Infection with Helicobacter pylori, a microaerophilic, gram-negative bacterium, is associated with type B gastritis and peptic ulcer disease and is a risk factor for gastric carcinomas in humans (8, 17, 27). It so prevalently affects the world population that H. pylori has been described as “the most common chronic infection” (http://www.cdc.gov/ncidod/dbmd/diseasereinf /hpylori_t.html), with some developing countries experiencing nearly 100% infection rates. While H. pylori infection is generally controlled with a cocktail of antibiotics and bismuth (60), the specter of emerging antibiotic resistance necessitates the search for alternative drug strategies and a clearer understanding of bacterial defense systems.

H. pylori colonizes the mucosal layer of the stomach and secretes immunogenic products that recruit macrophages and polymorphonuclear leukocytes to the site of infection (59). Here, the resulting oxidative burst by the phagocytes produces reactive oxygen species (ROS), such as superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and the hydroxyl radical (OH$^-$), that damage gastric tissues. Increased ROS levels are present in H. pylori patients (19), and it is thought that long-term exposure to ROS contributes to the development of cancerous gastric cells (16).

To resist oxidative damage from chronic inflammation, H. pylori relies on a variety of protective enzymatic systems, including catalase (the katA gene product) (47) and superoxide dismutase (the sodB gene product) (63), which eliminate H$_2$O$_2$ and O$_2^-$, respectively; however, it is unknown how H. pylori coordinates its oxidative stress response because the bacterium lacks the oxidatively activated regulatory genes, soxRS and oxyR, common to other eubacteria (67). We report herein that H. pylori also expresses a thioredoxin (Trx)-dependent alkyl hydroperoxide reductase (AhpC) protein, a member of the peroxiredoxin (Prx) family (62), whose activity can detoxify lipid hydroperoxides analogous to those created in membranes exposed to ROS (32). All Prx enzymes are dimers, decamers, or, in some cases, higher-order aggregates (1, 38, 61) with one essential, N-terminal Cys residue per subunit (Cys46 of Salmonella typhimurium AhpC) and was slightly more active with E. coli TrxR and Trx1 than was S. typhimurium AhpC, demonstrating the specialized catalytic properties of this peroxiredoxin.
To reduce AhpC, most bacteria, including *S. typhimurium*, express a specialized flavoprotein reductase, AhpF, which is homologous to *Escherichia coli* Trx reductase (TrxR), except that AhpF contains an additional N-terminal region directly involved in AhpC reduction (54, 55). Alternatively, some eukaryotic Prx systems utilize TrxR and Trx to reduce the peroxidase (sometimes referred to as Trx-dependent Prx or TrxPs), where TrxR catalyzes the reduction of Trx, which, in turn, reduces the Prx disulfide bond. In these cases, electron transfer proceeds along the following path: NADPH → TrxR → Trx → AhpC (TPx) → ROOH.

While eukaryotic examples of Trx-dependent Prx proteins abound (e.g., *Entamoeba histolytica* AhpC [52], *Saccharomyces cerevisiae* TPx [10], and multiple human Prx homologues [11]), to our knowledge, only one other bacterial Trx-dependent Prx, besides the *H. pylori* AhpC described herein, has been experimentally demonstrated [TPx from the cyanobacterium *Synechocystis* (75)]. A distinctly related Prx family member, *E. coli* bacterioferritin comigratory protein, has also been shown to exhibit low levels of Trx-dependent peroxidase activity (34). Inspection of the *H. pylori* genome yielded no *ahpF*, but an *E. coli* *trcA* homologue (trc1, HP0824 in the annotation of Tomb et al. [67]) encoding Trx1 was identified along with an *E. coli* trcB homologue (trxR, HP0825) encoding TrxR. Further examination uncovered HP1164, annotated as a TrxR locus due to its similarity to *Plasmidium falciparum* *trc* locus (21% identity). HP1164 does not, however, encode a putative catalytic motif (CXXC or CXXXCC) indicative of such a redox center and was therefore excluded from these studies. Further genomic searches revealed a second Trx locus (trx2, HP1458) encoding Trx2. Therefore, we considered TrxR (HP0825)-Trx1 (HP0824) and TrxR (HP0825)-Trx2 (HP1458) to be good candidates as AhpC-reducing systems.

Herein, we present the first example of a Trx-dependent alky hydroperoxide reductase system from a gastric pathogen and describe the cloning, purification, and kinetic characterization of AhpC, Trx1, Trx2, and TrxR from *H. pylori*. Along with the genetic characterization of *H. pylori* *ahpC*, we have also shown that AhpC plays a critical role in the defense against oxygen toxicity that is essential for survival and growth, even in microaerophilic environments.

(Abstracts reporting some of this information have been published earlier [5, 6].)

### MATERIALS AND METHODS

**Materials.** Sodium dodecyl sulfate (SDS), ultrapure glycerol, ultrapure urea, EDTA, dithiothreitol, Tris base, and other buffer reagents were purchased from Research Organics (Cleveland, Ohio). Bacteriological medium components were from Difco Laboratories (Detroit, Mich.). Ethanol was obtained from Warner Graham Company (Cockeysville, Md.). Isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) stocks were from Intrac (Milan, Italy). Vent DNA polymerase, Moloney murine leukemia virus reverse transcriptase, and T4 polynucleotide kinase. Total RNA concentration was determined at 260 nm. For the primer extension studies, an oligonucleotide (5'-end labeled in [γ-32P]ATP, 5,000 Ci/mmol; Amersham) and 2 U of T4 polynucleotide kinase. Total *H. pylori* RNA (100 μg) was incubated with the [32P]-labeled oligonucleotide and annealed under the following conditions: 80°C for 5 min, 65°C for 5 min, 42°C for 10 min, and 37°C for 20 min. Following annealing, the RNA was precipitated with ethanol, dried, and resuspended in 7 μl of diethylpyrocarbate-treated water and 13 μl of reverse transcriptase buffer (50 mM Tris-Cl, pH 8.3, 10 mM MgCl2, 1 μg of actinomycin D [Sigma], 1 mM dithiothreitol, 2.5 mM each deoxynucleoside triphosphate). One microliter of Moloney murine leukemia virus reverse transcriptase (50 U/μl) was added to the sample, and reverse transcription was carried out at 42°C for 60 min; 1 μl of 0.5 M EDTA and 1 μl of RNase (10 mg/ml) were added, and the reaction mixture was incubated at 37°C for an additional 30 min. The reaction was stopped by phenol-chloroform extraction, followed by ethanol precipitation. The sample was then dried and resuspended in 10 μl of sequencing loading buffer. In parallel, dideoxy DNA sequencing of the cloned *ahpC* gene and the upstream region was performed using the same oligonucleotide primer to determine the transcriptional start site. After denaturation at 95°C for 2 min, aliquots were subjected to 6% urea polyacrylamide gel electrophoresis (PAGE) and autoradiographed.

**Allelic replacement mutagenesis of *ahpC***. Genomic DNA isolated from *H. pylori* strain 26695 (67) was used to amplify *ahpC* with forward and reverse oligonucleotide primers (Table 1) under typical PCR conditions to generate a product of 930 bp long. Isolated *ahpC* was digested at flanking EcoRI and SacI sites and then inserted into pBlueScript-SK (+) plasmid vector (Stratagene, La Jolla, Calif.) digested with the same restriction enzymes. Vector derivatives of pBlueScript were stably maintained in *E. coli* strains but were not replicated in *H. pylori* hosts. The *ahpC*-containing plasmid was then digested with XhoI (located approximately 330 bp from the *ahpC* translational start site), and a chloramphenicol resistance cassette (camR) was inserted at the unique EcoRI site and then subjected to electroporation of *H. pylori* and *E. coli* strains. The same strains were also transformed with a construct in which the nitroreductase gene (*rdxA*) was interrupted with *camR*.

### TABLE 1. Primers used to amplify and subclone *H. pylori* structural genes

<table>
<thead>
<tr>
<th>Primer (5′−3”)</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>pPROK1-ahpC</td>
<td>CGCCCGGAGAGGGAAGAATGAGTTTCAAAAACCTGCC</td>
<td>CCGTGCAGCTACAGCTGATTAGGAAATTCTTTTAAAGAT</td>
</tr>
<tr>
<td>trx1</td>
<td>CGCCCGGAGAGGGAAGAATGAGCTGCAAATACGAACGGG</td>
<td>CCGAAGCTTCCCTTCAATAACGTTTGAAGC</td>
</tr>
<tr>
<td>trx2</td>
<td>CGCCCGGAGAGGGAAGAATGAGTTTCAAAAACCTGCC</td>
<td>CCGAAGCTTCCCTTCAATAACGTTTGAAGC</td>
</tr>
<tr>
<td>trxR</td>
<td>CGCCCGGAGAGGGAAGAATGAGCTGCAAATACGAACGGG</td>
<td>CCGAAGCTTCCCTTCAATAACGTTTGAAGC</td>
</tr>
<tr>
<td>pBluescript-SK-ahpC</td>
<td>TGGAAATCGCCCAATAACGATGAAACAG</td>
<td>GAGAGTCCGATTTAGCTTTAATGG</td>
</tr>
</tbody>
</table>

(Continued)
Cloning of \( \text{ahpC}, \text{trx1}, \text{trx2}, \) and \( \text{trxR} \) into expression vectors. The \( H. \text{pylori} \) genes were amplified for general nucleotide sequences for \( \text{ahpC}, \text{trx1}, \text{trx2}, \) and \( \text{trxR} \) (http://www.ncbi.nlm.nih.gov/). Colonies obtained after up to 7 days of incubation in selective medium were carried out as described elsewhere (57).

Cloning of \( \text{ahpC}, \text{trx1}, \text{trx2}, \) and \( \text{trxR} \) into expression vectors. The \( H. \text{pylori} \) genes were amplified for general nucleotide sequences for \( \text{ahpC}, \text{trx1}, \text{trx2}, \) and \( \text{trxR} \) (http://www.ncbi.nlm.nih.gov/). Colonies obtained after up to 7 days of incubation in selective medium were carried out as described elsewhere (57).
RESULTS

Amino acid sequence alignments. In an amino acid sequence comparison of the putative AhpC from H. pylori with the more extensively studied S. typhimurium enzyme, Cys49 in H. pylori AhpC aligns perfectly with the essential, conserved Cys46 from S. typhimurium AhpC (21). Cys49 in H. pylori AhpC is, therefore, most likely the site of interaction with peroxides and the site of sulfenic acid formation. The H. pylori AhpC amino acid sequence was also compared to the deduced amino acid sequences for other 1- and 2-Cys AhpC homologues from a wide range of organisms (Table 2). Of all the known homologues, H. pylori AhpC shares the most sequence identity with a select group of bacterial Prxs including AhpC from Campylobacter jejuni (67%), Tpx from Rickettsia prowazekii (54%), and AhpC from Legionella pneumophila (52%). Other than the proteins from these three bacterial sources, however, H. pylori AhpC is more similar to 2-Cys Prx protein sequences from higher organisms than to other bacterial AhpC proteins (Table 2). Of the sequences shown, S. cerevisiae Prx (2-Cys), C. elegans Prx, the human proliferation-associated gene (2-Cys), and Synchoctis sp. strain PCC6803 (a cyanobacterium) Tpx have all been shown experimentally to be recycled by a Trx-reducing system (10, 36, 52).

TrxR and Trx1 from H. pylori share a moderate amount of sequence identity (37 and 51%, respectively) with the corresponding E. coli homologues and a high percentage of identity (60 and 68%, respectively) with the homologues from the closely related species C. jejuni. As in H. pylori, the absence of ahpF in C. jejuni makes TrxR and Trx1 reasonable candidates as C. jejuni’s AhpC reductase system. H. pylori Trx2 shares the highest sequence identity with a Trx homologue from Archaeoglobus fulgidus (Trx4, 42%) (39) and is more closely related to E. coli Trx2 (41% identity) (45) than to E. coli Trx1 (33% identity) (29). Known redox centers and binding motifs of other Trx and TrxR proteins are completely conserved in the H. pylori proteins. Unlike the vast majority of TrxR-Trx1 systems from other bacteria, the gene encoding TrxR is positioned just downstream from trx1 in the H. pylori genome. The clustering of trx1 and trxR in this order has also been observed in Streptomyces clavuligerus (14); however, in mycobacteria (72) and in C. jejuni (49), the opposite orientation, trxR and then trx1, is observed. In most other species, trxR and trx1 are widely separated on the chromosome. Interestingly, H. pylori trx2 (HP1458) is not found near trx1 (HP0824) or trxR (HP0825).

Characterization of purified H. pylori proteins. AhpC, Trx1, Trx2, and TrxR expressed from pPROK1 in the respective E. coli strains were purified to homogeneity (Fig. 1). All proteins were obtained in high yields as soluble proteins after induction with IPTG at 37°C. Migration of the H. pylori proteins was compared to that of pure S. typhimurium or E. coli homologues during SDS-PAGE, which allowed the detection of each protein during purification and assessment of purity. Pure, reduced AhpC corresponded to an apparent molecular mass of 26 kDa, which is slightly higher than its expected molecular mass of 22,235 Da. When prepared in nonreducing sample buffer and analyzed by SDS-PAGE, AhpC migrated as a 50-kDa protein, indicating that the purified protein is oxidized and contains one or more intersubunit disulfide bonds (Fig. 1A, lane 6). TrxR migrated as a 33-kDa protein on SDS-PAGE under both reducing and nonreducing conditions (Fig. 1A, lanes 5 and 7; expected mass, 33,538 Da). Previously, an intersubunit disulfide bond between TrxR subunits was suggested by Windle et al. (74); our results, however, rule out such...
a linkage between subunits. Spectral analyses (data not shown) of TrxR revealed a strong $A_{454}$, which is typical of enzymes containing a bound flavin cofactor. Trx1 and Trx2 both migrated as monomers in the presence or absence of $\beta$-mercaptoethanol and gave masses of approximately 12 kDa, close to their expected masses of 11,854 and 11,744 Da, respectively.

**Kinetic characterization of Trx1, Trx2, and TrxR.** *H. pylori* TrxR and Trx1 were shown in a series of assays to act as a general disulfide reductase system analogous to their counterparts (data not shown). Using the DTNB-linked assay, our laboratory has demonstrated that peroxidase activity in *S. typhimurium* AhpC is reliant on an essential, conserved cysteine residue (Cys46) while a second active-site cysteine in *E. coli* peroxidase (Cys165) is not required. The replot of slopes with respect to Trx1 concentration (Fig. 3A) showed that lines representing five different concentrations of Trx1 were conducted in the absence of additional electron acceptors and were monitored on the stopped-flow spectrophotometer by the time-dependent decrease in NADPH fluorescence. A representative primary Hanes-Wolf plot of the initial-rate data over a range of NADPH concentrations (Fig. 3A) showed that lines representing five different Trx1 concentrations intersected at the $y$ axis, denoting a sub-stituted (i.e., ping-pong) reaction mechanism for TrxR (15).

The $k_{cat}$ and $K_m$ values for each substrate of *H. pylori* TrxR, assays varying NADPH at different fixed concentrations of Trx1 were conducted in the absence of additional electron acceptors and were monitored on the stopped-flow spectrophotometer by the time-dependent decrease in NADPH fluorescence. A representative primary Hanes-Wolf plot of the initial-rate data over a range of NADPH concentrations (Fig. 3A) showed that lines representing five different Trx1 concentrations intersected at the $y$ axis, denoting a sub-stituted (i.e., ping-pong) reaction mechanism for TrxR (15). The replot of slopes with respect to Trx1 concentration (Fig. 3B) yielded $k_{cat}$ and $K_m$ values, reported in Table 3, which were only slightly different from those for the corresponding *E. coli* proteins. Nonetheless, the catalytic efficiencies ($k_{cat}/K_m$) of both systems and both substrates are in a similar range, indicating that TrxR and Trx1 act as an effective general protein disulfide reductase system similar to the homologous *E. coli* system.

**AhpC cysteine thiol and disulfide quantification.** In previous work, our laboratory has demonstrated that peroxidase activity in *S. typhimurium* AhpC is reliant on an essential, conserved cysteine residue (Cys46) while a second active-site cysteine (Cys165) contributed by a different AhpC subunit stabilizes the oxidized protein through the formation of an intersubunit disulfide bond (21). Thiol quantification of reduced AhpC did not change the titl titer, indicating a high degree of accessibility of the cysteine thiol groups in the reduced protein. As isolated from *E. coli*, overexpressed *H. pylori* AhpC was in its oxidized...
form and lacked free thiol groups; NTSB assays revealed that oxidized AhpC contained one disulfide bond per monomer (0.90 ± 0.07). Taken together, along with the presence of intersubunit disulfide bonds in nonreducing SDS-PAGE gels of AhpC (see above), these results support a head-to-tail arrangement of monomers to form two active sites per dimer in *H. pylori* AhpC, as is the case with *S. typhimurium* AhpC.

**Peroxidase activity of AhpC, Trx1 or Trx2, and TrxR.** To test the ability of *H. pylori* AhpC to reduce hydroperoxides with *H. pylori* Trx1 and TrxR acting as the reducing system, the proteins were mixed with NADPH and cumene hydroperoxide and the change in $A_{340}$ was monitored. The maximal sustained rate of NADPH oxidation was observed when all three proteins, TrxR, Trx1, and AhpC, were included in the assay mixture (Fig. 4). Alone, TrxR possessed oxidase activity (Fig. 4, long, dashed lines), which was observed as a steady decrease in $A_{340}$ at 0.70 s$^{-1}$ in the absence of peroxide; *E. coli* TrxR exhibited a much slower rate of NADPH oxidation (0.01 s$^{-1}$) under these conditions. In the presence of peroxide, NADPH oxidation by *H. pylori* TrxR alone increased to 0.80 s$^{-1}$, which is indicative of weak peroxidatic activity of TrxR. In the absence of AhpC, a small burst in NADPH oxidation was first observed (between 0.3 and 1 s) at a rate of 5.1 s$^{-1}$ relative to the TrxR concentration, which tapers off to a rate similar to that of TrxR alone (0.9 s$^{-1}$) once all of the Trx1 had been reduced (reduction of 5 µM Trx1 accounts for a decrease in $A_{340}$ of 0.03). With all three *H. pylori* proteins present, an initial burst of NADPH oxidation (~6.6 s$^{-1}$ relative to TrxR), at a rate similar to that of TrxR and Trx1 alone, was observed. The somewhat slower but sustained rate observed between 10 and 20 s (2.5 s$^{-1}$) was not significantly changed when AhpC concentrations were varied, suggesting that both Trx1 reduction by TrxR and AhpC reduction by Trx1 were partially rate limiting under these conditions.

Investigation of the *H. pylori* genome yielded two Trx homologues, Trx1 and Trx2, both of which contain the catalytic WCXXC site required for reductase activity. To determine if Trx2 could act as a reductant for *H. pylori* AhpC, stopped-flow peroxidase experiments in which Trx2 replaced Trx1 in the

**FIG. 1.** SDS-PAGE analysis of purified AhpC, Trx1, Trx2, and TrxR from *H. pylori*. (A) Crude lysates and recombinant purified AhpC and TrxR were analyzed on the same 12% polyacrylamide gel in reducing sample buffer (except where noted) as follows: lane 1, molecular mass markers (Broad Range Molecular Weight Standards; Bio-Rad); lane 2, crude extracts of *E. coli* cells transformed with pPROK1/ahpC and induced with 0.4 mM IPTG for at least 3 h at 37°C; lane 3, pure, recombinant AhpC; lane 4, crude extracts of *E. coli* cells transformed with pPROK1/trxR and induced as described above; lane 5, pure, recombinant TrxR protein; lane 6, pure AhpC in nonreducing sample buffer; lane 7, pure TrxR in nonreducing sample buffer. (B) Crude lysates and recombinant Trx1 and Trx2 were analyzed on a 10% Tris-Tricine gel as follows: lane 1, molecular mass markers; lane 2, crude extracts of *E. coli* cells transformed with pPROK1/trx1 after induction with IPTG; lane 3, pure, recombinant Trx1; lane 4, crude extracts of *E. coli* cells transformed with pPROK1/trx2 after induction with IPTG; lane 5, recombinant purified Trx2 after purification. Equivalent protein masses (10 µg) were loaded in all lanes of both gels. Molecular masses (in kilodaltons) are indicated on the left.

**FIG. 2.** Comparative kinetic parameters of Trx1 and Trx2 from *H. pylori*. Trx activity assays using either 200 µM DTNB (black bars) or 80 µM insulin (gray bars) as a substrate for 0 to 50 µM Trx1 or Trx2 were conducted in a standard buffer of 100 mM potassium phosphate and 2 mM EDTA, pH 7.4, with 150 µM NADPH. Assays were started with the addition of 7.0 nM TrxR and were monitored at 340 nm when DTNB was used as the substrate to observe the release of TNB$^2^-$. Rates were determined from the first 10% of the reaction and then fitted to a hyperbola to determine the $V_{max(app)}$ and $K_{m(app)}$ values for each.
assay mixture were conducted (Fig. 5). Initially, NADPH oxidation rates were quite fast for reactions with Trx2 (6.0 and 14.7 s\(^{-1}\) for 5 and 10 μM Trx2, respectively, for data from 0.1 to 1 s), compared to the slower rates occurring at later time points in the same assays (0.8 s\(^{-1}\) from 3 to 10 s). After about 5 s, the rates observed for TrxR alone, for TrxR plus 5 μM Trx1, and for AhpC in the presence of TrxR plus 5 or 10 μM Trx2 were all about the same; only the TrxR-Trx1-AhpC system showed a significantly higher sustained rate of NADPH oxidation (Fig. 5). These data indicate that while Trx2 is a good substrate for TrxR, it fails to act as a reductase for \(H.\) pylori AhpC.

Proof that AhpC reduces peroxides was obtained using an endpoint assay in which the peroxide concentration was monitored over the course of the incubation. In an assay mixture containing TrxR, Trx1, AhpC, NADPH, and H\(_2\)O\(_2\), ferrithiocyanate complex formation with H\(_2\)O\(_2\) decreased over time (5.0 s\(^{-1}\) relative to TrxR), indicating that peroxide was continually consumed in the presence of \(H.\) pylori AhpC (Fig. 6). When AhpC was not included in the reaction mixture, no decrease in the peroxide levels was observed. Again, reaction rates were not linear with respect to AhpC concentrations due to partially rate-limiting reduction by TrxR and Trx1 (data not shown). Full kinetic characterization of AhpC with its reducing system TrxR-Trx1 is described in a later section.

**Specificity of \(H.\) pylori AhpC for TrxR-Trx1 or AhpF-like reductase systems.** In \(S.\) typhimurium and most other bacterial systems, the AhpC component is reduced by a specialized flavoprotein related to TrxR and known as AhpF, NADH oxidase, Nox-1, or PrxR (55). In a set of experiments designed to test the specificity of \(H.\) pylori AhpC for its own reductase system, \(S.\) typhimurium AhpF replaced \(H.\) pylori Trx1 and TrxR in the stopped-flow peroxidase assay mixture with \(H.\) pylori AhpC. No significant rate of NADH oxidation was observed in an anaerobic \(S.\) typhimurium AhpF and \(H.\) pylori AhpC system (Fig. 5, medium dashes). Higher concentrations of AhpF still showed no activity with \(H.\) pylori AhpC (data not shown), indicating a clear specificity of the cysteine-based peroxidase for reduction by Trx1 rather than by AhpF from a different bacterial source. \(S.\) typhimurium AhpC (10 μM) also exhibited
considerable specificity for its own reductase, AhpF (0.5 μM), compared with reduction by E. coli TrxR (0.5 μM) plus E. coli Trx1 (5 μM); turnover rates were 42 s⁻¹ with AhpF and 1.7 s⁻¹ with E. coli TrxR-Trx1 (data not shown). Nonetheless, the rate of turnover of S. typhimurium AhpC with the E. coli TrxR-Trx1 system was only about twofold lower than that of H. pylori AhpC with its own TrxR-Trx1 system (3.2 s⁻¹ under the same conditions) while H. pylori AhpC and S. typhimurium AhpF interaction was undetectable. Among the proteins under investigation, H. pylori TrxR and Trx1 and E. coli TrxR and Trx1 were the most interchangeable in peroxidase assays. H. pylori AhpC (2 μM) assayed with the E. coli proteins TrxR (2 μM) and Trx1 (25 μM) exhibited a rate of NADPH oxidation that was about the same as that obtained with H. pylori TrxR-Trx1 under the same conditions (1.9 versus 3.2 s⁻¹).

**Steady-state kinetics of AhpC.** To further investigate the peroxidase activity of H. pylori AhpC, reaction conditions were first established under which initial rates were directly proportional to AhpC at a Trx1 concentration (30 μM) that was at least 10-fold higher than the maximal concentration of AhpC (3 μM). Because the very low intrinsic NADPH oxidase activity of E. coli TrxR allowed the observation of low peroxidase rates above background NADPH turnover, E. coli TrxR replaced H. pylori TrxR using concentrations that were high enough (2 to 3 μM) to support rapid H. pylori Trx1 recycling (i.e., additional TrxR did not further increase observed rates of NADPH oxidation). AhpC-dependent rates of NADPH oxidation measured over a range of Trx concentrations (15 to 30 μM) suggested a simple bimolecular interaction between reduced Trx1 and oxidized AhpC at a rate of 1.0 × 10⁵ M⁻¹ s⁻¹ (data not shown).

Given the putative nonsaturable interaction between Trx1 and AhpC suggested by the studies described above and the need for information concerning interactions between reduced AhpC and hydroperoxide substrates, kinetic data were generated and analyzed as described previously by Dalziel (18). Because the secondary Dalziel plots (e.g., Fig. 7B) intersect the y axis at the origin (within experimental error), the Km values of AhpC for peroxides and Trx1 and Vmax values are infinite. Parallel lines in the primary plot (Fig. 7A) also indicate a substituted (ping-pong) mechanism for H. pylori AhpC by which AhpC does not form detectable enzyme-substrate complexes with either substrate and displays nonsaturating kinetics, a kinetic pattern that has been observed for other nonheme peroxidases, such as glutathione peroxidase (23) and tryptophan peroxidase (46).

With the above observations in mind, the rate data were plotted using the Dalziel equation for two-substrate reactions in which enzyme-substrate complexes are not observed: [E]f / v = φ1f [ROOH] + φ2f [Trx1], where [E]f is the concentration of AhpC and φ1f and φ2f are the Dalziel kinetic coefficients. Because enzyme-substrate complexes are not observed experimentally, the interactions of AhpC with its two substrates can be depicted as a sequence of consecutive, bimolecular, non-reversible reactions: AhpCred + ROOH → AhpCox + ROH + H₂O and AhpCox + TrxRed → AhpCred + Trx1ox. The apparent limiting rates for these two reactions are characterized by their kinetic rate constants, k1 and k2, respectively, which are the reciprocals of the Dalziel coefficients, φ1f and φ2f. To obtain the rate constants, the rate data were evaluated using the integrated Dalziel equation (24). After elucidating product and reactant amounts at various times, the data were substituted into the integrated rate equation (given in Materials and Methods) and plotted (Fig. 7A). The slopes of the lines in the primary plot are equal to φ1f; y intercepts from Fig. 7A replotted in Fig. 7B versus the reciprocal of the Trx1 concentration produced a line with a slope of φ2f. By taking the reciprocal of φ2f, the second-order rate constant, k₂, of 1.0 × 10⁵ M⁻¹ s⁻¹ was determined for the interaction between AhpC and Trx1, giving the same value as that obtained using the AhpC-dependent assays described above, in which the peroxide substrate...
was in excess. Using a similar strategy, the value for $k_1^\prime$ ($2.0 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$) was determined for the interaction of AhpC with H$_2$O$_2$ and was of a magnitude similar to that of $k_2$ for the oxidation of Trx1. Therefore, both steps are partially rate limiting under these conditions.

AhpC was also tested for the ability to reduce peroxides other than H$_2$O$_2$, including EtOOH, t-BOOH, CHP, and linoleic acid hydroperoxide (LOOH). Using one concentration of Trx1 (2.0 $\mu$M), the slopes from the primary plot were determined for each peroxide to yield the following apparent $k_1^\prime$ values: H$_2$O$_2$, $2.0 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$; EtOOH, $1.7 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$; t-BOOH, $1.6 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$; CHP, $1.2 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$; LOOH, $1.1 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$. AhpC was capable of reducing the different peroxides, including the more structurally complex compounds t-BOOH and LOOH, with similar apparent rate constants. No change in $k_1^\prime$ reflecting the interaction between Trx1 and AhpC was observed for the different hydroperoxide substrates.

**Essentiality of H. pylori ahpc.** Allelic replacement mutagenesis of ahpc was conducted to study the physiological role of AhpC in *H. pylori* and to determine the effect of AhpC removal on *H. pylori*’s responses to oxidative stress. When ahpc was interrupted with the camR cassette was introduced into *H. pylori* strains HP26695, SS1, HP1061, and HP1, there was no growth of colonies on selective medium after 7 days of incubation, whereas a few thousand colonies are normally obtained after 2 to 3 days for nonessential-gene knockouts carried out in this manner (e.g., with the rdxA knockout control). While single crossover events have been described in *H. pylori* using this method of transformation (57), no such phenomenon was observed even after several repeated transformation attempts. A similar construct was successfully generated in which the nitroreductase (rdxA) gene was knocked out with the same camR cassette (26) and introduced by transformation and homologous recombination into the same *H. pylori* strains. Both Cam’ and Mtz’ (metronidazole-resistant) colonies were formed, indicating that rdx is not essential for growth. Therefore, the loss of growth under microaerobic conditions with the loss of ahpc strongly suggests an essential role for AhpC in *H. pylori* viability.

**Genetic characterization of ahpc.** The gene encoding AhpC is located between cele (HP1562, encoding an iron III ABC transporter) transcribed in the opposite direction and a gene encoding an outer membrane protein (HP1564) in the same orientation as ahpc and containing endogenous promoter sequences (71). The location of the transcriptional start site of ahpc was determined by primer extension analysis. Two prominent reverse transcript bands migrate parallel to G and T residues, 96 and 94 bp upstream, respectively, from the AUG translation initiation codon (Fig. 8A). Sequences resembling the *H. pylori* $\sigma^{33}$ consensus sequence (25) were located at the expected distance from the transcriptional start site (Fig. 8B, bold). The potential $-10$ hexamer of the putative ahpc promoter, TATACT, displays a high degree of identity (5 of 6 bp) to the $-10$ consensus sequence for *H. pylori*, TATAAT (25). Other putative regulatory sequence elements were identified in the promoter region centered around $-40$ and $+10$ (underlined, Fig. 8B), underscoring the possibility that ahpc transcription is regulated. However, no Fur (ferric uptake regulator) binding site (69) was evident in the *H. pylori* ahpc promoter region and the *H. pylori* genome lacks a structural gene for OxyR, the redox-sensitive transcription factor which upregulates AhpC production in response to oxidative stress in other eubacteria (65). Although the deduced *H. pylori* AhpC amino acid sequence has already been published, the protein was not identified as an alkyl hydroperoxide reductase and the translational start site of ahpc was not correctly positioned by O’Ttole et al. (48).
DISCUSSION

Antioxidant systems critical to the defense of \textit{H. pylori} against ROS generated by the oxidative burst of macrophages and polymorphonuclear leukocytes are central to the ability of this organism to establish chronic infections in gastric tissues and to combat the high degree of inflammatory responses mounted by the host. To date, Fe-dependent superoxide dismutase (63), catalase (47), and the Trx-dependent peroxidase (AhpC) described herein have been characterized as \textit{H. pylori} antioxidant enzymes. Deletion of the gene encoding catalase (\textit{katA}) from the \textit{H. pylori} chromosome did not affect the viability of the organism (71), whereas we have found that \textit{ahpC} is essential for \textit{H. pylori} survival under microaerobic conditions. Previous studies with other organisms in which the \textit{ahpC} locus was deleted or mutated have not demonstrated AhpC essentiality. For example, in studies of the closely related, but nonpathogenic \textit{C. fasciculata}, deletion of \textit{ahpC} decreased its capacity to survive in macrophages (50), while deletion of \textit{ahpC} increased susceptibility to oxidative stress (4); nonetheless, viability under conditions of low oxygen tension was not affected for either mutant. In support of our findings, two other groups have recently reported attempts to knock out \textit{H. pylori ahpC} and have reached similar conclusions regarding its essentiality (42, 13). \textit{H. pylori} sensitivity toward oxidative damage is highlighted by its dependence on microaerophilic growth conditions. Its inability to grow in the absence of \textit{ahpC} is a clear reflection of the delicate redox balance required to support, yet not inactivate, key metabolic enzymes (37).

Given the apparent requirement of TrxR-Trx1 for AhpC activity, the TrxR-Trx1 reductase system is also likely to be indispensable for \textit{H. pylori} viability, as well, although this hypothesis has yet to be tested. The alternative, that an as-yet-unidentified reductase is also capable of AhpC reduction, is also a possibility. Interestingly, no homologues for the \textit{E. coli} glutathione reductase system, such as glutaredoxin (grx4), \gamma-L-glutamyl-L-cysteine synthetase (gshA), or glutathione reductase (gorA), can be found in the \textit{H. pylori} genome. Therefore, \textit{H. pylori} does not have the main other reductase system that can serve as a compensatory mechanism in most other organisms (56), highlighting the possible fundamental requirement for a functional TrxR-Trx1 system in \textit{H. pylori}.

Our demonstration that the 26-kDa protein previously described as an abundant “species-specific antigen” by O’Toole et al. (48) is a cysteine-based peroxidase reactivated by reduced Trx agrees nicely with the sequence-based comparisons of \textit{H. pylori} AhpC with a wide variety of other Prxs. A subset of these proteins, some of which have been classified as TPxs, are not reducible by bacterial AhpF proteins and rely on reducing equivalents from Trx to support turnover with cellular peroxides (10, 33, 36, 52). We have found that this capacity to accept electrons from reduced Trx is a property common to bacterial AhpC proteins as well, which nonetheless show much greater reactivity toward their specialized flavoprotein reductant, AhpF. Some eukaryotic Prxs, on the other hand, are incapable of turnover with reduced Trx proteins, at least under the conditions tested (22, 35). The specificity of such cysteine-based peroxidases for reduced Trx, as implied by the TPx designation, is therefore of questionable validity as a unique functional descriptor distinguishing this group among the diverse Prx proteins.

In cases in which the reactivation of Prx proteins by their electron donor proteins has been investigated in detail, two different kinetic patterns have been observed. For AhpF-AhpC interactions, reduction of AhpC by AhpF is a saturable phenomenon characterized by a \(k_{\text{cat}}\) for AhpC of around 15 \(\mu\)M and a \(k_{\text{cat}}/K_{\text{m}}\) for the flavoprotein of \(\sim 10^7\) \(M^{-1}\) \(s^{-1}\) (55). For the Prx system from \textit{C. fasciculata}, interaction of reduced tryptoperoxidin with its peroxidase is a bimolecular process with infinite \(k_{\text{cat}}\) and \(K_{\text{m}}\) values and a second-order rate constant of \(1.5 \times 10^6\) to \(3.5 \times 10^6\) \(M^{-1}\) \(s^{-1}\) (46). Here, we have demonstrated that the interaction of Trx1 with \textit{H. pylori} AhpC is also bimolecular (\(\sim 10^7\) \(M^{-1}\) \(s^{-1}\)) and that therefore no enzyme-substrate complexes between Trx1 and AhpC can be detected kinetically. Presumably, this kinetic pattern will hold for other Trx-dependent Prx proteins as well when such kinetic profiles are investigated.

Using kinetic studies akin to those previously applied to another Prx, tryparedoxin peroxidase from \textit{C. fasciculata}, the interaction of \textit{H. pylori} AhpC with peroxides was also shown to be a bimolecular process (like Trx-Prx interactions) lacking...
An essential role for AhpC in cellular peroxide reduction by *H. pylori* has been established with our *ahpc* mutagenesis experiments, and this is, to our knowledge, the only case in which an *ahpC* locus has been shown to be required for viability. The presence of this essential Trx-dependent peroxidase in *H. pylori* suggests important roles for TrxR, Trxl, and AhpC in the removal of alkyl hydroperoxides to protect against oxidative stress and in the preservation of the microaerobic environment required for *H. pylori* viability.

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**FIG. 9.** Pathway for transfer of reducing equivalents from NADPH to hydroperoxide in the alkyl hydroperoxide reductase system from *H. pylori*. Note that the enzyme species shown do not necessarily represent actual catalytic intermediates. FAD, flavin adenine dinucleotide.

detectable enzyme-substrate complexes. This ping-pong mechanism has also been observed for the distantly related glutathione peroxidases (23, 68). The higher rate of enzyme-peroxide interaction for glutathione peroxidase (−10³ M⁻¹ s⁻¹) has been attributed to the unique reactivity of the selenocysteine at the active site (64), although at 10³ to 10⁶ M⁻¹ s⁻¹, the rate of peroxide reduction is still quite high and indicative of an important role for *H. pylori* AhpC in cellular peroxide metabolism. Using k₁ to characterize the protein-peroxide interaction, our experiments demonstrated essentially no specificity when AhpC was tested with a wide variety of small, bulky, aromatic, or lipid hydroperoxide substrates, as was true of the *C. fasciculata* peroxidase (46). Differential reactivities toward particular hydroperoxide substrates have been reported for some other Prx enzymes based on less quantitative analyses (9, 33, 34). In addition, peroxynitrite (OONO⁻) has recently been shown to be a substrate for *H. pylori* AhpC, with the rate of decomposition occurring at a second-order rate constant of 1.21 × 10⁶ M⁻¹ s⁻¹ (7). These results are all consistent with a minimal binding site on AhpC for hydroperoxides (and peroxynitrite) consisting of little more than the catalytic residue (Cys49) at the active site.

On the basis of results reported here and mechanisms outlined for other 2-Cys AhpC homologues (10, 51), electrons from NADPH proceed along the path outlined in Fig. 9 for the reduction of peroxides to alcohols. This scheme is highly analogous to that for electron transfer through the *S. typhimurium* AhpC system, except that TrxR and Trxl replace AhpF in the *H. pylori* system. Nonetheless, one tightly bound flavin and three disulfide redox centers mediate electron transfer from pyridine nucleotide to peroxide in both cases.

Little information exists on potential redox or iron regulation of *H. pylori* AhpC expression, although in other studies of TrxR and Trxl from *H. pylori*, Windle et al. (74) observed that Trxl expression dramatically increased under conditions of oxidative stress; Trxl was therefore classified as a stress response protein in *H. pylori*. While the proximal location of trxl and trxl in the chromosome could provide the bacterium with a mechanism for a coordinated response eliciting expression of both proteins, an increase in Trxl expression did not accompany increased Trxl expression under oxidative stress conditions (74). Interestingly, reductase activity of the TrxR-Trxl reductase system was also reportedly present in the media of culture supernatants and could be available to support extra-cellular peroxide reduction by AhpC if the latter protein is also exported.

In conclusion, this is the first report of a Trx-dependent alkyl hydroperoxide system from a gastric pathogen. To our knowledge, AhpC from *H. pylori* is only the second known bacterial AhpC to be demonstrated experimentally to require the Trx-reducing system for reduction of the oxidized AhpC active site. An essential role for AhpC in *H. pylori* has been established with our *ahpc* mutagenesis experiments, and this is, to our knowledge, the only case in which an *ahpC* locus has been shown to be required for viability. The presence of this essential Trx-dependent peroxidase in *H. pylori* suggests important roles for TrxR, Trxl, and AhpC in the removal of alkyl hydroperoxides to protect against oxidative stress and in the preservation of the microaerobic environment required for *H. pylori* viability.


