Proteomic analysis of upregulated proteins in Helicobacter pylori under oxidative stress induced by hydrogen peroxide

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Abstract The development of gastric cancer was suggested to be associated with chronic inflammation as a consequence of Helicobacter pylori infection. Such inflammation-related oxidative stress induced by reactive oxygen species (ROS) in vivo may exert bidirectional effects on both hosts and H pylori. In this study, ROS-induced oxidative stress was mimicked by coculture of gastric epithelial cells with H pylori treated with hydrogen peroxide (H2O2). To investigate the effect of H2O2 on the proteome of H pylori, we performed two-dimensional polyacrylamide gel electrophoresis followed by liquid chromatography coupled with nano-electrospray ionization-tandem mass spectrometry (liquid chromatography mass spectrometry) and bioinformatics database analysis. The nine most overexpressed proteins consisted of three virulence factors, including cytotoxin-associated protein A (CagA), vacuolating cytotoxin (VacA), adherence-associated protein (AlpA), and two antioxidant enzymes alkylhydroperoxide reductase (AhpC) and catalase (KatA), plus one serine protease (HtrA), aconitate hydratase, and fumarate reductase. We have also confirmed the upregulation of virulence factors and antioxidant proteins in several H pylori strains isolated from patients of different clinical outcomes. Furthermore, it is noted that H pylori was found to decrease in infection rate and increase in proliferation after being exposed to H2O2. We also found that gastric epithelial cells can be protected from oxidative damage by H2O2 in the presence of H pylori. In conclusion, this study lends support to the supposition that ROS containing H2O2 as one of the major oxidative species can induce upregulation of virulence factors and
Introduction

Gastric cancer (GC) responsible for 10.4% mortality of all fatal cancer cases is the second most frequent cause of malignancy-related deaths worldwide [1]. In addition to the host genetic determinants and environmental factors, prolonged infection and host inflammatory response caused by persistent *Helicobacter pylori* colonization were regarded as major factors in the formation of GC [2]. Chronic infection by *H. pylori*, a Gram-negative and microaerophilic bacterium, can lead to various gastrointestinal diseases, including chronic gastritis, gastric ulcer, duodenal ulcer, and GC at different stages of infection [3–5].

Several signaling pathways involving *H. pylori* virulence factors related to the development of GC have been proposed. Cytotoxin-associated protein A (CagA), a 120–145 kDa protein with a carboxyl-terminal variable region, was shown to be delivered into gastric epithelial cells by the Type IV secretion system, where it activated the SH2 domain-containing protein tyrosine phosphatase 2 (SHP-2) oncoprotein, thereby increasing the potential of *H. pylori* to promote gastric tumorigenesis [6–8]. Another virulence factor participating in the development of GC is the vacuolating cytotoxin VacA, a multifunctional toxin [9–12]. VacA is expressed as a 140-kDa protoxin that undergoes proteolytic processing, yielding an 88-kDa mature toxin during secretion [13,14]. The pathogenesis of GC was suggested to be related to the ion-channel forming function of VacA. It facilitated the survival of *H. pylori* in the gastric mucosa, resulting in continual inflammation at the stomach niche environment [15]. Other virulence factors, such as the adhesion family, which included BabA [16], SabA [17], OipA [18], and HopZ [19] can all mediate the adhesion of *H. pylori* to the gastric epithelium. In addition to the adhesion factors, the adherence-associated proteins (AlpA and AlpB) were recently found to cause gastric injury by modulating intracellular signaling pathways involved in inflammation [20].

*H. pylori* infection on the gastric mucosa can activate immune system and inflammatory responses, leading to discharge of reactive oxygen species (ROS), such as hydrogen peroxide (H$_2$O$_2$), superoxide anion, and nitric oxide from phagocytes [21–24]. Consequently, such oxidative stress may bring about bidirectional effects on both hosts and *H. pylori*. The excessive ROS production may trigger oxidative stress to cause inflammatory damage to host gastric mucosa. To date, there appears to be very few reports on the physiological changes of *H. pylori per se* during infection under extreme ROS attack. Whether virulence factors of *H. pylori* can be correlated with gastric inflammation leading to the development of cancer remains elusive.

To investigate whether ROS can influence *H. pylori* infection and colonization, we cocultured AGS gastric epithelial cells and *H. pylori* under oxidative stress conditions at different concentrations of H$_2$O$_2$. We have also used a proteomics approach to characterize and compare protein expression profiles under oxidative stress in *H. pylori* strains of patients with GC. We have identified several proinflammation-related antigens, such as CagA, VacA, and AlpA plus proteins with antioxidant or chaperone activities, for example, AhpC, KatA, and HtrA, which were all found to be induced under stress conditions. The results point to the possibility that the increased expression of virulence and antioxidant proteins of *H. pylori* under oxidative stress induced by ROS may be the causative factors underlying the development of GC from *H. pylori* infection.

Materials and methods

**Bacterial and human cell cultures**

The *H. pylori* strains used in this study were isolated from the gastric biopsy specimens in three patients and were abbreviated as HC28 (from human GC), HD30 (from human duodenal ulcer), and HS65 (from human gastritis), respectively. These *H. pylori* strains were grown on Centers for Disease Control and Prevention anaerobic blood agar plates at 37°C in a modular atmosphere-controlled system (5% O$_2$/10% CO$_2$/85% N$_2$) and confirmed to be *H. pylori* because of their urease activity and helical morphology as determined by phase-contrast microscopy. AGS cells (American Type Culture Collection Number CRL-1739), a human cell line derived from gastric carcinoma, were maintained in Roswell Park Memorial Institute 1640 (RPMI 1640) medium.
supplemented with 10% fetal bovine serum and 100 U/mL penicillin-streptomycin at 37°C under 5% CO2-humidified atmosphere. The morphology of *H pylori* was visualized under phase-contrast microscopy after staining with the Gram stain (ECLIPSE TE2000-U, Nikon, Tokyo, Japan).

**Two-dimensional polyacrylamide gel electrophoresis image analysis**

After treating *H pylori* in the absence and presence of 10 mM H2O2 in RPMI medium for 3 hours, *H pylori* cells were solubilized in lysis buffer containing 8 M urea, 0.5% Triton X-100, and protease inhibitor cocktail. Around 250 µg total protein as estimated by protein-content determination using 2-D Quant Kit (Amersham Biosciences, Uppsala, Sweden) was loaded onto immobilized pH gradient (IPG) gel strips (pH 3–10, 13 cm, Amersham Biosciences). The IPG strips were rehydrated overnight. For the first-dimensional separation, isoelectric focusing (IEF) was carried out using Ettan IPGphor II (Amersham Biosciences) at 300–8000 V for 16 hours. After IEF, the IPG strips were equilibrated for 10 minutes each in two equilibration solutions (50 mM Tris-HCl, pH 8.8; 6 M urea; 2% sodium dodecyl sulfate (SDS); 30% glycerol containing 100 mg dithiothreitol; or 250 mg iodo-acetic acid), and the second-dimensional electrophoresis was conducted at 130–250 V for 5–6 hours. The gels were fixed in 10% methanol and 7% acetic acid for 30 minutes and stained by SYPRO Ruby (Invitrogen, Carlsbad, CA) overnight.

The two-dimensional polyacrylamide gel electrophoresis (2-DE) gel images were scanned using a fluorescence image scanner Typhoon 9400 (Amersham Biosciences) and analyzed by using PDQuest software (Bio-Rad, Hercules, CA, USA). Intensity levels were normalized between gels as a proportion of the total protein intensity detected for the entire gel.

**In-gel digestion and liquid chromatography mass spectrometry**

Based on the 2-DE gel analysis of samples under different oxidative stresses, we selected nine overexpressed proteins for further identification by liquid chromatography mass spectrometry (LC-MS/MS) (nano electrospray ionization-quadrupole time-of-flight) at the core facility laboratory of the Institute of Biological Chemistry, Academia Sinica. In-gel digestion with trypsin was carried out as described previously [25,26]. Proteins were identified in NCBI databases based on MS/MS ion search with the MASCOT program.

**RNA preparation and reverse transcription polymerase chain reaction**

Total RNA of HC28 was isolated using RNeasy mini kit and RNase-Free DNase Set (Qiagen, Valencia, CA). Total RNA from each sample was reverse-transcribed and polymerase chain reaction (PCR) was carried out by using Superscript One-Step reverse transcription (RT)-PCR kit (Invitrogen, Carlsbad, CA) [25]. The primers [27] used for RT-PCR are listed in Table 1. One PCR cycle comprised: 94°C for 1 minute, annealing temperatures of 50–55°C for 1 minute, and 72°C for 1 minute. The total cycle numbers were 35 with a final elongation step of 5 minutes at 72°C.

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**Figure 1.** Two-dimensional polyacrylamide gel electrophoresis (2-DE) protein profiles of HC28 under normal and oxidative conditions induced by hydrogen peroxide. Around 250 µg total protein in each sample was loaded onto immobilized pH gradient (IPG) gel strips (pH 3–10, 13 cm). For the first-dimensional separation, isoelectric focusing (IEF) was carried out using Ettan IPGphor II (Amersham Biosciences) at 300–8,000 V for 16 hours. After IEF, the IPG strips were equilibrated in sodium dodecyl sulfate (SDS)-urea buffer and placed onto the second-dimensional SDS-polyacrylamide gel electrophoresis. After electrophoresis, the gels were fixed in 10% methanol and 7% acetic acid and stained by Sypro-Ruby. (A) 2-DE gel image of HC28 under normal. (B) or oxidative stress in the presence of 10 mM H2O2. The nine differentially expressed protein spots were marked and further identified by LC-MS/MS. A series of spots (spots 1, 3, 4, 7, and 8) representing protein isoforms were indicated by two enclosed lines. The 2-DE gel images were scanned using a fluorescence image scanner Typhoon 9400 (Amersham Biosciences) and analyzed by using PDQuest software (Bio-Rad). Intensity levels were normalized between gels as a proportion of the total protein intensity detected for the entire gel.
Coculture of *H pylori* with AGS gastric cells

AGS cells were seeded into six-well plates, grown for 24 hours, and the medium was replaced with antibiotic-free RPMI 1640 medium before addition of *H pylori* isolates HC28. Bacterial colonies were harvested and washed with phosphate-buffered saline, and then bacteria were resuspended in antibiotic-free RPMI 1640 medium. AGS cells were cocultured with *H pylori* at a multiplicity of infection of 100. 

**H2O2** (Sigma-Aldrich, St. Louis, Mo, USA) was then added at concentrations varying from 0.1 mM to 10 mM for 3 hours at 37°C under a 5% CO2-humidified atmosphere, with concentrations similar to those used in other studies for examining the effects of ROS on gastrointestinal epithelial cells [28–30]. Following infection, AGS cells were analyzed by observing morphological changes (Eclipse TE2000-U, Nikon). Elongated cells were defined as cells that had thin needle-like protrusions that were 20 μm long and a typical elongated shape as previous reports. The infection rate was determined by calculating the ratio of *H pylori*-induced elongated cells (hummingbird phenotype) to total cell numbers [6,31]. All samples were examined in triplicate in three independent experiments.

Quantitative H2O2 assay

*H pylori* were cultured in RPMI medium containing 10 mM H2O2 for the indicated time. The concentration of H2O2 remaining in the medium was detected using the PeroXOuant Quantitative Peroxide Assay (Pierce Chemical Co., Rockford, IL, USA) according to the operational manual. The concentration of H2O2 was determined by measuring optical density (OD) at 560 nm with an Ultrospec 4000 spectrophotometer (Amersham Pharmacia, Uppsala, Sweden). Media containing only H2O2 (0 mM, 0.1 mM, 1 mM, and 10 mM) were used as controls.

Proliferation assay of *H pylori*

*H pylori* strains HC28 were grown on Centers for Disease Control and Prevention plates under normal condition (5% O2) for 48 hours and then were cultured in RPMI media containing different H2O2 concentrations for 3 hours. Before performing assays, harvested samples were quantified by measuring turbidity at OD600 (absorption at 600 nm). The cell proliferation assay was conducted by subculturing each test strain on fresh plates after stress treatment. The number of living cells was determined and represented as colony-forming units [25].

**Results**

Protein expression profiles of *H pylori* in response to H2O2

To investigate the change in protein expression of *H pylori* under oxidative stress, we analyzed 2-DE protein maps of *H pylori* under normal and high oxidative stress (10 mM H2O2). Figure 1 shows representative images of protein profiles from HC28 treated with or without 10 mM H2O2 for 3 hours. Approximately 390 spots in each gel can be visualized by PDQuest software (Bio-Rad, Hercules, CA, USA). The locations of the nine overexpressed protein spots were marked with numbers 1—9 in Fig. 1. Five of these nine proteins, including proteins 1, 3, 4, 7, and 8, contain a series of closely spaced spots, which were confirmed to be different isoforms by LC-MS/MS.

Herein, we identified these nine spots by in-gel digestion, followed by LC-MS/MS. A list of their sequence coverage and match scores is summarized in Table 2. All these nine protein spots were significantly overexpressed under oxidative stress in the presence of H2O2, four of which were virulence-related proteins, including cytotoxin-associated protein A (CagA), vacuolating cytotoxin (VacA), adhesin AlpA, and urease protein UreA. Three of which, alkylhydroperoxide reductase (AhpC), catalase (KatA), and a serine protease (HtrA), were proteins with antioxidant or chaperone activities. The other two proteins, being identified to be aconitate hydratase and fumarate reductase, were known to be involved in metabolism.

<table>
<thead>
<tr>
<th>Spot</th>
<th>Protein identified</th>
<th>NCBI accession number</th>
<th>Score/match coverage, %</th>
<th>pi/mass, kDa</th>
<th>Protein expression ratio (S/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>Cytotoxin-associated protein A</td>
<td>BAD51743</td>
<td>965/21</td>
<td>19</td>
<td>8.50/131.23</td>
</tr>
<tr>
<td>2a</td>
<td>Vacuolating cytotoxin</td>
<td>AA062539</td>
<td>407/10</td>
<td>10</td>
<td>9.18/90.190</td>
</tr>
<tr>
<td>3a</td>
<td>Alkyl hydroperoxide reductase (AhpC)</td>
<td>AA033162</td>
<td>665/17</td>
<td>65</td>
<td>5.98/22.216</td>
</tr>
<tr>
<td>4a</td>
<td>Catalase, chain A (KatA)</td>
<td>1QWLA</td>
<td>511/13</td>
<td>23</td>
<td>8.75/58.599</td>
</tr>
<tr>
<td>5a</td>
<td>Serine protease (HtrA)</td>
<td>NP223124</td>
<td>825/19</td>
<td>37</td>
<td>9.18/51.681</td>
</tr>
<tr>
<td>6a</td>
<td>Adhesin (AlpA)</td>
<td>BAF38006</td>
<td>208/4</td>
<td>13</td>
<td>9.25/56.263</td>
</tr>
<tr>
<td>7</td>
<td>Urease protein UreA</td>
<td>AAK69728</td>
<td>511/13</td>
<td>23</td>
<td>8.75/58.599</td>
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<tr>
<td>8</td>
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<td>889/16</td>
<td>22</td>
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<td>Fumarate reductase</td>
<td>NP228899</td>
<td>1060/26</td>
<td>31</td>
<td>6.67/80.069</td>
</tr>
</tbody>
</table>

* mRNA transcription levels were analyzed by reverse transcription polymerase chain reaction.

Protein expression ratios shown here are means of triplicate densitometric measurements of protein spots.

N = normal condition; S = stress condition.

Table 2 Proteins in *Helicobacter pylori* showing upregulation in response to hydrogen peroxide identified by nano-liquid chromatography mass spectrometry analysis
RT-PCR analysis of the transcription levels of the genes induced by H$_2$O$_2$

To verify the results of the protein-expression levels of the above-mentioned proteins, we performed RT-PCR analysis to determine whether the increased protein expression of the three virulent factors (CagA, VacA, and AlpA) and three stress-related proteins (AhpC, KatA, and HtrA) was manifested at the transcriptional level. These six proteins were previously shown to be associated with persistent colonization of $H$ pylori and progression of GC. As shown in Fig. 2A, an increase of the mRNA levels of six genes for HC28 was clearly observed after oxidative stress induced by H$_2$O$_2$. Quantitative analysis of the RT-PCR results are shown in Fig. 2B, corroborating the result obtained from proteomic analysis.

Verification of upregulation of virulence factors and antioxidant enzymes in $H$ pylori strains isolated from patients of different clinical outcomes in response to H$_2$O$_2$

Previous reports indicated that $H$ pylori appear to possess genetic plasticity under different environmental conditions. We have analyzed two $H$ pylori strains isolated from patients of duodenal ulcer (HD30) and gastritis (HS65), corroborating that H$_2$O$_2$ can also induce upregulation of the virulence factors and antioxidant enzymes in these two $H$ pylori strains. As shown in Fig. 3, the protein expression levels of these six proteins are consistently upregulated in these three different $H$ pylori isolates, suggesting that this effect is universal among varied $H$ pylori strains isolated from patients of different clinical manifestations. Although we used only one $H$ pylori GC strain to perform the proteomic analysis, the results of other strains isolated from patients of duodenal ulcer and gastritis also confirmed this phenomenon.

Exposure of $H$ pylori-infected AGS gastric epithelial cells to H$_2$O$_2$

To investigate whether ROS affects AGS cells infected with $H$ pylori during inflammation, we mimicked the condition by coculturing AGS cells and $H$ pylori under different H$_2$O$_2$ concentrations (0 mM, 1 mM, and 10 mM in Fig. 4). This experiment could monitor not only the state of AGS cells infected with $H$ pylori by observing changes of cell morphology under varied oxidative stress but also the response of $H$ pylori to ROS attack. As shown in Fig. 4, AGS cells alone could not withstand H$_2$O$_2$ concentration higher than 1 mM for 3 hours. Under this condition, H$_2$O$_2$ outside AGS cells can generate severe oxidative stress leading to cell death. However, they were able to resist the oxidative stress induced by H$_2$O$_2$ even at a concentration of 10 mM while coculturing with $H$ pylori. The cultures containing AGS cells and $H$ pylori maintained their living states in the presence of increasing H$_2$O$_2$ concentration.

To verify whether this novel protective effect may result from the antioxidant systems of the coculturing $H$ pylori operating under oxidative stress to safeguard AGS cells, we examined whether H$_2$O$_2$ in the medium was decomposed by the antioxidant enzyme system in $H$ pylori. The concentration of H$_2$O$_2$ in the medium without $H$. pylori was almost constant in 3 hours. However, H$_2$O$_2$ at 10 mM immediately disappeared within 1 minute after we cocultured $H$ pylori with AGS (data not shown). In addition, there was no detectable H$_2$O$_2$ produced by $H$. pylori after 3 hours. We observed that $H$. pylori possesses better antioxidant ability to detoxify excess H$_2$O$_2$ than gastric epithelial cells under severe oxidative stress.

Adaptabilities of $H$ pylori in response to H$_2$O$_2$

In addition to the protective effect of $H$ pylori against oxidative damage of H$_2$O$_2$, the infection rate of $H$ pylori...
decreased with increasing H₂O₂ concentration (Fig. 4). To obtain the infection rate of *H. pylori* under oxidative stress, we estimated the ratio of numbers of *H. pylori*-induced elongated cells (hummingbird phenotype) to total cell numbers under different H₂O₂ concentrations. As shown in Fig. 5A, there was about 40% decrease in *H. pylori* infection at 0.1 mM or 1 mM H₂O₂, whereas a drop of 80% infection rate was found at 10 mM H₂O₂. This observation indicated that *H. pylori* could not infect AGS cells under oxidative stress induced by H₂O₂.

The proliferation potential of *H. pylori* after oxidative stress with serial concentrations of H₂O₂ was also investigated by subculturing each test strain in fresh plates after treatments. Figure 5B indicated that the proliferation potential of *H. pylori* appears to increase under a short period of exposure to high concentrations of H₂O₂ in contrast to the inhibition of proliferation under long-term oxidative stress reported previously [25].

**Discussion**

Oxidative stress was reported to be associated with malignant transformation and chronic inflammation. It was also suggested to be the causative factor for many common cancers, including GC [32]. However, the agent inducing the gastric inflammation was not known until the discovery and successful culture of *H. pylori* by Marshall and Warren [3]. *H. pylori* has been classified as a Class I carcinogen by World Health Organization because of the abundant evidence indicating that *H. pylori* infection increased the risk of GC [33]. In the gastric carcinogenic process, H₂O₂ generation at the infection sites induced by *H. pylori* may result in DNA damage and apoptosis in host gastric epithelial cells [34,35]. Recently, many reports suggested that several virulence factors of *H. pylori*, including CagA, VacA, and adhesins, were also related to gastric carcinogenesis [15,36-38]. Nevertheless, how these two processes for the development of cancer in gastric niche environment can be related remains to be addressed.

In this study, we have made an attempt to investigate the changes in protein-expression profiles of *H. pylori* under oxidative-stress environment during infection. Coculture of AGS cells with *H. pylori* revealed that gastric epithelial cells can be protected from oxidative damage by H₂O₂ attack in the presence of *H. pylori*. Proposed explanation is that antioxidant systems of *H. pylori* decompose H₂O₂, thereby shielding AGS cells against oxidative stress. The antioxidant enzyme AhpC of bacterial 2-Cys peroxiredoxins family was known to be much sturdier than human 2-Cys peroxiredoxins under oxidative environment [39,40]. Furthermore, *H. pylori* catalase is also stable at very high concentration of H₂O₂ [41]. It is therefore not surprising that *H. pylori* shows higher resistance against oxidative stress than gastric epithelial cells.

To resist this kind of highly oxidative stress, *H. pylori* expresses not only antioxidant proteins to decompose ROS but also several chaperones to prevent the misfolding or unfolding of proteins under long-term stress condition. As shown in this study, AhpC, KatA, and HtrA were overexpressed by *H. pylori* under oxidative stress in the presence of H₂O₂. AhpC is the most abundant antioxidant protein in *H. pylori*. Previously, we reported that AhpC could shift from low-molecular weight oligomers with peroxide-reductase activity to high-molecular weight complexes with molecular-chaperone function under oxidative stress [42,43]. Catalase is a ubiquitous enzyme that protects cells from extracellular H₂O₂ attack by decomposition of H₂O₂ into water and oxygen [44]. Therefore, both AhpC and catalase are important for the colonization and persistent

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**Figure 3.** Comparative analysis of the protein expression levels of virulence factors and antioxidant proteins of *Helicobacter pylori* isolated from patients of different clinical outcomes in response to H₂O₂. The six overexpressed proteins were excised from two-dimensional polyacrylamide gel electrophoresis gel for comparative analysis. The *H. pylori* strains used here were isolated from the gastric biopsy specimens in three patients and denoted as HC28 (from human gastric cancer), HD30 (from human duodenal ulcer), and HS65 (from human gastritis), respectively. *H. pylori* from patients of different clinical outcomes consistently shows the upregulation of three virulence factors, CagA, VacA, AlpA and three proteins with antioxidant and chaperone activity, AhpC, KatA, and HtrA, in response to oxidative stress induced by H₂O₂. N = normal condition (without H₂O₂); S = stressed condition (10 mM H₂O₂).
Figure 4. Effects of H$_2$O$_2$ on cell morphology of AGS cells with or without *Helicobacter pylori* infection. (A) AGS cells were cocultured with *H. pylori* isolate HC28 from a patient with gastric patient in media containing H$_2$O$_2$ of different concentrations. AGS cells alone could not withstand H$_2$O$_2$ concentration higher than 1 mM (middle of left panels). However, they were able to resist oxidative stress induced by H$_2$O$_2$ even at 10 mM by the infection of HC28 (right panels). (B) Enlargement of AGS cells in panel A. Cells exhibiting hummingbird phenotype indicative of *H. pylori* infection are marked by arrows.
infection of *H. pylori* in gastric mucosa. Moreover, like AhpC, HtrA was also shown to possess dual functions, which can switch from chaperone to serine protease [45]. For diverse pathogenic organisms, HtrA is considered as a virulence factor and required for oxygen tolerance [46–48]. Although the specific role of HtrA in *H. pylori* remains to be elucidated, our results confirm the fact that HtrA is overexpressed when *H. pylori* encounters oxidative stress, indicating that its multiple functionality may be required under various stress conditions.

In addition to those factors that protect *H. pylori* itself from injury, the novel finding in our study was the observation that the protein-expression levels of *H. pylori* virulence factors, including CagA, VacA, and AlpA, were upregulated in response to oxidative stress (Figs. 1 and 5). CagA is a well-known and highly immunogenic protein encoded by the *cagA* gene. On its delivery into gastric epithelial cells by the Type IV secretion system, CagA being a bacterial oncoprotein can cause a series of cellular alterations by influencing signal transduction pathways in host cells [8]. The higher risk of GC was found to increase among patients infected with *cagA*+ than *cagA*– strains, and a higher inflammatory response was also associated with the former strain [49]. VacA, which has a vacuolating effect, also plays an important role in the pathogenesis of peptic ulceration and GC, especially with its ability to block proliferation of T cells and interfere with downstream signaling of the T-Cell receptor and IL-2 receptors [12]. Moreover, the adherence-associated proteins (AlpA and AlpB) also modulate cellular signaling pathways, including the activation of MAPKs, c-Fos, and c-Jun, CREB, AP-1, and NF-kappaB-related pathways [20].

By comparing the protein expression levels of these virulence factors and antioxidant enzymes in three *H. pylori* strains isolated from patients of different gastroduodenal diseases, we found that these proteins could be induced consistently under severe oxidative stress but the inherent protein expression levels were different (Fig. 3). This result indicates that there is a strong correlation between *H. pylori* virulence and environmental oxidative stress. Although there is a similar mechanism in regulating virulence factors and antioxidant enzymes of *H. pylori* from patients of different gastroduodenal diseases, expression levels of these genes are quite different because of distinct oxidative-stressed conditions associated with different pathological outcomes of *H. pylori* infection in the gastroduodenal environment. The same phenomenon was also corroborated and proved by our concurrent study, which demonstrated AhpC as a useful biomarker for monitoring different stages of inflammation associated with *H. pylori* infection in different gastrointestinal diseases [50].

Furthermore, it is noted that *H. pylori* was found to decrease in infection rate and increase in proliferation after being exposed to *H_2O_2*. Proposed explanation is that *H_2O_2* induced metabolism-related enzymes, fumarate reductase andaconitate hydratase (Table 1), leading to increased proliferation of *H. pylori*. On *H. pylori* proliferation, they may stop infecting gastric epithelial cells.

Thus herein, we propose a likely mechanism underlying the changes of protein expression levels of these six proteins in *H. pylori* under long-term oxidative stresses. The progression of *H. pylori* infection to various clinical manifestations starts with an acute infection and continues persistent colonization on gastric mucosa. When the oxidative stress around the gastric niche where *H. pylori* colonizes is too severe because of the presence of excessive ROS, the increased protein expression of virulence factors coupled with the antioxidant enzymes may be triggered. Moreover, the generation of large amount of virulence factors may lead to gastric inflammation, resulting in more ROS to engender high induction of these proteins in *H. pylori*. Finally, this vicious cycle may activate gastric carcinogenesis through...
repeated inflammation and the generation of cancer-related proteins by H pylori.

Our findings reveal that H pylori from patients of different clinical manifestations consistently shows the upregulation of three virulence factors, CagA, VacA, AlpA and three proteins with antioxidant and chaperone activity, AhpC, KatA, and HtrA, in response to oxidative stress induced by H2O2. This study lends support to the supposition that ROS with H2O2 as one of its major oxidative stress species may be the causative factor underlying the development of GC from H pylori infection.

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