Oxidative-Stress Resistance Mutants of *Helicobacter pylori*

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Within a large family of peroxidases, one member that catalyzes the reduction of organic peroxides to alcohols is known as alkyl hydroperoxide reductase, or AhpC. Gene disruption mutations in the gene encoding AhpC of *Helicobacter pylori* (*ahpC*) were generated by screening transformants under low-oxygen conditions. Two classes of mutants were obtained. Both types lack AhpC protein, but the major class (type I) isolated was found to synthesize increased levels (five times more than the wild type) of another proposed antioxidant protein, an iron-binding, neutrophil-activating protein (NapA). The other class of mutants, the minor class (type II), produced wild-type levels of NapA. The two types of AhpC mutants differed in their frequencies of spontaneous mutation to rifampin resistance and in their sensitivities to oxidative-stress chemicals, with the type I mutants exhibiting less sensitivity to organic hydroperoxides as well as having a lower mutation frequency. The *napA* promoter regions of the two types of AhpC mutants were identical, and primer extension analysis revealed their transcription start site to be the same as for the wild type. Gene disruption mutations were obtained in *napA* alone, and a double mutant strain (*ahpC napA*) was also created. All four of the oxidative-stress resistance mutants could be distinguished from the wild type in oxygen sensitivity or in some other oxidative-stress resistance phenotype (i.e., in sensitivity to stress-related chemicals and spontaneous mutation frequency). For example, growth of the NapA mutant was more sensitive to oxygen than that of the wild-type strain and both of the AhpC-type mutants were highly sensitive to paraquat and to cumene hydroperoxide. Of the four types of mutants, the double mutant was the most sensitive to growth inhibition by oxygen and by organic peroxides and it had the highest spontaneous mutation frequency. Notably, two-dimensional gel electrophoresis combined with protein sequence analysis identified another possible oxidative-stress resistance protein (HP0630) that was up-regulated in the double mutant. However, the transcription start site of the HP0630 gene was the same for the double mutant as for the wild type. It appears that *H. pylori* can readily modulate the expression of other resistance factors as a compensatory response to loss of a major oxidative-stress resistance component.

Helicobacter pylori is a spiral bacterium that colonizes the gastric mucosae of humans, leading to a variety of gastric inflammatory responses, including peptic ulcer disease and chronic gastritis. Prolonged infections and inflammation due to persistent *H. pylori* colonization lead to severe tissue damage and sometimes to adenocarcinomas of the stomach (4). The pathogenesis of *H. pylori* depends on its persistence, which in turn depends on its survival in a harsh environment, which can be characterized by acidity, peristalsis, and attack by phagocytes and their released oxygen species (19, 25). These and other host defenses are undoubtedly counteracted by the successful pathogen, and some of these host responses are even beneficial to further colonization (5). Even outside of the host, it is clear that a microaerobic environment is important for the survival of *H. pylori*. Consequently, there are prevailing views (12, 19, 24) that O_2-mediated damage is commonly encountered by the bacterium and that protective pathways to deal with toxic O_2-derived products may be key to the organism's ability to colonize the mucosal environment of the host.

Alkyl hydroperoxide reductase (AhpC) belongs to a family of peroxidases that are beginning to receive intense research focus due to their roles in dissipating damaging hydrogen peroxides (10) and related hydroperoxides. The specific role of AhpC in catalyzing the reduction of organic hydrogen peroxides to their respective alcohols and the roles of accessory enzymes in these reductions are being determined (13, 33, 34). In addition, AhpC of *Salmonella enterica* serovar Typhimurium was shown to confer resistance to reactive N intermediates (9). *H. pylori* AhpC is closely related to the peroxiredoxin of higher organisms, such as those found in *Saccharomyces cerevisiae* and *Caenorhabditis elegans*, but it also has considerable sequence identity to five bacterial AhpCs (2). The reductase system for pure *H. pylori* AhpC was identified and consists of thioredoxin and thioredoxin reductase; the flavoprotein reductant (AhpF) used by most bacteria to reduce AhpC was shown to be inactive for the *H. pylori* AhpC system (2). The complete system (AhpC with thiol-specific reductant) was capable of reducing a variety of hydroperoxide substrates that include both small aromatic and lipid hydroperoxides. It was concluded that *H. pylori* alkyl hydroperoxide reductase is essential for growth of the bacterium, since gene-disrupted deletion mutants could not be recovered (2, 8, 23).

Based on the genome sequence, *H. pylori* contains a battery of enzymes to combat oxidative stress, but the importance of these enzymes for the growth of the organism via targeted mutagenesis approaches has been addressed for only a few of these (24, 29; R. W. Seyler and R. J. Maier, Abstr. 101st Gen. Meet. Am. Soc. Microbiol., abstr. D-185, 2001). To address the role of AhpC in *H. pylori*, we obtained strains with mutations in the *ahpC* gene, encoding AhpC, by screening the allelic-exchange transformants under 2% O_2 conditions. Surprisingly, two types of mutants were recovered. Both classes lack AhpC,

3186

TABLE 1. Strains and plasmids

Strain, plasmid, or primer	Description	Source
Strains		
H. pylori		
ATCC 43504	Parent strain for all *H. pylori* strains	ATCC[a]
ahpC::Kan type I	*aphA3* insertion within *ahpC*	This study
ahpC::Kan type II	*aphA3* insertion within *ahpC*	This study
napA::Cm	*cat* insertion within *napA*	This study
ahpC::*napA*	*ahpC napA* double mutant	This study
E. coli DH5α	Cloning strain	Lab stock
Plasmids		
pBluescript KS+	Cloning vector	Stratagene
pKSΔS	pBluescript KS+ with destroyed *Sac*I site	This study
pAhpC	*ahpC* inserted into *Sma*I site of pBluescript KS+	This study
pAhpCKan	*aphA3* inserted into *Afl*II site of pAhpC	This study
pNapA	*napA* cloned from ATCC 43504 into pBluescript KS+	This study
pNapAT1	*napA* cloned from *ahpC*::Kan type I mutant	This study
pNAΔS	*napA* cloned into pKSΔS	This study
pNapA::Cm	*cat* inserted in *Sac*I site of pNAΔS	This study
Primers		
ahpCF	5′ AGCCACGCCCAATAACGATG 3′	IDT[b]
ahpCR	5′ TCGCCTTTTCTCCAACCTGCTG 3′	IDT
NapAF	5′ AACCACTAAATTAAAGGGTAACGGC 3′	IDT
NapAR	5′ CGCTGTAATCTCTCATGCTGGC 3′	IDT
NapART	5′ ACACGATCGCATCCGCTTGC 3′	IDT
HP0630RT	5′ AGAATGCCCGAACGCTTTGG 3′	IDT

[a] ATCC, American Type Culture Collection.
[b] IDT, Integrated DNA Technologies, Coralville, Coraville, Iowa.

but the major class of mutant recovered was found to up-regulate another proposed antioxidant protein, an iron-binding (31), neutrophil-activating protein (NapA) (14). Consequently, we created single mutant strains with individual mutations in the gene for NapA and a double mutant strain with mutations in the genes for NapA and AhpC. Of the four types of mutants, the double mutant was the most sensitive to oxidative stress and by far had the highest frequency of spontaneous mutation. However, all the mutants could be distinguished from the wild type in some oxidative-stress resistance phenotype. The phenotypic results are consistent with the conclusion that *H. pylori* has multiple oxidative-stress-related proteins and that compensatory mutations occur to circumvent the loss of AhpC.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *H. pylori* strain ATCC 43504 was used as the wild type. Cultures of *H. pylori* were grown microaerobically at 37°C in a 5% CO_2 incubator (model 3130; Forma Scientific) under different continuously controlled levels of oxygen (between 2 and 12% partial pressure, as indicated). Plates coated with brucella agar (Difco) supplemented with 10% defibrinated sheep blood (Gibson Laboratories, Inc.) are called BA plates and were used for growth of *H. pylori*. Kanamycin (at 30 μg/ml) or chloramphenicol (at 20 μg/ml) was added as indicated. All strains were confirmed to be *H. pylori* because of the presence of urease activity and catalase activity and the helical morphology of the organisms as determined by phase-contrast microscopy (28). Genetic manipulations were performed with *Escherichia coli* strain DH5α. Luria-Bertani agar supplemented with ampicillin (100 μg/ml), chloramphenicol (20 μg/ml), or kanamycin (30 μg/ml) was used for growing various *E. coli* strains, as noted. Strains and their sources are given in Table 1.

Cloning of *ahpC* and construction of an *ahpC*::Kan mutant. Oligonucleotide primers for cloning the *ahpC* gene (ahpCF and ahpCR) (Table 1) were designed from the sequenced strain J99 (1). PCR amplification was performed with *Taq*

DNA polymerase (Fisher Scientific, Pittsburgh, Pa.) using chromosomal DNA from *H. pylori* strain ATCC 43504 as a template. The PCR product (851 bp) was then treated with T4 DNA polymerase (Promega) and phosphorylated with T4 polynucleotide kinase (Promega). The fragment was then cloned into a *Sma*I site of pBluescript KS+ (Stratagene) to give pAhpC. This construct was then sequenced by the University of Georgia Molecular Genetics Instrumentation Facility. The *ahpC*::Kan construct was created via insertion of kanamycin resistance cassette AphA3 (25) into the *Afl*II site of pAhpC. Restriction analysis revealed that the Kan^r cassette was in the orientation opposite that of the *ahpC* gene.

Cloning of *napA* and construction of pNapA::Cm. A fragment of *H. pylori* DNA corresponding to the *napA* gene was amplified from genomic DNAs obtained from both *H. pylori* strain ATCC 43504 and the *ahpC*::Kan (type I) strain with the primers NapAF and NapAR (Table 1) and *Taq* DNA polymerase (Fisher Biotech). This 809-bp fragment contains the entire *napA* coding region as well as 339 bp upstream of the *napA* start codon. The PCR fragment was treated with T4 DNA polymerase to ensure blunt ends and phosphorylated with T4 polynucleotide kinase. The fragments were then ligated with pBluescript KS+ digested with *Sma*I. The resulting clones, pNapA (*napA* cloned from ATCC 43504) and pNapAT1 (*napA* cloned from the *ahpC*::Kan type I strain), were then sequenced at the University of Georgia Molecular Genetics Instrumentation Facility. In order to utilize the *Sac*I unique restriction site within the *napA* coding region, it was necessary to excise the fragment with *Eco*RI and *Bam*HI and clone it into a derivative of pBluescript KS+ in which the *Sac*I site had been destroyed (pKSΔS), yielding pNAΔS. *Sac*I-digested pNAΔS was then filled in with T4 DNA polymerase and ligated to the chloramphenicol resistance cassette (*cat*). The resulting plasmid, pNapA::Cm, was then electroporated into competent ATCC 43504 cells to yield the *napA* mutant *napA*::Cm and into competent *ahpC*::Kan type I cells to yield the *ahpC napA* double mutant. Insertion of the cassette was confirmed by isolation of chromosomal DNAs from the mutant strains and PCR amplification of the *napA* locus, followed by agarose gel electrophoresis, which was used to monitor the increase in size of *napA* due to the *cat* insertion.

Transformation of *H. pylori*. Preparation of competent cells involved harvesting and washing cells four times in a solution of (ice-cold) sucrose-glycerol, followed by the immediate storage of the cells at −80°C. *H. pylori* cells were transformed with pAhpCKan by electroporation (27). During transformation, 2

TABLE 2. Growth sensitivity to oxygen

Strain	Growth sensitivity to oxygen concn (%)[a]:						
	2	3	4	5	7.5	10	12
Wild type	++	+++	+++	+++	+++	+++	+++
ahpC::Kan type I	++	+	+/−	+/−	+/−	−	−
ahpC::Kan type II	++	+	+/−	+/−	+/−	−	−
napA::Cm	++	+++	++	+	+	+/−	+/−
ahpC napA	+	+	+/−	+/−	−	−	−

[a] Score on day 3. Symbols: −, no growth; +/−, slight growth at the site of greatest inoculum, no individual colonies; +, significant growth at the site of greatest inoculum, no individual colonies; ++, significant growth and development of isolated colonies (pinpoint-like colonies, less than 0.5 mm in diameter); +++, healthy growth with individual colonies (more than 0.5 mm in diameter).

μl of the plasmid was added to 50 μl of competent cells and electroporated with Transporator Plus (BTX, San Diego, Calif.) with a pulse of 2.5 kV. Immediately after the pulse, 50 μl of Mueller-Hinton broth (Difco) was added to the cuvette containing the competent cells. Aliquots of these cells were then immediately plated on nonselective BA plates, and the plates were incubated in the CO_2 incubator under partial pressure (2% O_2). After a 48-h incubation period, the cells from this nonselective medium were transferred to BA plates containing kanamycin. Kan^r colonies were selected by incubation in a 2% O_2 incubator. Genomic DNA isolated from Kan^r strains were shown to have an increase in the size of the PCR product of ahpC from 851 bp to approximately 2,251 bp, as anticipated. Further, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and two-dimensional (2D) gels were used to confirm the lack of synthesis of AhpC protein in the gene-directed mutants.

Gel electrophoresis. Plate-grown cells (2% O_2) were harvested with a swab from the plate and resuspended in 50 mM Tris (pH 7.4) buffer containing 50 mM NaCl. The cells were collected by centrifugation (10,000 × g for 10 min), resuspended in Tris-NaCl buffer, and broken by two passages through a French pressure cell at 138,000 kPa (SLM Instruments, Inc.). Crude extracts were then cleared of unbroken cells by centrifugation at 10,000 × g for 10 min. The protein concentrations of the cell extracts were determined with a bicinchoninic acid protein assay kit (Pierce, Rockford, Ill.). For SDS-PAGE, 5 μg of cell extract was placed into SDS buffer, boiled for 5 min, and applied to a denaturing 12.5% acrylamide gel as described previously (21). For 2D gel electrophoresis, 50 μg of total protein (crude extract) was added to 1.125× immobilized-pH-gradient (IPG) buffer (1× concentration: 8 M urea, 2% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, and 40 mM Tris base). The IPG strips (pHs 3 to 10; Bio-Rad, Hercules, Calif.) were then rehydrated overnight at room temperature with the IPG buffer-treated protein. Proteins were focused for 5 h at 3,000 V by using a Multiphore II isoelectric-focusing apparatus (Pharmacia Biotech, Piscataway, N.J.). The strips were then placed onto a 12.5% acrylamide gel, and the proteins were separated in the second dimension by SDS-PAGE, which was performed according to the recommendations of the manufacturer (Bio-Rad). Protein spots were visualized by silver staining as described previously (26).

Protein fractionation and sequencing. Crude extracts of H. pylori wild-type cells were subjected to partial purification by fast-protein Q-Sepharose ion-exchange chromatography (Pharmacia Biotech). Proteins were prepared for sequencing by subjecting the starting material (partially purified ahpC::Kan type I crude extract for NapA, or the ahpC napA crude extract for HP0630) to SDS-PAGE (12.5% acrylamide), followed by electroblotting onto a polyvinylidene difluoride membrane (32). The appropriate bands were then excised and subjected to eight cycles of N-terminal protein sequencing. Protein sequencing was performed by the Molecular Genetics Instrumentation Facility at the University of Georgia.

RNA isolation and primer extension analysis. Plates were streaked and incubated at 2% O_2 for 3 days. The harvested cells of the wild type, napA single mutant, and ahpC napA double mutant were resuspended in 1 ml of phosphate-buffered saline (PBS). RNA was isolated as described previously (2). For the primer extension analysis, 10 pmol each of either primer NapART or HP0630RT (Table 1) was 5′-end labeled in the reaction with 5 μl of [γ-^{32}P]ATP (6,000 Ci/mmol; Amersham) and 10 U of T4 polynucleotide kinase (Promega). An RNA sample (11 μl at a concentration of 200 μg/ml) was incubated with a 3-μl solution of the ^{32}P-labeled primers and allowed to anneal at 70°C for 5 min, followed by slow cooling to 50°C for 3 h. A mixture containing 1 μl of avian myeloblastosis virus reverse transcriptase (Promega), 4 μl of 5× reverse transcriptase buffer (supplied with enzyme), and 1 μl of a 10 mM concentration of the deoxynucleoside triphosphates was added to the annealed primers for 1 h at

42°C. DNA-sequencing reactions of napA and HP0630 were performed simultaneously with the same primers and the fmol DNA-sequencing system (Promega). Aliquots of primer extension and DNA-sequencing reaction mixtures were heated prior to being loaded onto a 6% PAGE–7 M urea gel. Gels were run for 2 h at 65 W and dried, and bands were visualized by exposure to film.

Growth sensitivity to oxygen. H. pylori mutant and wild-type strains were streaked for individual colony observation by use of three-way streaks onto BA plates. These plates were placed into the CO_2 incubator under controlled O_2 concentrations. The incubators were adjusted to different (but continuously controlled) oxygen levels (2, 3, 4, 5, 7.5, 10, or 12% partial pressure), and the plates were observed for the presence of isolated colonies in the area of the final streak after 3 days in the incubator. Individual colony sizes described in Table 2 are from the third streak, when individual colonies were the farthest apart.

Tolerance to oxygen. BA plates were heavily streaked and incubated at 2% O_2 for 3 days to obtain confluent growth. ahpC::Kan and wild-type cells from these plates were harvested, and cell suspensions were prepared in PBS. The optical density of the cells was measured (at 600 nm) and adjusted to 0.1; 5-ml aliquots were exposed to either anaerobic or aerobic conditions at 37°C, as previously described (29). Undiluted samples from each set of conditions were plated hourly for a period of 6 h onto BA plates. CFU (survivors) were counted after 3 to 5 days of incubation with 2% O_2.

Frequency of spontaneous mutations. The frequency of spontaneous mutations was monitored by quantitating mutation to rifampin resistance (29). The strains were plated onto BA plates with or without rifampin (5 μg/ml). Cells were first harvested from the plates and then resuspended in PBS buffer. Optical density (at 600 nm) was measured, and dilutions were plated to determine the number of viable cells in the original (undiluted) suspension. The mutant was plated onto BA plates containing kanamycin, and the wild type was plated onto the same medium but without kanamycin. The original suspensions of wild-type and mutant cells were also plated onto BA plates supplemented with rifampin. The numbers of wild-type and mutant cells were then compared with the numbers of colonies identified on rifampin plates, and the mutation frequency was calculated (29).

Peroxide sensitivity. Sensitivity to three different cytotoxic agents was evaluated by disk assays (29). Sterile filter paper disks 7.5 mm in diameter containing one of the agents in water (5% [vol/vol] cumene hydroperoxide, 5% [vol/vol] t-butyl hydroperoxide, or 2 mM paraquat) were placed on BA plates (100 by 15 mm, 25-ml volume) that had previously been streaked for confluent growth with either mutant or wild-type cells. The plates were then placed in the 2% O_2 incubator. Following a 2-day incubation period, the clear zones surrounding the disks were measured. The data given represent the distances from the edge of the disk to the end of the clear zone, where growth begins. No growth inhibition was seen in disks containing H_2O.

RESULTS

Sequencing and cloning of the ahpC gene. The H. pylori ahpC gene no. HP1563 (gene numbers are from reference 30) encodes an alkyl hydroperoxide reductase, known as AhpC (30). Our goal was to clone this gene to enable the generation of gene-directed mutants for use in physiological studies. The cloned gene was then used to make a Kan^r ahpC-disrupted mutant by allelic exchange (see Materials and Methods). Importantly, the mutant strains were obtained by screening the
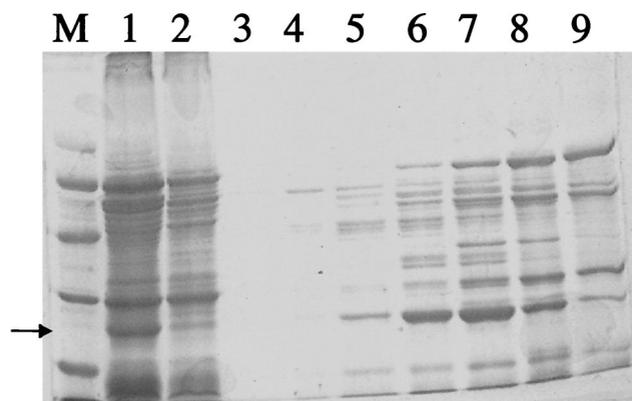
FIG. 1. Fractionation of AhpC via Q-Sepharose chromatography. The arrow delineates the protein of interest, AhpC. Lane M, low-range standards composed of phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa); lane 1, *H. pylori* crude extract; lane 2, flowthrough eluent; lanes 3 through 9, fractions 20 through 26.
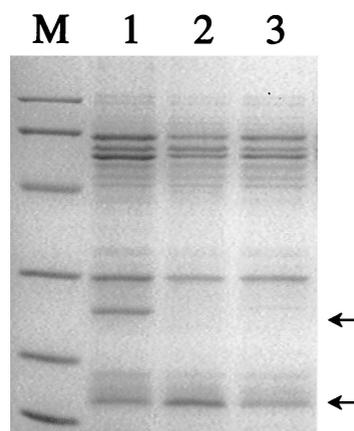

FIG. 2. SDS-PAGE of *H. pylori* strains. Five micrograms of crude extract was loaded into each lane. Lane M, low-range markers (sizes are indicated in the legend to Fig. 1); lane 1, strain ATCC 43504; lane 2, *ahpC*::Kan type I strain; lane 3, *ahpC*::Kan type II strain. The arrows indicate proteins AhpC (26 kDa) and NapA (17 kDa).

transformants with O_2 at low pressure and Kan^r transformants were not recovered when the transformants were screened in 12% O_2. SDS-PAGE was done to compare AhpC levels in wild-type and mutant cells. Alkyl hydroperoxide reductase was previously shown to be a predominant protein (third most abundant) in *H. pylori*, and it could be readily identified in 2D gels (18). In single-dimension gels of crude extracts of the wild type, we observed a predominant peptide that migrated at 26 kDa (Fig. 1); this peptide represented the putative AhpC absent in extracts from *ahpC*::Kan mutants.

Fast protein Q-Sepharose ion-exchange chromatography was used to fractionate AhpC from extracts of the wild type (Fig. 1). Fraction 23 contained an abundance of the 26-kDa protein, which was well separated from other bands, so the polypeptides in this fraction were separated by SDS-PAGE and transblotted onto a polyvinylidene difluoride membrane. The excised 26-kDa band was subjected to eight cycles of N-terminal sequencing. The sequence result was in excellent agreement with that predicted for AhpC, with complete identity in the first 8 amino acids (MLVTKLAP). Based on SDS-PAGE analysis, all mutants chosen (as Kan^r colonies) lacked the 26-kDa protein (i.e., AhpC). However, from the gel analysis, we also observed that the AhpC mutant (Fig. 2, lane 2) contained levels of another protein (of an approximate molecular mass of 17 kDa) higher than those in the wild type. This type of mutant was common (see below) and is referred to as type I. Sequencing of the first 8 amino acids of the up-regulated protein (by transblotting and excising) revealed 100% identity to *H. pylori* NapA (neutrophil-activating protein). Densitometric scanning of all the proteins in each lane of Fig. 2 showed that the NapA band (normalized to all protein bands) was fivefold greater in intensity than that determined for the wild type. Apparently, the loss of AhpC caused the concomitant overproduction of another oxygen stress-related protein, NapA. NapA in *H. pylori* is a ferritin-like iron-binding protein (31), and it may play a role in reducing toxic oxygen radical production via free-iron removal.

Two types of mutant strains. Upon further analysis of a series of transformant colonies, it was observed that we had obtained another type of AhpC mutant in which NapA was not up-regulated. Therefore, two types of mutants were discernible. To determine the prevalence of the two types of mutants, 36 individual colonies were chosen from three complete and independent transformation procedures (selecting for kanamycin insertions). The Kan^r transformants from all three procedures were selected under the partial-pressure (2% O_2) condition. Out of the 36 *ahpC*::Kan mutants, only 2 lacked the up-regulation of the NapA phenotype. These two isolates still made NapA, but they did so at the wild-type level. These isolates are referred to as type II mutants, and SDS gels of a representative of each of the two types of mutants are shown in Fig. 2. As a control, *modA*::Kan mutants were obtained by screening for transformants in the same manner (in 2% O_2). In this case, no up-regulation of NapA was visible for any of the six mutants chosen; all *modA* mutants had normal (like wild-type) NapA levels. Therefore, the up-regulation phenotype was associated with the loss of AhpC, even though the second class of mutants was obtained in the same screening procedure. As further confirmation of the correlation between the lack of AhpC and a concomitant up-regulation of NapA, we prepared samples of the wild-type cells and both types of mutant cells and subjected them to 2D gel electrophoresis. The result (Fig. 3) confirmed the loss of AhpC in the *ahpC* gene-directed mutants and documented the up-regulation of NapA in the type I mutant and the normal levels of NapA in type II strains (data not shown).

The difference in NapA levels between the parent strain and the type I mutants was not due to a change in the *napA* promoter region. The DNA sequences of the intergenic region (209 bp) between HP0244 and the start of *napA* were sequenced from clones derived from both the wild-type strain ATCC 43504 and the *ahpC*::Kan type I mutant, and they were determined to be identical.

Due to the observed up-regulation of NapA and our desire to identify the roles of oxidative-stress resistance proteins, we created more mutant strains. These were a gene disruption
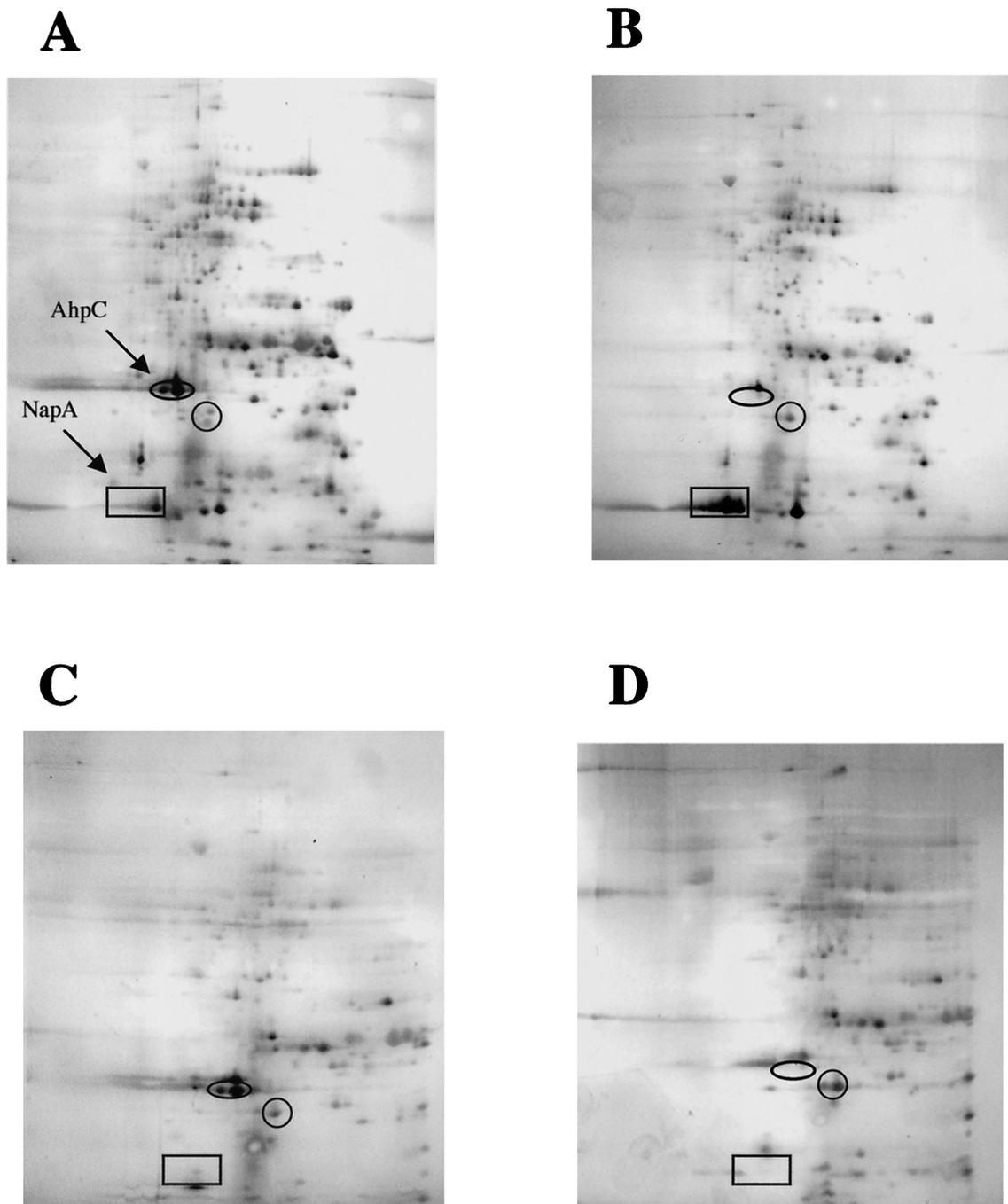
FIG. 3. Separation (2D) of crude extracts of the wild-type (ATCC 43504) (A), *ahpC*::Kan type I (B), *napA*::Cm (C), and *ahpC napA* (D) strains. The first dimension (isoelectric focusing) was carried out with the pH range between 3 (left) and 10 (right). The identified proteins are AhpC (within the ovals), NapA (within the rectangles), and HP0630 (circled).

mutant with a mutation in *napA*, designated the *napA*::Cm strain, and a double mutant with mutations in both *napA* and *ahpC*, designated the *ahpC napA* strain. Conferring additional stress on *H. pylori* via mutation of oxidative-stress resistance genes created another compensatory change: in the double mutant strains (*ahpC napA* mutant), the 2D gels revealed the induction of another protein of about 22 kDa in size. N-terminal sequencing of this protein showed 100% homology with

HP0630 of *H. pylori* 26695 (30), a 21.8-kDa protein. Interestingly, a conserved domain search of the Pfam database (U.S. version 6.6) (3) revealed that this protein is part of a family of NAD(P)H dehydrogenases (pfam02525) which, in eukaryotic cells, catalyze the two-electron reduction of quinone and are involved in cellular protection against damage by free radicals and reactive oxygen species (22). The only bacterial member of the family to be characterized is the Mda66 protein of *E. coli*,

A


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+1 C
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B

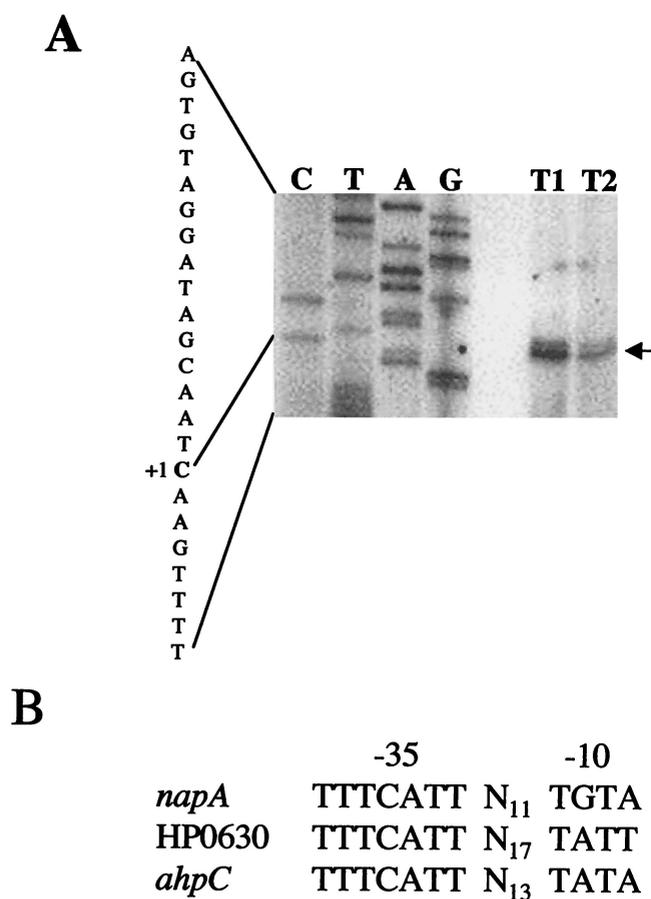|	-35		-10
napA	TTTCATT	N$_{11}$	TGTA
HP0630	TTTCATT	N$_{17}$	TATT
ahpC	TTTCATT	N$_{13}$	TATA

FIG. 4. (A) Primer extension analysis of *napA* in *ahpC*::Kan type I and type II mutants. The extended product (designated by the arrow) is located 53 bp upstream from the translation initiation codon AUG and is indicated by "+1." (B) Alignment of −35 and −10 promoter regions of *napA* and HP0630 with the previously identified *ahpC* regions (2). The −35 heptamers begin 33, 30, and 33 bp upstream from the transcription start sites of *napA*, HP0630, and *ahpC*, respectively.

which has been shown to be a NADPH quinone reductase but to which no physiological role has been assigned (16). Further analysis of this protein will bring a deeper understanding of its role in *H. pylori*, perhaps as an oxidative-stress resistance factor.

Primer extension analysis of *napA* and HP0630. The transcription start sites of the *napA* and HP0630 genes were identified by primer extension analysis. Two possible transcription start sites of *napA* were identified 53 and 54 bp upstream of the translation initiation codon. The most prominent band indicating base C, located at 53 bp upstream, was designated +1 (Fig. 4A). All the strains analyzed (wild-type, *ahpC*::Kan type I, and *ahpC*::Kan type II strains) utilized the same transcription start site for *napA*. Similarly, the wild-type, *ahpC*::Kan type I, and *ahpC napA* strains had the same start site for the HP0630 gene, at 16 bp upstream of the translation start site (data not shown). The extended product for the *ahpC*::Kan type I mutant was more intense than that of the type II mutant (Fig. 4A), in agreement with the increased amount of protein observed for the type I mutant. Analysis of the promoter region for the two new genes of interest along with that of the previously determined *ahpC* promoter (2) revealed strong homology in the −35 regions among the three genes and some (but less) similarity in the location and sequence of the −10 regions among the three genes (Fig. 4B). The inverted repeat identified in the *ahpC* promoter (2) did not appear to be conserved in either *napA* or HP0630.

Sensitivity to oxygen. Oxidative-stress responses are usually related to an organism's ability to tolerate oxygen and to combat the toxic oxygen radicals and other related oxidative species that may arise. One way in which we sought to characterize mutants was to determine their sensitivity to oxygen. Initially, we measured the growth of the wild-type and mutant cells in environments having different levels of oxygen. BA plates were incubated at 2, 3, 4, 5, 7.5, 10, or 12% O$_2$. The presence of isolated colonies, as well as growth at the site of inoculation (initial streak site) at each O$_2$ level, was evaluated after 3 days (Table 2). In the wild-type strain of *H. pylori*, isolated colonies were visible at all concentrations of O$_2$. On plates streaked with either of the AhpC-type mutants, small, isolated colonies were visible from the 2% O$_2$ incubator. No isolated colonies were visible when the plates were incubated at pressures above 2% O$_2$, and slight growth (but not individual colonies) was visible under partial-pressure conditions of 3, 4, 5, and 7.5% O$_2$. Still, this growth was observed only at the site of greatest inoculum. No growth was visible (for any of the AhpC mutants) at 10% O$_2$ or higher, O$_2$ levels fully permissible for the growth of wild-type cells. NapA-deficient cells showed growth with the appearance of individual colonies under microaerobic conditions up to 3% O$_2$, like wild-type cells, and this strain began to suffer growth inhibitions at 4% O$_2$. The double mutant (*ahpC napA*) cells were highly oxygen sensitive. We observed no individual colonies of this strain at any O$_2$ concentration; however, there was growth at the site of greatest inoculum. We previously reported that a *sodB* mutant was considerably more sensitive to oxygen killing than the wild-type strain in experiments measuring the viability loss of nongrowing cells exposed to air. Interestingly, unlike the *sodB* mutant (29), both types of *ahpC*::Kan mutants showed only slightly greater viability loss than the wild type when they were subjected to these non-growth-promoting, air exposure conditions (data not shown).

Sensitivity to cytotoxic agents. A previously studied *sodB* mutant of *H. pylori* was shown to have increased susceptibility to hydrogen peroxide (29). To test whether our *ahpC*::Kan mutant showed increased sensitivity to an oxidative-stress reagent (such as peroxide exposure), a series of disk inhibition assays were performed. Inhibition zones were measured around agent-saturated disks (Table 3). Both types of AhpC mutants were more sensitive to cumene hydroperoxide than the wild type. However, with *t*-butyl hydroperoxide, the type I mutant was less sensitive than type II, as shown by a slight decrease in the size of the inhibition zone. This tolerance of *t*-butyl hydroperoxide by the type I mutant is probably related to the increased NapA level, since the type II mutant was considerably more sensitive to this peroxide than the wild type. Both types of AhpC mutants were more sensitive to paraquat than the wild-type strain. The *napA*::Cm mutant was similar to the wild type in its sensitivity to peroxides and paraquat, presumably because this strain contains abundant AhpC. The double mutant (*ahpC napA*) showed the greatest sensitivity of all the mutants to both *t*-butyl hydroperoxide and to paraquat.

TABLE 3. Disk sensitivity assay[a]

Strain	Disk sensitivity with the following treatment[a]:		
	5% Cumene hydroperoxide	5% t-Butyl hydroperoxide	2 mM paraquat
Wild type	7.3 ± 1.5	13.5 ± 1.2	1.1 ± 0.1
ahpC::Kan type I	11.7 ± 2.4	9.0 ± 0.2	9.5 ± 0.5
ahpC::Kan type II	12.9 ± 2.3	19.8 ± 1.0	10.4 ± 1.1
napA::Cm	7.3 ± 1.3	14 ± 1.7	1.1 ± 0.1
ahpC napA	9.9 ± 1.3	20.5 ± 1.7	13.7 ± 0.9

[a] Zones of inhibition were measured (in millimeters) around filter paper disks saturated with 10 μl of the indicated compounds. Water as a control did not yield any zones of growth inhibition. Results are the averages ± standard deviations from five independent experiments.

Spontaneous rifampin resistance. Mutant strains of *H. pylori* lacking superoxide dismutase had higher frequencies of spontaneous mutation than that of the wild type (29). *H. pylori* deprived of functional AhpC may be more prone to DNA damage caused by reactive oxygen species as well. In three independent experiments on the AhpC mutants, we found that both of the mutants had significantly elevated spontaneous mutation frequencies compared with that of the wild-type strain. The average frequencies of Rif^r mutation were 133 and 20 times greater for the type II (40 Rif^r CFU per 10^8 cells) and type I (6 Rif^r CFU per 10^8 cells) mutants, respectively, than that for the wild-type strain. The *ahpC napA* double mutant strain's frequency was 350-fold greater than that of the wild type, whereas the *napA*::Cm single mutant had a mutation frequency no greater than that of the wild type. Lipid hydroperoxides are known to undergo metal ion-mediated decomposition which results in modification of DNA and formation of so-called adducts. This type of alteration can cause frequent mutations (7) and could be one explanation for why the double mutant had a much higher mutation frequency than the other strains. The double mutant strain clearly has a deficiency in dissipating organic hydroperoxides (Table 3).

DISCUSSION

In order to combat reactive oxygen species and therefore persist in the gastric mucosa, pathogenic *H. pylori* is equipped with a number of putative detoxifying proteins (11, 24, 25). These include not only enzymes to detoxify oxygen-related radicals but N-containing reactive species as well. One of the enzymes observed in great abundance in *H. pylori* cells grown outside of the host is alkyl hydroperoxide reductase (18), and several groups have concluded that the organic-peroxide-detoxifying activity is important to *H. pylori* survival, since strains with mutations in *ahpC* could not be recovered (2, 8, 23). From our results, such mutants could be recovered under low-O_2 conditions. AhpC clearly plays a role in oxidative-stress resistance, but whether this is due primarily to minimizing the level of organic peroxides (17) is unclear. For example, it was recently suggested that H_2O_2 may be the primary substrate for AhpC (10).

Two classes of mutants, both lacking AhpC, were obtained. The type I mutants contained increased levels of NapA protein, whereas the type II mutants did not. This increased NapA expression was associated with an increased resistance to *t*-butyl hydroperoxide and a lower spontaneous mutation frequency than that of the type II mutants. Previously, it was found that overexpressed levels of alkyl hydroperoxide reductase were able to overcome the high frequency of spontaneous mutations, which suggests that the reductase plays an important role in protecting against oxidative DNA damage (17). The same may be true for our (*ahpC*::Kan type I) mutants, where NapA appears to play a role in minimizing the spontaneous mutation frequency.

In other systems, a loss of oxidative-stress resistance function has been reported to cause a concomitant increase in levels of another oxidative-stress resistance function. Mutation of AhpCF of *Bacillus subtilis* led to increased expression of some factors that may help confer H_2O_2 resistance (6). Similarly, the antioxidant protein Dpr of *Streptococcus mutans* was identified due to its increased production upon mutation of other antioxidant-encoding genes (33). Dpr, a homologue of NapA, was proposed to help confer aerotolerance to *S. mutans* by keeping free-iron levels low, thus minimizing hydroxyl radical production (34).

Increased expression of an oxidative resistance factor appears to be a compensatory response used by oxidative-stress-deficient *H. pylori* to ensure that oxidative damage resistance is maintained. The peroxide sensitivity phenotype associated with AhpC mutants supports this compensatory up-regulation conclusion. Our results on the up-regulation phenomena prompted us to obtain additional mutants, namely, a *napA*::Cm mutant and a strain with a double gene disruption (*ahpC napA*). NapA is an iron-binding protein that may protect the cells by sequestering ferric iron released from iron sulfur clusters (20). The need for sequestering this type of iron is related to Fenton reactions that produce DNA-damaging hydroxyl radicals (15). Most of the results on the *H. pylori napA*::Cm mutants do not clearly define an oxidative-stress role for this protein, but the *napA*::Cm mutant strain was O_2 sensitive compared to the wild type. However, the high spontaneous mutation frequency and marked sensitivity to organic peroxides of the double mutant are in agreement with the conclusion that both proteins (AhpC and NapA) play roles in oxidative agent detoxification. Further experiments on the role of the protein (HP0630) that is up-regulated in the *ahpC napA* mutant may prove to be useful in furthering our knowledge of oxidative-stress resistance in gastric pathogens.

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