S1 Supporting Information for Manuscript- Shelake et al

A novel mechanism of “metal gel-shift” by histidine-rich Ni\(^{2+}\)-binding Hpn protein from *Helicobacter pylori* strain SS1

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Material and Methods section

A. *H. pylori* strain and growth conditions

The mouse-adapted strain of *Helicobacter pylori* Sydney strain (*H. pylori* SS1) was used in this study (Lee *et al*. 1997). *H. pylori* SS1 was grown on Tripticase Soy agar with 5% sheep blood (TSA II) (Becton Dickinson, Franklin Lakes, NJ) for three days at 35ºC in 12% carbon dioxide condition. A single colony was isolated and sub-cultured on TSA II agar at 35ºC in 12% carbon dioxide condition.

B. Purification of DNA from *H. pylori* strain SS1

The genomic DNA from strain SS1 was extracted based on phenol-chloroform method as described previously (Lee *et al*. 1997) with minor modifications. Briefly, bacteria were harvested from TSA II agar and suspended in 3 ml of Tris-buffered saline (TBS). After washing with TBS once by centrifugation at 3,800 g for 5 min at 4ºC, 5 x 10^8 colony forming unit (CFU) of bacteria were re-suspended in 500 μl of lysis buffer (50 mM Tris-HCl, pH 8.0; 100 mM EDTA, pH 8.0; 1% SDS, 100 mM NaCl) containing 0.2 mg/ml proteinase K (Amaresco, Solon, OH) and incubated at 37ºC for 12 h. Subsequently, UltraPure™ buffer-saturated phenol (Invitrogen, Carlsbad, CA) was added, and the mixture was gently rotated for 15 min. After centrifugation, the aqueous phase was transferred to a tube containing chloroform/isoamyl alcohol (24:1, Sigma-Aldrich, St. Louis, MO) and agitated gently for 10 min. The aqueous phase was collected to a new tube by centrifugation. The DNA was precipitated by addition of isopropyl alcohol and 0.3 M sodium acetate, and then the DNA pellet was rinsed with 70% ethanol. After centrifugation, pellet was air-dried and dissolved in Tris-EDTA buffer (pH 8.0).

C. Enzyme-linked immunosorbent assay (ELISA) analysis

Green fluorescent protein with artificial His.tag (*gfp-His*<sub>6</sub>) and GFP fused with Hpn (*gfp-
Hpn) were cloned in pET21b. IPTG-induced over-expression of both the proteins was done with or without Ni\textsuperscript{2+} added in the culture (Fig S3-A and B). Pellets of 60 µl bacterial cultures were dissolved in 60 µl sample buffer and incubated for 3 min at 100°C. Final volume of 15 µl loaded in each lane for SDS-PAGE. ELISA experiment was performed using previously described protocol with some modifications (Miura et al. 2008). Different buffers were prepared before starting ELISA experiment (buffer components summarized in Table D).

Flat-bottom 96-well ELISA plates (untreated 96-well microplates from Falcon) were used for coating. Concentration of each protein (Hpn, GFP-Hpn and GFP-His\textsubscript{6}) was adjusted to 1 µg by dilution with coating buffer to the final volume of 50 µl. Plates were incubated at 4°C for overnight. Next day, solution was thrown away and 200 µl blocking buffer into each well was added. Then, plate was incubated at 37°C for 1hr. After incubation, solution was discarded and plate was washed three times by washing buffer. His.Tag\textsuperscript{®} antibody was diluted to standardized concentration (1:500) with dilution buffer [C-terminal specific-anti 6xhistidine monoclonal antibody (9F2) (Wako Japan, product code: 010-21861)] and plate incubated at 37°C for 1hr after adding 100 µl in each well. After three washes with wash buffer, plate was incubated with horseradish peroxidase-conjugated anti-mouse IgG (GE Healthcare, product code: NA931VS, diluted to 1:1000 in wash buffer) for 1 hr at 37°C. After similar washing, the substrate ABTS [2,2’-Azinobis(3-ethylbenzothiazoline-6-sulfonic Acid Ammonium Salt) from Wako Japan] dissolved in 0.1 M citrate buffer and hydrogen peroxide (0.03%) was added. Then the plate was incubated for 20 minutes at room temperature. Reactions were stopped by adding stop buffer (100 µl) in each well. The absorbance at 415 nm was measured using a Spectramax M3 microplate reader (Molecular Devices Co., Sunnyvale, CA). Values obtained (absorbance at 415 nm) for Ni\textsuperscript{2+}-treated samples (from average of at least three replications) were normalized against untreated samples and plotted in graph.
Result section

Metal-binding to Hpn changes Hpn-antibody interaction

Interaction of His.Tag® antibody with Hpn may not necessarily show similar results for other protein having artificial His.Tag or Hpn conjugated with another protein owing to differential co-ordination geometry of metal-binding and its chemical surrounding. We investigated this possibility using GFP- His$_6$ and GFP-Hpn (Fig S3-A and B). GFP does not interact with His.Tag® antibody by its own. GFP is comparatively large protein (26.7 kDa) but still positional shift was observed in case of GFP-Hpn expressed in LB medium supplied with Ni$^{2+}$. Western blot data of GFP-His$_6$ and GFP-Hpn showed almost equal intensity signals in both the cases i.e. with or without Ni$^{2+}$ (Fig S3-C).

Recognition site for His.Tag® antibody is His$_6$ peptide attached at C-terminal of a recombinant protein and it is a linear epitope. Hence, conformational change upon Ni$^{2+}$-binding to Hpn may lead to altered binding of His.Tag® antibody. This was further examined by ELISA using His.Tag® antibody (Fig S3-D). The SDS-treated and non-treated Hpn protein shown similar results which is compatible with previous observations for several other proteins (Lechtzier et al, 2002; Burgass et al, 2008). The relative detection sensitivity of Ni$^{2+}$-treated protein with His.Tag® antibody in ELISA give order of untagged Hpn < GFP-Hpn < GFP-His$_6$. Hence, the variability that we observed in detection of apo- and metalated-Hpn on western blots may have resulted not only from membrane-binding efficiency but also from differential exposure of His-rich region upon metal-binding.

These data signify that the metal-binding to Hpn causes altered binding of His.Tag® antibody, possibly due to change in protein confirmation.
Fig A. Comparison of DNA sequence of hpn from *Helicobacter pylori* strain SS1 (this study) with strain 26695 (NCBI data).

The promoter region and hpn gene of strain SS1 was PCR amplified and nucleotide sequence was compared with NCBI data of strain 26695 (GenBank accession number U26361). Putative promoter elements are shown in a box. The hpn gene region is shown in uppercase letters and mutations at the nucleotide level are shaded in gray. The putative terminator region of transcription is underlined. The promoter region was highly conserved in strain SS1 including Shine-Dalgarno sequence (GGAG) and promoter elements (-10 and -35).
**Fig B. Separation of purified Hpn on non-denaturing blue native-PAGE.**

High molecular weight marker (GE Healthcare), abbreviated as HMW, was used in all gels [Thyroglobulin (669 kDa), Ferritin (440 kDa), Catalase (232 kDa), Lactate dehydrogenase: (140 kDa) and Albumin (66 kDa)].

Recombinant Hpn (200 µM) was treated with either 1 mM of EDTA or Ni$^{2+}$ independently and then applied to 10% native-gel. Apparent multimeric complexes of >670, ~500, ~230 kDa were observed in presence or absence of Ni$^{2+}$ and EDTA.
Fig C. Western blot and ELISA analysis of Hpn, GFP-His₆ and GFP-Hpn.

A. Schematic diagram of gene construct consisted of gfp fused with artificial His.tag (left panel) and GFP-His6 protein expression confirmed by SDS-PAGE (right panel).

B. Schematic diagram of gfp fused with hpn at C-terminal followed by stop codon (left panel) and recombinant protein of GFP-Hpn fusion analyzed by SDS-PAGE (right panel).

D. Differential binding affinity of Anti-His antibody ELISA assay
C. Western blot of untagged Hpn, GFP-Hpn and GFP-His$_6$ on PVDF membrane either treated with or without Ni$^{2+}$ solution (1mM of NiSO$_4$).

D. ELISA of denatured untagged Hpn, GFP-Hpn and GFP-His$_6$ Equal amount of protein (1 µg) was coated on ELISA plate. His.Tag® antibody was diluted to 1:500 for detection. Values obtained (absorbance at 415 nm) for Ni$^{2+}$-treated samples (from average of at least three replications) were normalized against untreated samples and plotted in graph. Paired t-test was performed to compare the metal ion effect, ** indicate p<0.01.
Table A.
Proteins showing apparent SDS-resistant oligomeric forms upon denaturing SDS-PAGE

| Protein                                              | Oligomeric form                   | Remark                                                                                                   | Reference                      |
|------------------------------------------------------|-----------------------------------|-----------------------------------------------------------------------------------------------------------|--------------------------------|
| Na⁺/H⁺ exchanger protein 1                          | High-molecular-weight aggregate   | Aggregated when subjected to elevated temperature                                                        | Bullis et al. 2002              |
| Amyloid β (Aβ) protein                               | Dimer, trimer, tetramer           | Copper induced oligomerization on SDS-PAGE                                                                 | Atwood et al. 2000; 2004        |
| Inducible nitric oxide synthase (iNOS)               | Dimer                             | Human iNOS expressed in vivo only form undisruptable dimer                                                | Kolodziejski et al. 2003        |
| Acid phosphatase                                     | Tetramer                          | Retained in partially denatured conditions                                                               | Tham et al. 2010                |
| Bovine α-lactalbumin (α-LA), hen egg lysozyme (LYS) and human serum albumin (HSA) |
| Superoxide dismutase                                 | Several forms depending on Pt concentration | Oligomerization of proteins was promoted by Pt-protein adduct formation                                   | Pinato et al. 2013              |
| Hpn                                                  | Hetermeric form of ~125 kDa       | Retained a small fraction of a multimeric form along with monomer                                         | Nowakowski et al. 2014          |
|                                                      | High-molecular-aggregate          | Observed in elution buffer containing higher imidazole                                                   | Present study                   |
Table B.
List of proteins retaining metal ion on SDS-PAGE.

| Protein                                                                 | Detection method                                                                 | Metal ion retained | Remark                                                                 | Reference                      |
|------------------------------------------------------------------------|----------------------------------------------------------------------------------|--------------------|----------------------------------------------------------------------|--------------------------------|
| **A. Denaturing SDS-PAGE**                                              |                                                                                  |                    |                                                                      |                                |
| Sub-unit of proteins from a photochemical center                        | SDS-PAGE, Particle induced X-ray emission                                         | Fe                 | Direct detection in the gel bands.                                    | Solis et al. 1998              |
| Ca²⁺-dependent protein kinase from Soybean                              | SDS-PAGE                                                                         | Ca                 | Ca²⁺-dependent shift of electrophoretic mobility                     | Li et al. 1998                 |
| Ca²⁺-dependent protein kinase from Tobacco                             | SDS-PAGE                                                                         | Ca                 | Ca²⁺-dependent shift of electrophoretic mobility                     | Yoon et al. 1999               |
| AtCaM8 and AtCaM9 from Arabidopsis                                      | SDS-PAGE                                                                         | Ca                 | Ca²⁺-dependent electrophoretic mobility shifts                       | Köhler and Neuhaus, 2000       |
| Cisplatin-treated *E. coli* cells                                       | LA-ICP-MS, RP-ESI-Q-TOF                                                          | Pt                 | Identification of Pt-containing proteins                             | Allardyce et al. 2001          |
| Proteins from human liver cytosol                                       | Synchrotron radiation X-ray fluorescence                                           | Zn, Cu, Fe         | Gel was dried immediately after electrophoresis. Measured directly from bacterial cell extracts resolved on SDS-gel | Gao et al. 2003                |
| Cyanobacterial SmtA                                                     | LA-ICP-QMS                                                                        | S, Zn, Cd          | Zinc detected in CBB-stained protein band.                           | Binet et al. 2003              |
| Apoazurin                                                              | Synchrotron radiation X-ray fluorescence                                           | Zn                 |                                                                                                                                | Welsoeh et al. 2004            |
| Proteins from *in vitro* callus of Citrus                               | SDMS-PAGE with Synchrotron radiation X-ray fluorescence splinter X-ray fluorescence mapping with micro-XANES | Ca, Cu, Fe, K, Mg, Na and Zn | Microwave oven used for protein bands decomposition                     | Verbi et al. 2005              |
| Albumin-depleted bovine serum proteins                                  |                                                                                  | Cr                 | Chromium-protein interactions allowed better separation                | Finney et al. 2010             |
| Ca²⁺-dependent protein kinase from Arabidopsis                          | SDS-PAGE                                                                         | Ca                 | Ca²⁺-dependent shift of electrophoretic mobility                      | Romeis et al. 2011             |
| Bovine α-lactalbumin (α-LA), hen egg lysozyme (LYS) and human serum albumin (HSA) | SDS-PAGE                                                                         | Pt                 | SDS-resistant protein oligomers                                       | Pinato et al. 2013             |
| Superoxide dismutase                                                    | LA-ICP-MS                                                                        | Cu                 | Minor fraction retained                                               | Nowakowski et al. 2014         |
| Ferredoxin II in *Mesorrhizobium loti*                                  | SDS-PAGE                                                                         | Ca                 | Ca²⁺-dependent shift of electrophoretic mobility                     | Moscatiello et al. 2015        |
| Hpn                                                                    | SDS-PAGE, MALDI-TOF, ICP-OES                                                      | Ni                 | Ni²⁺-dependent shift of electrophoretic mobility                     | Present study                  |
| **B. Modified SDS-PAGE conditions**                                     |                                                                                  |                    |                                                                      |                                |
| Human brain proteins                                                    | MALDI-FT-ICR-MS and LA-ICPMS                                                     | Zn, Cu             | Second dimension IEF (SDS-PAGE)                                      | Becker et al. 2005             |
| Yeast mitochondrial ATPase                                              | LA-ICP-MS and MALDI-FT-ICR-MS                                                    | Fe, Zn, Cu         | Second dimension IEF (SDS-PAGE)                                      | Krause-Buchholz et al. 2006    |
| Superoxide dismutase, alcohol dehydrogenase                            | SDS-Tris-Tricine-PAGE-LA-ICP-MS                                                  | Cu, Zn             | Lower current for separation and without CBB-staining SDS-PAGE in the absence of BME or DTT | Jiménez et al. 2010            |
| Human serum albumin (HSA)                                               | OFFGEL isoelectric focusing and ICP-MS                                            | Pt                 | TCEP (Tris 2-carboxyethyl)                                           | Mena et al. 2011               |
| Protein fractions of a rat kidney cytosol treated with                  | nLC–ESI-LTQ-MS/MS                                                                | Pt                 |                                                                      | Mena et al. 2013               |
| oxaliplatin               | phosphine)-based SDS-PAGE |
|-------------------------|---------------------------|
| SOD, Yeast ADH, Bovine  |                            |
| AP, Carbonic anhydrase, β-| Lower SDS in buffer       |
| galactosidase           | (0.0375%) plus no         |
|                         | EDTA in buffer            | Nowakowski et al. 2014 |
| LA-ICP-MS, in-gel Zn-protein staining | Cu, Zn |
| Peptide                        | Metal | Matrix solution                                      | Combined method | Reference               |
|-------------------------------|-------|-----------------------------------------------------|-----------------|-------------------------|
| (GHHPH)$_2$G peptide          | Cu    | DHB                                                 | TOF-MS          | Hutchens et al. 1991   |
| Human glycoprotein            | Cu    | DHB in 0.1% aqueous TFA                             | TOF-MS          | Nelson and Hutchens    |
|                               |       |                                                     |                 | 1992                   |
| Zinc finger peptides          | Zn    | HCCA in 1:1 ammonium bicarbonate (1M)-ethanol.      | -               | Woods et al. 1995      |
| Prion protein                 | Cu    | DHB and 6,7-dihydroxycoumarin                       | TOF-MS          | Hornshaw et al. 1995   |
| Ferrichrome                   | Fe    | DHB in methanol                                     | TOF-MS          | Kaltashov et al. 1997  |
| Luteinizing hormone releasing | Ni, Cu, Zn | Paranitroaniline in ethanol (10 mg/mL)            | Fourier Transform-MS | Masselon et al. 1999 |
| hormone                                                                 |                                                |                 |                        |
| Zinc finger peptide           | Zn    | 6-aza-2-thiothymine or DHB in Tris (10 mM), ammonium bicarbonate (20 mM) or 0.1% TFA | TOF-MS          | Lehmann et al. 1999    |
| Bradykinin                    | Cu, Ag, Co, Ni, Zn | HCCA matrix saturated in water and acetonitrile (70:30 v/v) containing 0.1% TFA. | TOF-MS          | Cerda et al. 1999      |
| Prion proteins                | Cu    | Sinapinic acid in 20% acetonitrile                  | TOF-MS          | Qin et al. 2002        |
| Human brain proteins          | Cu, Zn | DHB in acetonitrile and 0.1% TFA in water (2:1)    | FT-ICR-MS       | Becker et al. 2005     |
| Human tau proteins            | Cu, Zn | DHB in acetonitrile and 0.1% TFA in water (2:1)    | FT-ICR-MS       | Susanne Becker et al.  |
|                               |       |                                                     |                 | 2007                   |
| Human brain proteins          | P, Cu, Zn, Fe | DHB in acetonitrile: 0.1% TFA in water (2:1)      | FT-ICR-MS       | Becker et al. 2007     |
| Angiotensin I                 | Cu, K | HCCA, 3-aminoquinoline and glycerol                 | TOF-MS          | Hortal et al. 2008     |
| Rat tissues                   | Zn, Cu, Fe, Cr, Cd, Pb | HCCA in acetonitrile:0.1% TFA in water (2:1)  | TOF-MS and LA-ICP-MS | Becker et al. 2008 |
| Compound                  | Metal | Solvent Details                                    | Detection Method | References          |
|--------------------------|-------|---------------------------------------------------|------------------|---------------------|
| a-Crystallin Zn           |       | HCCA in 1:2 acetonitrile and 0.1% TFA             | TOF-MS           | Karmakar and Das 2012 |
| Protein fraction from     | Cu    | HCCA in 50% acetonitrile and 0.1% TFA             | TOF-MS           | Jayasinghe and Caruso 2013 |
| Brazil nuts               |       |                                                   |                  |                     |
| Hpn Ni                   |       | Sinapinic acid (100% ACN, 0.01% TFA, and distilled water; v:v, 50:10:40) | TOF-MS           | Present study       |

(DHB: 2,5-dihydroxybenzoic acid; HCCA: α-cyano-4-hydroxycinnamic acid; FT-ICR-MS: Fourier transform-ion cyclotron resonance-mass spectrometry; LA-ICP-MS: Laser ablation-inductively coupled plasma-mass spectrometry)
| Buffer type     | Components                                                                 |
|----------------|-----------------------------------------------------------------------------|
| Coating buffer | Phosphate buffer saline, [1.16 g Na$_2$HPO$_4$, 0.1 g KCl, 0.1 g K$_3$PO$_4$, 4.0 g NaCl (500 ml distilled water) pH 7.4] |
| Dilution buffer| 0.1% BSA with 0.05% Tween-20 in PBS                                          |
| Blocking buffer| 1% BSA with 0.05% Tween-20 in PBS                                            |
| Washing buffer | 0.05% Tween-20 in PBS                                                        |
| Stop buffer    | 5N Sodium Hydroxide in distilled water                                       |
Annexure A.

Protocol followed for analysis of apparent MW of Hpn on SDS-PAGE in Fig. 5

(Important commands are mentioned in bracket)

1. Picture file opened in ImageJ software (File – open or drag to icon).
2. Brightness/contrast was adjusted for easy comparison (Image - Adjust-) and picture was Inverted (Edit - Invert).

3. Shape for selection was chosen as straight line (width 30) by double click on the icon.
4. Measurement parameters were selected (Analyze – Set Measurements – Select parameters – click add to overlay and length).
5. Measurements were done by selecting area from top of the gel till the specific protein separated on gel (Analyze – Measure).

6. All values saved to excel file.
7. Apparent MW on SDS-gel was calculated on the basis of known theoretical MW for two marker proteins - lysozyme (14.4 kDa) and trypsin inhibitor (21.5 kDa). Formulae used as follows -

A. Difference in migration distance (DMD) compare to (14.4 kDa) marker protein = MD from top of gel by Lysozyme (14.4 kDa) – MD from top of gel of respective Hpn band.

B. MW difference compare to 14.4 kDa band

\[
= \frac{(DMD \text{ compare to 14.4 kDa for Hpn band}) \times 7.1}{(DMD \text{ compare to 14.4 kDa for 21.5 kDa band})}
\]

Factor 7.1 is the MW difference between two marker proteins i.e. lysozyme and trypsin inhibitor (21.5–14.4=7.1)

C. Apparent MW (kDa) = 14.4 kDa + MW Difference for (EDTA or Ni) Hpn (kDa)

For example, for apparent MW for Ni^{2+}-Hpn on 12.5% gel = 14.4 + 0.70 = 15.1 kDa

| Gel % | Protein band on SDS-gel | Distance from top of gel | DMD compare to 14.4 kDa band | MW difference compare to 14.4 kDa band | Apparent MW (kDa) |
|-------|------------------------|-------------------------|-------------------------------|----------------------------------------|-------------------|
| 15.0% | 14.4 kDa               | 411                     | 86                           | 7.10                                   | 14.40             |
| 15.0% | 21.5 kDa               | 325                     | 4                            | 0.33                                   | 14.73             |
| 15.0% | Apo-Hpn                | 407                     | 4                            | 0.33                                   | 14.73             |
| 15.0% | EDTA-Hpn               | 407                     | 4                            | 0.33                                   | 14.73             |
| 15.0% | Ni^{2+}-Hpn            | 449                     | -38                          | -3.14                                  | 11.26             |
| 18.0% | 14.4 kDa               | 332                     | 73                           | 7.10                                   | 14.40             |
| 18.0% | 21.5 kDa               | 259                     | -28                          | -2.72                                  | 11.68             |
| 18.0% | Apo-Hpn                | 360                     | -28                          | -2.72                                  | 11.68             |
| 18.0% | EDTA-Hpn               | 360                     | -28                          | -2.72                                  | 11.68             |
| 18.0% | Ni^{2+}-Hpn            | 396                     | -64                          | -6.22                                  | 8.18              |
| 20.0% | 14.4 kDa               | 300                     | 76                           | 7.10                                   | 14.40             |
| 20.0% | 21.5 kDa               | 224                     | -50                          | -4.67                                  | 9.73              |
| 20.0% | Apo-Hpn                | 350                     | -50                          | -4.67                                  | 9.73              |
| 20.0% | EDTA-Hpn               | 350                     | -50                          | -4.67                                  | 9.73              |
| 20.0% | Ni^{2+}-Hpn            | 386                     | -86                          | -8.03                                  | 6.37              |
| 22.5% | 14.4 kDa               | 235                     | 61                           | 7.10                                   | 14.40             |
| 22.5% | 21.5 kDa               | 174                     | -82                          | -9.54                                  | 4.86              |
| 22.5% | Apo-Hpn                | 317                     | -82                          | -9.54                                  | 4.86              |
| 22.5% | EDTA-Hpn               | 317                     | -82                          | -9.54                                  | 4.86              |
| 22.5% | Ni^{2+}-Hpn            | 352                     | -117                         | -13.62                                 | 0.78              |
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