generate the pBBR*H.cinaedi*AhpC. Authenticity of the nucleotide sequence of the insert was confirmed by Applied Biosystems Model 3730 capillary DNA sequencer with Big Dye Terminator Cycle Sequencing Kit . The pBBR*H.cinaedi*AhpC was then transformed by heat shock into the *E.coli ahpC* mutant, kindly provided by Dr. Leslie Poole, as well as the parental *E. coli* strain; the *E.coli ahpC* pBBR*H.cinaedi*AhpC and *E.coli* pBBR*H.cinaedi*AhpC were created, respectively (37). These two strains were used to ascertain their oxidant susceptibility in the inhibition zone assay.

Inhibition zone assay. Exponential phase bacterial cells (OD_{600} nm of 0.1) were mixed with 10 ml of semi-soft agar (Brucella agar containing 10 % fetal bovine serum) held at 50^{o}C, and overlaid onto Brucella agar plates, which were then held at room temperature for 15 min to let the top agar solidify. Sterile 6 mm diameter paper discs soaked with 10 ml of either 1 M H_2O_2 or 0.1 M *tert*-butyl hydroperoxide (TBH) or 0.02 M cumene hydroperoxide (CHP) or 10 mM menadione superoxide generator (MD), were placed on the surface of the cell lawn. The diameter of inhibition zones was measured after 48 h of microaerobic incubation at 37^{o}C (4).

Organic hydroperoxide degradation assay (FOX assay). Log-phase cultures were adjusted to OD_{600} of 0.1 with fresh medium prior to the addition of TBH to achieve a

concentration of 10 mM. Residual organic hydroperoxide concentrations were determined at different intervals by a xylenol orange–iron reaction (21, 29). At indicated time intervals, 1 ml of the culture was removed and centrifuged at 10,000 rpm for 5 min, and 100 µl of clear supernatant was added to 400 µl of 25 mM sulphuric acid in a 1 ml cuvette. Subsequently, 500 µl of freshly prepared reaction buffer (200 mM ammonium ferrous sulphate, 200 mM xylenol orange, and 25 mM sulphuric acid) was added to the mixture. After a 30 min incubation at room temperature, the A_{540nm} was recorded, and the concentration of residual organic peroxide was calculated from a standard curve of organic hydroperoxide in Brucella broth.

Macrophage co-cultures. RAW 264.7 murine macrophages were grown at 37^{o}C in 5% CO_2 in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen, US). Following removal of antibiotics by washing with PBS, macrophages were infected with either the parental strain of *H. cinaedi* or *H. cinaedi ahpC* mutant at MOIs of 10 and 100. Bacterial survival was assessed at intervals of 10, 30, 60, 180, and 360 minutes by colony counting. After centrifugation of the co-culture supernatant at 12,000 rpm for 5 min, bacterial cells were resuspended in fresh broth, serially diluted, plated on TSA blood agar plates, and incubated as previously described. Bacterial survival was enumerated as colony-forming units.

***In vivo* studies.** All animal experiments were conducted in accordance with protocols approved by the Committee on Animal Care at MIT. Male and female BALB/cJ and BALB/c-IL10 null (C.Cg-*Il10*^{tm1Cgn}) mice (27) bred and housed at MIT were used for characterization of the bacterial strains *in vivo*. Mice were fed a standard rodent diet, provided water *ad libitum*, housed in microisolator cages, and maintained specific-

pathogen-free, including all known *Helicobacter* spp.in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care. At 6-8 weeks of age, mice were inoculated with a 0.2 ml of 10^9 cells/ml bacterial suspension by oral gavage every other day for 3 doses. In each experiment with BALB/cJ or BALB/c-IL10$^{-/-}$, mice were divided into three treatment groups: uninoculated (*n* = 10; 5 male and 5 female), inoculated with *H. cinaedi* WT (*n* = 10; 5 male and 5 female), and inoculated with *H. cinaedi ahpC* mutant (*n* = 10; 5 male and 5 female). Mice were euthanized 6 weeks post-inoculation. Two independent experiments were performed.

***H. cinaedi* colonization.** Successful colonization was confirmed by endpoint PCR of feces at 1-week post last dose of inoculation using *Helicobacter* genus-specific primers (C05; 5′ACTTCACCCCAGTCGCTG 3’ and C97; 5′GCT ATG ACG GGT ATC C 3’) as previously described (10). Additionally, bacterial colonization in the cecum was quantified by real-time PCR. Briefly, DNA was extracted from cecum using a High Pure PCR template preparation kit (Roche Molecular Biochemicals, Indianapolis, IN). Then, relative DNA concentrations of WT and mutant strains were determined by real-time quantitative PCR using the ABI Prism TaqMan 7700 sequence detection system (PE Biosystems, Foster City, CA) with specific primers for *H.cinaedi* 16S-23S intergenic spacer region (forward primer HciSPF; 5’-ATG AAA ATG GAT TCT AAG ATA GAG CA-3’ and HciSPR; 5’-AAG ATT CTT TGC TAT GCT TTT GGG GA-3’ (35).

ELISA for anti-*H. cinaedi* antibody responses. Enzyme linked immunosorbent assays (ELISA) were used to measure serum concentration of Th1-associated IgG2a and Th2-associated IgG1 antibodies to *H. cinaedi,* as previously described (35). Briefly, aliquots of sonicated *H. cinaedi* used as an antigen preparation were analyzed for

protein content using a BCA assay (Pierce, USA). Sample wells on Immulon II 96-well plates were coated with 10 µg/ml of antigen in 0.1 M sodium phosphate buffer and incubated overnight. After blocking and washing, serum (1:100 dilution) was incubated with the antigen for 2 h at room temperature. Biotinylated secondary antibodies and extravidin peroxidase were used for detecting IgG1 and IgG2a, which was developed with hydrogen peroxide and ABTS [2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)] as the substrate system. The reaction was stopped by the addition of ABTS stop solution (20% SDS/50% dimethylformamide in water), and A_{405nm} was determined. IgG1 and IgG2a concentrations were calculated from a standard curve generated on the same plate.

Histology. Formalin-fixed tissues were routinely processed, embedded in paraffin, sectioned at 4 mm thickness, and stained with hematoxylin and eosin. Histologic abnormalities in the large bowel were assigned scores based on the size and frequency of hyperplastic and inflammatory lesions, on a scale of 0 to 4 (0, none; 1, minimal; 2, mild; 3, moderate; and 4, severe). Epithelial dysplasia and neoplasia were graded using a scale of 0 to 4: 0, normal; 1, mild dysplastic changes; 2, moderate or severe dysplasia; 3, gastrointestinal intraepithelial neoplasia; and 4, invasive carcinoma, as previously described (15, 35). Stomach and liver sections were examined, but no histological lesions were found.

Statistical analysis. *In vivo* data from two independent experiments were combined. Both histological lesion scores and colonization by *H. cinaedi* WT were compared with that by *H. cinaedi ahpC* mutant using one-way ANOVA with Bonferroni post tests. Serology data and in vitro data were analyzed by two-tailed Student *t*-tests. All

analyses were performed using GraphPad Prism, version 4.0. Values of $P < 0.05$ were considered significant.

Results

Analysis of *H. cinaedi ahpC* primary structure

Screening of the annotated genome sequence of *H. cinaedi* revealed an *ahpC* open reading frame identified as HCCG_1660.3. Its deduced amino acid sequence shares high scores of identity to *H. hepaticus* (93%), *H. pylori* (71%), and *Campylobacter jejuni* (68%). The putative *ahpC* gene is located downstream of HCCG_1661.3 which encodes a nitrilase/cyanase hydratase and upstream of HCCG_1659.3 which encodes a conserved hypothetical protein HCCG_ 1658.3 encodes acetate permease. AhpC, a member of the peroxiredoxin family of peroxide metabolizing enzymes, is categorized into three types: 1-Cys, typical 2-Cys and atypical 2-Cys PRX, according to the number of cysteine residues involved in the enzymatic mechanism at the catalytic site. *H. cinaedi ahpC* is a typical 2-Cys PRX based on its primary structure, which contains two conserved cysteine residues, C49 (peroxidatic cysteine) and C170 (resolving cysteine) (9). An open reading frame homologous to *E. coli* ahpF was not identified. Thus, unlike *E. coli* in which ahpF is required for regeneration of reduced *ahpC* in a $FADH_2$-dependent manner, *H. cinaedi* likely utilizes thioredoxin as a reductant for *ahpC* in a manner previously described for *H. pylori* (34).

Inactivation of *H. cinaedi ahpC* alters bacterial resistance to oxdative stress

AhpC is responsible for direct detoxification of organic peroxides to their corresponding alcohols and water. Upon exposure to peroxides, the peroxidatic cysteine is oxidized to a cysteine sulfenic acid intermediate that, in turn, reacts with the resolving cysteine residue to form an intramolecular disulfide bond. We used an *ahpC* mutant to investigate its physiological function in *H. cinaedi*. Levels of resistance to

oxidative stress-producing agents in WT *H. cinaedi* and the isogenic *ahpC* mutant were compared using inhibition zone assays. As illustrated in Fig. 1a, the mutant was less resistant to *t*-butyl hydroperoxide (TBH) than its parental wild-type strain, with an inhibition zone of 18.7 + 1.2 mm for the mutant and 11.0 + 1.0 mm for the WT strain. The mutant also showed decreased resistance to cumene hydroperoxide (CHP), as its inhibition zone was 24.0 + 1.0 mm compared with 12.0 + 1.0 mm for the parental strain. Resistance to the intracellular superoxide generator menadione (MD) was also evaluated, but no significant difference between the mutant and WT strain was observed (Fig 1a).

Unexpectedly, the *ahpC* mutant was more resistant to H_2O_2 relative to the WT strain (Fig 1a), having an inhibition zone of 17.7 + 0.6 mm compared to the parental strain with a zone of 21.0 + 1.0 mm. Increased resistance to H_2O_2 attributable to a lack of functional *ahpC* has previously been observed in both gram negative and positive bacteria including *Xanthomonas campestris*, *Bacillus subtilis*, *Helicobacter hepaticus* and *C. jejuni* (2, 4, 17, 30). In most cases, enhanced H_2O_2 resistance was shown to be due to a compensatory increase in activity of catalase, the enzyme catalyzing decomposition of H_2O_2 to water. We therefore measured catalase activity to assess its role in the observed increase in resistance to H_2O_2 in the *H. cinaedi ahpC* mutant. Total catalase activity was 33.0 ± 0.9 in U/mg protein in the mutant, compared to 13.4 ± 0.5 U/mg protein in the WT strain, as shown in Fig 1b, indicating that compensatory enhancement of total catalase activity was indeed responsible for the observed effect. However, this result is in contrast to the *H. pylori ahpC*, which is more susceptible to H_2O_2. *H. pylori ahpC* mutant was more susceptible to H_2O_2, and had decreased

catalase activity due to the unique structure of *H. pylori* catalase which uses *ahp*C as its heme chaperon and is highly sensitive to inactivation by organic hydroperoxides which accumulate in *ahpC* mutant cells (5, 43).

Reduced ability to degrade organic hydroperoxide in the *H. cinaedi ahpC* mutant.

Since the *H. cinaedi ahpC* mutant had decreased resistance to killing by organic hydroperoxides, we tested whether this phenotype arose from reduced capacity for detoxification. The ability of mutant and WT strains to degrade TBH was measured using a modified FOX assay (28), in which bacterial cultures were exposed to 150 mM TBH and residual peroxide was measured at prescribed time intervals thereafter (Fig 1c). The capacity of the mutant to degrade the substrate was significantly decreased compared to that of the WT strain ($P < 0.05$). After 30 min, less than 10% of TBH was degraded by the mutant whereas approximately 30% TBH was degraded by WT *H. cinaedi*, indicating that the lower resistance of the mutant strain was attributable, at least in part, to reduced capacity to degrade the oxidant.

The functional AhpC of *H.cinaedi* complements the organic hydroperoxide sensitive phenotype in *E.coli ahpC*

Since assays for complementation of *H.cinaedi* mutants are not available, complementation was performed in an *E. coli* mutant lacking AhpC (37). The complemented strain *E.coli ahpC* pBBR*H.cinaedi*AhpC and *E.coli*/ pBBR*H.cinaedi*AhpC were tested for oxidant resistance levels by an inhibition zone assay. The *E. coli ahpC* mutant showed a hypersensitive phenotype against organic hydroperoxide (500 µM CHP) with a inhibition zone of 31.1 ± 1.0 mm, compared to the parental *E. coli* strain with a zone of 27.7 ± 0.45 mm. The functional *H.cinaedi*AhpC complementated strain

(*E.coli ahpC* pBBR*H.cinaedi*AhpC) ablated the hypersensitive phenotype of the *E.coli ahpC* mutant to levels comparable for the *E. coli* parental strain as evidenced by an inhibition zone of 27.72 ± 0.83 mm (Figure 5). Therefore, it suggested that the *H.cinaedi* AhpC functions as an organic hydroperoxide detoxification system in *H.cinaedi*.

Reduced survival of *H. cinaedi ahpC* mutant in an aerobic atmosphere

Although microaerophilic bacteria such as *Helicobacter* spp. are human pathogens that colonize the gastrointestinal tract where the oxygen level is low, oxygen tolerance is important for these bacteria to survive in an aerobic environment during their transmission via feces to a susceptible host. Therefore, we tested whether *ahpC* deficiency in *H. cinaedi* affected oxygen tolerance by exposing bacterial cultures to atmospheric oxygen, and enumerating cells surviving this environment at the times indicated in Fig 2a. After 3 h exposure, the number of viable mutants was slightly decreased compared to WT cells, but after 6 h exposure a significant reduction ($P < 0.001$) in survival of the mutant was observed. These results suggest that *ahpC* is required for prolonged survival of *H. cinaedi* under ambient oxygen conditions and may therefore be important for host infection via fecal-oral transmission with this bacteria.

AhpC plays an important role in survival of *H. cinaedi* within murine macrophages

Macrophages are primary responders to mucosal bacterial pathogens, and production of ROS is an important defense mechanism macrophages employ against microbes. On the other hand, microorganisms have developed systems to protect themselves from toxic oxygen radicals. We performed experiments to test whether the absence of *ahpC* in *H. cinaedi* affected bacterial susceptibility against macrophage

killing. RAW264.7 cells were infected with a MOI 100 of *H. cinaedi ahpC* mutant or WT cells for up to 6 h, after which surviving bacteria were enumerated by dilution plating. At 3 h post-infection, a dramatic decrease (50%) in bacterial survival was observed in mutants compared with 90% survival in the WT strain (Fig 2b); significant differences were also observed 6 h post-infection (Fig 2b). These *in vitro* data demonstrate that *ahpC* contributes to the survival of *H. cinaedi* in murine macrophages.

Reduced cecal colonization in mice by *ahpC* mutant compared to WT *H. cinaedi*.

The above *in vitro* results show that *ahpC* plays an important role in the survival of *H. cinaedi* to stress induced by organic hydroperoxides and in resistance to killing by macrophages. We hypothesized that it also plays an important role in survival and colonization *in vivo*. Given the reported ability of *H. cinaedi* to colonize and cause intestinal disease in IL10$^{-/-}$ C57BL/6 mice (15, 35) we tested the ability of WT and mutant *H. cinaedi* to colonize BALB/c mice and BALB/c IL10$^{-/-}$ mice. Initial colonization by *H. cinaedi* was confirmed by PCR on fecal samples at 1-week post-infection (WPI). Additionally, at 6 WPI colonization levels in the cecum were determined using quantitative PCR with oligonucleoide primers specific to *H. cinaedi* and data obtained were expressed as femtogram (fg) of *H. cinaedi* DNA per picogram (pg) of mouse cecal DNA. Initially, data obtained *in vivo* were analyzed by gender, based on earlier observations that inflammatory responses to infections by other *Helicobacter* spp. in mice were related to gender (19, 33). In our experiments, WT *H. cinaedi* exhibited comparable cecal colonization levels in female and male BALB/c mice, whereas in IL10$^{-/-}$ mice the level of cecal colonization of male mice was significantly higher than that of females (P = 0.0008, Fig. 3). Similar results, in which the colonization level of *H.*

313 *hepaticus* in female mice was less than that in male mice were described by Ge et al.
314 (14). In males and females of both strains of mice, we found that the levels of cecal
315 colonization by the *H. cinaedi ahpC* mutant were significantly lower than those by the
316 WT ($P < 0.001$ for IL10$^{-/-}$ mice and $P < 0.01$ for BALB/c mice); in contrast to WT *H.*
317 *cinaedi*, the mutant was only detectable in female BALB/c-IL10$^{-/-}$ mice. The inability of
318 the *H. cinaedi ahpC* mutant to colonize murine cecal tissue at comparable levels as the
319 WT, suggests that *H. cinaedi ahpC* may be required for persistent colonization in the
320 lower bowel of mice.

321 **Inactivation of *ahpC* does not affect the degree of intestinal pathology during *H.***
322 ***cinaedi* infection.**

323 Clinical disease was not evident at 6 WPI in either BALB/c or BALB/c-IL10$^{-/-}$
324 mice. However, the histological activity index (sum of all lesion scores) of the cecum
325 was higher in infected BALB/c-IL10$^{-/-}$ mice compared to that of infected BALB/c mice of
326 both genders (Table 1). Comparison of WT and mutant *H. cinaedi* with respect to
327 histologic activity indices in the cecum and colon showed no significant differences
328 (Table 1, and data not shown).

329 ***H. cinaedi ahpC* mutant failed to induce robust antibody responses, while**
330 **infection by wild type induced both Th1 and Th2-associated serum antibodies**

331 Successful colonization by a pathogen is commonly associated with induction of
332 pathogen-specific host immune responses. The levels of pathogen-specific IgG2a and
333 IgG1 have been used as markers for mucosal Th1 and Th2 responses, respectively. To
334 investigate whether inactivation of *ahpC* in *H. cinaedi* affects these immune responses,
335 levels of serum IgG1 and IgG2a were measured in infected mice at 6 WPI. BALB/c

mice infected with WT bacteria developed a mixed antibody response with comparable levels of *H. cinaedi*-specific IgG2a and IgG1 (Fig. 4a, b), similar to responses previously reported for C57BL/6J mice (35). Both IgG2a and IgG1 responses were similar in $IL10^{-/-}$ and BALB/c mice (Fig. 4). Additionally, gender did not affect the antibody responses in either mouse strain, but infection with *H. cinaedi ahpC* resulted in significant reductions in *H. cinaedi*-specific IgG2a and IgG1 in both male and female BALB/c mice (Fig. 4a, b). $IL10^{-/-}$ mice infected with mutant *H. cinaedi* displayed a similar reduction in IgG1 in both genders (Fig. 4c), but only females showed a reduction in IgG2a (Fig. 4d).

Discussion

The protective role of *ahpC* against oxidative stress has been reported in several bacterial species, including *Escherichia coli*, *Salmonella typhimurium*, *Helicobacter pylori*, *H. hepaticus* and *Campylobacter jejuni* (2, 4, 17, 30). The fact that *ahpC* is highly conserved both in eukaryotic and prokaryotic organisms suggests that it serves an important biological function. The *ahpC H. cinaedi* mutant exhibited reduced resistance to organic hydroperoxides, a feature commonly observed in other bacterial *ahpC* mutants (24, 26). Because no shuttle vectors are currently available, gene complementation of *H. cinaedi* mutants could not be undertaken. We instead performed a *H. cinaedi* AhpC complementation assay in an *E. coli ahp*C mutant. In this experiment, we observed that the *H. cinaedi* AhpC restored resistance to the organic hydroperoxide in the *E. coli ahp*C mutant (Fig. 5). The adjacent genes of *ahp*C, HCCG_01661.3; HCCG_01558.3 which encode known functions for nitrilase/cyanide hydratase and acetate permease were tested for their gene expression levels by

quantitative PCR in wt *H. cinaedi* and the *ahp*C mutant. There were no significant changes in the expression of the mRNA levels between wt and the mutant strain in those two genes (data not shown). This indicates that the phenotypes resulting from construction of *ahp*C mutant was not due to a polar effect. To rule out the possibility that the altered phenotypes observed in the *ahpC* mutant did not arise from coincident mutations in other genes, experiments were conducted in two independently constructed mutants and data from representative mutants were similar.

H. pylori ahpC disruption leads to a decrease in catalase activity, with a purported role of AhpC as a heme chaperone (5, 30). Interestingly, however, the *ahpC H. cinaedi* mutant had an increased resistance to H_2O_2, which corresponded to a concomitant increase in total catalase activity, suggesting compensatory elevation of catalase gene expression upon inactivation of *ahpC*. This mechanism has been observed previously in both gram positive and negative bacteria including *Staphylococcus aureus* (7), *Bacillus subtilis* (22), *Xanthomonas campestris* (4), *Helicobacter hepaticus* (17), and *Campylobacter jejuni* (30). In most cases, the mechanism is modulated by a transcription regulator that concurrently controls the expression of both *ahpC* and catalase genes. Lack of *ahpC* causes an intracellular accumulation of peroxides resulting in regulator activation, and up-regulation of the catalase gene. However, the peroxide stress response in *H. cinaedi* has not been characterized; thus the precise regulatory mechanism causing increased catalase production in *H. cinaedi ahpC* mutants is unknown and requires further investigation. Although we have not found other phenotypic changes besides catalase activity in the *H. cinaedi ahpC* mutant strain when exposed to H_2O_2, it is possible that other genes related to bacterial antioxidant

properties could also be affected due to the AhpC mutation. However, a *H. cinaedi* microarray (which is not available right now) will be needed to fully elucidate this possibility. This microarray strategy has been successfully used to determine oxidative stress genes that are effected in the transcriptome of WT *Moraxella catarrhalis* and *M. catarrhalis* Δoxy R mutant which has increased sensitivity to H_2O_2 when exposed to high levels of H_2O_2 (18).

The *H. cinaedi ahpC* mutant showed roughly 3-fold reduction in the rate of organic hydroperoxide degradation, which severely affected the mutant's ability to degrade organic hydroperoxides. This reduced reaction rate likely accounted for the relative sensitivity to the adverse effects observed in the mutant, indicating that *ahpC* is an important component of the cellular defense mechanisms against exogenous organic hydroperoxides. We also noted that approximately 10% of added organic hydroperoxide was degraded in the *ahpC* mutant, suggesting the existence of other peroxide detoxification pathways in *H. cinaedi*. The contributions of organic hydroperoxide resistance (Ohr) enzyme and other peroxiredoxins to organic hydroperoxide degradation have been shown in several soil bacteria (1, 6, 21, 25, 31, 32). Based on the genome sequence, *H. cinaedi* does not contain *ohr*, but it does possess two peroxiredoxins; namely bacterioferritin comigratory protein (HCCG 00844.3) and thiol peroxidase (HCCG 01386.3).

Fecal-oral transmission is probable given *H. cinaedi* has been commonly isolated in fecal samples (20, 39, 41, 42). The ability of *H. cinaedi* to survive and persist in the environment with ambient oxygen is an important characteristic for both its transmissibility and its pathogenic potential. Though exposed to low levels of oxygen in

its intestinal niche (44), *H. cinaedi* must survive exposure to an aerobic atmosphere during fecal-oral transmission to susceptible hosts. The *ahpC* mutant exhibited significantly reduced survival under atmospheric oxygen conditions, indicating that *ahpC* appears not only to play a primary role in scavenging of harmful peroxides, but also is crucial for bacterial survival and persistence in the extraintestinal environment.

Macrophages upon activation respond by producing a bactericidal arsenal of reactive oxygen and nitrogen species. Our finding that *H. cinaedi ahpC* mutants were more vulnerable to macrophage killing than the isogenic WT strain suggests that *ahpC* plays a critical role in neutralizing toxicity from free radicals generated within macrophages. The fact that the mutants contain increased levels of total catalase activity implies that H_2O_2, compared with organic hydroperoxides, is not the major radical responsible for *H. cinaedi* cell death from macrophage killing.

IL-10 is an important anti-inflammatory cytokine, and IL-$10^{-/-}$ mice develop chronic lower bowel inflammation when infected with several *Helicobacter* spp. (3, 11, 27, 35). Recently, C57BL/6 and C57BL/6-IL$10^{-/-}$ mice were used to evaluate *H. cinaedi* pathogenicity. *H. cinaedi* was able to colonize the gastrointestinal tract and cause typhlocolitis in C57BL/6-IL$10^{-/-}$ but not WT C57BL/6 mice (30). Consistent with the previous study, WT *H. cinaedi* colonized the cecum of both BALB/c and BALB/c-IL$10^{-/-}$ mice, but BALB/c-IL$10^{-/-}$ mice were more susceptible than WT mice. *H. cinaedi ahpC* mutants however, lost the ability to persistently colonize male or female BALB/c mice and male BALB/c-IL$10^{-/-}$ mice; they colonized female BALB/c-IL$10^{-/-}$ mice sparingly. These *in vivo* results agree with *in vitro* experiments in which *ahpC* mutants showed drastically reduced survival within macrophages, and suggest that *ahpC* contributes to

the ability of *H. cinaedi* to persistently colonize the intestine. It is not know whether over-expression of alkyl hydroperoxide reductase will enhance the ability of *H. cinaedi* to survive and colonize the intestines. However, it has been suggested by Croxen et al (2007) that in *H. pylori* a high level of AhpC is not required for primary gastric colonization in mice; in their experiment, using knockdown techniques to reduce AhpC activity, they proved that 70% or even 25% of WT AhpC function provided sufficient antioxidant protection allowing the knockdown strains to colonize (8).

IgG1 and IgG2a serological markers for Th2 and Th1 responses, respectively, were significantly increased in mice infected with WT *H. cinaedi* compared with uninfected controls, indicating the development of specific immunity despite variable colonization. Similar to our earlier report, induction of a serological Th1 type response to *H. cinaedi* infection was more predominant than a Th2 type response (35). As expected, the *ahpC* mutants induced minimal Th1 and Th2 type responses, presumably due to their inability to persistently colonize the gastrointestinal tract.

Even though clinical disease was not observed, typhlocolitis was more extensive in BALB/c-IL10$^{-/-}$ mice than in BALB/c mice infected with either WT *H. cinaedi* or the *ahpC* mutant (Fig 6). However, it is important to note that the period of infection was limited to 6 WPI, and a longer period of colonization of BALB/c-IL10$^{-/-}$ mice may have resulted in more robust lower bowel inflammation. Also, strain differences have been observed in both gastric and intestinal models of infection by *Helicobacter* spp., in which BALB/c mice have been shown to respond to infection with a more marked non-inflammatory Th2 response than the Th1 response noted in C57BL/6 mice (35).

In summary, we describe the functional characterization of *ahpC* in *H. cinaedi*. Our results suggest that this gene provides protection of *H. cinaedi* from both exogenous and endogenously generated organic hydroperoxide toxicity, and also from macrophage killing. Moreover, *H. cinaedi* plays a role in colonization, particularly in a host lacking immune modulation by IL-10. AhpC offers potential as a drug target for effective therapy against *H. cinaedi* infections.

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Figure and Table legends

Figure 1. *H. cinaedi* ahpC detoxifies peroxides *in vitro*. (A) Inhibition zone assay. The *H. cinaedi* ahpC mutant (dark bars) was tested for oxidant sensitivities and compared to WT *H. cinaedi* (light bars). TBH, t-butyl hydroperoxide; CHP, cumene

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Figure 2. AhpC aids in aerobic atmosphere and intracellular survival of *H. cinaedi*. (A) Oxygen sensitivity in *H. cinaedi* WT strain (○) and ahpC mutant (□) were analyzed for survival under atmospheric condition. (B) Susceptibility of WT *H. cinaedi* (●) and ahpC mutant (■) to killing in RAW 264.4 murine macrophages. The experiment was repeated three times and representative data are shown.

Figure 3. Loss of ahpC alters the ability of *H. cinaedi* to colonize mice. Bacterial burdens of WT and ahpC mutant *H. cinaedi* in cecal tissue in BALB/c mice (A) and BALB/c-IL10$^{-/-}$ mice (B). Results presented as mean ± SEM. *$P < 0.01$, **$P < 0.001$. N.D. is not detectable.

Figure 4. **Diminished host adaptive immune response upon infection with *H. cinaedi* lacking ahpC**. *H. cinaedi*-specific Th2-associated immunoglobulin IgG1 was measured in BALB/c WT (A) and BALB/c-IL10$^{-/-}$ (C) mice, as well as Th1-associated immunoglobulin IgG2a in BALB/c WT (B) and BALB/c-IL10$^{-/-}$ (D) mice. *P<0.01; Data presented as mean ± SEM.

Figure 5. *H. cinaedi* ahpC complements the hypersensitive phenotype of an *E. coli* ahpC mutant. Inhibition zone assay with CHP, cumene hydroperoxide, was performed on the parental *E.coli* strain (light grey bar), *E.coli* ahpC knockout strain (dark grey bar), and *E. coli* ahpC/p*H. cinaedi* AhpC (grey bar), which is the E. coli ahpC knockout strain

628 transformed with functional AhpC of *H. cinaedi*. Mean and standard deviations are from

629 triplicate experiments. $P < 0.05$

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631 **Table 1.** Histological disease indices of cecal tissue at 6 weeks post-infection of BALB/c

632 and BALB/c-IL10$^{-/-}$ mice gavaged with PBS (uninfected), WT *H. cinaedi,* or *ahpC* mutant

633 *H. cinaedi.*

Infection	BALB/c Female	BALB/c Male	BALB/c Combined	BALB/c-IL10$^{-/-}$ Female	BALB/c-IL10$^{-/-}$ Male	BALB/c-IL10$^{-/-}$ Combined
PBS	1.25 (0.5-1.5)	0.75 (0.5-2.0)	1.0 (0.5-2.0)	1.0 (0.5-1.5)	1.0 (0.5-1.5)	1.0 (0.5-1.5)
H. cinaedi	1.0 (0.5-2.5)	1.0 (0.5-2.0)	1.0 (0.5-2.5)	3.25 (2.5-5.5)	4.0 (3.0-6.5)	3.5 (2.5-6.5)
H. cinaedi ahpC	2.5 (0.5-2.5)	1.5 (0.0-2.0)	1.0 (0.5-2.5)	2.0 (0.5-4.5)	4.0 (4.0-5.0)	4.0 (0.5-5.0)

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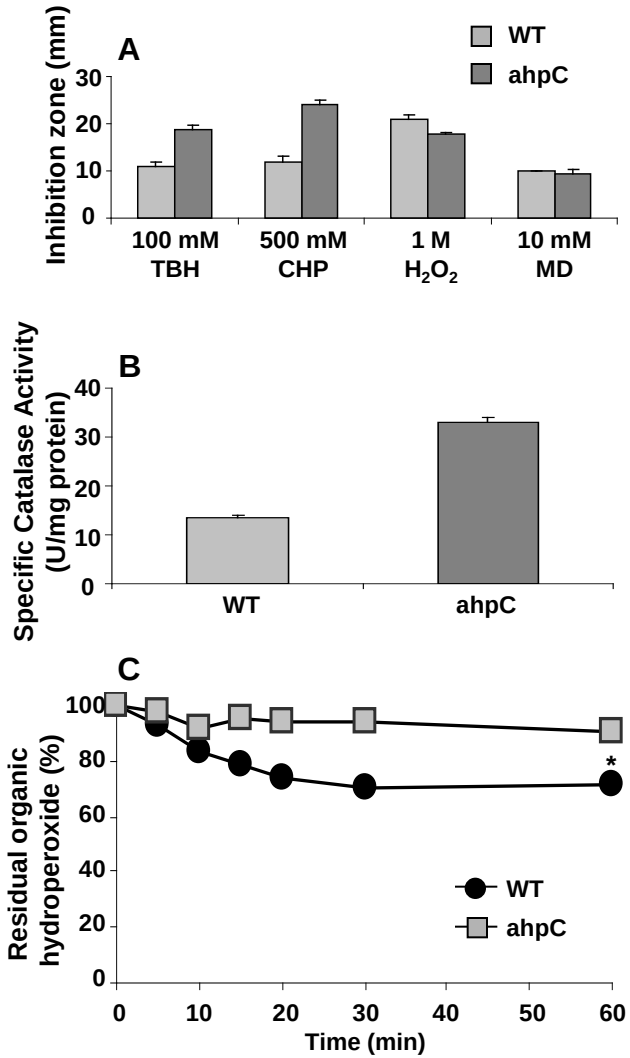

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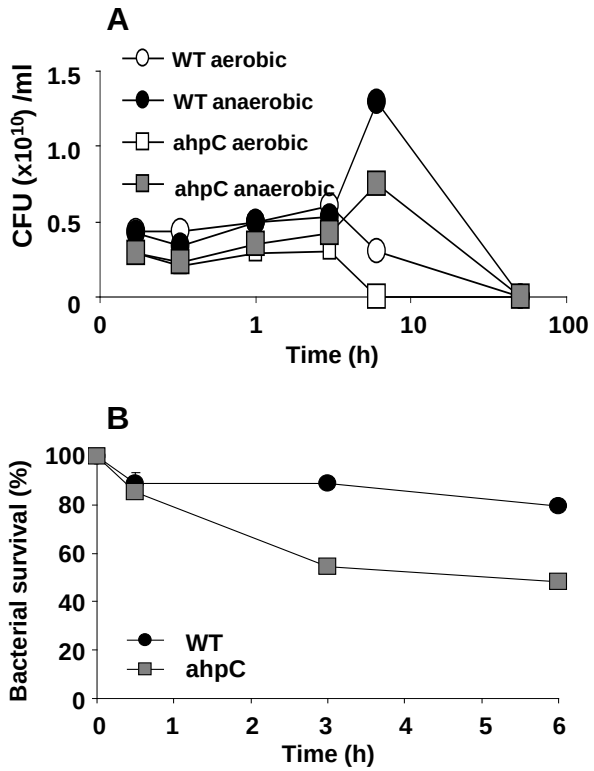

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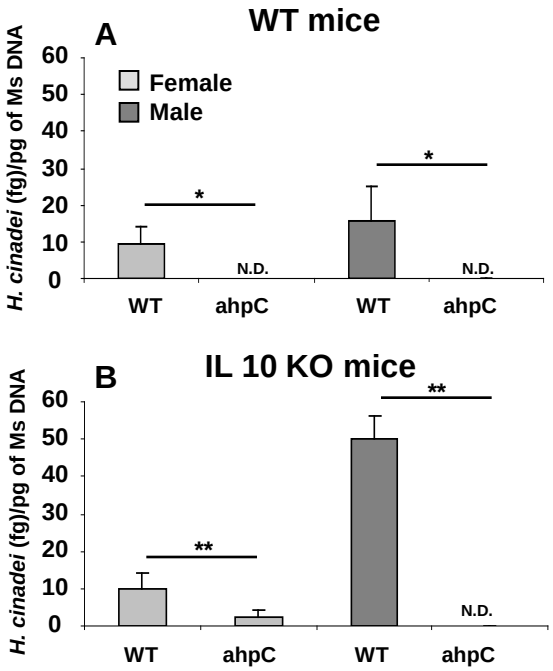

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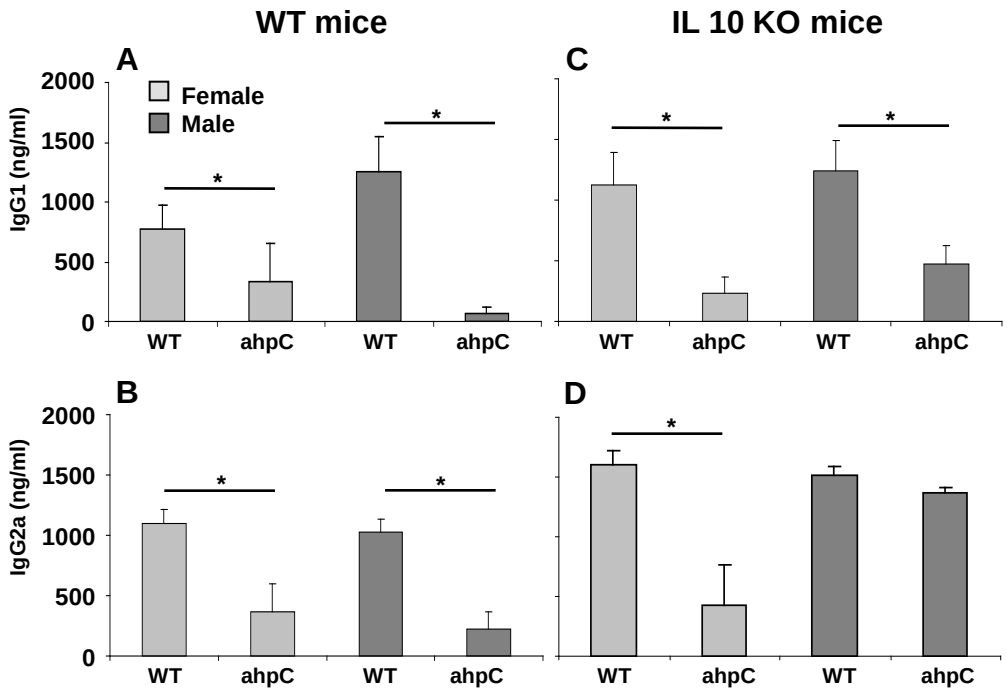

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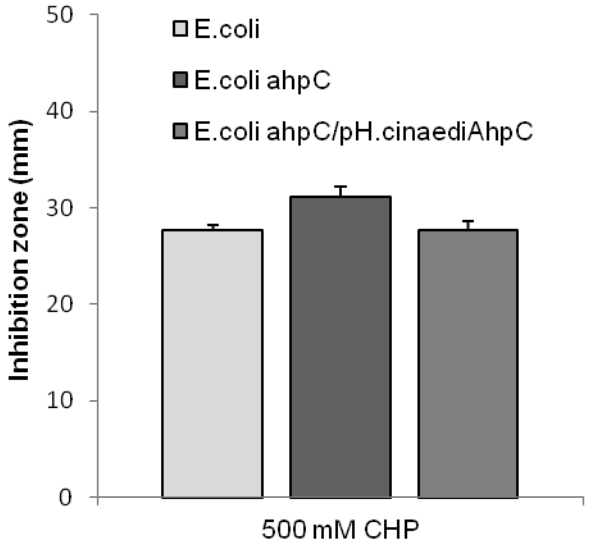

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