



Mutation of cytotoxin-associated gene A affects expressions of antioxidant proteins of *Helicobacter pylori*

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Abstract

AIM: To determine if disruption of the *cagA* gene of *Helicobacter pylori* (*H. pylori*) has an effect on the expression of other proteins at proteome level.

METHODS: Construction of a *cagA* knock out mutant *Hp27_ΔcagA* (*cagA*⁻) via homologous recombination with the wild-type strain *Hp27* (*cagA*⁺) as a recipient was performed. The method of sonication-urea-CHAPS-DTT was employed to extract bacterial proteins from both strains. Soluble proteins were analyzed by two-dimensional electrophoresis (2-DE). Images of 2-DE gels were digitalized and analyzed. Only spots that had a statistical significance in differential expression were selected and analyzed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS). Biological information was used to search protein database and identify the biological function of proteins.

RESULTS: The proteome expressions between wild-type strain and isogenic mutant with the *cagA* gene knocked-out were compared. Five protein spots with high abundance in bacteria proteins of wild-type strains, down-regulated or absently

expressed in bacteria proteins of mutants, were identified and analyzed. From a quantitative point of view, the identified proteins are related to the *cagA* gene and important antioxidant proteins of *H. pylori*, including alkyl hydroperoxide reductase (Ahp), superoxide dismutase (SOD) and modulator of drug activity (Mda66), respectively, suggesting that *cagA* is important to maintain the normal activity of antioxidative stress and ensure *H. pylori* persistent colonization in the host.

CONCLUSION: *cagA* gene is relevant to the expressions of antioxidant proteins of *H. pylori*, which may be a novel mechanism involved in *H. pylori* *cagA* pathogenesis.

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Key words: *Helicobacter pylori*; Cytotoxin-associated gene A; Knock-out; Antioxidant protein; Two-dimensional electrophoresis

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INTRODUCTION

Helicobacter pylori (*H. pylori*), a spiral-shaped bacterium that colonizes the human gastric mucosa, is estimated to inhabit at least half of the world's human population^[1]. Infection with *H. pylori* is associated with development of peptic ulcer, gastric carcinoma and gastric mucosa-associated lymphoid tissue lymphomas^[2-4]. However, little is known about the molecular mechanisms of pathogenesis induced by *H. pylori* and the fundamental causes of diversity in infection outcomes. Cytotoxin-associated gene A protein (CagA) is a *H. pylori* immuno-

dominant antigen with its gene residing in the *cag* pathogenicity island, which is a 40-kilobase insertion containing genes involved in virulence^[5]. Recent studies indicate that CagA is delivered from *H. pylori* into the cytoplasm of *H. pylori*-attached gastric epithelial cells via the type-IV secretion system^[6]. Upon membrane localization, translocated CagA interacts with a number of host proteins involving cell signaling in a tyrosine phosphorylation-dependent and -independent manner^[7,8]. Epidemiological studies have shown that *cagA* positive *H. pylori* strains are associated with higher grades of gastric mucosal inflammation as well as severe atrophic gastritis and gastric carcinoma^[9,10]. CagA is considered a marker of increased pathogenic potential and may play an important role in the pathogenesis induced by *H. pylori*.

China is one of the nations with the highest *H. pylori* infection incidence^[11]. More than 90% isolated strains possess *cagA* gene^[12]. However, the biological activity of *cagA* gene still remains unclear. In the present study, we analyzed two related *H. pylori* strains, *Hp27* and *Hp27_ΔcagA*, through a proteomic approach. Wild type strain *Hp27* is a *cagA* gene possessor, while strain *Hp27_ΔcagA* is an isogenic *cagA* knock out mutant. Proteins with altered expressions can be separated by two-dimensional electrophoresis (2-DE) and conclusively identified by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis of the peptide digests. This study was to determine if disruption of the *cagA* gene has an effect on the expression of other proteins of *H. pylori* at proteome level.

MATERIALS AND METHODS

Bacterial strains

Hp27 (cagA⁺) isolated from a patient with chronic atrophy gastritis was grown on Brucella agar plates containing 10% sheep blood supplemented with 10 mg/L vancomycin, 2500 U/L polymyxin B, 2 mg/L amphotericin and 5 mg/L trimethoprim, in an anaerobic jar consisting of 50 mL/L O₂, 100 mL/L CO₂, and 850 mL/L N₂ at 37°C for 3 d. Kanamycin (25 mg/L) was added for mutated strains selection.

Construction of *Hp27_ΔcagA* isogenic mutant

Isogenic *Hp27_ΔcagA* mutant was obtained from strain *Hp27* as follows. In brief, we produced a *Hp27_ΔcagA* isogenic mutant harboring a total *cagA* deletion as previously described^[13]. *H. pylori* were grown on blood agar plates under microaerophilic conditions for 3 d and genomic DNA was extracted with a genomic DNA purification kit (Takara). An upstream (U fragment) and a downstream region (D fragment) of the *cagA* gene were amplified over the genomic DNA as homologous arms using the *P1/P2* and *P3/P4* primer pairs, respectively. A kanamycin-resistant gene was amplified as a screening marker from *pEGFP-N2* vector (Clontech) using the *P5/P6* primer pair. The primer sequences are as follows (restriction sites are underlined): *P1-Xba* I :

5'-GCGCTCGAGACTTCTTGTAGCTGTC-3'; *P2-Hind* III: 5'-GGCAAGCTTTGTTTCTCCTTTACT-3'; *P3-Pst* I: 5'-GGCTGCAGAGGATTGAGGAATAC-3'; *P4-Xba* I: 5'-CGTCTAGATTTTATGCGATCAAACAAC-3'; *P5-Hind* III: 5'-GCAAGCTTATGATTGAACAAGATGGATTG-3'; *P6-Pst* I: 5'-GGCTGCAGTCAGAAGAACTCGTCAAGAAG-3'.

After digestion of PCR products with *Xba* I /*Hind* III (U fragment) and *Pst* I /*Xba* I (D fragment), the kanamycin resistance gene digested with *Hind* III /*Pst* I was introduced between the U and D fragments. The resulting chimera was cloned into the *Xba* I /*Xba* I -digested *pBluescript SK II* (-) vector (Stratagene) to obtain the construct targeting vector: *pBSKΔcagA_kan*. *Hp27* served as a recipient strain was electrotransformed with *pBSKΔcagA_kan*. Bacteria were grown on blood agar plates for 48 h and then streaked on fresh selective plates supplemented with 25 μg/mL of kanamycin. A real *cagA* isogenic mutant (*Hp27_ΔcagA*) was selected from kanamycin-resistant colonies and the corresponding insertional DNA region was checked by PCR to confirm the exact recombination.

Protein extraction

A wild-type strain of *Hp27* and an isogenic mutant of *Hp27_ΔcagA* were harvested from Brucella agar plates and then washed 3 times with ice-cold PBS. Total protein was extracted with an appropriate volume (300 μL) of lysis buffer containing 8 mol/L urea, 65 mmol/L DTT, 2% CHAPS, 2 mmol/L PMSF, 0.5% IPG buffer, and protease inhibitor mixture. The extraction mixture was sonicated with parameters of 120 W, 5 min, pulse: 1S, 2S. The protein mixture was centrifuged at 12000 r/min for 40 min. After transferred to a clean tube, the supernatant was stored at -70°C as aliquots. The protein concentration was determined by Bradford dye-binding assay with bovine serum albumin as the standard.

Two-dimensional electrophoresis

Two-dimensional electrophoresis was carried out as follows. Precast IPG strips (pH 3-10 linear, 18 cm, Amersham Pharmacia Biotechnology Inc.) were used in the first dimension. A total amount of 1000 μg protein was diluted to a total volume of 350 μL with the buffer containing 8 mol/L urea, 20 g/L CHAPS, 5 g/L IPG buffer 3-10, 20 mol/L DTT and a trace of bromophenol blue. After loaded on IPG strips, IEF was carried out on IPGphor (Bio-Rad, USA) according to the following protocol: rehydration for 16 h at 50 V, 1 h at 200 V, 1 h at 500 V, 1 h at 1000 V, 5 h at 10000 V and 60000 V h at 10000 V (total 112.5 kVh). The current was limited to 50 μA per gel. After IEF separation, the strips were immediately equilibrated for 2 × 15 min with an equilibration solution containing 50 mmol/L Tris-HCl (pH 6.8), 6 mol/L urea, 300 g/L glycerol and 20 g/L SDS. Then, 20 mmol/L DTT was included in the first equilibration solution, and 20 g/L iodoacetamide was added in the second equilibration step to alkylate thiols. Electrophoresis in the second dimension was carried out

on 15% SDS-PAGE gels (18 cm × 20 cm × 0.1 cm). The strips were held in place with 5 g/L agarose dissolved in a SDS/Tris running buffer and electrophoresis was carried out at a constant power (2.5 W/gel for 40 min and 15 W/gel for 5 h) using a Protean II xi cell gel SDS-PAGE system (Bio-Rad, USA).

Image processing and analysis

After electrophoresis, gels were stained with Coomassie brilliant blue, equilibrated in a solution containing 500 mL/L methanol, 50 mL/L acetic acid and 25 g/L Coomassie brilliant blue R-250 for at least 2 h, and rinsed in 300 mL/L ethanol containing 70 mL/L acetic acid. Digitalized images were obtained by ImageScanner GS-800 (Bio-Rad, USA) scanning of the gels, and analyzed qualitatively and quantitatively by the PDQuest gel image analysis software 7.1 (Bio-Rad, USA). To determine variation, three gels were prepared for each sample. The computer analysis allowed automatic detection and quantification of protein spots as well as matching. The normalized volume of protein spots was used to analyze the differential level of protein expression. Only those spots that had a statistical significance in differential expression were selected for further investigation.

Protein identification by MALDI-TOF-MS

Differential protein spots were cut out from the gel. After being washed with 300 μ L milliQ water for 15 min, each protein spot was decolorized with the successive action of 50 μ L of 15 mmol/L potassium ferricyanide and 50 mmol/L sodium thiosulphate for 5-10 min. Faded gel pieces were dried in a vacuum centrifuge tube for 5 min. Cysteine reduction and alkylation were performed and incubated with 10 mmol/L DTT, 100 mmol/L NH_4HCO_3 at 56°C for 1 h in the dark. Gel pieces were dried again and incubated with 50 mmol/L fresh iodoacetamide in 100 mmol/L NH_4HCO_3 at room temperature for 30 min and rehydrated in digestion buffer containing 20 μ L of 12.5 μ g/mL modified trypsin and 20 mmol/L NH_4HCO_3 for 30 min in ice. Excess liquid was removed and the gel pieces were digested continuously at 30°C overnight (> 16 h). The resulting peptide mixture was extracted from the digested solution by centrifugation and resuspended in 10 μ L of 50% CH_3CN and 0.1% trifluoroacetic acid (TFA) for 10 min at 30°C on a shaking platform. Peptide mass maps were generated by Applied Biosystems Voyager System 6192 MALDI-TOF-mass spectrometry (ABI, USA). Peptide masses were analyzed using the MS-Fit search program (<http://prospector.ucsf.edu/ucsfhtml4.0u/msfit.htm>). The searching parameters were set up as follows: acquisition mass ranges 900-3500 Da, the mass tolerance was ± 0.5 Da; the number of missed cleavage sites was allowed up to 1; the minimum number of matched peptide was four; species was set as bacteria; and the searching range was within the experimental pI value ± 0.5 pH unit and experimental Mr $\pm 20\%$.

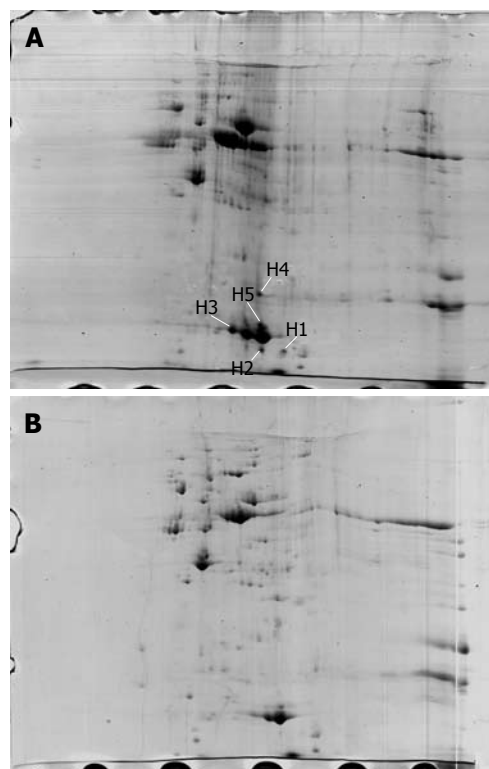


Figure 1 Differential two-dimensional maps of wild-type strain *Hp27* (A) and mutant strain *Hp27_ΔcagA* (B) stained with Coomassie blue.

RESULTS

The proteome maps of *H. pylori* strains *Hp27* and *Hp27_ΔcagA* were produced and compared (Figure 1). After spot detection, background subtraction, volume normalization, differentially expressed proteins were detected in wild-type strain versus isogenic mutant. The arrows indicate five protein spots whose expression levels were significantly differently represented in strain *Hp27* as compared to strain *Hp27_ΔcagA*.

Expression levels of the two proteins were obtained by calculating the relative spot volume of each protein *versus* the total amount of protein in the gel. Segments of 2-DE gel map for five proteins derived from strains *Hp27* and *Hp27_ΔcagA* are shown in Figure 2.

Five protein spots differentially expressed in strains *Hp27* and *Hp27_ΔcagA* were excised from 2-DE gels and identified by peptide mass fingerprinting (Figures 3 and 4).

Mascot searches using the peptide mass fingerprinting data indicated that the differentially expressed proteins were alkyl hydroperoxide reductase (Ahp), superoxide dismutase (Sod) and modulator of drug activity (Mda66) (Table 1). These three proteins are all important antioxidant proteins of *H. pylori*. From a quantitative point of view, these proteins are novel, and have not been reported previously in relation to *cagA* gene.

DISCUSSION

A comparative proteome analysis was carried out between the two *H. pylori* strains: *Hp27*, a wild-type

Table 1 Mascot search results of PMFs in 5 protein spots

Spot	Accession No.	MW	PI	Description	MOWS E score	Sequence coverage (%)
H1	gi 2313748	21 591	6.59	Modulator of drug activity (Mda66)	62	46
H2	gi 2314747	22 221	5.88	Alkyl hydroperoxide reductase (AhpC)	111	67
H3	gi 2314747	22 221	5.88	Alkyl hydroperoxide reductase (AhpC)	140	76
H4	gi 2313490	24 602	5.77	Superoxide dismutase (SOD)	86	62
H5	gi 2313490	24 602	5.77	Superoxide dismutase (SOD)	132	69

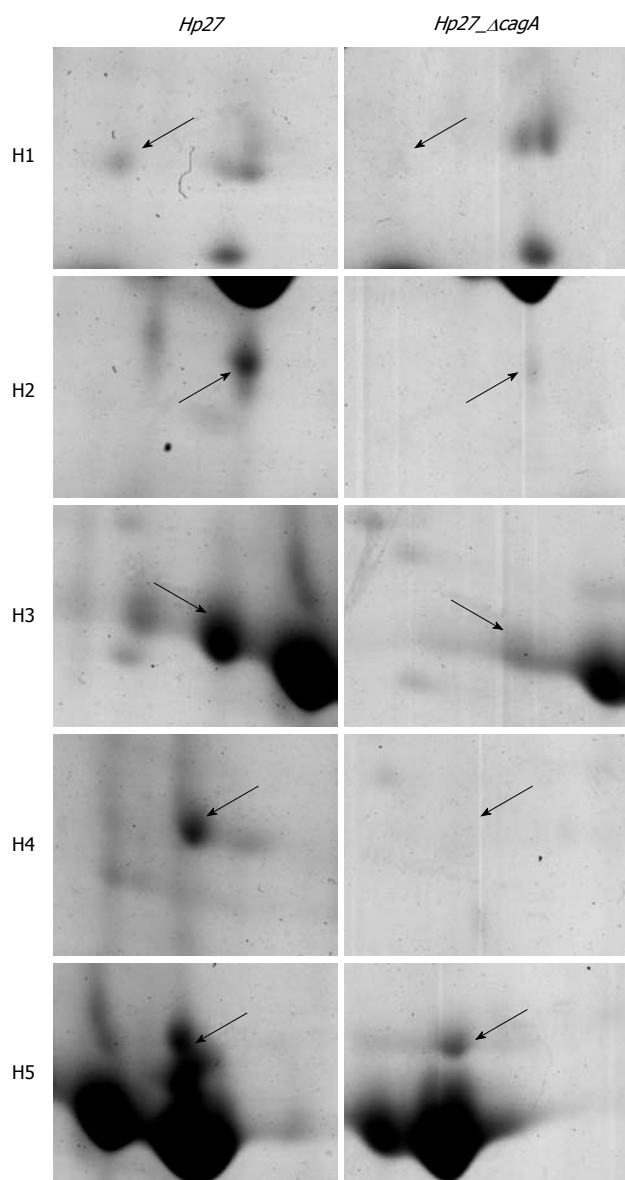


Figure 2 Magnified segments of 2-DE gel map of protein spots including H1-H5.

strain and *Hp27_ΔcagA*, a *cagA* isogenic mutant. Five differential protein spots, which are abundant in bacteria proteins of wild-type strains and are down-regulated or absent-expressed in bacteria proteins of mutants, were selected to perform in-gel trypsin digestion and MALDI-TOF-MS-based PMF analysis. The three identified proteins are Ahp, Sod and Mda66. Of the 5 position identifications, spots H2 and H3 were identified as the same protein Ahp, and spots H4 and H5 were

also identified as the same protein Sod, indicating that these gene products are present as isoforms with post-translational modification^[14].

Alkyl hydroperoxide reductase (AhpC), a thioredoxin (Trx)-dependent AhpC, is a member of the 2-Cys peroxiredoxins family (2-Cys Prxs). A group of thiol-specific antioxidant enzymes, which catalyze the reduction of hydrogen peroxide and organic hydroperoxides, are ubiquitous proteins that protect organisms from damage by reactive oxygen species^[15]. *H. pylori* are oxygen-sensitive microaerophilic bacteria, and contain many antioxidant proteins, among which AhpC is most abundant. The function of AhpC is to protect *H. pylori* from a hyperoxidative environment by reducing toxic organic hydroperoxides^[16]. Wang *et al.*^[17] reported that mutant cells defective in AhpC are more sensitive to oxidative stress conditions, accumulate more free (toxic) iron, and suffer more DNA fragmentation compared to wild type cells. Olczak *et al.*^[18] tested the ability of strains with mutation in *ahpC* (encoding alkyl hydroperoxide reductase) to colonize the stomachs of mice, and showed that the mutant is clearly more sensitive than the parent strain to both oxygen and cumene hydroperoxide and unable to colonize mouse stomachs, whereas 78% of the mice inoculated with the parent strain become *H. pylori* positive. Recently, Chuang *et al.*^[19] revealed that AhpC of *H. pylori* acts as a peroxide reductase in reducing organic hydroperoxides and as a molecular chaperone in preventing protein misfolding under oxidative stress. Besides, AhpC could also influence the activity of other proteins. Catalase in *ahpC* mutant partially inactivated (approximately 50%) in comparison with the parent strain, indicating that organic hydroperoxides (the substrate of AhpC), which accumulate in *ahpC* mutant cells, are responsible for the inactivation of catalase^[20]. In this study, the expression of AhpC was down-regulated in the *cagA* gene knocked-out mutant (*Hp27_ΔcagA*), suggesting that AhpC, one of the most important anti-oxidative stress proteins of *H. pylori*, is related with the *cagA* gene.

Superoxide dismutase (SOD), a nearly ubiquitous enzyme in organisms exposed to toxic environments, is able to catalyze the conversion of superoxide radicals to hydrogen peroxide and molecular oxygen. Single SOD in *H. pylori*, encoded by the *sodB* gene, has been suspected to be a virulence factor for this pathogenic microaerophile^[21]. Seyler *et al.*^[22], who first isolated mutants with interruptions in the *sodB* gene, found that the *sodB* mutants are devoid of SOD activity, and more sensitive to O₂ and H₂O₂ for both growth and viability.

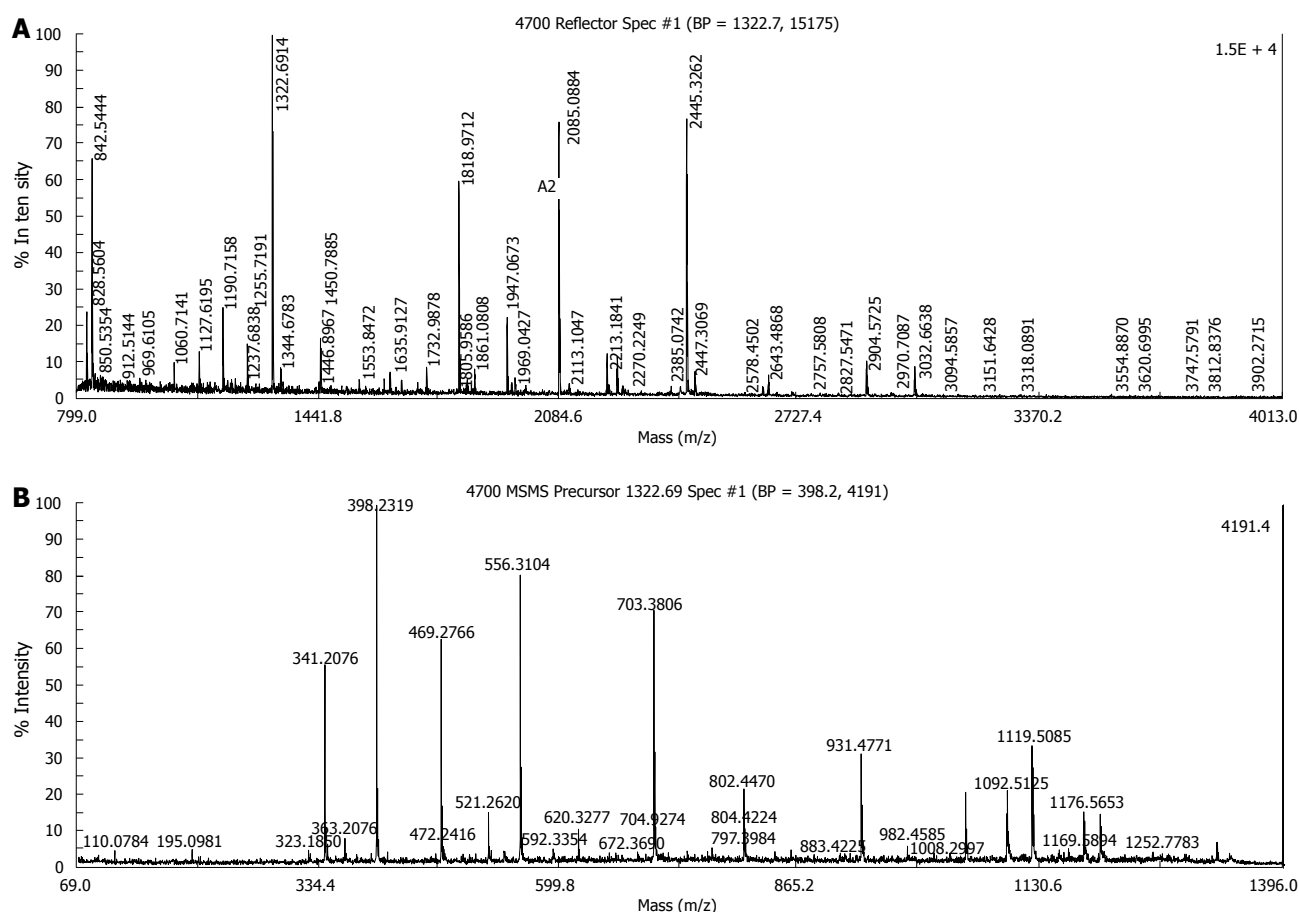


Figure 3 Mass spectrometry (MS) maps of one protein spots. A: MS of H1; B: MS/MS of H1.

Since oxidative stress is correlated with DNA damage, they studied the frequency of spontaneous mutation to rifampin resistance. The frequency of mutagenesis of the *sodB* mutant strain is about 15-fold greater than that of the wild-type strain. Wang *et al*^[17] also reported that mutant cells defective in SOD are more sensitive to oxidative stress conditions and suffer more DNA fragmentation compared to wild type cells, and that a significant proportion of cells of *sodB* mutant strains develop into stress-induced coccoid form or lysed as well as that they also contain a significantly higher amount of 8-oxo-guanine associated with their DNA, compared to wild type cells. Seyler *et al*^[22] observed that only 1 out of 23 mice inoculated with a SOD-deficient mutant of a mouse-adapted strain became *H. pylori* positive, while 15 out of 17 mice inoculated with the wild-type strain harbored the organism, in a mouse colonization model, indicating that SOD is a virulence factor affecting the ability of *H. pylori* to colonize the mouse stomach and is important for the growth and survival of *H. pylori* under oxidative stress conditions.

Mda66, identified in the *ahpC napA* double mutant by two-dimensional gel electrophoresis combined with N-terminal protein sequencing, is another possible antioxidant protein^[23]. Single bacterial homologue is a MdaB protein of *Escherichia coli* (*E. coli*), first identified as a modulator of drug activity (named *mda66*) because the gene is mapped at 66 min on the *E. coli* chromosome^[24]. Wang *et al*^[25] demonstrated that, like its homologue in

E. coli^[26], Mda66 protein is a NADPH quinone reductase and able to reduce quinone to quinol. Quinone metabolism within cells has a direct effect on the cell's ability to deal with oxidative stress^[27]. Therefore, the reduced status of *H. pylori* Mda66 protein plays an important role in the management of oxidative stress. Wang *et al*^[25] reported that the wild-type strain could tolerate 10% oxygen, but the growth of *mdaB* mutant was significantly inhibited by 10% oxygen. The *mda66* mutant was also more sensitive to H₂O₂, organic hydroperoxides, and paraquat, an agent generated by superoxide. Although the wild-type strain survived more than 10 h after air exposure, exposure of the mutant strain to air for 8 h resulted in no recovery of viable cells. It was reported that oxidative stress sensitivity of *mda66* mutant can reduced the ability of *mda66* mutant to colonize mouse stomachs^[25]. *H. pylori* were recovered from 10 of 11 mouse stomachs inoculated with the wild-type strain, with about 5000-45000 CFU/g of stomach, while only 3 of 12 mice inoculated with the *mdaB* mutant strain did not harbor any *H. pylori*, and contained less than 2000 CFU/g of stomach^[25]. Therefore, the physiological function of *H. pylori* Mda66 protein is similar to that of NADPH quinone reductase that plays an important role in the management of oxidative stress and contributes to successful colonization of the host.

Oxidative stress resistance is one of the key properties enabling pathogenic bacteria to escape the effects of reactive oxygen produced in the host.

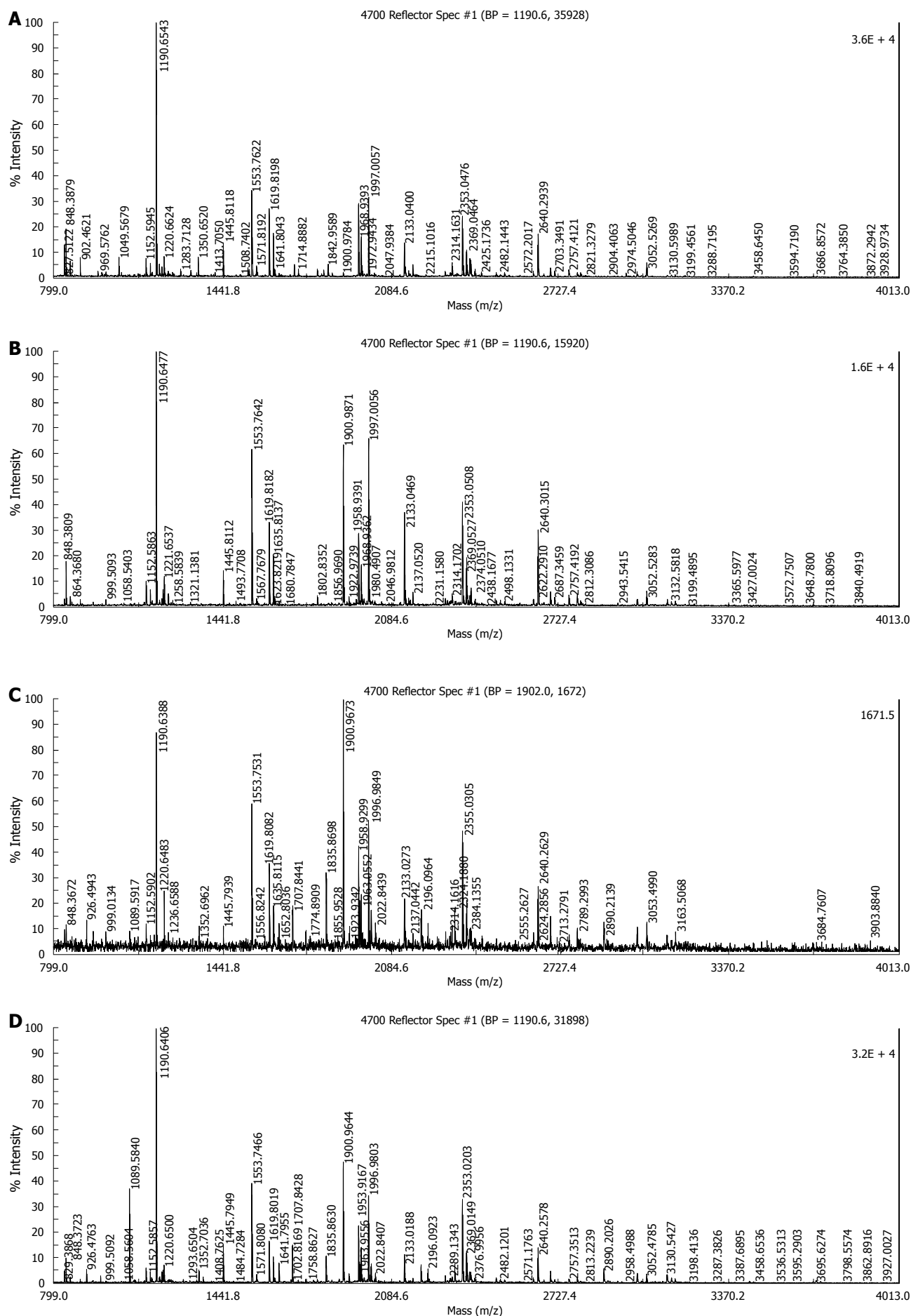


Figure 4 Mass spectrometry (MS) maps of 4 protein spots. A: MS of H2; B: MS of H3; C: MS of H4; D: MS of H5.

Therefore, proteins (enzymes) involved in oxidative stress resistance are the important factors for bacterial colonization and pathogenesis^[28]. Microaerophilic organisms, such as *H pylori*, are particularly vulnerable to the detrimental effects of oxygen and oxidative stress. Nevertheless, enzymes including AhpC, catalase (KatA), SOD, thioperoxidase (Tpx), *etc.*, can maintain persistent infection by using a variety of protective enzymatic systems that eliminate or minimize toxic oxygen-derived products.

In this study, three important antioxidant proteins of *H pylori* including AhpC, SOD and Mda66 were identified from the 2-DE maps showing differentially expressed proteins between the wild-type strain (*Hp27*) and isogenic *cagA* knock out mutant (*Hp27_ΔcagA*), indicating that the *cagA* gene is relevant to the expressions of antioxidant proteins of *H pylori*. Disruption of the target gene was found to have a certain effect on the expression of other genes that its encoded protein was shown to play a direct or indirect role in the regulation of protein biosynthesis, suggesting that *cagA* gene quantitatively influences *ahpC*, *sodB* and *mda66* transcription or their subsequent translation and correct folding.

Regulatory genes are usually located in the genome upstream of genes. With reference to the genomic sequence of *H pylori* 26695, *cagA* is only located in the upstream of *mda66*, and it is difficult to explain how CagA regulates other antioxidant proteins. Since there are no DNA-binding motifs or motifs suggestive of a two-component regulatory system in CagA, the protein may act as a signal transducer by means of other proteins^[8,29]. It was recently reported that a ferric uptake regulator (Fur) and a post-transcriptional regulator CsrA play a key role in the regulation of antioxidative stress enzymes^[21]. We presumed that *cagA* might be related to Fur and/or CsrA, through which *cagA* influences the expression of antioxidant protein. However, the correlation between *cagA* and Fur and/or CsrA needs to be further studied.

In this study, *cagA* was found to be correlated with the three stress-resistant enzymes, suggesting that *cagA* gene may be of importance for *H pylori* to maintain the normal activity of antioxidative stress and to keep long-term persistence in the host, which is a novel mechanism involved in *cagA* pathogenesis. Based on our results and the reported linkage between *cagA* and motility^[30], our conclusion is that *cagA* has virulence effects and may play a specific role in *H pylori* pathogenesis by influencing the expression of other proteins (enzymes).

COMMENTS

Background

Helicobacter pylori (*H pylori*) is a causative agent of gastritis, peptic ulcer and gastric cancer. CagA, a major virulence factor of *H pylori*, is considered a marker of increased pathogenic potential and may play an important role in the pathogenesis of *cagA* gene. However, little is known about the molecular mechanism and multiple biological functions of *cagA*.

Research frontiers

CagA is a *H pylori* immunodominant antigen whose gene resides in the *cag* pathogenicity island, a 40-kilobase insertion containing genes involved in

virulence. A previous study showed that *cagA* is related with the motility of *H pylori*. However, its exact mechanism still remains unclear. In this study, a proteomic approach was employed to determine if disruption of the *cagA* gene has some effects on the expression of other proteins of *H pylori*, through which various functions of the *cagA* gene can be recognized.

Innovations and breakthroughs

A proteomic approach combined with MALDI-TOF-MS was employed to investigate changes in expression of the wild-type strain of *Hp27* (*cagA*⁺) and the *cagA* gene knock out isogenic mutant. Three antioxidant proteins were identified, including alkyl hydroperoxide reductase (Ahp), superoxide dismutase (SOD) and modulator of drug activity (Mda66), respectively. It is implied that *cagA* is essential to maintain the normal activity of antioxidative stress and ensure *H pylori* persistent colonization in the host, which may be a novel mechanism involved in *cagA* pathogenesis.

Applications

These results provide a novel mechanism involved in *H pylori cagA* pathogenesis. The *cagA* gene may be of importance for *H pylori* to maintain the normal activity of antioxidative stress and keep long-term persistence in the host.

Terminology

CagA: Cytotoxin-associated gene A protein encoded by cytotoxin-associated gene pathogenicity island, a major virulence factor of *H pylori*. Two-dimensional electrophoresis: A two-step method to separate soluble proteins. Soluble proteins were first separated by isoelectric focusing (IEF) electrophoresis, and then according to the difference in molecular weight by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Antioxidant protein: Proteins (enzymes) involved in oxidative stress resistance, which are important factors for bacterial colonization and pathogenesis.

Peer review

The results of this study are interesting. The findings are novel. However, how the absence of antioxidant proteins should be further studied.

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