Dynamic HypA zinc site is essential for acid viability and proper urease maturation in *Helicobacter pylori*

Ryan C. Johnson[a], **Heidi Q. Hu**[b], **D. Scott Merrell**[a,*], and **Michael J. Maroney**[b,*]

[a]Microbiology and Immunology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Rd., Bethesda, MD 20814, USA

[b]Department of Chemistry and Program in Molecular and Cellular Biology, University of Massachusetts Amherst, 240 Thatcher Rd. Life Sciences Laboratory N373, Amherst, MA 01003, USA

Abstract

Helicobacter pylori requires urease activity in order to survive in the acid environment of the human stomach. Urease is regulated in part by nickelation, a process that requires the HypA protein, which is a putative nickel metallochaperone that is generally associated with hydrogenase maturation. However, in *H. pylori*, HypA plays a dual role. In addition to an N-terminal nickel binding site, HypA proteins also contain a structural zinc site that is coordinated by two rigorously conserved CXXC sequences, which in *H. pylori* are flanked by His residues. These structural Zn sites are known to be dynamic, converting from $Zn(Cys)_4$ centers at pH 7.2 to $Zn(Cys)_2(His)_2$ centers at pH 6.3 in the presence of Ni(II) ions. In this study, mutant strains of *H. pylori* that express zinc site variants of the HypA protein are used to show that the structural changes in the zinc site are important for the acid viability of the bacterium, and that a reduction in acid viability in these variants can be traced in large measure to deficient urease activity. This in turn leads to a model that connects the $Zn(Cys)_4$ coordination to urease maturation.

Introduction

Helicobacter pylori (H. pylori) is a Gram negative bacterium that colonizes the human gastric mucosa[1]. Infection causes chronic inflammation and is a major risk factor for development of peptic ulcers and cancer[2]. *H. pylori* is a prevalent pathogen that colonizes approximately one-third to one-half of the worldwide adult population[3]. Successful colonization of the acidic environment of the stomach requires the activity of urease[4], a nickel-dependent enzyme that catalyzes the hydrolysis of urea to ammonia and carbon dioxide[5]. Urease is highly expressed in *H. pylori*, representing up to 10% of the total

protein.[6] However, the enzyme remains inactive without maturation through nickel insertion into the active site.[5]

H. pylori urease maturation is dependent on accessory proteins UreIEFGH.[5] Additionally, HypA and HypB, accessory proteins normally involved in the maturation of NiFe-hydrogenases, also aid in urease maturation; deletion of *hypA* or *hypB* results in urease deficiency in *H. pylori* despite the presence of the full *ureIEFGH* cascade.[7] Urease deficient phenotypes in *hypAB* deletion mutants can be compensated for by addition of Ni(II) to the media, implicating the role of HypA and HypB in nickel delivery to the urease maturation pathway.[7a, 7c] The traditionally hydrogenase-specific nickel metallochaperone, HypA, has been shown to interact directly with a urease-specific nickel metallochaperone, UreE, *in vitro*, providing a possible link for the role of HypA in urease maturation.[7c] Several studies have interrogated the interaction between HypA and UreE. Biophysical characterizations show that HypA can outcompete UreG for interaction with UreE.[8] Additionally, nickel transfer from HypA to UreE has been shown to involve the interaction of the HypA nickel-binding domain and the UreE nickel-binding C-terminus.[9]

As shown in Fig.1, the overall protein structure of HypA has been shown by nuclear magnetic resonance (NMR) to have a distinct nickel-binding domain and structural zinc-binding domain [PDB: 2KDX].[10] In addition to the two conserved CXXC motifs in the HypA structural zinc site, the *H. pylori* zinc binding motifs also have His residues closely flanking the conserved motifs. The structural zinc site has also been extensively characterized by X-ray absorption spectroscopy (XAS), demonstrating that the WT HypA protein structural zinc site is dynamic.[11] The average Zn coordination at pH 7.2 is $Zn(Cys)_4$, which changes to $Zn(Cys)_2(His)_2$ at pH 6.3 (the estimated internal pH of *H. pylori* under acid shock conditions) with nickel bound in the nickel site.[11] The dynamic nature of the HypA zinc site is lost when any one of the four cysteine residues in the CXXC motif is mutated to alanine or aspartate, and results in locking of the zinc coordination in the Zn-Cys_2His_2 structure (acidic conformation).[11b] The dynamic nature of the HypA zinc site is also lost when either of the histidine residues flanking the CXXC motifs is mutated to alanine; this change results in locking of the zinc coordination in the Zn-Cys_4 structure (neutral conformation).[11b] These structure-locking mutants of HypA are ideal for interrogation of the role of the HypA zinc site and corresponding protein conformations in urease maturation and acid resistance in *H. pylori*, and are the focus of this study. Herein we create a series of isogenic *H. pylori* strains that each express zinc site mutant *hypA* variants under the control of the endogenous promoter, and then utilize these mutants to define the contribution of the conserved HypA motifs to *H. pylori* acid viability and urease activity. These studies connect the point mutations in the structural zinc site of a nickel metallochaperone, HypA, to changes in acid viability and urease maturation *in vivo*.

Materials and methods

Bacterial growth conditions

All *H. pylori* strains were maintained at −80°C in brain heart infusion broth (Becton Dickinson) supplemented with 10% fetal bovine serum (FBS) and 20% glycerol and were cultivated on horse blood agar (HBA) medium containing 4% columbia agar base (Neogen

Corp), 5% defibrinated horse blood (HemoStat Laboratories, Dixon, CA), 0.2% β-cyclodextrin (Sigma), 10 μg/mL of vancomycin (Amresco), 2.5 U/mL of polymyxin B (Sigma), 5 μg/mL of trimethoprim (Sigma), and 5 μg/mL of amphotericin B (Amresco). Where required, 5% sucrose was added to HBA for selection of sucrose sensitive strains. Liquid growth of *H. pylori* was performed in brucella broth (Neogen Corp) with 10% FBS and 10μg/mL of vancomycin. All *H. pylori* cultures were grown under microaerobic conditions (5% O_2, 10% CO_2, and 85% N_2) at 37°C with 100rpm shaking for liquid cultures. *H. pylori* strain G27 was used for all experiments[12]. *Escherichia coli* Top10 cells were either grown on LB agar or in LB liquid medium with shaking at 225rpm. Kanamycin (25μg/mL) and ampicillin (100μg/mL) were used for bacterial selection.

hypA mutant construction

H. pylori G27 genomic DNA was used as the template for mutant construct development. A *hypA* mutant strain containing a *kan-sacB* cassette insertion was constructed as follows. The HypA_Up_F and HypA_Up_R primers (Table 1) were used to amplify a 556 base pair segment of DNA that spanned 398 base pairs upstream of *hypA* (HPG27_832) and 158 base pairs into the *hypA* coding region. Additionally, the HypA_Dn_F and HypA_Dn_R primers (Table 1) were used to amplify a 532 base pair region that began at nucleotide position 160 of *hypA* and spanned 337 base pairs downstream of the *hypA* stop codon. The HypA_Up_R and HypA_Dn_F primers were designed to contain identical flanking sequences, which harbored XhoI and XbaI restriction enzyme sites. Thus, the upstream and downstream amplicons were next fused together utilizing splicing by overlap extension (SOE) PCR[13]. The resulting spliced product was cloned into the pGEM-T easy vector (Promega) and transformed into *E. coli* Top10 cells. The *kan-sacB* cassette[14], which confers resistance to kanamycin and sensitivity to sucrose, was amplified from pKSF-II[14] using primers Kan_SacB_F and Kan_SacB_R, which were designed to incorporate a XhoI and XbaI restriction site, respectively. The resulting fragment was digested with XhoI and XbaI and was then inserted between the spliced *hypA* fragments that had been similarly digested. The resulting construct was then transformed into *H. pylori* G27 and transformants were selected on HBA plates containing kanamycin. Successful insertion of the *kan-sacB* cassette into the *hypA* gene was confirmed by PCR and sequencing using the HypA_Confirm_F/R and HypA_seq_F/R primers, respectively. The resulting mutant strain was named DSM1283.

Site-directed mutagenesis of *hypA* Zn-binding site

Mutations of the cysteines in the HypA CXXC motifs (Cys71, Cys74, Cys91 and Cys94) to alanine or aspartic acid and the flanking histidines (His79 and His95) to alanine were constructed by polymerase chain reaction (PCR) using the PJI110 plasmid (wild type *hypA* sequence carried on the pET-22b(+) vector, Table 1) as a template.[11b] The PJI110 plasmid was transformed into NovaBlue (Novagen) competent cells and then re-isolated using the Axyprep Plasmid MiniPrep Kit (Axygen) and used as the DNA template in all subsequent PCR reactions. PCR primers were designed to incorporate the desired mutations and are listed in Table 1. Reactions were carried out in 50μL volumes using 1ng of template DNA and 2.5ng or 2μM of each primer per reaction. Successful PCR amplifications were confirmed by 0.8% agarose gel electrophoresis, and then methylated PJI110 template was digested with 20 units of DpnI (New England Biolabs) for 1 hour at 37°C. The resulting

PCR mixture was transformed into NovaBlue competent cells. Single colonies were selected and grown to saturation in 5mL liquid cultures of LB-Miller (Fisher Scientific) media supplemented with ampicillin at 37°C. Cells were pelleted by centrifugation at 6,000 g for 5 minutes and plasmids were isolated using the Axyprep Plasmid MiniPrep Kit. Successful mutations were confirmed by plasmid sequencing (GENEWIZ, Inc.).

We utilized the wild-type or mutant HypA pET-22b(+) plasmids, which each contained the entire HypA coding region, to move the mutant constructs of interest into the *H. pylori* chromosome. Each of the constructs was individually transformed into DSM1283, and double crossover events in which the *kan-sacB* cassette was replaced by the mutagenized *hypA* gene carried on the pET-22b(+) vector were selected for based on sucrose resistance. Sucrose resistant transformants were screened for kanamycin sensitivity and proper integration of the *hypA* construct was confirmed by PCR and sequencing using the HypA_Confirm_F/R and HypA_seq_F/R primers, respectively. In total, *H. pylori* mutant strains were made that contained the following *hypA* mutations: C74A (DSM1296), C74D (DSM1301), C77A (DSM1363), C77D (DSM1364), H79A (DSM1365), C91A (DSM1298), C91D (DSM1299), C94A (DSM1366), C94D (DSM1297), and H95A (DSM1300). In addition, a *hypA*-restorant (*hypA*-R; DSM1295) in which the *kan-sacB* cassette was replaced by the wild type *hypA* gene was also created in order to control for any defects that may have resulted due to genetic manipulation.

Acid resistance testing

Each of the ten *hypA* Zn-site *H. pylori* mutants were tested for acid resistance. Additionally, the wild type *H. pylori* strain, the *hypA::kan-sacB* mutant, and the *hypA* restorant were included as controls. Furthermore, a urease-deficient mutant of *H. pylori* (DSM43) in which the kanamycin resistance cassette replaced the *ureB* subunit was used as a positive control for acid sensitivity; DSM43 was created by transforming wild type *H. pylori* with vector pEJ22 as previously described.[15] Assays were conducted as follows: 20mL liquid cultures of *H. pylori* were inoculated to an optical density (600nm) of 0.05 from overnight liquid grown bacterial cells and then allowed to grow for approximately 19 hours. 1mL aliquots were removed from the culture and pelleted by centrifugation. The supernatants were removed and the bacterial pellet was re-suspended in 1mL of phosphate buffered saline (PBS) at pH 6 or 2.3, with or without supplementation with 5mM urea. Immediately after the bacterial pellets were re-suspended, an aliquot was removed, serially diluted in brucella broth and plated on HBA plates to determine colony forming units (CFU) per milliliter. The cultures were then incubated in 1.5mL capped tubes for 1 hour at 37°C. At this point, a second aliquot was removed from the cultures, immediately serially diluted, and plated to determine CFU/mL as described above. Percent survival after 1-hour incubation in the various PBS solutions was determined for each bacterial strain. At least three biological replicates were performed for each strain.

Urease activity assay

Urease activities were determined for each of the ten *hypA* Zn-site variants, the wild type *H. pylori* strain, the *hypA::kan-sacB* mutant, and the *hypA* restorant strain. For each strain, 8mL liquid cultures of *H. pylori* were inoculated to an optical density (600nm) of 0.05 from

overnight liquid grown bacterial cells and then allowed to grow for approximately 22 hours. At that point, 1mL aliquots were removed from the culture and pelleted by centrifugation (~10^8 cells). The supernatants were removed and the bacterial pellets were stored at $-20°C$ until ready for urease assays. The frozen cells were thawed and then re-suspended in 750μL of ice-cold HEPES buffer (pH 7.0), 1mM phenylmethanesulfonyl fluoride (PMSF) (MP Biomedicals, LLC), and 1× protease inhibitor cocktail (Sigma-Aldrich) and then lysed by sonication at 70% power for 6 pulses (2-second each) on ice. Lysate was centrifuged at 15,000-g for 10 minutes to remove insoluble fractions from soluble whole cell extracts. Soluble whole cell extracts were kept at 4°C for up to one month and insoluble fractions were stored at $-20°C$. Total protein concentration in soluble whole cell extract was assessed by Bradford Assay using the Coomassie Protein Assay Kit (Thermo Scientific).

Urease activities for each strain were determined using a modified phenol-hypochlorite method to assay the amount of ammonia released in the soluble whole cell extract of *H. pylori* lysate in the presence of urea[16]. For each strain, 5μL of whole cell extract was added to 245μL of urease reaction buffer (50mM HEPES, 25mM Urea, pH 7.0), and incubated at 37°C for 20 minutes to allow for ammonia production. The reaction was quenched with the sequential addition of 375μL of phenol-hypochlorite buffer A (100mM phenol, 167.8μM sodium nitroprusside) and then the addition of 375μL of phenol-hypochlorite buffer B (125mM NaOH, 0.044% NaClO); samples were mixed with quick vortexing after the addition of each buffer. The assay mixture was incubated at 37°C for 30 minutes to allow for color development (the conversion of ammonia to indophenol) and the absorbance was evaluated at 625nm. Assays were performed alongside a standard curve created using known amounts of ammonium chloride (0.24 – 500nmol) in place of whole cell extract. The urease activity of ureB strain was set as background and subtracted from the activity of all other strains. Urease activity for the various mutants was normalized to the *hypA*-restorant (*hypA*-R; DSM1295) *H. pylori* as 100%. All experiments were performed in triplicate with two independently grown cultures.

Results

HypA Zn-binding sites are important for acid survival

To determine whether changes in the zinc binding sites of HypA affected acid resistance of *H. pylori*, we created strains of *H. pylori* in which the Cys residues found in the two CXXC motifs associated with zinc binding (Cys74, Cys77, Cys91 and Cys94) and the two His residues (His79 and His95) that flank these two CXXC motifs were mutated. Cys residues were changed to Asp and Ala, while His residues were changed to Ala. We then utilized these 10 HypA mutant strains in combination with the wild type strain, a *hypA* mutant (*hypA::kan-sacB*), a *hypA*-restorant (*hypA*-R) and the *ureB* mutant (ureB) control strains to assess acid resistance. All 14 of these *H. pylori* strains showed similar robust survival profiles when exposed to pH 6 in the presence or absence of 5mM urea (Fig. 2A, B). Conversely, when exposed to pH 2.3, all of the *H. pylori* strains, including the wild type, showed a dramatic decrease in viability; less than 0.01% of the inoculum survived at pH 2.3 in the absence of urea (Fig. 2C). However, when urea was supplemented to the pH 2.3 buffer, we began to detect differences in acid resistance across the various strains (Fig. 2D).

As expected, the wild type *H. pylori* strain and the *hypA*-restorant were able to efficiently utilize the supplemented urea as a substrate for the urease system and survive the acidic challenge. Also as expected, the urease deficient *ureB* strain was incredibly acid sensitive; no surviving bacteria were detected (limit of detection 500 CFU/mL equating to 0.0001% survival). The *hypA* strain that carried an insertion in the *hypA* coding sequence was also deficient in its ability to resist acidic stress (< 3% survival). This result confirmed previous studies that indicated that HypA is necessary for efficient urease activity[7]. For the various Zn-binding site mutants, mutation of Cys77, His79, and His95 of HypA resulted in no changes in acid resistance. Conversely, significant decreases in acid resistance, as compared to the wild type strain, were observed for the C74D, C91A, and C94A mutant strains: 17.6%, 2.1% and 1.7% average survival, respectively. This finding suggests that these zinc-binding site residues play a critical role in acid viability, presumably by affecting the ability of HypA to provide nickel to the urease maturation pathway.

Mutation to HypA Zn-binding sites impacts urease activity

The acid survival data suggested that the C74, C91 and C94 residues play a critical role in the ability of HypA to facilitate urease maturation.[7] We next directly investigated urease activity in each of the ten Zn-binding site mutants of *hypA*, as well as in the *ureB*, the *hypA::kan-sacB*, the *hypA*-restorant and the wild type strains. The *hypA*-restorant showed three-fold less urease activity than wild type *H. pylori*, which may be a consequence of genetic manipulation. Consequently, urease activities were normalized to the *hypA*-restorant strain. Since the *ureB* strain of *H. pylori* is missing the nickel-containing β subunit of the urease enzyme and should have no urease activity, the average urease activity of the *ureB* strain was subtracted from measurements of all strains to correct for any background levels of ammonia present in whole cell extract.

As shown in Fig. 3, we identified two classes of mutations of the HypA structural zinc site, those that had no effect on urease activity (WT-like), and those that decreased urease activity. This latter class can be subdivided into moderately deficient and severely deficient (*hypA::kan*-sacB-like) subclasses. Mutations of His residues (H79A and H95A) flanking the HypA CXXC motifs resulted in strains that retained WT-like activity, suggesting that these His residues are not important for urease maturation. In contrast, all of the mutations involving Cys residues in the HypA CXXC motifs were deficient in urease activity to some extent (less than 15% compared with the *hypA*-restorant strain), indicating the importance of the Cys residues in the proper function of HypA in the urease maturation pathway. Comparisons of the urease activities in the Cys mutant strains with the *hypA* stain revealed more subtle phenotypes. The activity of C77D, C91D, and C94D were statistically different ($p < 0.05$) from the *hypA::kan*-sacB strain (12 – 13% compared with the *hypA*-restorant strain) constituting a moderately deficient phenotype (Fig. 3). The remaining Cys mutants, C74A, C74D, C77A, C91A, and C94A, showed activity levels similar to the *hypA* strain (> 4% compared with *hypA*-restorant strain). These urease activity results corroborate the acid survival phenotypes, where C74D, C91A, and C94A mutants were found to be both acid-sensitive and severely deficient in urease activity. Contrary to the acid survival results, C74A and C77A mutants were not acid sensitive under our test conditions, but were found to be severely deficient in urease activity. These findings suggest that, although all of the

Cys residues in the CXXC motifs are important for proper HypA function in the urease maturation pathway, there is a position-dependent gradient of importance based on the residue position as well as the type of mutation.

Discussion

The *H. pylori* HypA protein is a putative nickel metallochaperone that is involved in both hydrogenase maturation (as are all HypA proteins) and urease maturation, a function that is unique to *H. pylori.* NMR structural studies of a monomeric construct of *H. pylori* HypA revealed that it contains a structural zinc site associated with two rigorously conserved CXXC motifs in addition to a nickel-binding site associated with the N-terminus.[10] The amino acid sequence of *H. pylori* reveals that the zinc-binding CXXC motifs are unique among other HypA proteins in that each is flanked by a His residue.[11b] The structural Zn-sites of HypA were previously shown to change coordination in a pH and nickel-dependent manner, adopting a $Zn(Cys)_4$ conformation at neutral pH (7.2), and a $Zn(Cys)_2(His)_2$ conformation at more acidic pH (6.3) (Fig. 1) in the presence of Ni(II).[11] These two structures were correlated with other physical properties of dimeric HypA, including nickel binding stoichiometry and thermal stability, with the lower pH form associated with greater thermal stability.[11b] Mutagenesis studies revealed that altering any Cys residue in the two CXXC motifs (to Ala or Asp) resulted in adoption of the acidic conformation $(Zn(Cys)_2(His_2))$, and any mutation of the flanking His residues resulted in the neutral conformation $(Zn(Cys)_4)$, in the presence of Ni(II) at both pH values, as determined by EXAFS analysis of data collected at ~ 50K.[11b] These pH-insensitive and structure-locking mutants of HypA were important tools employed in the present study to probe the role of HypA in urease maturation and acid viability *in vivo*.

In vivo urease activities of *H. pylori hypA* Zn site variant strains correlate well with previous *in vitro* studies employing addition of purified HypA proteins to whole cell extract from a *hypA* mutant of *H. pylori*.[11b] Results of the *in vitro* studies show a general decrease in urease activity upon addition of the zinc site variants relative to addition of wild type HypA protein, and a generally greater effect associated with Cys mutants as compared to His mutants.[11b] The *in vivo* studies presented here reveal two distinct phenotypes: WT-like urease activity, represented by His mutants, and urease-deficient strains associated with Cys-mutants. These two classes of mutants correspond to the $Zn(Cys)_4$ or $Zn(Cys)_2(His)_2$ conformations, respectively. Our data suggest that the all the Cys residues in the HypA Zn site are important in the maturation of urease in *H. pylori*, a result that is consistent with a model in which the conformation associated with $Zn(Cys)_2(His)_2$ sites, or in which access to the $Zn(Cys)_4$ structure is inhibited in a dynamic sense, leads to lower levels of urease activation. Among the Cys mutations, there were two levels of urease activity observed, a moderate decrease associated with C77D, C91D, and C94D, all Cys→ Asp mutations, and a severely deficient level associated primarily with Cys→ Ala mutations, which have activity levels similar to the *hypA::kan*-sacB strain (C74A, C74D, C77A, C91A, and C94A). With the exception of C74D, Cys→ Asp mutations give rise to more active variants than Cys→ Ala mutations, suggesting that at least part of the difference lies in coordination of the CXXC motifs. A Cys→ Ala mutation results in a loss of a ligand in the CXXC motifs, while a Cys→ Asp mutation results in the potential substitution of a Cys thiolate ligand by a

carboxylate ligand. Thus, the Cys→ Asp substitution provides a mechanism to access a $Zn(Cys)_3Asp$ site that favors the wild type-like $Zn(Cys)_4$ conformation, which appears to preserve greater HypA function in moderately affected mutants-- C77D, C91D, and C94D, but not in C74D (*vide infra*).

A similar trend can be discerned in the study of acid viability: His mutations have no effect, whereas C91A and C94A mutations are associated with low acid survival (comparable to the *hypA::kan*-sacB strain), and C91D and C94D have wild type survival under the assay conditions employed. Interestingly, mutations in the more N-terminal CXXC motif lead to strains that are significantly less acid sensitive. Taken together with the urease activity assays, this suggests that the ability of HypA to deliver Ni to the urease maturation pathway may lie with the dynamic nature of the structural zinc sites. While no clear reason is apparent for the functional differences observed for the two CXXC motifs, it is noteworthy that two Cys residues are substituted by His at low pH, whereas two are retained and anchor the Zn site. This pair-wise difference in function is one possible explanation for the differences between the more N- and C- terminal CXXC sequences. Further elucidation of this difference awaits structural studies of the low pH form of HypA.

It is clear from both the urease assay data and acid viability data that Cys74 is a special position among the Cys variants. It is the only position where the Cys→ Asp mutation results in a less acid viable strain than the Cys→ Ala mutation, and the only position where urease activity is not higher for the Asp variant than for the Ala variant. Examination of the NMR structure (Fig 1) shows that the Zn site is formed by a loop that begins with Cys74 and ends with Cys94. However, the terminal Cys residues in the loop differ in that Cys94 is flanked by a His residue and Cys74 is not. In the HypA dimer crystal structure from *Thermococcus kodakarensis KOD1* (PDB ID: 3A44),[17] Cys74 and 94 have a similar relationship, though these residues are now derived from different monomers in the strand-swap dimer.[17] The lack of an alternative ligand could make Cys74 a more vulnerable position, particularly if it plays an anchoring role, the loss of which may result in a more severe phenotype due to destabilization of the entire structural zinc site.

HypA and HypB are accessory proteins that are generally associated with hydrogenase maturation, but in *H. pylori* have been shown to be important for the maturation of both NiFe-hydrogenase[7a, b] and urease[7]. In addition to interacting with HypB[7b, 18], HypA has also been shown to interact directly with the urease-specific Ni metallochaperone, UreE[7c, 8–9]. Interaction of HypA with both HypB[18] and UreE[9] has been demonstrated to involve the N-terminal Ni-binding domain of HypA and Ni-transfer between proteins. In this study we demonstrated that altering the HypA structural zinc site can also severely affect acid viability and urease maturation *in vivo*. These findings suggest that protein structural dynamics, reflected in the HypA zinc site structure, may result in subtle alterations of the overall HypA fold and differential interactions between HypA and partnering proteins, ultimately leading to a gradient in urease and hydrogenase enzymatic activities that is a function of cellular pH and nickel availability. Since mutations of the His residues do not lead to a large effect on urease activity, it is possible that the conformation associated with $Zn(Cys)_2(His)_2$ coordination is involved in supporting Ni trafficking to hydrogenase under acidic conditions. This possibility is the focus of ongoing studies. In this regard, it is worth

noting that the stoichiometry of Ni binding associated with $Zn(Cys)_4$ coordination is two per protein, while $Zn(Cys)_2(His)_2$ coordination supports binding of only a single Ni ion,[11b] and that the active sites of urease and hydrogenase require incorporation of two and one Ni ion, respectively.

Acknowledgments

Notes and references

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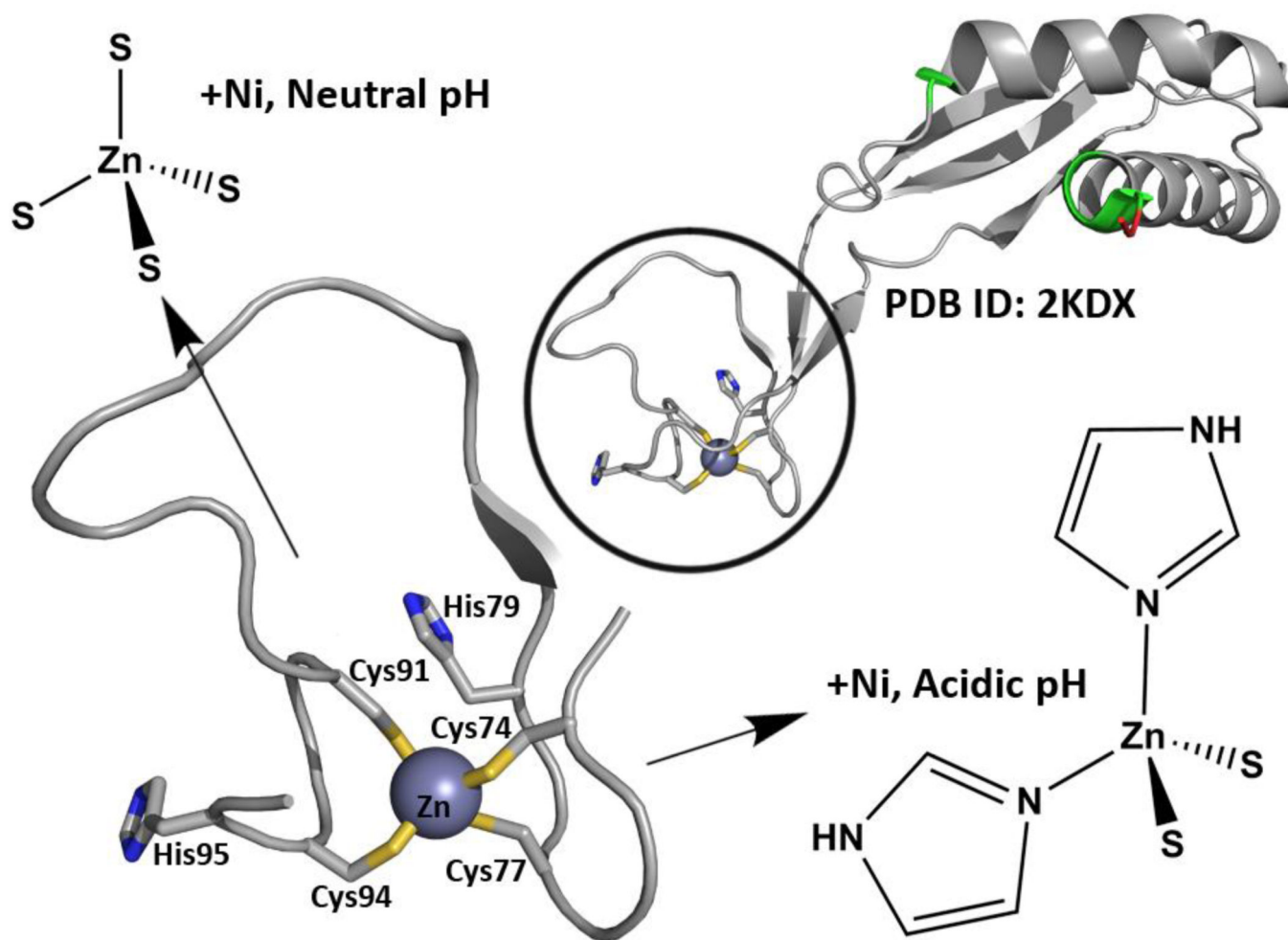
Fig. 1.
The structure and dynamics of the HypA zinc site have been characterized. The NMR structure of HypA[10] (PDB ID: 2KDX) shows distinct Ni- and Zn-binding domains, where known Ni-binding residues are colored in green (non-native residues leftover from affinity-tag processing shown in red). Close-up look of the Zn site reveals two CXXC motifs with flanking His residues (Zn shown as gray sphere). The Zn site has also been shown to alter its average structure depending on pH and Ni-binding; at acidic conditions with Ni-bound, two His imidazoles replace two of the sulphur donors from the Cys residues.
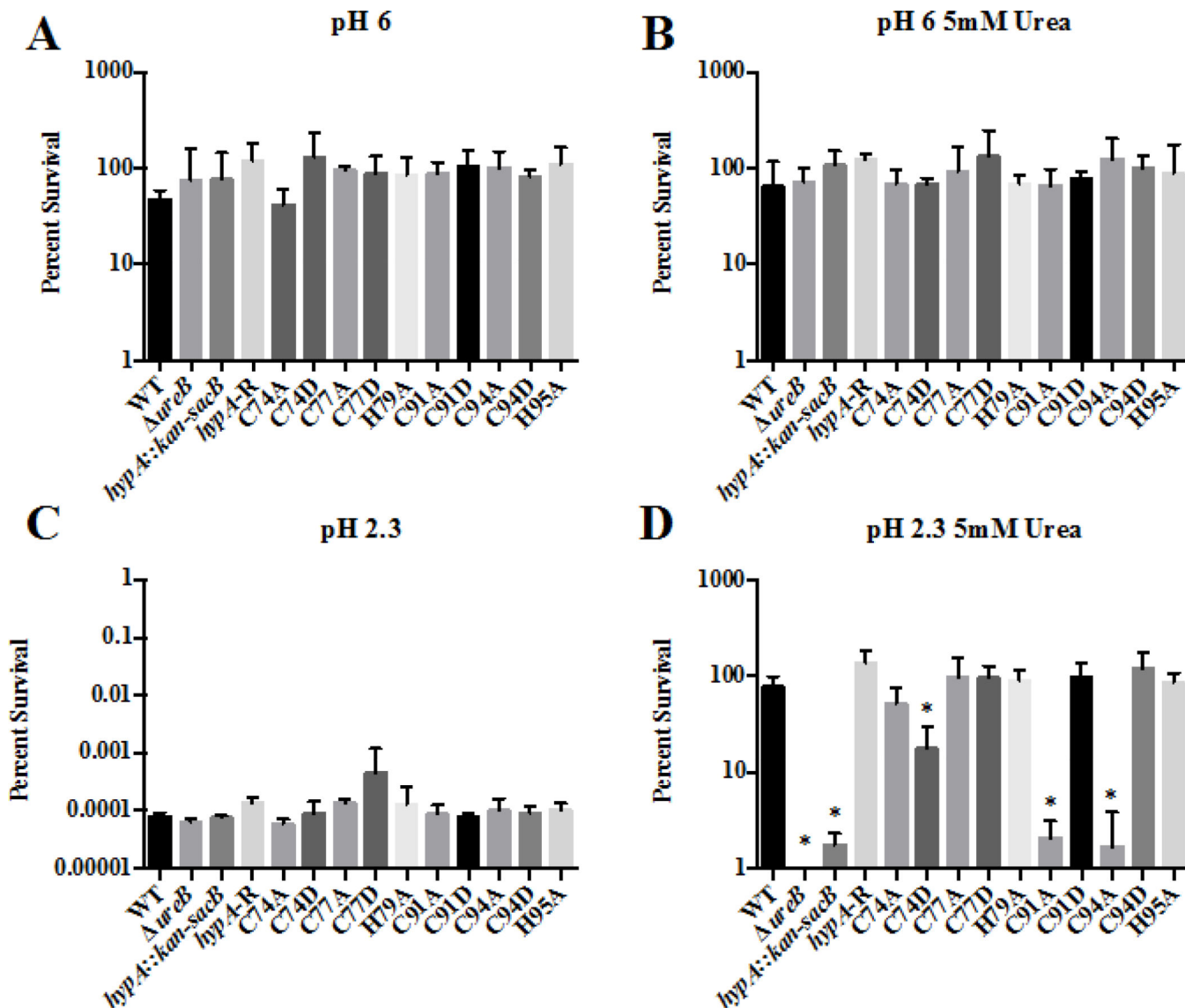
Fig. 2.
Specific amino acid mutations within the zinc-binding site of the HypA protein in *Helicobacter pylori* result in decreased acid resistance. The ten *hypA* mutants, as well as the wild type (WT), *ureB* knockout (*ureB*), *hypA* interrupted mutant (*hypA::kan-sacB*), and the *hypA*-restorant (*hypA*-R) were exposed to various environments for 1 hour: pH 6 (A), pH 6 containing 5mM urea (B), pH 2.3 (C), and pH 2.3 containing 5mM urea. Percent survival was calculated for each strain. Data represent mean ± standard deviation. * = Acid resistance was significantly reduced when compared to wild type (p < 0.01, one-way ANOVA followed by Dunnett's test for multiple comparisons).
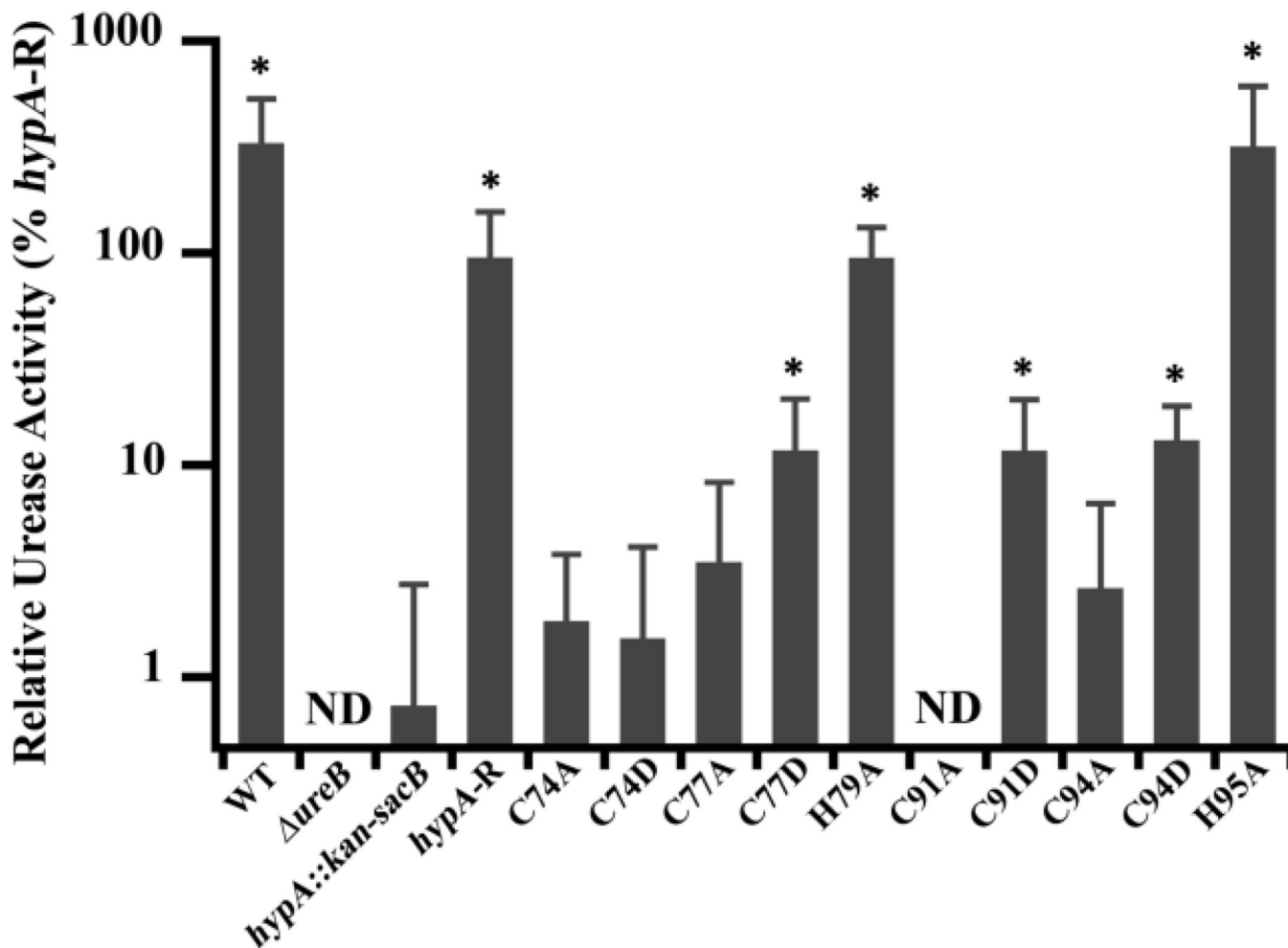
Fig. 3.
Zn-site mutations of *hypA* in *H. pylori* lead to deficiencies in urease activity. Soluble whole cell extracts were obtained from lysis of ten *hypA* Zn-site mutant strains, as well as the wild type (WT), *ureB* knockout (ΔureB), *hypA* interrupted mutant (*hypA::kan-sacB*), and the *hypA*-restorant (*hypA*-R) strains. The phenol-hypochlorite method was used to assay the ammonia production (urease activity) of the strains. Amount of ammonia produced was quantified by comparison with a standard curve constructed with known amount of ammonium chloride. The ΔureB strain activity was subtracted from each strain as background ammonia in cell extracts and all activity was normalized to *hypA*-R strain. * = Urease activity was significantly different from the *hypA::kan-sacB* strain ($p < 0.05$). ND = none detected.

Table 1

Strain, plasmids, and primers used in this study

Strains	Description	Reference
DSM1	G27 WT	[12]
DSM43	G27 *ureB* Kan[R]	[15], This study
DSM1283	G27 *hypA*::*kan-sacB* Kan[R] Suc[S]	This study
DSM1295	G27 *hypA* restorant	This study
DSM1296	G27 *hypA* C74A	This study
DSM1297	G27 *hypA* C94D	This study
DSM1298	G27 *hypA* C91A	This study
DSM1299	G27 *hypA* C91D	This study
DSM1300	G27 *hypA* H95A	This study
DSM1301	G27 *hypA* C74D	This study
DSM1363	G27 *hypA* C77A	This study
DSM1364	G27 *hypA* C77D	This study
DSM1365	G27 *hypA* H79A	This study
DSM1366	G27 *hypA* C94A	This study

Plasmids	Description	Reference
pDSM3	pKSF-II	[14]
pDSM32	pEJ22	[15]
pJI110	pET22b(+) vector with *hypA* WT coding sequence	[11], This study
pET22b-HypA(C74A)	pET22b(+) vector with *hypA* C74A coding sequence	This study
pET22b-HypA(C74D)	pET22b(+) vector with *hypA* C74D coding sequence	This study
pET22b-HypA(C77A)	pET22b(+) vector with *hypA* C77A coding sequence	This study
pET22b-HypA(C77D)	pET22b(+) vector with *hypA* C77D coding sequence	This study
pET22b-HypA(H79A)	pET22b(+) vector with *hypA* H95A coding sequence	This study
pET22b-HypA(C91A)	pET22b(+) vector with *hypA* C91A coding sequence	This study
pET22b-HypA(C91D)	pET22b(+) vector with *hypA* C91D coding sequence	This study
pET22b-HypA(C94A)	pET22b(+) vector with *hypA* C94A coding sequence	This study
pET22b-HypA(C94D)	pET22b(+) vector with *hypA* C94D coding sequence	This study
pET22b-HypA(H95A)	pET22b(+) vector with *hypA* H95A coding sequence	This study

Primers	Sequence (5'-3')	Reference
HypA_Up_F	CCGCTTTGATTCAGATGGGGTG	This study
HypA_Up_R[*]	*TCTAGA*AGCTTGCGATCGCTCGAGACTCTAAAAGTCTCAAACGCGCTC	This study
HypA_Dn_F[*]	*CTCGAG*CGATCGCAAGCTTCTAGAGAATCTTTGGTGTGTAAAGACGC	This study
HypA_Dn_R	GCAAAACGCTGCGGTATTGC	This study
Kan_SacB_F[*]	GTGGG*CTCGAG*CCCGGGCGAACCATTTGAGGTGA	This study
Kan_SacB_R[*]	GCGCG*TCTAGA*TATAAGCCCATTTTCATGC	This study
HypA_Confirm_F	GGCTAACGAGCGTGGATAAG	This study

HypA_Confirm_R	GCACTCACTAAAATCGTGGGC	This study
HypA_seq_F	CTAAAGCGGTAACCACATCCG	This study
HypA_C74A_F	GGTTGAATTAGAA<u>GC</u>CAAGGATTGTTCGCATGTTTTTAAGCCTAACGCG	[11b], This study
HypA_C74A_R	CGCGTTAGGCTTAAAAACATGCGAACAATCCTTG<u>GC</u>TTCTAATTCAACC	[11b], This study
HypA_C74D_F	GGTTGAATTAGAA<u>GA</u>CAAGGATTGTTCGCATGTTTTTAAGCCTAACGCG	[11b], This study
HypA_C74D_R	CGCGTTAGGCTTAAAAACATGCGAACAATCCTTG<u>TC</u>TTCTAATTCAACC	[11b], This study
HypA_C77A_F	GAATTAGAATGCAAGGAT<u>GC</u>TTCGCATGTTTTTAAGCCTAACGCGC	[11b], This study
HypA_C77A_R	GCGCGTTAGGCTTAAAAACATGCGAA<u>GC</u>ATCCTTGCATTCTAATT	[11b], This study
HypA_C77D_F	GAATTAGAATGCAAG<u>GA</u>TGATTCGCATGTTTTTAAGCCTAACGCGC	[11b], This study
HypA_C77D_R	GCGCGTAGGCTTAAAAACATGCGAATCA<u>TC</u>CTTGCATTCTAATTC	[11b], This study
HypA_H79A_F	GCAAGGATTGTTCG<u>GC</u>TGTTTTTAAGCCTAACGCGCTAG	[11b], This study
HypA_H79A_R	GTTAGGCTTAAAAACA<u>GC</u>CGAACAC	This study
HypA_C91A_F	GCGCTAGATTATGGGGTG<u>GC</u>TGAGAAATGCCACAGC	[11b], This study
HypA_C91A_R	GCTGTGGCATTTCTCA<u>GC</u>CACCCCATAATCTAGCGC	[11b], This study
HypA_C91D_F	CGCCGTAGATTATGGGGTG<u>GA</u>TGAGAAATGCCACAGC	[11b], This study
HypA_C91D_R	GCTGTGGCACTTTCTCAT<u>CC</u>ACCCCATAATCTAGCGCG	[11b], This study
HypA_C94A_F	GGTGTGTGAGAAA<u>GC</u>CCACAGCAAG	This study
HypA_C94A_R	AACATTCTTGCTGTGG<u>GC</u>TTTCTCAC	This study
HypA_C94D_F	GGGGTGTGTGAGAAA<u>GA</u>CCACAGCAAGAATGTTATTATCAC	[11b], This study
HypA_C94D_R	GTGATAATAACATTCTTGCTGTGG<u>TC</u>TTTCTCACACACCCC	[11b], This study
HypA_H95A_F	GTGTGTGAGAAATGC<u>GC</u>CAGCAAGAATGTTATTATC	[11b], This study
HypA_H95A_R	GATAATAACATTCTTGCTG<u>GC</u>GCATTTCTCACACAC	[11b], This study

[*] Restriction enzyme sites are italicized (XhoI or XbaI); <u>Underline</u> denotes nucleotide changes made to the pJI110 (wild type *hypA*) plasmid