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The DNA-Binding Protein HU has a Regulatory Role in the Acid Stress Response Mechanism in *Helicobacter pylori*

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Abstract

Background: Bacterial genomes are compacted by association with histone-like proteins to form a complex known as bacterial chromatin. The histone-like protein HU is capable of binding and bending the DNA molecule, a function related to compaction, protection, and regulation of gene expression. In *Helicobacter pylori*, HU is the only histone-like protein described so far. Proteomic analysis from our laboratory showed that this protein is overexpressed under acidic stress.

Materials and Methods: We used a purified recombinant wild-type protein and two mutant proteins with the amino acid substitutions K3A/S27D and K62R/V63N/P64A to characterize the function of the N-terminal domain and the flexible arm of HU.

Results: In vitro assays for DNA protection, bending, and compaction were performed. We also designed a *H. pylori* hup::cat mutant strain to study the role of HU in the acid stress response. HU_{wt} protein binds DNA and promotes its bending and compaction. Compared with the wild-type protein, both mutant proteins have less affinity for DNA and an impaired bending and compaction ability. By using qRT-PCR, we confirmed overexpression of two genes related to acid stress response (*ureA* and *speA*). Such overexpression was abolished in the hup::cat strain, which shows an acid-sensitive phenotype.

Conclusions: Altogether, we have shown that HU_{wt}–DNA complex formation is favored under acidic pH and that the complex protects DNA from endonucleolytic cleavage and oxidative stress damage. We also showed that the amino-terminal domain of HU is relevant to DNA–protein complex formation and that the flexible arm of HU is involved in the bending and compaction activities of HU.

Approximately 50% of the world population is chronically infected with *Helicobacter pylori* [1]. Colonization and the inflammatory response induced by this Gram-negative human pathogen have been related to peptic ulcers, chronic gastritis, MALT lymphoma, and gastric cancer [2,3]. For colonization, *H. pylori* needs to survive the hydrolytic enzymes found in the oral cavity, in the esophageal tract, and in the extremely acidic pH of the stomach [4,5]. To survive, this bacterium has developed an effective acid response mechanism based on the urease enzyme. The enzymatic activity of urease turns the urea molecule into ammonia and carbon dioxide. These products can diffuse through the inner membrane, thus buffering pH in the periplasmic space and even in the microenvironment that surrounds the bacterial cell [6].

In addition to urease, it has been suggested that in *H. pylori*, proteins such as the bacterial ferritin Pfr and the neutrophil-activating protein NapA could be part of a global acid stress response mechanism. Under acid stress conditions, these two proteins increase their DNA-binding abilities and ferroxidase activity. These changes protect DNA against reactive oxygen species (ROS) that are generated by the inflammatory response induced by the host immune cells, and by the Fenton reaction, a chemical process that is favored by the acidic pH in the cell cytoplasm [7,8].

As previously described in our laboratory, *H. pylori* cells overexpress a set of ~49 proteins when it is subjected to an acid stress. Among those proteins, we have identified the urease subunits UreA and UreB, Pfr, NapA, and the DNA-binding protein HU [9,10]. We have also shown that the Fur-regulated (ferric uptake regulator) arginine decarboxylase gene, *speA*, is overexpressed under acid stress conditions [11,12]. The *speA* gene is homologue to *adiA* from *Escherichia coli*, a gene that participates in the acid response system 3 (AR3). In this system, the arginine molecules are internalized by the membrane protein AdiC to serve as substrate for the arginine decarboxylase enzyme AdiA. This leads to the formation of agmatine. Thus, the proton consumption that is involved in this reaction reduces the overall abundance of protons in the cell cytoplasm [13]. In *E. coli*, this system appears to be regulated by the histone-like protein HU. In that regard, an *E. coli ΔhupA/B* mutant strain showed impairment in the functionality of AR3, thus resulting in an acid-sensitive phenotype [14]. Recently, it has been proposed that *H. pylori* has developed an AR3-like acid adaptation system on the basis of the overexpression of the arginine decarboxylase (*speA*) mRNA, the increased SpeA protein abundance, and the agmatine production by cells under acidic conditions [11].

The histone-like protein HU is member of the DNA-binding protein family II (DNABII), a group of proteins related to the organization and compaction of the bacterial chromatin [15]. These nucleoid-associated proteins (NAPs) have also been related to the formation of DNA–protein complexes in the site-specific recombination process, initiation of replication, and regulation of gene expression [16]. The amino acid sequence of HU is well conserved among homologous proteins. Structurally, HU has an α-helix structure in the N-terminal domain together with a β-sheet structure and random coils toward the C-terminal end of the protein. HU is a dimer, with a compact core of intertwined monomers. The C-terminal domains of each monomer unit form arms that intercalate the DNA double helix, thus inducing two sharp DNA kinks [17,18]. The ability of HU to bend DNA has implications for the compaction of the bacterial nucleoid, a feature that has been also related to DNA protection. In vivo experiments showed that an *E. coli* HU-deficient mutant strain is highly sensitive to DNA-damaging factors, such as oxidative stress and gamma or UV radiations [19,20]. Besides its role in DNA compaction and protection, the HU protein has a regulatory role upon the "HU regulon," which is composed of genes related to anaerobic growth, acid stress, high osmolarity, and the SOS response [21].

Genome analysis of *H. pylori* has led to the identification of only 32 genes with suspected functions in gene regulation mechanisms [22]. Such low number of gene regulation elements suggests the participation of other molecules or strategies, such as the global gene regulation mechanisms that involve the activity of NAPs including HU. The aim of this work was to study the DNA-binding, compaction, and bending abilities of the HU protein in *H. pylori*; the role of some amino acids in those protein functions; and also the role of HU in the mechanism of acid stress response.

Experimental Procedures

Bacterial Strains and Culture Conditions

Helicobacter pylori was grown at 37 °C, 5% CO_2, and 75% relative humidity in TSA plates (trypticase soy agar media) supplemented with 5% v/v horse serum, with 0.4% *H. pylori* selective supplement Dent (Oxoid, Basingstoke, Hampshire, UK), 0.3% Vitox (Oxoid), and 5% horse serum (HyClone, Logan, UT, USA). *Escherichia coli* strains were grown at 37 °C on Luria–Bertani (LB) broth or LB agar plates, containing ampicillin (100 μg/mL), kanamycin (30 μg/mL), or chloramphenicol (20 μg/mL).

Gene Cloning and Protein Expression

The *hp0835* ORF (*hup* gene) was PCR-amplified from *H. pylori* genomic DNA using the Platinum taq DNA polymerase enzyme (Life Technologies, Carlsbad, CA, USA) and cloned into *Nde*I/*BamH*I sites of the pET15b expression vector (Merck KGaA, Darmstadt, Germany) to generate a pET-*hup* plasmid. The *E. coli* BL21 (DE3) strain harboring the pET-*hup* plasmid was grown to OD_{600} 0.6, and expression of the recombinant His_6–HU fusion protein was induced by adding 2 mmol/L IPTG followed by a 3-hour incubation. The cells were harvested by centrifugation (6000 ×*g*, 10 minutes, 4 °C) and resuspended in ice-cold lysis buffer (10 mmol/L imidazole, 300 mmol/L NaCl, 10 mmol/L HEPES pH 7.9, 2 mmol/L PMSF, and 0.1% v/v Triton X-100). The bacterial cells were sonicated and centrifuged (12 000 ×*g*, 45 minutes, 4 °C), and the supernatant was filtered through 0.45-μm cellulose filter to remove all the cellular debris [23]. The N-terminal His-tagged protein was purified using Ni-Sepharose™ 6 Fast Flow (GE Healthcare, Buckinghamshire, UK) pre-equilibrated in binding buffer (10 mmol/L imidazole, 500 mmol/L NaCl, 10 mmol/L HEPES pH 7.9) and incubated with the cellular lysate supernatant for 1 hour at 4 °C with gentle shaking. The packed sepharose column was washed

twice with binding buffer (containing 30 mmol/L imidazole), and the proteins were recovered using elution buffer (500 mmol/L imidazole, 500 mmol/L NaCl, 10 mmol/L HEPES pH 7.9). The eluted fractions were analyzed in SDS-PAGE and Western blot using an anti-His mouse monoclonal primary antibody (Life Technologies). The fractions with the purified HU protein were pooled; dialyzed overnight against 50 mmol/L NaCl, 10% v/v glycerol, 10 mmol/L HEPES pH 7.9; and finally stored at −20 °C. Protein concentration was quantitated using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA) [24]. The HU mutant proteins were constructed using the overlap extension method [25] with the pET-*hup* vector as template. We designed a double-mutant protein (HU_{m2}) with K3A and S27D substitutions, and a triple-mutant protein (HU_{m3}) with K62R, V63N, and P64A substitutions. After cloning and sequencing, both proteins were purified as previously described.

Protein–Protein Cross-Linking Assay

It is well known that the HU protein has a dimeric conformation. Cross-linking assays were performed to confirm whether the recombinant wild-type or the mutant HU proteins used in this study preserve such oligomerization pattern. Purified proteins were dialyzed against double-distilled H_2O for 1 hour at room temperature. Each protein was diluted in buffer A (50 mmol/L KCl, 0.1 mmol/L DTT, 0.01 mg/mL BSA, 10% glycerol, 0.05% Brij58, and 20 mmol/L HEPES) adjusted to pH 7.9 or buffer A adjusted to pH 5.5 (buffer B) and incubated in the presence of 0.5% glutaraldehyde for 30 minutes at 37 °C. The reaction was stopped with 4× protein Laemmli sample buffer and analyzed in 12% SDS-PAGE. Protein–DNA complexes were visualized with Brilliant Blue G stain.

Electrophoretic Mobility-Shift Assay

Each purified protein (10 μg) was dialyzed and diluted in Electrophoretic Mobility-Shift Assay (EMSA) buffer (50 mmol/L KCl, 0.5 mmol/L EDTA, 0.1 mmol/L DTT, 0.01 mg/mL BSA, 5% glycerol, 0.05% Brij58, and 20 mmol/L HEPES pH 7.9) with 40 ng of circular/supercoiled pHel3 plasmid DNA and incubated for 1 hour at 37 °C. The pHel3 plasmid DNA is derived from pHel1, a cryptic plasmid isolated from *H. pylori* [26]. A control sample containing a HU–DNA complex was treated with proteinase K before gel analysis. Another control sample consisted of plasmid DNA incubated with BSA instead of HU. All samples were analyzed by 1% agarose gel electrophoresis (2.5 hour, 40 V, and 4 °C) using TAE solution (40 mmol/L Tris/acetate pH 7.0, 1 mmol/L EDTA) as running buffer. To assess the influence of pH on the DNA-binding ability of HU, we performed the EMSA by changing the pH of the running buffer to pH 6.0, 7.0, or 8.0.

DNA Protection Assay

To perform a DNA protection assay against hydroxyl radical damage, the pHel3 plasmid DNA was incubated in EMSA buffer for 1 hour at 37 °C in the presence of each of the HU proteins. Then, $FeSO_4$ and H_2O_2 were added to a final concentration of 20 and 5 mmol/L, respectively [27,28]. The DNA protection from endonucleolytic digestion was assessed by adding 0.7 units of DNase I to the previously formed HU–DNA complex [29]. In both cases, after incubation for 3 minutes at room temperature, the reactions were terminated by the addition of 10% SDS and 40 mmol/L EDTA and then loaded onto a 1% agarose gel. All gel image analysis and band quantitations were made using the ImageJ software [30].

DNA Cyclization Assay

The pHel3 plasmid DNA (40 ng) was linearized by *Xho*I digestion, diluted in ligation buffer (10 mmol/L $MgCl_2$, 1 mmol/L ATP, 10 mmol/L DTT, and 50 mmol/L Tris–HCl pH 7.5), and incubated with T4 DNA ligase and each of the HU proteins (10 μg) for 1 hour at room temperature [31]. A control sample containing only linearized plasmid and T4 DNA ligase was included. Finally, 5 μL of proteinase K stock solution (0.25 mg/mL) was added to each sample, incubated for 1 hour at 37 °C, and then analyzed in 1% agarose gel electrophoresis.

DNA Supercoiling Assay

Circular supercoiled pHel3 plasmid DNA (40 ng) was relaxed with 10 U of Vaccinia Topoisomerase I (Epicentre, Madison, WI, USA) and incubated for 2 hour at 37 °C in buffer containing 0.15 mol/L NaCl, 0.1% BSA, 0.5% glycerol, 0.1 mmol/L EDTA, 0.1 mmol/L spermidine, and 10 mmol/L Tris–HCl pH 7.5. The reaction was ended by adding 5 μL of stop buffer (1% SDS, 0.025% bromophenol blue, and 5% glycerol). The relaxed DNA was incubated with 10 ng of each protein (HU_{wt}, HU_{m2}, or HU_{m3}) for 2 hour at 37 °C in EMSA buffer, and then the proteins were digested by adding 5 μL of proteinase K (0.25 mg/mL stock solution) at 37 °C for 30 minutes. Finally, 5 μL of stop buffer was added to each sample before the analysis of DNA conformers by 1% agarose gel electrophoresis for 16 hour at 3 V/cm and 4 °C in

TAE buffer. DNA was visualized by ethidium bromide staining.

Construction of a *Helicobacter pylori* hup Mutant

A 720-bp DNA fragment containing the *hup* gene was PCR-amplified from *H. pylori* genomic DNA using the primer pair promHP-FW and hupR2 (Table 1) and cloned into the *BamH*I/*Hind*III sites of the pUC19 plasmid. Using the overlap extension method, we created a *Bcl*I restriction site in the *hup* gene using primers R-Hu-*Bcl*I and F-Hu-*Bcl*I (Table 1). Subsequently, a ~1-kb DNA fragment containing the chloramphenicol acetyl transferase gene from *Campylobacter coli* and its promoter region (*cat* cassette) was inserted in the *Bcl*I site to interrupt the *hup gene*, thus obtaining pUC-*hup::cat*. This suicide vector was introduced into *H. pylori* cells by natural transformation to replace the chromosomal *hup* gene via allelic exchange as described elsewhere [32]. Briefly, bacterial cells were scraped from a 48-hour culture and incubated for 15 minutes in ice with 0.5 μg of the transforming plasmid pUC-*hup::cat*. The mixture was spread on TSA plates. After overnight incubation, cells were resuspended in PBS pH 7.0 and plated in fresh TSA media with chloramphenicol (10 μg/mL). The resulting transformed *H. pylori hup::cat* (cam^R) colonies were isolated and stored at −20 °C. Disruption of the *hup* gene was confirmed by PCR (Fig. 1), using purified genomic DNA and by sequencing.

Effect of the hup Mutation on *Helicobacter pylori* Cell Growth Under Acid Condition

Helicobacter pylori wild-type and *hup* mutant strains were inoculated at an initial OD_{600} 0.2 in Brucella broth supplemented with 50 mL/L fetal bovine serum, 10 mL/L Vitox, and 1.7 mL/L Dent adjusted to pH 7.0 or 5.5. The cultures were incubated for 48 hour at 37 °C, 180 rpm, and 75% relative humidity in a 2.5-L hermetic container

Table 1 Oligonucleotide primers used in this study

Primer	Sequence
promHP-FW	5′ GGTGGTCCGGATCCCGCTAAAGATAAAAAAAGCGC 3′
hupR2	5′ GGTGGTCCAAGCTTGGCTTGTTTTTTGCGAGATCT 3′
F-Hu-*Bcl*I	5′ GGAGTTGATCAGTTTTGGCAAAT 3′
R-Hu-*Bcl*I	5′ ATTTGCCAAAACTGATCAACTCC 3′
F-ureA	5′ TGATGCTCCACTATGCTGG 3′
R-ureA	5′ GTCTTTTTACCAGCTCTCGC 3′
F-spea	5′ TCCACATAGGCTCTCAAATCAG 3′
R-spea	5′ CTGGGTGTATTCTACGGCTAAC 3′
F-16S	5′ GGAGTACGGTCGCAAGATTAAA 3′
R-16S	5′ CTCAATGTCAAGCCTAGGTAAGG 3′

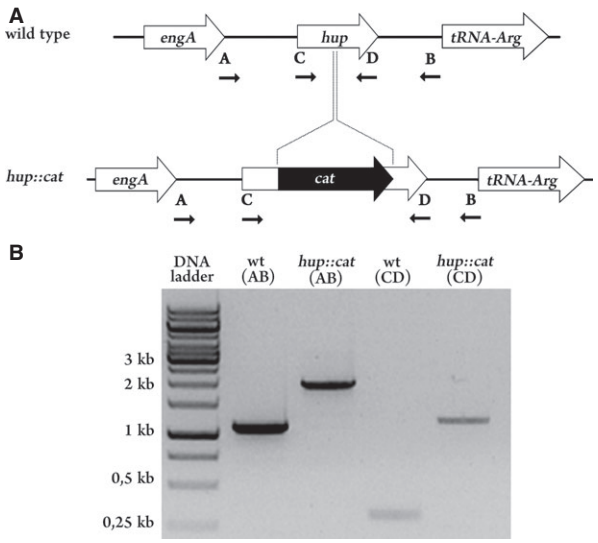

Figure 1 Confirmation of the *Helicobacter pylori hup::cat* genotype. (*A*) Schematic representation of the allele replacement event in the *hup* (HP0835) gene. (*B*) Verification of the *cat* gene insertion in *hup* by PCR.

(AnaeroJar™; Oxoid) using Campygen (Oxoid) to generate microaerophilic conditions. To determine progression of the bacterial culture, 1 mL of sample was taken every 12 hour to measure changes in OD_{600} and pH.

qRT-PCR Analysis of ureA and speA gene Expression Under Acid Stress Condition

The *H. pylori* wild-type and the *hup::cat* mutant strains were grown for 48 hour on TSA plates. The bacterial cells were scraped and resuspended in PBS at pH 7.4. Several aliquots containing $\approx 1.8 \times 10^9$ cells/mL (OD_{600} 3.0 U) were deposited in six microtubes, each one with 1 mL of RPMI 1640 liquid culture medium adjusted to pH 7.4, and in other six tubes with 1 mL of the same medium adjusted to pH 5.5. All microtubes were incubated at 37 °C, 5% CO_2, and 75% relative humidity, and samples were taken at 0, 15, 30, 45, 60, and 120 minutes of incubation. Bacterial cells were collected and then washed by centrifugation with PBS, pelleted again, resuspended in 50 μL of RNA*later* (Qiagen, Hilden, Germany), and incubated for 15 minutes at room temperature. After a final centrifugation, the pellets were stored at −80 °C until processing. Total RNA was extracted using the RNeasy mini kit (Qiagen), and the remaining DNA in the sample was digested with DNase I (Thermo Scientific, Suwanee, USA) according to the manufacturer's protocol. cDNA synthesis was performed in a 20 μL mixture containing 1 μg of RNA, 60 ng of random hexamer primers, 20 U of Ribolock RNase inhibitor, 1 mmol/L of each dNTP, 4 μL

of reverse transcription buffer 5×, and 200 U of reverse transcriptase (Life Technologies) according to the manufacturer's protocol. The qRT-PCR was carried out with the SYBR sensimix kit (Bioline, London, UK), 2 μL of cDNA, and each of the primer pairs (F-ureA, R-ureA, F-speA, R-speA, F-16S, and R-16S) (Table 1) using a 96-well reaction plate in the ABI7300 thermocycler (Applied Biosystems, Foster City, CA, USA). The mRNA levels of the *speA* and *ureA* genes in cells exposed to pH 5.5 relative to those at pH 7 were calculated with the absolute quantitation method, using the *rRNA 16S* gene as internal control.

Results

Structural Analysis of HU

We were able to obtain purified fractions containing each of the HU proteins used in this study. Cross-linking assays performed with these protein samples showed that the wild-type (HU_{wt}) and the two mutant proteins (HU_{m2} and HU_{m3}) form dimers in solution. To a lesser extent, we also observed formation of tetramers, all this in a pH-independent manner (data not shown). We also performed a secondary/tertiary structure simulation in silico, using the Phyre server [33] and the 3D-Mol Viewer, Vector NTI Advance ver. 10.3.0 software (Life Technologies). These analyses showed that all the three proteins display a similar folding pattern, thus implying that the amino acid replacements experimentally introduced in the protein sequence did not alter the apparent protein structure (data not shown).

DNA-Binding Ability of the HU Protein

The DNA-binding ability of the HU protein was analyzed by EMSA using supercoiled pHel3 plasmid DNA. Formation of stable complexes with different electrophoretic mobilities, and its dependence on the amount of protein, was observed (Fig. 2A, lanes 2, 3). Protein digestion of the DNA–protein complex by proteinase K resulted in recovery of the DNA migration pattern (Fig. 2A, lane 4). The ability of the HU mutant proteins (HU_{m2} and HU_{m3}) was also examined. As shown in Fig. 2B, the wild-type HU protein binds DNA more effectively than either HU_{m3} or HU_{m2}.

Effect of Acidic pH on the DNA-Binding Ability of the HU Protein

To assess the influence of pH on the DNA-binding ability of HU, DNA and the protein were incubated in EMSA buffer at different pH conditions. As shown in Fig. 2C,D, formation of the DNA–HU_{wt} complex is higher at pH 6.0 than at alkaline pH. At pH 6.0, interaction of HU_{wt} with supercoiled DNA produced large complexes, which were unable to migrate into the agarose gel. When pH was risen to 7.0 or 8.0, protein affinity for DNA was progressively lowered, on the basis that a lower retardation of complexes in the agarose gel was observed. Migration distances of these complexes (expressed as percentage of retardation) at different pH conditions were statistically different ($p < .01$). When HU_{m2} and HU_{m3} proteins were assayed at the same pH conditions, no statistical difference was observed (data not shown).

DNA Protection by HU Against Degradation

To determine whether the nucleoid compacted structure is part of a cellular mechanism involved in the protection of DNA from potentially hazardous elements, the ability of HU to protect DNA from hydroxyl-radical-mediated damage or endonucleolytic cleavage was examined. In this study, we observed that each of the HU proteins, the wild type and both mutants, did protect DNA from the hydroxyl radical attack. However, different levels of protection were observed for each of the three HU variants, as it was previously observed with the EMSA. The highest DNA protection was achieved with the HU_{wt} protein, followed by HU_{m3} and HU_{m2} with a much lesser protection (Fig. 3A). The treatment with DNase I resulted in full digestion of the naked DNA. Preincubation in the presence of the proteins HU_{wt} and HU_{m3} resulted in higher DNA protection against the endonucleolytic cleavage as compared to the effect of the HU_{m2} protein (Fig. 3B). In both conditions, the BSA protein (control) failed to protect DNA (data not shown).

HU Protein Mediates DNA Bending

The ability of HU to bend the DNA molecule was tested in the presence of T4 DNA in a cyclization assay. Agarose gel analysis showed that the *Xho*I-linearized pHel3 plasmid was represented by a single band (Fig. 4, lane 2) while incubation of the linearized plasmid with T4 DNA ligase resulted in formation of closed and nicked circular DNA structures, among other undefined conformers (Fig. 4, lane 3). When the HU_{wt} protein was included, it greatly facilitated the interaction between the DNA loose ends and promoted formation of a unique closed-circle DNA structure (Fig. 4, lane 4). Moreover, when each of the two HU mutant proteins was used, no induction of cyclization was observed, thus resulting in the same pattern observed in the absence of HU (Fig. 4, lanes 5–6).

HU Protein Induces DNA Compaction

The ability of HU to constrain DNA compaction was tested by incubating topoisomerase I-relaxed plasmid DNA with each one of the purified HU proteins (wild type and mutants). In the absence of HU, topoisomerase I fully relaxed the pHel3 plasmid, thus resulting in a slower migration in the agarose gels (Fig. 5, lane 2). However, when DNA was incubated with HU_{wt}, it became diversely compacted and appeared as a set of DNA topoisomers displaying a higher mobility in the gels (Fig. 5, lane 3). This was also observed (to a lesser extent) when relaxed DNA was incubated in the presence of the HU_{m2} protein, but not compaction was observed when DNA was incubated in the presence of HU_{m3} (Fig. 5, lanes 4–5).

Helicobacter pylori hup Mutant Construction and the Effect of Acidic pH on Its Growth

When the *H. pylori* 43504 wild-type and the *hup::cat* mutant strains were tested for growth, both cultures

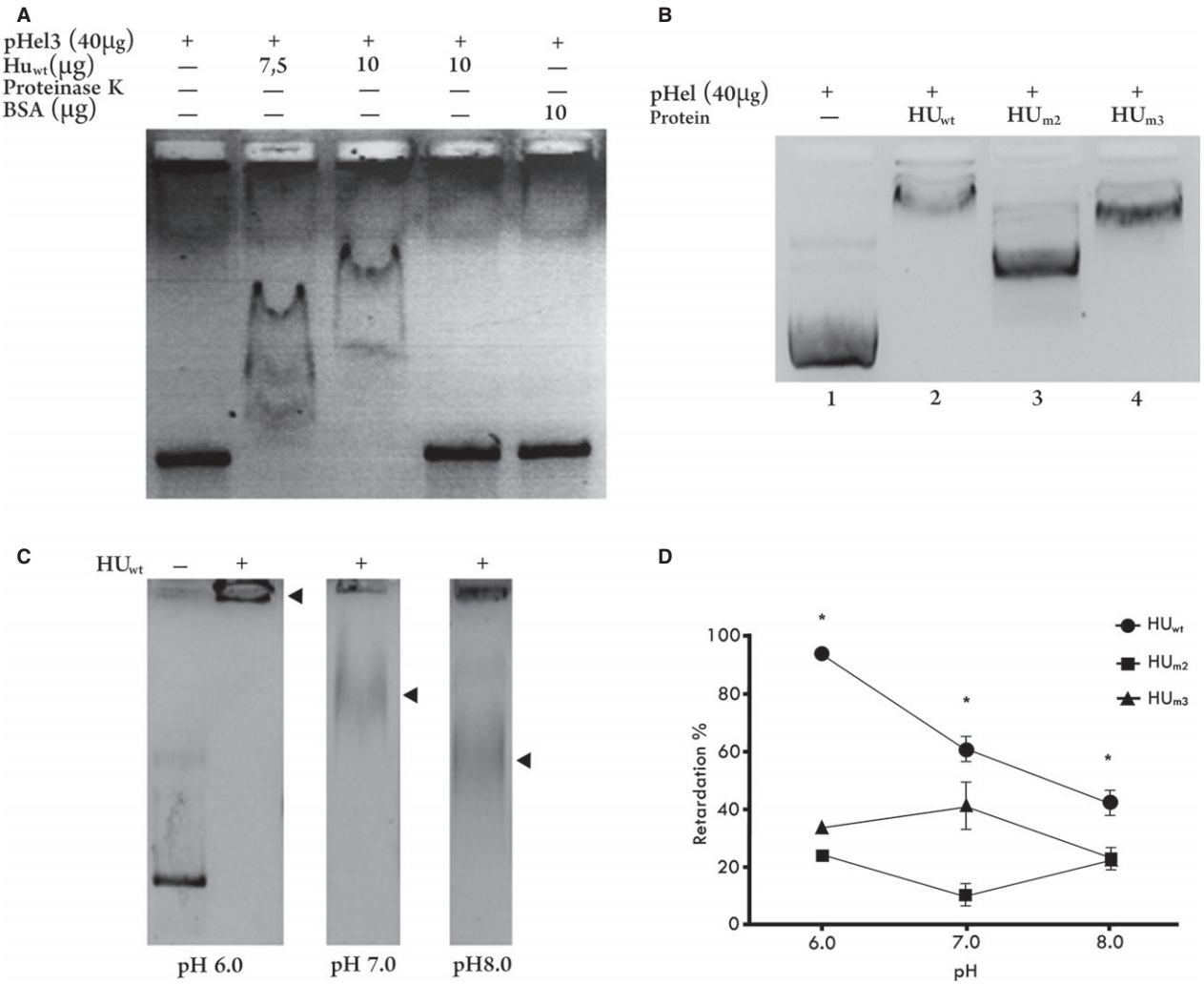

Figure 2 Electrophoretic Mobility-Shift Assay (EMSA) analysis of complex formation between the HU protein and plasmid DNA. (Panel *A*) pHel3 plasmid DNA and HU_{wt} were incubated to allow the DNA–protein complex formation, lane 1: protein-free DNA; lanes 2–3: DNA incubated with increasing concentrations of HU_{wt}; lane 4: HU_{wt}–DNA complex treated with proteinase K; lane 5: plasmid DNA incubated with BSA. (Panel *B*) DNA–protein complex formation using HU_{wt}, HU_{m2}, and HU_{m3} proteins. (Panel *C*) Effect of pH on the HU_{wt}–protein complex formation. The first lane shows naked DNA migration, whereas the following lanes show HU_{wt}–DNA complex formation at pH 6.0, 7.0, and 8.0. Arrows indicate relative positions of the complex after agarose gel electrophoresis. (Panel *D*) Analysis of percentage of retardation from data shown in Panel *C*. Statistical differences (*$p < .05$), for the wild-type HU protein, are indicated.

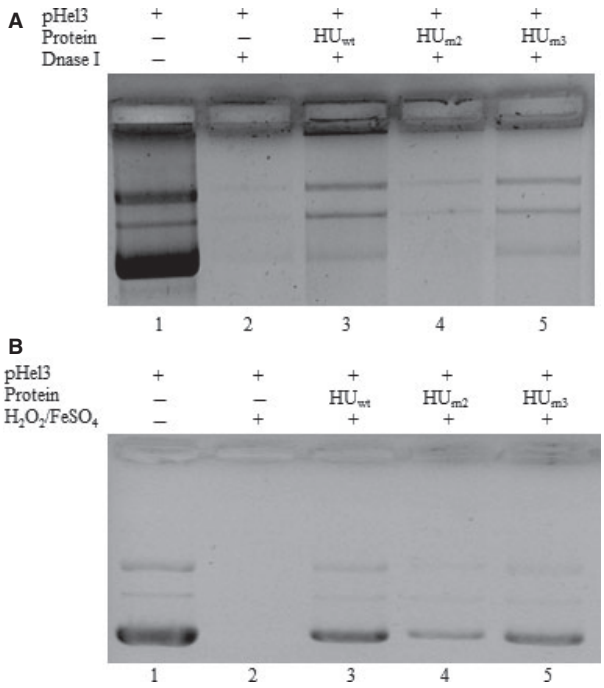

Figure 3 DNA protection ability of HU. The pHel3 plasmid DNA was preincubated with HU_{wt} (lane 3), HU_{m2} (lane 4), or HU_{m3} (lanes 5) and treated with either (*A*) 0.7 units of DNase I, or (*B*) 20 mmol/L $FeSO_4$/ 5 mmol/L H_2O_2. In each gel, the first lane shows DNA without treatment and the second lane naked treated DNA. After protein digestion, all the samples were analyzed by 1% agarose gel electrophoresis and stained with ethidium bromide.

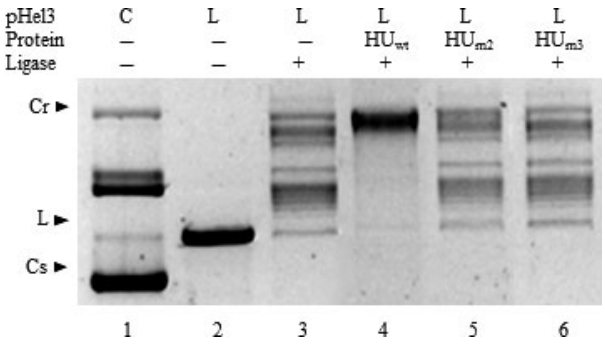

Figure 4 Cyclization of linearized plasmid DNA mediated by HU. The pHel3 plasmid DNA (lane 1) was linearized with *Xho*I restriction enzyme (lane 2). For the cyclization assay, the linearized plasmid was incubated with T4 DNA ligase (lane 3) or with T4 DNA ligase and HU_{wt}, HU_{m2}, or HU_{m3} proteins (lanes 4–6). After digestion with proteinase K, resulting DNA conformations were analyzed by 1% agarose gel electrophoresis. Circular DNA (C), linearized DNA (L), circular relaxed DNA (Cr), and circular supercoiled DNA (Cs).

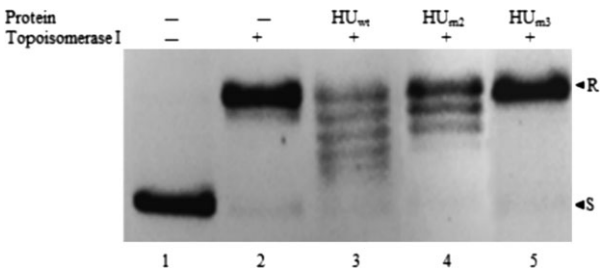

Figure 5 Effect of HU on DNA compaction. Agarose gel electrophoresis analysis of supercoiled (lane 1) or topoisomerase I-relaxed plasmid DNA (lanes 2–5). Circular relaxed DNA was incubated with HU_{wt}, HU_{m2}, or HU_{m3} (lanes 3, 4, and 5, respectively). After digestion with proteinase K, each sample was analyzed in 1% agarose gels and stained with ethidium bromide. S, supercoiled DNA; and R, relaxed DNA.

reached the same OD_{600} after 48 hour of incubation in Brucella broth media adjusted to pH 7.0. However, when pH was adjusted to 5.5, the *hup* mutant strain showed an acid-sensitive phenotype, as shown in Fig. 6A,B. Growth of the wild-type strain culture under acidic conditions was accompanied by a progressive rise in the pH value of the culture medium (from pH 5.5 to pH 6.8). By contrast, when the experiment was carried out with the *hup* mutant strain, just a marginal change in the pH of the medium was observed (Fig. 6C,D).

Helicobacter pylori speA and ureA Gene Expression Under Acid Stress Conditions

To study the physiological response in terms of expression of two selected genes that are involved in the acid stress response in this bacterium (*ureA* and *speA*), both the wild-type and *hup* mutant strains were exposed to an acid stress (pH 6.0) for a short period of time (≤120 minutes). qRT-PCR analysis showed that in the wild-type strain starting at 45 minutes after exposure to acidic pH, the *ureA* gene was overexpressed (four- to eightfold). The *speA* gene was also overexpressed (four fold) at 120 minutes after the acid challenge. Such overexpression was unexisting when the acid-sensitive *hup::cat* mutant strain was exposed to the acid stress challenge (Fig. 7).

Discussion

HU, the major histone-like protein in *E. coli* and also found in many eubacteria, is the only NAP shown in *H. pylori* [34,35]. This protein binds DNA with no sequence specificity and plays a key role in the processes of site-specific recombination, DNA replication, and DNA repair mechanisms [28]. In microorganisms subjected to extremely harsh acid environmental conditions, the highly compacted structure of the nucleoid may be a survival strategy aimed to prevent diffusion of

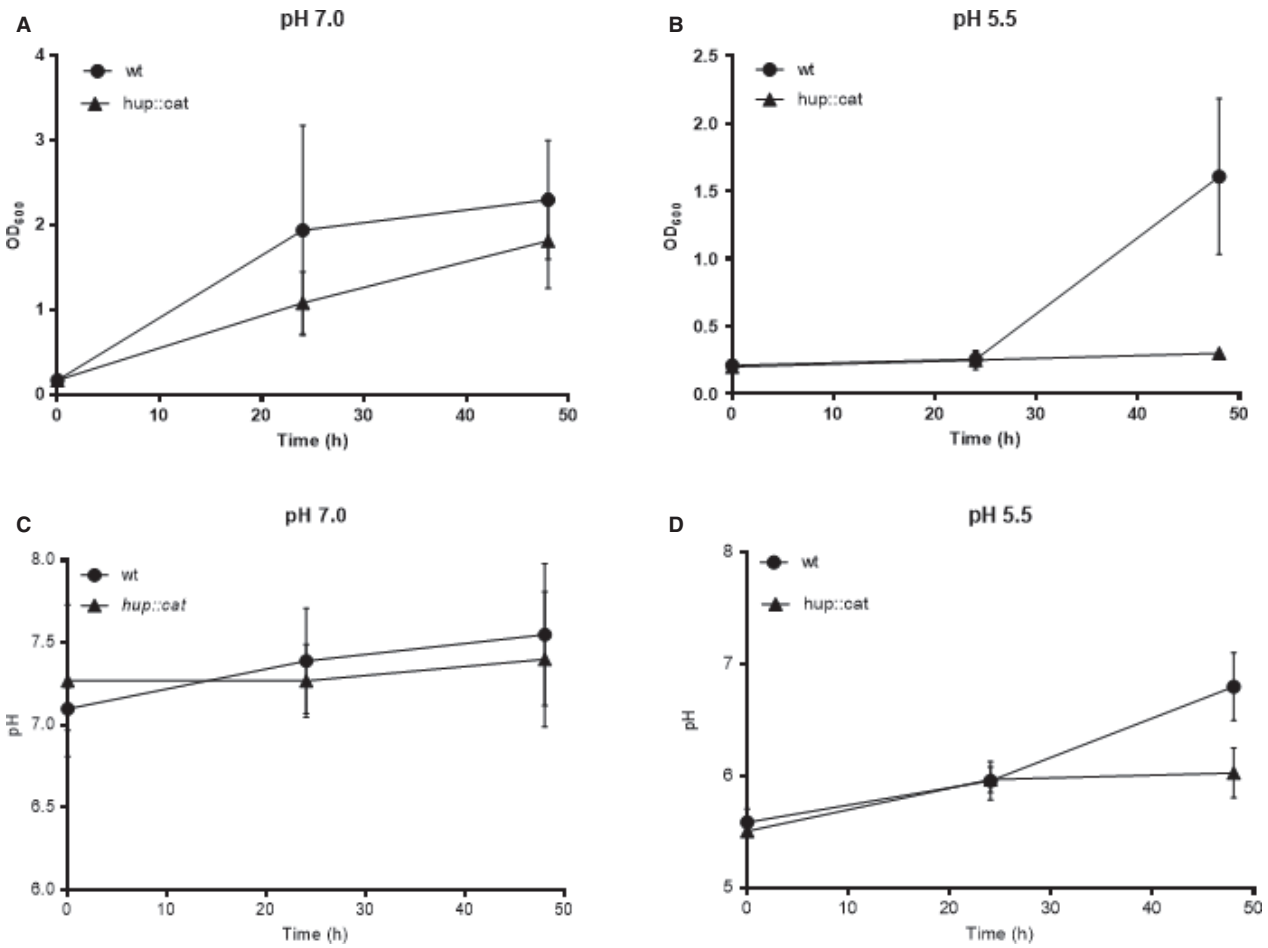

Figure 6 Growth of *Helicobacter pylori hup* mutant strain. Growth of the *H. pylori* wild-type and the *hup*::cat mutant strains under either (*A*) neutral or (*B*) acidic pH conditions. Variation in the pH value of the culture media during bacterial growth progression. (*C*) Initial pH 7.0 and (*D*) initial pH 5.5. Optical densities (600 nm) and pH values (averages and standard deviations) from three independent experiments are shown.

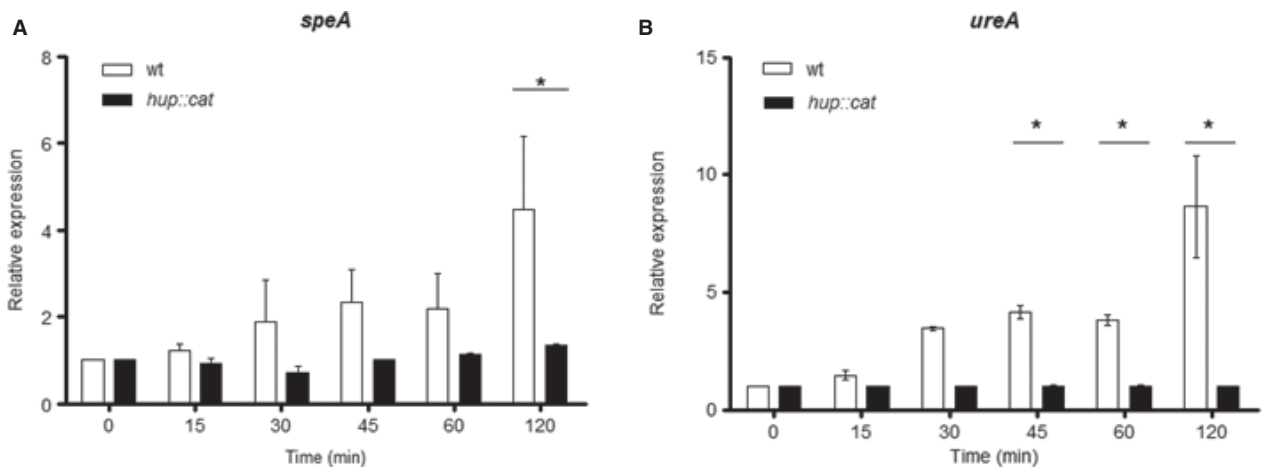

Figure 7 qRT-PCR analysis of *ureA and speA* gene expression in *Helicobacter pylori* under acid stress. mRNA expression analysis of (*A*) *ureA* and (*B*) *speA* genes. *H. pylori* wild-type (empty bars) and *hup* mutant strains (black bars) challenged at pH 5.5. Relative expression levels normalized to 16S rRNA levels from three independent experiments are shown. Statistical differences (*$p < .05$) are indicated.

DNA fragments generated by DNA-damaging agents and to contribute to oxidative resistance in these species [36].

Despite having a secondary structure similar to that of other HU proteins from eubacteria, the amino acid sequence of the wild-type HU protein occurring in *H. pylori* shows some remarkable differences with the overall consensus. One of those differences is the (amount) number and location of the arginine and lysine amino acid residues. This might be highly important considering that this histone-like protein uses an indirect readout mechanism that is based on conformational changes of DNA involving the width of the DNA groove. Penetration of arginines into the minor groove at sites where DNA bends and where that groove is narrowed provides an important stabilizing interaction [37].

On the other hand, the lysine residues are also involved in protein–DNA complex formation. Lysine interaction is enhanced by its protonation under physiological and acidic pH values, as it was described for the Dps protein of *E. coli* [38]. In contrast to the three well-defined arginine residues found in the general consensus sequence, the *H. pylori* HU protein has only two arginines, located in very different positions. This deficit of arginine residues interacting with DNA may be compensated by the predominance of lysine residues distributed throughout the whole protein surface. *H. pylori* HU protein has 18 lysines, whereas in other bacteria, such as *E. coli, Salmonella enterica,* or *Bacillus subtilis*, only 8–11 lysine residues do exist per HU protein monomer.

The in silico structure prediction performed in this study suggests that the two mutant proteins designed in this work (HU_{m2} and HU_{m3}) have the same folding pattern displayed by the wild-type HU_{wt} protein. Accordingly, the differences in the DNA-binding and bending abilities observed in all the three HU proteins might be an effect of particular and local point interactions between well-defined amino acids and the DNA backbone.

It has been previously suggested that changes in stability of the DNA–protein complex can be explained by variations in the length of the HU-binding site. These variations are, in part, determined by the presence or absence of amino acids capable of forming surface salt bridges distal to the sites of DNA kinking. Grove et al. [36,39] demonstrated that folding of the N-terminal domain of the HU protein in *Thermotoga maritima* is critical for formation of a stable DNA–protein complex. The authors showed an interaction (through salt bridge formation) between K3 and D26 in the N-terminal domain of this protein. On that ground, we mutated both amino acids in the *H. pylori* HU protein to preclude any eventual interaction between them and to elucidate the role of the N-terminal domain in the DNA–protein interaction. This could explain the lower stability of the DNA–HU complex and the decreased protective and compaction effect on DNA observed with our designed HU_{m2} mutant protein (which carries the K3 substitution). This effect was displayed only when the K3A substitution was accompanied with the S27D substitution in the *H. pylori* HU protein, thus suggesting that the N-terminal domain could play a critical role in the DNA–protein complex formation.

On the other hand, deficient complex formation by HU_{m3} can be explained by the relevance of the universally conserved proline-64 amino acid for the DNA-bending ability of all the HU homologues. This observation fits well with previous results showing that the HU protein with amino acid replacements in the area near proline-64 has an impaired ability to form a DNA–protein complex [35,40]. In this study, we also showed that three amino acids (K62, V63, and P64) found at the tip of the flexible protein arm that interacts with DNA are also relevant for the DNA-bending ability. Proline-64 would interact directly with the DNA minor groove, thus inducing bending of the DNA molecule, and the surrounding amino acids could act by stabilizing the DNA–protein complex.

For HU_{wt}, protein–DNA complex formation was increased under acidic conditions, and the complex provided protection against endonucleolytic cleavage (DNase I) and against the ROS generated in the Fenton reaction, a chemical process that occurs to a large extent under acid stress in the cell cytoplasm [41]. This ability is similar to the protective effect described for NapA and Pfr proteins [7,8]. Both proteins have a dual protective effect, acting through complex formation with DNA and also by sequestrating the iron ions involved in the Fenton reaction [42]. But unlike these proteins, HU may act only as a physical barrier to protect DNA from damage and degradation. The lower DNA-binding ability displayed by the double-mutant protein (HU_{m2}) underlies the formation of a less stable complex, which is in turn reflected in less DNA shifting in the EMSA and, therefore, a diminished protection against enzymatically or chemically induced DNA damage.

The ability of the HU protein to bend and to introduce compaction in a relaxed DNA molecule also suggests a role for HU in the compacted structure of the nucleoid. In bacterial cells, such as *E. coli*, this compacted structure is maintained through the interaction of DNA with a number of factors, including RNA and NAPs, such as HU, H-NS, and IHF [15,43,44]. In that regard, a deletion of one of these NAPs resulted in only minor phenotypical changes in the bacteria, which suggests overlapping of functions in these NAPs [45]. Although the *hup* gene

codes for the only histone-like protein described to date in *H. pylori*, the *hup::cat* mutant strain was successfully constructed, thus implying that *hup* is not an essential gene, as it has been reported to be in *B. subtilis* [46]. Viability of this *hup* mutant strain suggests the presence of other NAPs that remain unidentified in the *H. pylori* proteome. This *H. pylori hup* mutant strain showed impaired growth in the supplemented trypticase soy broth used in this study. This aspect was sorted out by using supplemented Brucella broth, which indicates that the *hup::cat* mutant strain also has different metabolic requirements for its proper growth.

In *E. coli*, the HU protein has been involved in the regulation of a specific set of genes related to growth and survival in acidic environments, anaerobiosis, high osmolarity, and SOS induction [14,23]. In the present study, we then assessed the expression of two genes related to the acid response in *H. pylori*, namely *ureA* and *speA*. Gene expression analysis of the *H. pylori* wild-type strain under acid stress condition showed the induction of the *ureA* gene. This was an expected result considering that this gene is an important part of the pH buffering mechanisms in *H. pylori* [5,6,47]. In this study, we also demonstrated the induction of the *speA* gene under acid stress, a phenotype that was not observed when the acid-sensitive *hup::cat* mutant strain was tested.

Several observations suggest that chromatin remodeling contributes to gene expression, which is thought to make regulatory regions accessible to the transcription machinery [48]. Also, it is known that a number of promoters require negative supercoiling to be activated for transcription [49]. Sangjin et al. [50] demonstrated that the interaction of the DNA with proteins induces allosteric changes in the double helix, distorting the length of the minor and major grooves of the DNA molecule. This structural change determines more or less accessibility of the RNA polymerase or transcription factors to the regulatory sequences. This implies that not only the linear distribution of genetic elements but also the spatial distribution of this elements throughout the genome and its relation with the compaction level of the DNA are important for gene regulation (like in bacterial operons). In *E. coli*, the histone-like protein HU is essential for the galactose operon regulation. In this case, HU protein mediates the formation of a loop in the DNA structure, allowing the GalR protein interaction with the two operator sites in this operon (O_E and O_I) [23]. The higher HU expression mediated by the acid stress in *H. pylori* could play a role in the remodeling of the bacterial chromosome, constraining negative supercoils [51] and allowing the access of the transcription machinery to the promoter regions of ureA, speA, and other genes involved in the acid stress response, leading to adaptation to the acid condition.

It was recently proposed that *H. pylori* has an acid resistance system that is similar to AR3 in *E. coli* [11]. In *E. coli*, the AR3 system is one of the three acid resistance mechanisms (named as AR1, AR2, and AR3) that contributes to regulating pH in the cytoplasm [13]. The pH-dependent induction of *speA* shown in this study complements recently reported data from our laboratory by providing new evidence that *speA* is homologous to the *adiA* gene in *E. coli*, even though the role of the SpeA protein in arginine decarboxylation as well as its contribution to the protection of this bacterium against acid stress remains to be elucidated.

In summary, we have demonstrated the ability of the *H. pylori* HU_{wt} protein to bind, bend, and compact the DNA molecule. The increased ability of HU to bind DNA under acid conditions, as well as protection against endonucleolytic cleavage and against damage by hydroxyl radicals, suggests an active role of this protein in the protection of DNA in an acidic environment. We also demonstrated that the *hup* mutant strain has an acid-sensitive phenotype and an altered expression profile of two genes related to an acid stress response, *ureA* and *speA*. All these results support the view that the HU protein is part of the acid stress response mechanism in *H. pylori* by protecting DNA and by facilitating the expression of some genes that are directly involved in the stress acid response.

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