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Upregulation of a non-heme iron-containing ferritin with dual ferroxidase and DNA-binding activities in *Helicobacter pylori* under acid stress

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ACCESSION NUMBER.
Nucleotide sequence accession number
Sequence data determined in this study have been deposited in the GenBank database under the accession number DQ312265 (*pfr* gene of HD103 strain from *Helicobacter pylori*)

***Helicobacter pylori* is a spiral Gram-negative microaerophilic bacterium. It is unique and distinctive among various bacterial pathogens for its ability to persist in the extreme acidic environment of human stomachs. To address and identify changes in the proteome of *H. pylori* in response to low pH, we have used a proteomic approach to study the protein expression of *H. pylori* under neutral (pH 7) and acidic (pH 5) conditions. Global protein-expression profiles of *H. pylori* under acid stress were analysed by two-dimensional polyacrylamide gel electrophoresis (2-DE) followed by liquid chromatography (LC)-nanoESI-mass spectrometry (MS)/MS and bioinformatics database analysis. Among the proteins differentially expressed under acidic condition, a non-heme iron-containing ferritin of *H. pylori* (HP-ferritin) was found to be consistently upregulated at pH 5 as compared to pH 7. It was also found that HP-ferritin can switch from an iron-storage protein with ferroxidase activity to a DNA-binding/protection function under *in vitro* conditions upon exposure to acidic environment. Prokaryotic ferritins, such as non-heme iron-binding HP-ferritin with dual functionality reported herein, may play a significant urease-independent role in the acid adaptation of *H. pylori* under physiological conditions *in vivo*.**

Keywords: Acid-amplified free radicals/acid stress/Fenton reaction/*Helicobacter pylori*/non-heme iron-containing ferritin of prokaryotes/urease.

Abbreviations: *H. pylori*, *Helicobacter pylori*; 2-DE, two-dimensional polyacrylamide gel electrophoresis; LC-MS/MS, liquid chromatography coupled tandem mass spectrometry; RT-PCR, reverse transcriptase-polymerase chain reaction; HP-ferritin, non-heme iron-containing ferritin from *H. pylori*.

Helicobacter pylori (*H. pylori*), a Gram-negative, slow-growing, flagellated microaerophilic (5% O_2) bacterium, is an important causative agent for gastrointestinal disorders, such as chronic gastritis, gastric and duodenal ulcers and gastric cancer (*1–3*). *Helicobacter pylori* possesses a large number of virulence proteins for colonizing human gastric mucosa and causing tissue damage to host cells (*4*, *5*). The bacterium is well adapted to the acidic conditions of pH 1–2 in human stomach lumen as evident by the fact that *H. pylori* infection usually lasts for decades or more (*2*, *6*). Survival of *H. pylori* under severe acid shock has been extensively studied and generally proposed to depend on urease activity, which is an essential cytosolic enzyme for survival and colonization of *H. pylori* in gastric lumen by releasing ammonia from urea, thereby neutralizing the gastric acid in stomachs (*7*, *8*). While acid is known to increase partial activity of urease, the increased and constitutive expression of urease is considered to be more direct and effective (*9*, *10*). A few other possible contributors to acid survival include acid-induced expression of several protein factors such as Hsp70 (*11*, *12*) and CagA (*13*). Another possible strategy for acid protection may be the production of cholesteryl glucosides that decrease membrane fluidity and enhance stability of the membrane in the presence of acid (*14*). Thus the inhabitance of *H. pylori* under the extreme acidic conditions of stomachs is intriguing because *H. pylori* is considered to belong to a member of neutrophilic bacteria.

Several recent reports analysed differential gene expression in *H. pylori* under acidic conditions by employing DNA microarray technology (*15–17*). However, there are still no definitive conclusions regarding how the expressed protein factors function in the anti-acid stress mechanism of *H. pylori*. In this study, we performed all experiments in the absence of urea to minimize the effect of urease even though it does not seem to play an important role in the survival of this micro-organism in a pH range between 4 and 7 (*17*). Due to the difficulty of *H. pylori* to proliferate below pH 4 *in vitro*, we have applied a proteomic approach (*18*, *19*) to examine the protein expression profiles of HD103 (a strain isolated from a patient of

duodenal ulcer) under neutral (pH 7) and mildly acidic (pH 5) conditions. Among the proteins differentially expressed under acidic condition, a non-heme iron-containing ferritin of prokaryotes was found to consistently increase at pH 5 as compared to pH 7. By performing analytical ultracentrifugation and fluorescence spectroscopy, we found that the molecular size of HP-ferritin remained unchanged but the conformational change had occurred when the protein was exposed to acid. It was also found that a change in the function of HP-ferritin from an iron-storage role with ferroxidase activity to that of DNA-binding and protection under acid stress. A likely mechanism involving acid-induced ferritin expression and the corresponding iron uptake by these prokaryote ferritins possessing dual functionality is proposed to account for the roles of iron storage/DNA-binding proteins in the binding and protection of bacterial DNA against acid-amplified free-radical damage from Fenton reaction under acid-stress environment of gastric lumen (*20*, *21*).

Materials and Methods

Bacterial strains, growth conditions

HD103 is a clinical isolate obtained from gastrointestinal biopsy specimens of a patient with duodenal ulcer. *Helicobacter pylori* strains were grown on Centers for Disease Control and Prevention (CDC) anaerobe blood plates at 37°C in a modular atmosphere controlled system (5% O_2/10% CO_2/85% N_2) (Don Whitley Scientific, Shipley, UK) and confirmed to be *H. pylori* because of their urease activity and helical morphology as determined by phase-contrast microscopy. Cultured cells were washed with PBS buffer (pH 7.5) or citrate buffer (pH 5.0) and used to culture in the medium of Brain Heart Infusion (BHI) with 1% β-cyclodextrin. All cultures were grown in the modular atmosphere controlled system for 24 h, centrifuged for 5000 r.pm. and the pellet was collected for later experiments.

2-DE and image analysis

Cell pellets of *H. pylori* grown under neutral or acidic conditions in BHI media for 24 h were solubilized in lysis buffer containing 8 M urea, 0.5% CHAPS or Triton X-100. After estimation of protein content by using a 2-D Quant Kit (Amersham Biosciences, Uppsala, Sweden), same amounts of 250 µg total protein were loaded onto IPG gel strips (pH 3–10, 13 cm, Amersham Biosciences, Uppsala, Sweden). The IPG strips were re-hydrated overnight according to the operation guidelines of the manufacturer (Amersham Biosciences, Uppsala, Sweden). For the first-dimensional separation, IEF was carried out using Ettan IPGphor II (Amersham Biosciences, Uppsala, Sweden) at 20°C with 50–3500 V for 19 h. After IEF, the IPG strips were equilibrated for 10 min each in two equilibration solutions [50 mM Tris–HCl, pH 8.8, 6 M urea, 2% SDS, 30% glycerol containing 100 mg dithiothreitol (DTT) or 250 mg iodoacetic acid (IAA), respectively], attached to a 14% SDS-polyacrylamide gel of Laemmli's buffer system, then covered by 0.5% agarose gel. 2-DE was conducted at 130–250 V for 5–6 h until the bromophenol blue reached the bottom of the gel. The gels were stained by Sypro-Ruby overnight. The protein profiles of the gels were scanned using a Typhoon 9400 scanner (Amersham Biosciences, Uppsala, Sweden). Gel image matching was done using PDQuest software (Bio-Rad, Richmond, 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 coupled tandem mass spectrometry

Based on the 2-DE gel analysis of samples under different pH conditions, differentially expressed proteins were selected for further identification by liquid chromatography coupled tandem mass spectrometry (LC MS/MS). The protein spots were cut from 2-DE gels, and then destained three times with 25 mM ammonium bicarbonate buffer (pH 8.0) in 50% acetonitrile (ACN) for 1 h. The gel pieces were dehydrated in 100% ACN for 5 min and then dried for 30 min in a vacuum centrifuge. Enzyme digestion was performed by adding 0.5 µg trypsin in 25 mM ammonium bicarbonate per sample at 37°C for 16 h. The peptide fragments were extracted twice with 50 µl 50% ACN/0.1% TFA. After removal of ACN and TFA by centrifugation in a vacuum centrifuge, samples were dissolved in 0.1% formic acid/50% ACN and analyzed by LC-nanoESI-MS/MS at the core facility laboratory of the Institute of Biological Chemistry, Academia Sinica. Proteins were identified in NCBI databases by use of MS/MS ion search with the search program MASCOT as described previously (*18*).

RNA preparation and reverse transcriptase-polymerase chain reaction

Batches of HD103 total RNA were isolated from the harvest on BHI medium at appropriate time intervals under acidic stress (pH 5.0) using RNeasy mini kit (Qiagen, Valencia, CA, USA). Subsequently, RNA (500 ng) from each sample was reverse-transcribed and polymerase chain reaction (PCR) was performed using Superscript reverse transcriptase (RT)-PCR kit (Invitrogen, Carlsbad, CA, USA). One PCR cycle comprised: 94°C for 30 s, 55°C for 1 min and 72°C for 1 min. The total cycle numbers were 28 with a final elongation step of 7 min at 72°C. Primer sequences were designed as follows.

For 23s rRNA: 5′-TGTGTGCTACCCAGCGATGC-3′ and 5′-GCGTTGAATTGAAGCCCGAG-3′;

For non-heme iron-containing ferritin (HP-ferritin): 5′-GCGCATATGTTATCAAAAGACATC-3′ and

5′-CTCGAGAGATTTCCTGCTTTTAG-3′. PCR products (178 bp for 23 S rRNA, 510 bp for HP-ferritin) were checked on 1.5% agarose gel and stained with 0.4 µg/ml ethidium bromide. Stained bands were visualized under ultraviolet (UV) light and photographed with a camera (Nikon E4500, Tokyo, Japan).

Expression and purification of HP- ferritin

Oligonucleotide primers that incorporated base changes necessary to produce *Nde*I and *Xho*I restriction sites were used to amplify the entire HP-ferritin gene by PCR. The HP-ferritin gene was cloned into the vector pET21b under control of the bacteriophage T7 promoter and used in an inducible T7 expression system. Correct insert orientation in the expression vector was confirmed by restriction enzyme analysis and partial DNA sequence analysis. *Escherichia coli* BL21 (DE3) was then transformed with pET21b-ferritin (carrying the HP-ferritin gene) and used for induced expression of the protein in LB medium. For the purification of *H. pylori* ferritin expressed in *E. coli* BL21(DE3), *E. coli* containing the insert of pET21b-ferritin was grown as described above, washed, re-suspended in 20 mM Tris buffer (pH 7.6), and lysed by sonication. Cell debris was cleared by centrifugation. The supernatant was then filtered through a 0.2-µm pore size filter unit (Millipore, Billerica, MA, USA) prior to purification on Ni-chelating column.

The ferroxidase activity of HP-ferritin

Ferrozine, a specific indicator of ferrous ion, was applied to trace the oxidation of ferrous under atmosphere. HP-ferritin as well as the corresponding buffer was diluted into assay buffer to give 1 µM final concentration, then 100 µM ferrous chloride was added to start the reaction. At the indicated time point, 250 µl aliquot was taken from the reaction and mixed with 25 µl of 30 mM ferrozine solution dissolved in 1 M ammonium acetate to stop the reaction. The absorbance at 570 nm was measured in a 96-well plate reader. The molar extinction coefficient 27,900 $M^{-1}\,cm^{-1}$ was used to calculate the concentration of the ferrous and ferrozine complex.

Analytical ultracentrifuge analysis

Sedimentation equilibrium experiments were performed using an Optima XL-I analytical ultracentrifuge (Beckman Instruments, Palo Alto, CA, USA) in an An-60 Ti rotor equipped with a standard six-channel cell. The protein samples were rotated between speeds 6,000, 8,000 and 10,000 r.pm. at 20°C until equilibrium was attained. Absorbance was monitored at 280 nm. The apparent molecular weight (MW_{app}) was obtained by global fitting of multiple scans acquired at 8,000 r.p.m. using the sedimentation analysis software supplied by the manufacturer.

Fluorescence analysis

All ferritin samples were equilibrated at 37°C for 12 h before measurements. For the wavelength scan from 400 to 600 nm, oligomeric HP-ferritin was diluted to a concentration of 0.025 μg/μl in scan solutions (pH 7.0 or pH 5.0 phosphate buffers). For measurements of extrinsic fluorescence using the fluorescent probe 1-anilino-8-naphthalene sulphonate (ANS) and its closely-related derivative 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulphonate (Bis-ANS), 50 μl of ANS or Bis–ANS in methanolic stock solution (0.1 M) was added to 1 ml ferritin solution and incubated at 37°C for 1 h. The extrinsic fluorescence spectra were then measured with a Jasco FP-6300 spectrofluorometer (Jasco, Tokyo, Japan) fitted with a thermostatted cell cuvette by setting the excitation wavelength at 370 nm, a light slit of 5 nm for both excitation and emission modes.

Gel-retardation Assay

pUC19 DNA was mixed with HP-ferritin at a DNA/protein ratio of 1:2 (w/w) in 50 mM MOPS buffer (pH 7.0) or 50 mM acetate buffer (pH 5.0), respectively. The incubation was carried out at 37°C for 30 min. The DNA–protein mixtures were then resolved on a 1.0% agarose gel. The electrophoresis was carried out at a constant voltage of 100 V. The unbound and protein-bound DNA were then detected by ethidium bromide staining.

DNA-scission assay

After incubating HP-ferritin and DNA in pH 5 or pH 7 buffer for 1 h, we treated incubated samples with 50 μM ferrous ammonium sulphate and 10 mM H_2O_2 (final concentrations, Fenton reagent). The DNA-scission reaction mixture was incubated at 37°C as described previously (*22*, *23*). We also purified plasmid substrate after DNA-scission experiments by GFX^{TM} DNA purification kit (Amersham Biosciences, Uppsala, Sweden) to separate plasmid DNA from HP-ferritin before running on 1% agarose gel.

Results

2-DE analysis of protein expression profiles of H. pylori *under acid stress*

A global protein-expression profile of HD103 under neutral (pH 7.0) and acidic (pH 5.0) conditions for 24 h were analysed using high-resolution 2-DE. The p*I* range for the first-dimension IEF strips was 3–10 and the second-dimension SDS-PAGE was run at 14% polyacrylamide gel (Fig. 1A and B). The 2-DE profiles of *H. pylori* grown under neutral or acidic conditions showed more than 412 distinct protein spots as revealed by image analysis. The proteomic analysis showed that about 10 proteins were significantly found to be differentially expressed under acidic conditions, with increased amounts in eight proteins, decreased in two proteins as shown in Table I. In our analysis we have found three protein spots being identified as urease-related proteins (protein spots No. 1–3 of Table I), confirming the role of urease in the acid adaptation of *H. pylori* to low pH. We also detected the upregulation of the expression levels of a non-heme iron-containing ferritin, *i.e.* HP-ferritin (protein spot No. 4 of Table I) and a free radical scavenger protein, adhesin–thiol peroxidase at pH 5 (protein spot No. 7). In addition, thioredoxin reductase (a member of the pyridine nucleotide-disulphide oxidoreductase family) (*24*) was downregulated under acid-stress conditions. HD103 was also found to have high degrees of upregulation in four proteins under acid stress, which included transcription anti-termination protein NusG, peptidyl-prolyl *cis–trans* isomerase, γ-glutamyltranspeptidase (a novel apoptosis-inducing and immunosuppressive factor of *H. pylori*) (*25*, *26*)

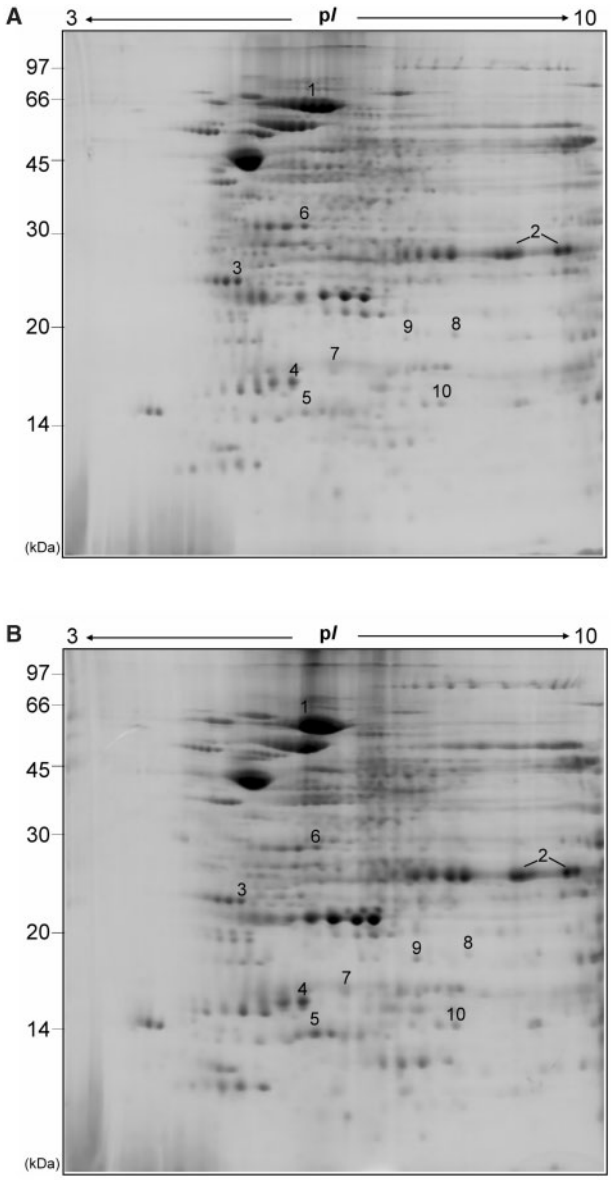

Fig. 1 Comparative 2-DE gel patterns of *H. pylori* cultured under neutral (pH 7) and acidic (pH 5) conditions in BHI culture media. Two-hundred fifty micrograms of total proteins from lysates of *H. pylori* cells were loaded on IPG gel strips (pH 3–10, 13 cm). The IPG strips were rehydrated and, after IEF, subjected to 2-DE. 2-DE protein profiles of HD103 under neutral (A) and acidic (B) conditions. Protein spots marked by No. 1–10 on the maps were found to be differentially expressed; these were further identified by nano LC-MS/MS and listed in Table 1. The result is representative of three independent experiments.

and the neutrophil-activating protein NapA (*27*). Thus, the effect of a simple chemical stress such as pH change reported herein involved at least 10 proteins with their roles scattered around cellular oxidoreduction, free radical scavenging, inhibition of T-cell proliferation and human neutrophil recruitment. Among these 10 protein markers, we noticed that HP-ferritin was more consistently upregulated (1.87-fold as shown in Table I) than the other identified proteins. It is likely and conceivable that HP-ferritin may play a urease-independent role in the acid adaptation of *H. pylori* (see 'Discussion' section).

Table I. Proteins in *H. pylori* showing up- or downregulation under acid stress identified by nano-LC-MS/MS analysis.

Spot	Protein name	Gene name	Swiss-Prot entry	Match	Sequence coverage, %	pI/mass, kDa	Protein expression ratio (S/N)*
Urease-related protein							
1	Urease B	ureB	Q9S0Q5	34	47	5.64/61.632	1.83
2	Urease protein UreA	ureA	Q93NI8	50	56	8.47/26.537	1.60
3	Urease accessory protein UreG	ureG	Q9ZMZ7	16	45	5.02/21.927	0.77
Oxidoreductase							
4	Non-heme-iron ferritin	pfr	O86247	8	10	5.51/19.333	1.87
5	Neutrophil activating protein NapA	napA	P43313	97	67	6.83/16.825	2.34
6	Thioredoxin reductase	HPAG1_0811	Q1CT44	23	60	5.76/33.490	0.68
7	Adhesin-thiol peroxidase	tagD	Q5XL90	26	52	7.68/18.224	1.69
Others							
8	Transcription anti-termination protein NusG	nusG	P55976	11	48	6.99/20.249	2.40
9	Gamma-glutamyltranspeptidase		Q9F5N9	9	15	9.27/61.060	3.05
10	Peptidyl-prolyl *cis-trans* isomerase	ppiA	O25982	6	22	6.00/17.610	4.18

*S, stress condition; N, normal condition.
Protein expression ratios shown here are means of triplicate densitometric measurements of protein spots.

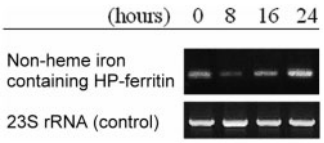

Fig. 2 RT-PCR analysis on mRNA transcription corresponding to HP-ferritin gene in *H. pylori*. Batches of HD103 total RNA were isolated from the harvest at appropriate time intervals from BHI culture media under pH 5 conditions. RT-PCR products were run on 1.5% agarose gel and stained with 0.4 μg/ml ethidium bromide. 23S rRNA was used as control gene for the quantification of RT-PCR products. The numbers (0, 8, 16 and 24) indicate harvest times in hours of HD103 under pH 5 conditions. Note that mRNAs of HP-ferritin of HD103 were transiently downregulated during the initial 0–8 h incubation and upregulated at the end of 24 h at pH 5. The result is representative of duplicate experiments.

Therefore, we focus on the function(s) of this prokaryotic ferritin (protein spot 4) under different pH in this study.

RT-PCR analysis on H. pylori mRNA to monitor the transcription level of HP-ferritin induced by acid stress

We further determined whether the increased protein expression of HP-ferritin was manifested at the transcriptional level. As shown in Fig. 2, a time-dependent increase of HP-ferritin mRNA transcriptional level was clearly observed after exposure of HD103 to acidic condition for 24 h. Therefore, we have corroborated the result of proteomic analysis by detecting the increase of HP-ferritin at the transcription level.

Ferroxidase activity and iron-incorporation ability of HP-ferritin under acidic condition

We further investigated whether the inherent ferroxidase activity of most eukaryotic ferritins (*28*) still existed in HP-ferritin under acidic condition. As shown in Fig. 3, HP-ferritin possesses a very low ferroxidase activity at pH 5, in contrast to that of maintaining such activity under neutral condition at pH 7.

Analysis of HP-ferritin under neutral and acidic conditions by analytical ultracentrifuge

To clearly identify whether the molecular size of HP-ferritin changed under acid stress, we performed the sedimentation equilibrium analysis by analytical ultracentrifugation. The MW_{app} of HP-ferritin were determined to be 493,000 (mean of 483,000 and 503,000) at pH 7 and 487,500 (mean of 478,000 and 497,000) at pH 5 by duplicate measurements (Fig. 4). The result indicated that a native molecular mass of HP-ferritin was about 490 kDa at both pH 7 and pH 5, like most ferritins (*28*), corresponding to ~24 ferritin subunits in the native HP-ferritin crystal structure as reported recently (*29*). Therefore, the loss of ferroxidase activity at pH 5 is probably not caused by the dissociation of HP-ferritin under acid stress.

Conformational difference of HP-ferritin under neutral and acidic conditions

Fluorescence spectroscopy was used to examine the tertiary structural differences of HP-ferritin incubated at pH 5 and 7 in phosphate buffers. The fluors can be introduced into HP-ferritin by simple incubation. Such extrinsic protein fluorescence generated by the use of added fluors is sensitive and very useful for structural comparison of homologous proteins studied under different conditions (*30*). The extrinsic flour used in our study is Bis–ANS. It becomes fluorescent when bound to the hydrophobic area on the surface of various macromolecules. Bis–ANS was thus used to investigate whether there is any surface hydrophobicity difference between HP-ferritin incubated at pH 5 and pH 7. Two emission maxima locating at 425 and 485 nm were clearly indicated in the scanned spectra of fluorescence emission measurements of HP-ferritin under both acidic and neutral conditions. Although there is no shift in the emission maxima for HP-ferritin at pH 5 and pH 7, there is a decrease at 425 nm and an increase at 495 nm in emission intensity under acid stress (Fig. 5). This observation should point to the fact that the conformational change had occurred when the protein was exposed to acid. It also indicated that the hydrophobic region of HP-ferritin

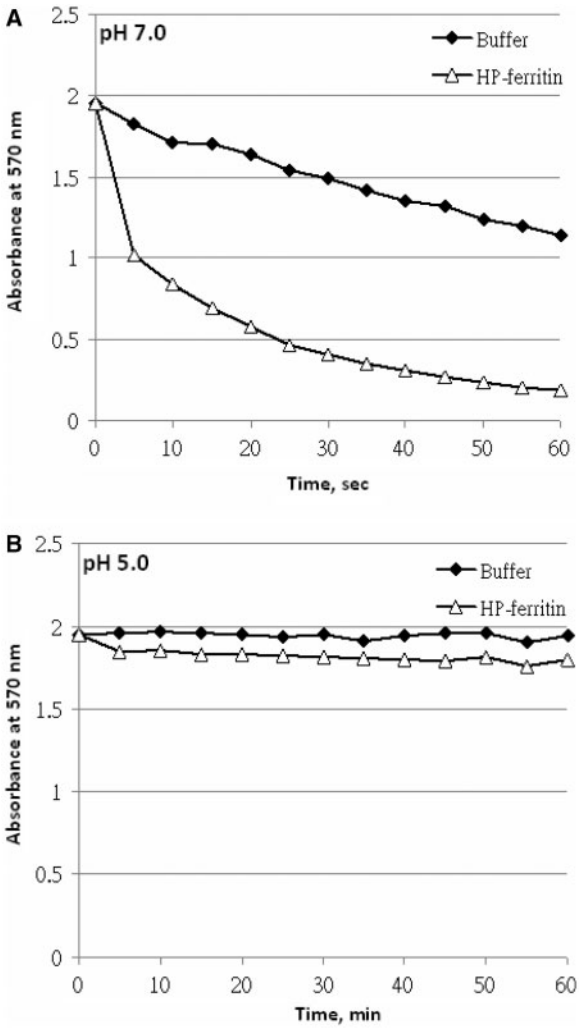

Fig. 3 Ferroxidase activity of recombinant HP-ferritin under neutral (pH 7) and acidic (pH 5) conditions. Protein and ferrous sulphate were mixed in the phosphate-buffered saline of pH 7 (A) or pH 5 (B). At each indicated time point, ferrozine (the ferrous ion indicator) was added to stop the reaction. The concentration of the complex of ferrozine and ferrous ion was measured by absorbance at 570 nm. The sharp drop in the concentration of the complex indicated the high enzyme activity of ferroxidase at pH 7. It is noted that HP-ferritin showed no ferroxidase activity at pH 5.

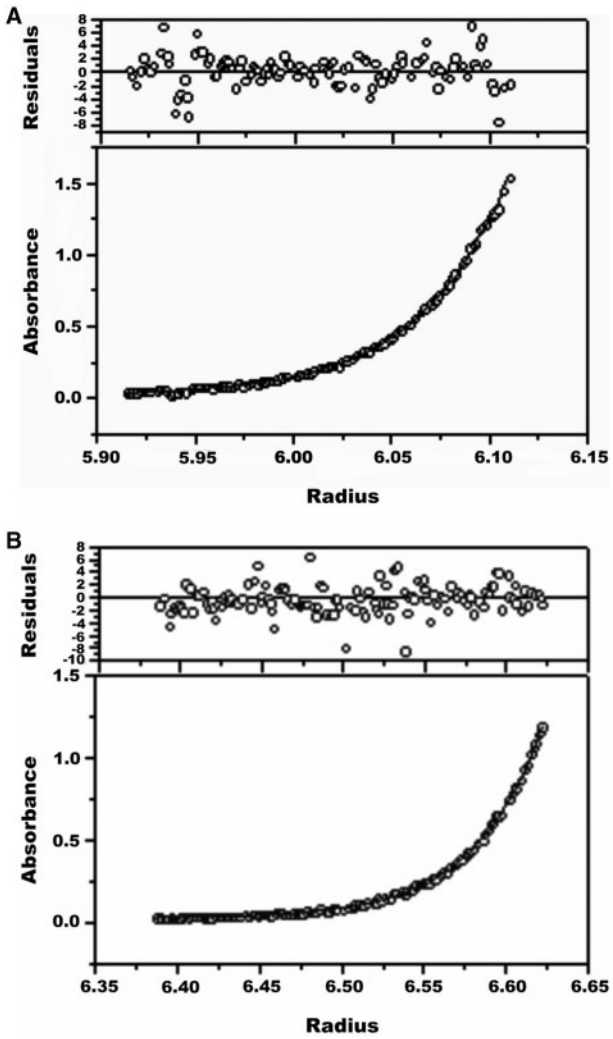

Fig. 4 The molecular size analysis of HP-ferritin under neutral (pH 7) and acidic (pH 5) conditions by analytical ultracentrifuge. (A) Representative sedimentation equilibrium distributions of HP-ferritin in the buffer containing 10 mM sodium phosphate and 150 mM sodium chloride at pH 7.0. The molecular weight of ferritin was determined to be 493,000 (mean of 483,000, 503,000) by duplicate measurements. (B) Representative sedimentation equilibrium distributions of HP-ferritin in the buffer containing 10 mM sodium phosphate and 150 mM sodium chloride at pH 5.0. The molecular weight of ferritin was determined to be 487,500 (mean of 478,000, 497,000) by duplicate measurements.

may become more exposed to exterior environment at pH 5 than at pH 7, resulting in a higher binding of fluorescent dye under acidic condition (*30*). Therefore HP-ferritin indeed exhibits conformational change upon acid exposure.

DNA-binding and DNA-scission protection of HP-ferritin

As shown in Fig. 6, in contrast to the incubation under the neutral condition at pH 7, HP-ferritin under acid stress at pH 5 did bind to plasmid DNA, forming a complex which is expected to move with a lower mobility than that of free DNA.

One important aspect of HP-ferritin is that by its binding to DNA under acid stress, HP-ferritin is able to protect DNA from oxidative damage caused by free radicals in the presence of metal ions such as iron and copper (*22*, *23*). We performed such DNA-scission assays in the presence of Fenton reagents (H_2O_2 plus ferrous ions). As shown in Fig. 7, plasmid DNA used in the above gel-retardation assay can be cleaved extensively in a short time period by the addition of Fenton's reagents. By the DNA-binding ability of HP-ferritin, the DNA–ferritin complex has indeed avoided the powerful DNA-scission damage from the Fenton reaction.

Discussion

The virulence of pathogenic bacteria is dependent on their adaptation and survival under the stressful conditions encountered in their hosts. *Helicobacter pylori* colonizes exclusively in the gastric lumen and mucosa, making it an ideal model system to study the molecular

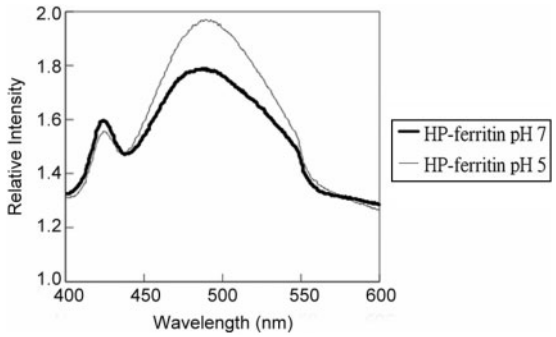

Fig. 5 Fluorescence analysis of HP-ferritin under neutral (pH 7) and acidic (pH 5) conditions. HP-ferritin samples were equilibrated at 37°C for 12 h before fluorescence measurements. Samples of 1 ml each in phosphate buffers of pH 7 or pH 5 were added 50 µl of ANS in methanolic stock solution (0.1 M) and incubated at 37°C for 1 h. The extrinsic fluorescence spectra were then measured with a Jasco FP-6300 spectrofluorometer fitted with a thermostatted cell cuvette by setting the excitation wavelength at 370 nm. The emission spectra were scanned from 400 to 600 nm in a cuvette thermostatted at 30°C. HP-ferritin was diluted to a final concentration of 0.025 µg/µl in scan solutions (pH 7 or pH 5 phosphate buffers) and spectra were obtained at pH 5 (thin line) and at pH 7 (thick line).

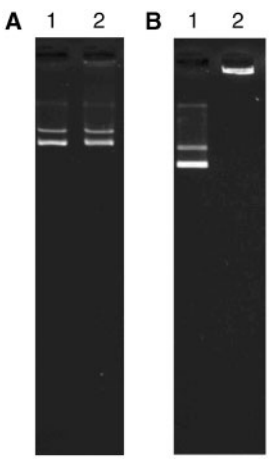

Fig. 6 DNA gel-retardation assays of HP-ferritin under neutral (pH 7) and acidic (pH 5) conditions. HP-ferritin was incubated with plasmid DNA in 50 mM MOPS buffer pH 7 (A) or 50 mM acetate buffer pH 5 (B), respectively. Lane 1, 0.5 µg DNA only; lane 2, 0.5 µg DNA mixed with 1 µg HP-ferritin. The incubation was carried out for 30 min at 37°C. Reaction mixtures were then loaded onto 1% agarose gel and DNA resolved by agarose electrophoresis at 100 V for 15 min. Agarose gels were stained by ethidium bromide. The fastest migrating band corresponds to the plasmid of supercoiled form; the slower migrating bands correspond to plasmid of lesser degrees of supercoiling or circular forms. It is noted that the band at the top of the gel in lane 2 of (B) corresponds to the complex of HP-ferritin and plasmid DNA formed at pH 5.

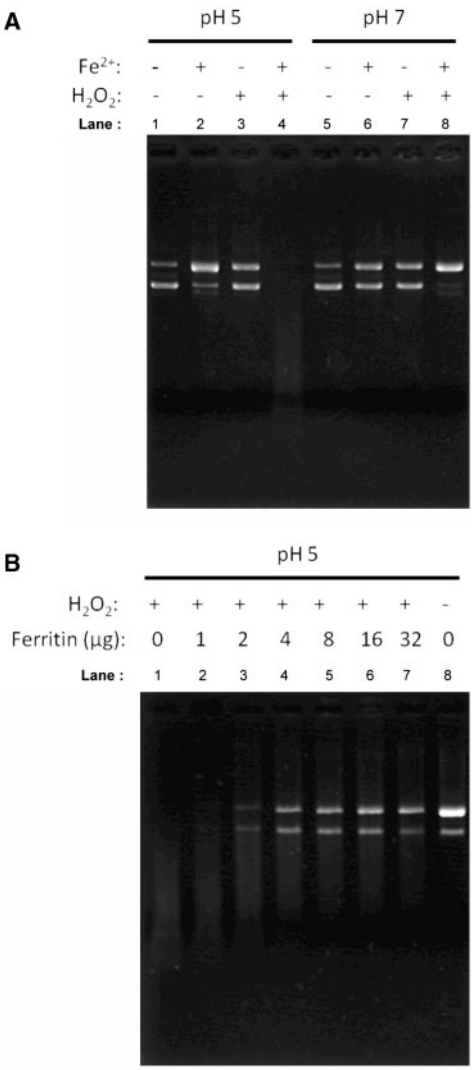

Fig. 7 DNA-scission protection by HP-ferritin. (A) DNA scission by means of Fenton reaction at pH 5 and pH 7. (B) Protection of DNA cleavage from Fenton reaction by HP-ferritin at pH 5. In (A), pUC19 was diluted into the buffer of 100 mM sodium acetate, pH 5 (lanes 1–4) or the buffer of 100 mM MOPS, pH 7 (lanes 5–8). Fenton reagent consisting of ferrous ammonium sulphate and hydrogen peroxide were then added to start reactions. After incubation at 37°C for 1 h, plasmid DNA in each reaction was analysed by 1% agarose gel electrophoresis. Note that the smeared pattern in lane 4 indicated strong DNA scission under acid-amplified Fenton reaction at pH 5 as compared to less DNA cleavage at pH 7. (B) pUC19 and various amounts of HP-ferritin were mixed in 100 mM sodium acetate, pH 5 and then incubated at 37°C for 30 min. Subsequently, 50 µM ferrous ammonium sulphate and 10 mM H_2O_2 (final concentrations) were added (except for the lane 8 without hydrogen peroxide). Note that protection of DNA from Fenton reaction increases with HP-ferritin concentration. In (B) plasmid substrate after DNA-scission experiments was purified by GFX™ DNA purification kit to remove HP-ferritin before running on 1% agarose gel.

mechanism of acid adaptation of various microorganisms in human stomachs (*1*, *2*). Intracellular urease activity of *H. pylori* cells has long been proposed to be a defence mechanism for the neutralization of gastric acids from gastric lumen (*10*). However, other regulatory proteins and molecular mechanisms cannot be ruled out for the acid adaptation of *H. pylori* in human stomachs. Moreover, little is known about how *H. pylori* responds to the moderately acidic conditions encountered at the gastric mucus layer (~pH 4–5) because of the difficulty of *H. pylori* to proliferate below pH 4 *in vitro* (*18* and unpublished data). Therefore, in this study, we determined the role of acid stress by adopting neutral (pH 7) and mildly acidic (pH 5) conditions.

A previous study compared gene expression profiles of *H. pylori* grown at neutral and acidic pH by DNA

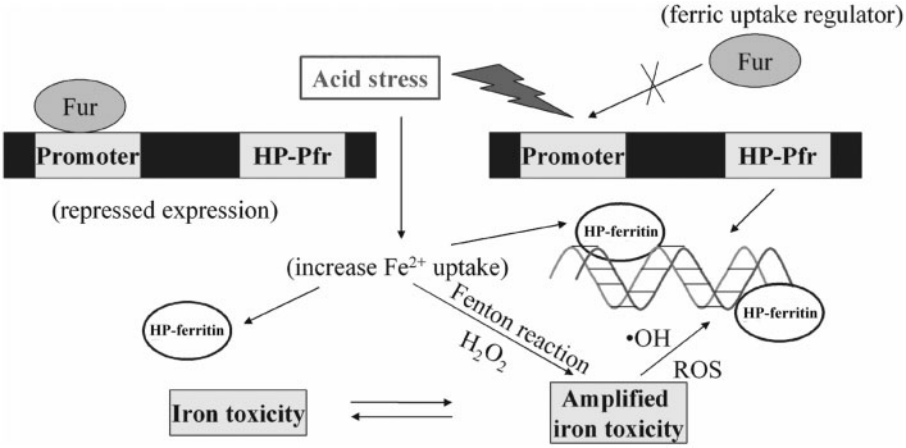

Fig. 8 A schematic representation of the interaction and correlation between acid stress, iron uptake/storage, HP-ferritin and ferric uptake regulator (Fur) in *H. pylori*. Under normal physiological condition at pH 7, HP-ferritin expression is inhibited by the Fur protein with transcription repressor function. Fur repressor would generally reduce its binding to HP-ferritin promoter under acid stress, resulting in an increased expression of HP-ferritin from Pfr (prokaryote ferritin) gene. Simultaneously, the amplified metal-toxicity induced by Fe^{2+}-mediated Fenton reaction in the presence of ferrous ions (from acid-induced Fe^{2+} uptake) and H_2O_2 would force *H. pylori* to exert some protection on its own vital DNA, triggering a change in the function of HP-ferritin from an iron-storage role with ferroxidase activity to that of DNA binding and protection.

microarrays (*31*). During growth under acidic conditions, 56 genes were found to be upregulated and 45 genes downregulated. It supported the claim that acidity is a signal modulating the expression of multiple genes related to metal ion homeostasis. The report revealed that protective mechanisms involving diminished transport and enhanced storage of metal ions are activated under acid stress. It is of interest to note that five of the upregulated genes encoded proteins controlling intracellular ammonia synthesis, including urease, amidase and formamidase, underlining the major role of urease and ammonia in the protection against acidity in *H. pylori*. In our proteomic analysis, we found three protein spots being identified as urease-related proteins (protein spots No. 1–3 of Table I), confirming the role of urease in the acid adaptation of *H. pylori* at low pH.

The most salient functional feature of HP-ferritin studied in this report lies in the detection of its DNA-binding activity as revealed by gel-retardation assay (Fig. 6). It is reminiscent of some recent reports concerning non-specific DNA-binding proteins of bacteria. In the stationary phase cultures of *E. coli*, the existence of a novel protein Dps (DNA-binding protein from stationary phase cells) was discovered (*32*). It is a non-specific DNA binding protein with a three-dimensional structure very similar to that of ferritins. Dps-like proteins have also been identified in distantly related bacteria such as *E. coli*, *Bacillus subtilis*, *Listeria innocua* and *Synechococcus sp.* (*33–35*). These non-specific DNA-binding proteins have been shown to protect cells from oxidative stress (*36*, *37*), although the mechanism for such protection remains unclear. It is believed that the protection of DNA by preventing DNA-scission (Fig. 7) from reactive oxygen species (ROS) generated by the cellular metabolite hydrogen peroxide in the presence of reactive metal ions is afforded by the ferroxidase activity of HP-ferritin. In general, animal ferritin utilizes oxygen as an oxidant for ferroxidase activity to convert active ferrous ions to the more innocuous ferric ions and then deposits ferric ions into the protein matrix of ferritin aggregate (*28*, *38*). In contrast, HP-ferritin was shown to be essential for iron storage in subcellular granules and enabled *H. pylori* to multiply under severe iron starvation, resulting in protecting the bacteria from acid-amplified iron toxicity (*39–41*) presumably caused by iron-mediated Fenton reaction (*42*, *43*). It is also noteworthy that this DNA-binding activity was not detected in the horse ferritin (data not shown), which is supposedly to be more functionally related with our HP-ferritin. Tertiary structural difference as revealed by extrinsic fluorescence induced by Bis-ANS may reflect the change in the surface hydrophobicity of HP-ferritin under acid stress. Therefore, we considered that the exposed hydrophobic region upon acid exposure might facilitate HP-ferritin to bind DNA under acidic conditions. Site-specific mutagenesis on some residues locating on the outside of protein molecules may shed some light on the mechanism underlying this important property. Interestingly, HP-ferritin possesses DNA-binding ability similar to the Dps protein family even though they share low sequence homology.

The physiological requirement of iron in a non-toxic and available form necessitates the evolution of ferritins in all living cells (*28*). Ferritins with iron-storage property are tailored to accommodate the fluctuation of the mobilizable iron inside the cells. Prokaryote ferritin of *H. pylori* has been shown to be involved in protection against metal toxicity (*44*, *45*). However, the detailed mechanism for such protection is not clear. In prokaryotes, iron-responsive regulation has been proposed to be mediated through the ferric uptake regulator (Fur) protein. The Fur protein controls iron homeostasis (*46*) and it is known to act as a Fe^{2+}-dependent transcriptional repressor that binds to the Fur-box of bacterial promoters and downregulates

gene expression in many Gram-negative and some Gram-positive bacteria (*47*). The transcription level of Fur has been shown to decrease under acidic condition (*48*) in contrast to that of HP-ferritin. Fur was also shown to play a role in the regulation of acid resistance (*49*). Fur and HP-ferritin may be interacting directly or indirectly to play some intricate role in the adaptation of *H. pylori* to iron limitation and low pH within the gastric mucosa. Therefore, the anti-acid mechanism for the survival of *H. pylori* may involve several sequential steps by coordinating the inherent functions of HP-ferritin and Fur as shown in the tentative scheme of Fig. 8. When *H. pylori* encounters the acidic environment in the gastric lumen or mucosa, the amplified metal-induced free radicals generated in the presence of ferrous ions and H_2O_2 would force *H. pylori* to exert some protection on its own vital DNA, triggering an increasing protein expression level of HP-ferritin and a change in the function from an iron-storage role with ferroxidase activity to that of DNA binding and protection. Therefore, this HP-ferritin possesses dual function under acid stress as does alkylhydroperoxide reductase (AhpC) under oxidative stress reported previously in our laboratory (*50*). Further analysis of DNA–ferritin complex of *H. pylori* and a detailed characterization of the protein–protein interaction between HP-ferritin and Fur regulator may prove to be essential in providing some insights into the anti-acid mechanism of *H. pylori* under acid stress.

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Conflict of interest

None declared.

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