TOPIC HIGHLIGHT

Nickel trafficking system responsible for urease maturation in *Helicobacter pylori*

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Abstract

Helicobacter pylori (*H. pylori*) is a common human pathogen responsible for various gastric diseases. This bacterium relies on the production of urease and hydrogenase to inhabit the acidic environment of the stomach. Nickel is an essential cofactor for urease and hydrogenase. *H. pylori* has to uptake sufficient nickel ions for the maturation of urease, and on the other way, to prevent the toxic effects of excessive nickel ions. Therefore, *H. pylori* has to strike a delicate balance between the import of nickel ions, its efficient intracellular storage, and delivery to nickel-dependent metalloenzymes when required. The assembly and maturation of the urease enzyme is a complex and timely ordered process, requiring various regulatory, uptake, chaperone and accessory proteins. In this review, we focus on several nickel trafficking proteins involved in urease maturation: NikR, NixA, HypAB, UreEFGH, HspA, Hpn and HpnI. The work will deepen our understanding of how this pathogenic bacterium adapts to severe habitant environments in the host.

Core tip: *Helicobacter pylori* (*H. pylori*) is responsible for various gastric diseases. The nickel containing urease and hydrogenase are essential for the successful infections of *H. pylori* in the stomach. Nickel is an essential cofactor for urease and hydrogenase. In this review we discussed the various regulatory, uptake, chaperone and accessory proteins involved in the maturation of urease, especially the proteins NikR, NixA, HypAB, Ure-EFGH, HspA, Hpn and HpnI. The work will deepen our understanding of how this pathogenic bacterium adapts to severe habitant environments in the host.

INTRODUCTION

Helicobacter pylori (*H. pylori*), a micro-aerophilic Gram-negative spirobacterium, infects around half of the people worldwide and is responsible for gastric diseases
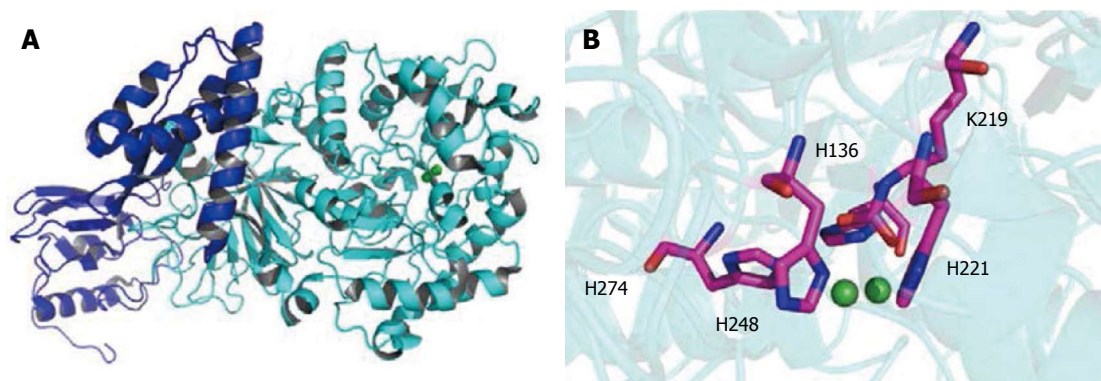
Figure 1 Structure of *Helicobacter pylori* urease. A: The urease enzyme subunit UreAB (UreA, blue; UreB, cyan; PDB code: 1E9Z); B: The active sites of *H. pylori* urease with the side chains of the enzyme involved in the chelation of the catalytic di-nickel center shown (Ni, green; O, red; N, blue; C, pink). *H. pylori*: *Helicobacter pylori*.

such as chronic gastritis, peptic ulcer and gastric cancer[1]. The bacterium is widely present in the mucus layer of the stomach, the mucus glands in the stomach cavity and the surface of gastric epithelial cells as well as within the cells. Due to the wide presence in the differential parts of the stomach, it is difficult to completely eradicate the pathogen during gastric disease therapy[2]. The commonly used treatment for *H. pylori* related diseases is the so-called triple therapy, which consists of two antibiotics and either a proton pump inhibitor (PPI) or one kind of bismuth-based colloidal drug[3,4]. In some countries, standard triple therapy combining one PPI, amoxicillin and clarithromycin is the best option. However, in countries where clarithromycin resistance rate is over 20%, bismuth-containing quadruple therapy, or non-bismuth sequential or concomitant therapies are the preferred option. The medical and social impact of the discovery of *H. pylori* was acknowledged by the award of the 2005 Noble Prize in Physiology and Medicine to Marshall and Warren.

Around 80% of *H. pylori* cells inhabit the moderately acidic gastric mucus. Once entry into the stomach, the first hurdle for *H. pylori* is to be quickly transmitted through the extremely acidic gastric lumen, exhibiting a median pH of approximately 1.4[5]. *H. pylori* multiplies in an environmental pH from 6.0 to 8.0[6], and cannot survive when the pH < 4.0 or > 8.2[7]. In order to live in the gastric environment, *H. pylori* has developed various acid-resistant mechanisms. Time-independent acid resistance depends on the high isoelectric points of the inner and outer membrane proteins to reduce proton permeability[8]. Acute acid resistance depends on the constitutive synthesis of urease that catalyzes the hydrolysis of urea to ammonia and carbamate, the latter of which is further degraded to ammonia and carbonic acid. The end products are in an equilibrium between their protonated and de-protonated forms, leading to an elevation of the surrounding pH from absolutely acidic to approximately neutral[9]. Urease is an oligomeric Ni^{2+}-containing heterodimer of UreA and UreB subunits and is essential for *H. pylori* to infect in all animal models so far examined[10-12]. The substrate gastric juice urea is able to rapidly access intrabacterial urease through a pH-gating urea channel, UreI[13], when the periplasmic pH falls < 6.2.

H. pylori urease is produced in a high level, accounting for up to 10% of total cellular proteins[14]. Expression of urease protein is constitutive[15], primarily due to the housekeeping σ^{80}-dependent promoters for the transcription of both *ureAB* and *ureEFGHI*[16,17]. Under *in vitro* growth conditions without additionally added Ni^{2+}, only 2% of the active sites were filled with Ni^{2+}[18,19]. Urease produces NH_3 from gastric juice urea with maximal efficiency at millimolar concentrations[14,20], 10^{14} times faster than uncatalyzed reactions. The enzymatic hydrolysis of urea causes an abrupt overall pH increase, resulting in negative side effects for human and positive effects in the buffering of the periplasm and maintenance of a proton motive force adequate for ATP synthesis of the bacterium[21]. *H. pylori* urease was shown to be a giant 1.1 MDa complex containing 12 subunits of UreA and UreB (Figure 1), with two Ni^{2+} needed for enzyme activity[6,22]. The assembly of the urease enzyme is a complex, timely ordered process, and the UreEFGH accessory proteins are absolutely necessary[23,24]: UreH stabilizes the apoprotein[25]; UreF facilitates carbamylation of the Ni^{2+}-bridging lysine residue and blocks premature Ni^{2+} binding to the active site[26]; UreG provides energy during urease assembly[27]; and UreE facilitates Ni^{2+} incorporation into the active center[28]. The hydrogenase accessory proteins HypA and HypB are also necessary to maintain the urease activity, indicating that the bacterium utilizes both maturation systems for the activation of its urease[18]. This present review intends to cover the reports and discoveries in the field of nickel trafficking system in urease maturation of *H. pylori*, which may deepen our understanding of how this pathogenic bacterium adapts to severe habitant environments in the host.

NICKEL REGULATORY PROTEIN NIKR

Bacteria have developed sophisticated mechanisms to regulate levels of intracellular nickel ions, to ensure sufficient nickel for enzyme processes in one way and to

prevent excessive toxic free ions in the other way[29]. NikRs, a novel class of ribbon-helix-helix nickel regulatory proteins, are homotetrameric transcription factors that repress and/or activate specific genes in response to nickel availability. *H. pylori* NikR, a tetrameric protein made of two dimeric N-terminal DNA-binding domains (DBD) and C-terminal domains for tetramerization and metal binding (MBD), binds stoichiometric nickel with picomolar affinities[30,31], comparable to NikRs from other species[32-34]. The DBD and MBD are connected by a flexible linker, allowing for differential conformations (open, trans and cis) of NikR. In *E. coli* and *Pyrococcus horikoshii*, the apo-NikRs adopt an open conformation, whereas the apo-NikR shows an unusual closed *trans*-conformation and asymmetrical quaternary arrangement, where the DBDs are on the opposite sides of the transmembrane domain[35]. Computational and NMR studies suggest that NikR is interconverting among the open, trans and cis forms in solution and nickel binding facilitates the interconversion[36].

At non-physiologically low pH (4.6-5.6), NikR had three types of nickel-binding sites: the final high affinity site (F) with square-planar geometry, the intermediate site (I) involving residues belonging either to the F or external site, and the external sites (X) with an octahedral geometry[35,37]. Whereas in physiological conditions (pH 5.6-7.5), NikR binds four low-spin Ni^{2+} at the protein tetramerization interface, although differential nickel coordination modes are proposed. Michel's group suggests that two nickels are bound at 4-coordinate square-planar sites with His_3Cys ligands (*i.e.*, 4-sites) and the other two are coordinated by $His_3(H_2O)_{2-3}$ in square pyramidal or octahedral geometries (*i.e.*, 5/6 sites)[37]. Ciurli's group reports a structure with all four nickel ions bound to 4 sites[38], and the four binding sites are classified into two sets (2/2), with binding affinities differing by one order of magnitude[39]. The findings may suggest that an equilibrium exists between the two nickel-bound forms of the protein.

The biological role of NikR is to regulate the transcription of multiple genes as a function of nickel availability[40,41]: up-regulated genes in nickel metabolism (*nikABCDE*, *nixA*, *ureA*, *ureB*, *hpn* and *hpn-like*); down-regulated genes in iron uptake and storage (*pfr*, *fur* and *exbB/exbD*), motility (*cheV*, *flaA* and *flaB*), and stress responses to outer membrane proteins (*omp11*, *omp31* and *omp32*)[40]. The nickel-responsive binding of NikR to target promoters *pUreA*, *pNikR*, *pexbB* and *pFur* have been characterized by the *in vitro* gel shift and DNase I footprinting studies. Michel's[42] group proposed a mechanism for nickel-mediated DNA recognition by NikR. NikR prefers binding Ni at 5/6 sites. Upon addition of two Ni, the ligands are rearranged to two 4-sites. Addition of two more Ni results in mixed coordination geometry (two 4-sites and two 5/6-sites) and makes the protein binding to target DNA. The binding to DNA changes the orientation of the DBD from trans to cis, an orientation that is stabilized at the MBD/DBD interface[42].

Controversial opinions exist for the roles of NikR in urease activation as a function of pH. One opinion goes that under acidic conditions, the greater availability of Ni^{2+} leads to the formation of Ni^{2+}-NikR complexes which further increase the expression of urease, Ni^{2+} transporter NixA and iron regulator Fur[43,44]. Whereas, Pflock *et al*[45] found that a two-component system ArsRS (acid responsive signaling) regulated urease expression in response to low pH, and further proposed that urease expression is mediated by two distinct mechanisms: one in response to increasing Ni^{2+} concentration (NikR) and one in response to decreasing pH (ArsR).

NICKEL UPTAKE

Due to the essential stasis of Ni^{2+}-containing urease for the host colonization and infection of *H. pylori*, a constant supply of Ni^{2+} into *H. pylori* is required. The concentration of nickel ions in the environment is relatively low: around 30 nM in seawater and 5 nM in freshwater, a condition requiring highly specific importers of Ni^{2+} ions for *H. pylori*[46]. Thus far, two types of nickel uptake strategies have been identified in *H. pylori*[46]: (1) NixA[47], a member of the nickel-cobalt transporter family (Ni-CoT)[48]; and (2) the multiple-component ATP-binding protein cassette (ABC)-transporters, which are believed to be a four-gene operon designated as *abc*ABCD[49].

NixA is required for effective *H. pylori* colonization, as disruption of the gene led to reduced colonization[50]. NixA is predicted to have eight transmembrane-spanning helices, and transports Ni^{2+} with a V_{max} of 1750 pmol Ni^{2+}/min per 10^8 cells and a K_m of 11.3 nmol[51,52], thus enabling *H. pylori* to efficiently scavenge nickel ions in the range of 2-11 nmol from the human body[53]. NixA transcription was shown to be repressed by NikR in a nickel-dependent manner to prevent excess toxic *in vivo* nickel[44,54].

NixA deletion mutants still retained urease activity in some levels (up to 50% in some strains)[50,55], indicating the existence of an alternative nickel transporter. Further analysis identified the *abcABCD* genes, a component of the ATP-dependent nickel transport system to be potentially involved in NixA-independent nickel uptake, as mutations in *abcCD* decreased urease activity[49]. Another work identified FrpB4 to be a potential outer membrane nickel uptake protein as energized by the TonB/ExbB/ExbD machinery[56], indicating that the established iron uptake machinery may be involved in nickel uptake. However, further work is needed to confirm their role and mechanism in nickel transport.

CHAPERONES

Similar to other bacteria, *H. pylori* has to maintain a delicate balance between the import of nickel ions, its efficient intracellular storage, and delivery to nickel-dependent metalloenzymes when required. Metals, such as nickel, pose problems for the cell because they are required for the growth, whereas they inhibit growth and
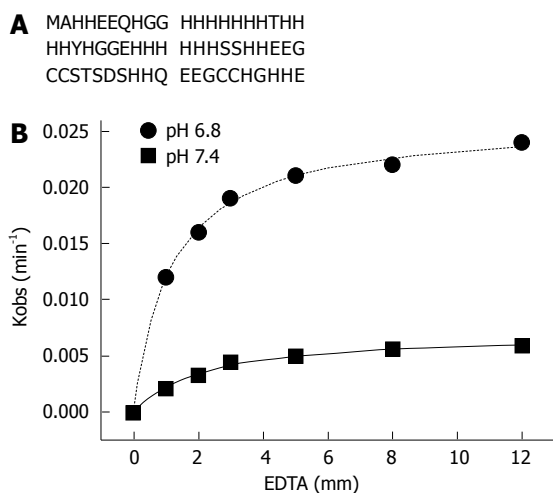
A MAHHEEQHGG HHHHHHHTHH
HHYHGGEHHH HHHSSHHEEG
CCSTSDSHHQ EEGCCHGHHE

B

Figure 2 Amino acid sequence of Hpn (A) and the apparent rate of nickel release *vs* concentrations of ethylenediaminetetraacetic acid at pH 6.8 and 7.4 and the best nonlinear fit of the data (B). EDTA: Ethylenediaminetetraacetic acid.

exhibit toxic effects when present in excess. In this section, we would like to discuss the proteins involved in metallocenter assembly in urease.

HypA and HypB

HypA and HypB are named to emphasize their roles in the maturation and activation of NiFe hydrogenase (*hyp*, hydrogenase pleiotropic). However HypA and HypB are also found to be accessory proteins for urease[57], as reflected by the reduced urease activity (40-200 folds) upon *hypA* or *hypB* disruption[18] and the competition between HypA and UreG for UreE (see below) recognition[58]. HypA binds nickel and zinc ions and HypB is a P-loop GTPase to provide energy during nickel insertion in hydrogenase. HypA and HypB exist as homodimers in solution and form heterodimers with each other[59,60] with a low affinity (K_d of 52.2 ± 8.8 μmol)[61]. HypA and HypB also make heterodimers with UreE[62] and SlyD[63], respectively in solution. The NMR structure of zinc-bound HypA monomer indicates that the nickel binding site is located at the N-terminus and nickel is bound to four nitrogens in a square planar geometry[64]. A thermodynamic study indicates that the zinc binding site has a much higher affinity to zinc than nickel and zinc binding induces a great change in the secondary structure of HypA to exert its structural role in the metalloprotein[65]. Further study with XAS showed that HypA dimer has a unique structural flexibility of the zinc site and has roles in sensing nickel binding and pH[66,67]: a decrease of pH from 7.2 to 6.3 induces a change of the zinc binding ligands from Cys_4 to Cys_2His_2 and results in a change of the nickel binding stoichiometry from one Ni per monomer to one Ni per dimer[66]. Cys106 and His107 of HypB are required for nickel binding and metal-dependent dimerization[68]. Nickel binding of HypB is possibly facilitated by SlyD *via* its IF (insert-in-flap) domain[63]. Zinc binding significantly inhibits the GTPase activity of

HypB[68]. Nickel binding is reported to either slightly[68] or highly[61] stimulate the activity of HypB, with reasons for these discrepancies yet unknown. The regulation of HypB activities by metal binding may contribute to the maturation of the hydrogenase and urease.

UreEFGH

UreEFGH is a group of accessory proteins involved in the synthesis of the urease active site[41], which has been excellently covered recently in a review by Farrugia *et al*[69]. This review will only briefly discuss their respective roles. The information about UreH is quite limited primarily due to its insolubility in solution, although it is believed to be the first protein to bind to apo-urease[70]. UreE is the chaperone to deliver nickel to urease and UreF activates the GTPase activity of UreG[29,41]. UreE is capable of binding Ni and Zn (K_d of 0.15 and 0.49 μmol, respectively) in a stoichiometry of one per dimer[71,72]. Apo-UreE is a dimer and the metal-bound protein is a tetramer (dimer of dimer) formed by the coordination of the metal ion by His104 from each subunit[73]. A second UreE crystal structure indicates that Ni is six-coordinate (His102 from one monomer, His102, His152, Glu4 from the other, a water molecule and one unidentified ligand)[74]. His152 is disordered in the crystal and could be replaced by UreG residues, thus leading to the transfer of nickel from UreE to UreG. In the calculated structure of UreDEFG through computational modeling, the convex surface of the UreG dimer is in direct contact only with the shallow crevice at the interface of the two UreF monomers through weak van der Waals and polar interactions[75]. UreF and UreH can form dimer of heterodimers in solution with concomitant conformational changes in two distinctive regions of UreF[76]: (1) the flexible C-terminus becomes ordered to form an extra helix α10 and a loop stabilized by hydrogen bonds involving Arg250; and (2) the first turn of helix α2 uncoils to expose a conserved residue Tyr48. Both Arg250 and Tyr48 are critical for the heterotrimeric formation of UreG-UreF-UreH and urease maturation[76]. One crystal structure of UreGFH indicates that UreFH facilitates UreG dimerization and assembles its metal binding sties by juxtaposing two Cys66-Pro67-His68 motifs at the interface to form the (UreGFH)₂ complex[77].

HspA, Hpn and Hpn-like

HspA, Hpn and Hpn-like (Hpnl) proteins in *H. pylori* are histidine-rich in full or in part. HspA is a bacterial GroES homologue with a unique cysteine- and histidine-rich C-terminal domain[78]. HspA binds 2 Ni per monomer with a dissociation constant of 1.1 μmol *in vitro*[79]. The *in vivo* work showed that HspA is involved in intracellular nickel sequestration and detoxification, and plays a role as a specific nickel chaperone in the maturation of hydrogenase, while not for urease[80]. Hpn (Figure 2A) is a histidine rich protein (accounting for around half of its amino acids) and highly abundant in the cell cytoplasm (approximately 2% of all protein synthesized)[81].
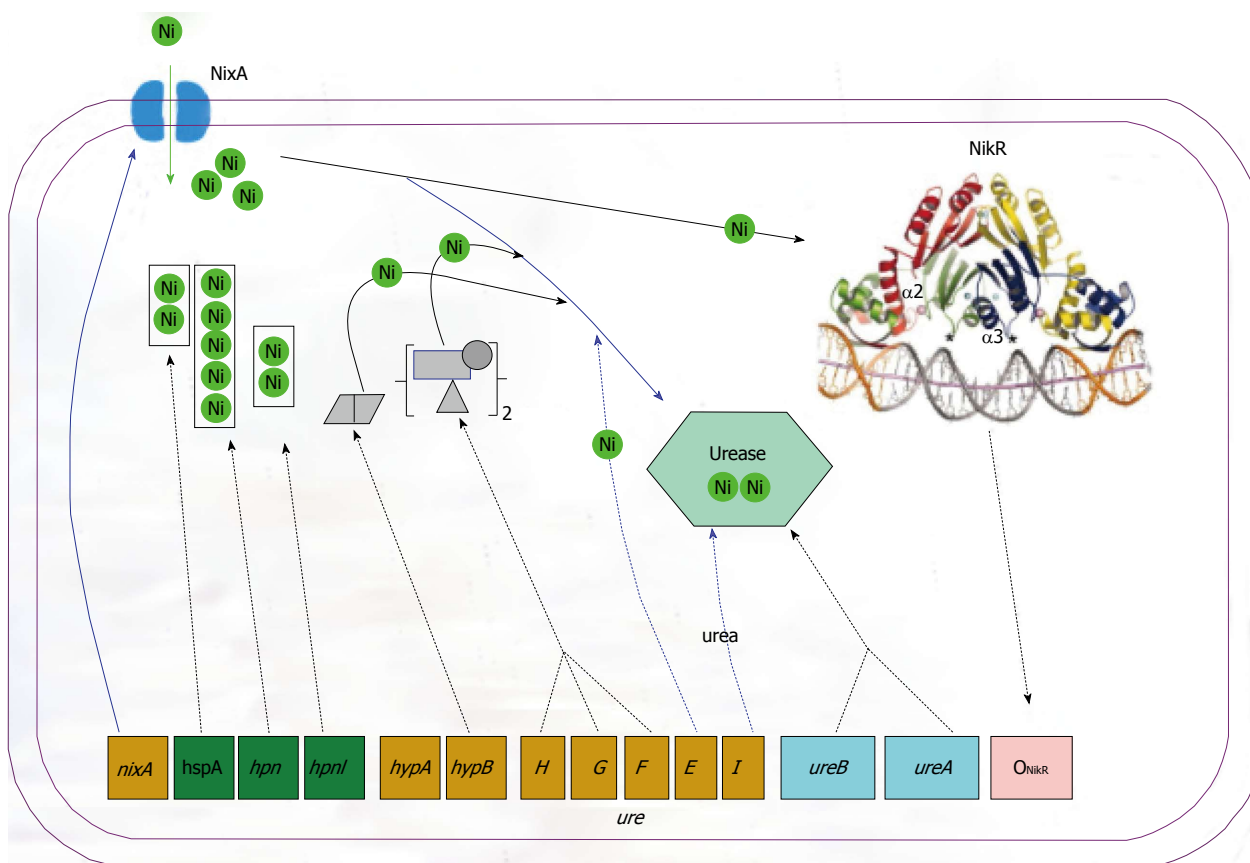
Figure 3 Complex network controlling urease synthesis and activity in *Helicobacter pylori*. The different levels of control comprise (1) expression of the UreAB structural subunits fine-tuned by acidity and the nickel-dependent transcriptional regulator NikR; (2) nickel uptake into cells *via* NixA importer; (3) nickel storage in histidine-rich proteins such as Hpn, Hpnl and HspA; (4) nickel incorporation into urease as mediated by accessory proteins UreEFGH and HypAB; and (5) urea substrate entry *via* Urel. *H. pylori*: *Helicobacter pylori*.

The majority of histidines are located within the central part of the protein and include two separated stretches of 6 and 7 consecutive histidine residues. There are two internal short repeats of Glu-Glu-Gly-Cys-Cys, four sets of paired histidine residues and an X-X-His motif at the N-terminus. All these sequence features indicate that this protein would strongly bind metal ions. Mutated strains of *H. pylori* lacking the *hpn* gene are four times more sensitive to ranitidine bismuth citrate, a metal-containing drug widely used to treat *H. pylori* infections, than the wild type[3,82,83]. Hpn exists in solution as a range of multimeric forms with the 20-mer to be potentially physiologically relevant[84]. The protein can bind nickel in a stoichiometry of five Ni per monomer with a K_d of 7.1 μmol. Therefore it is possible that nickel may be transferred from Hpn to stronger nickel binding proteins, such as HypA (K_d of 1.3 μmol) and HspA (K_d of 1.8 μmol). Nickel can be released from Hpn by decreasing pH (pH$_{1/2}$ of 6.3) or by adding nickel chelating agent EDTA[84,85], which indicates that Hpn could provide stored nickel ions to the relevant chaperone proteins for the subsequent urease maturation upon intracellular pH decrease. The nickel release from Hpn by EDTA is a two-step process consisting of a rapidly established equilibrium (formation of Hpn-Ni•EDTA, *K*) followed by a rate-determining step (dissociation of Hpn-Ni•EDTA to Ni-EDTA and apo-Hpn,

k_2)[85]. The data was fitted in Figure 2B which suggests that lower pH favors both the formation of the Hpn-Ni•EDTA intermediate and its decomposition to the Ni-exchanged products[85]. Later work by our group showed that this His-rich protein can form amyloid-like structures and exhibit some cytotoxic effects to gastric epithelia cells[86], indicating that Hpn may be involved in the pathological roles of *H. pylori* other than the nickel storage role in the maturation of nickel specific enzymes[87]. Hpnl is a histidine- and glutamine-rich protein in *H. pylori*, the N-terminus (46 residues) of which shows 56% identity to Hpn. Hpnl binds two nickel ions per monomer in the histidine-rich domain with a dissociation constant of 3.8 μmol[88]. Nickel release experiments established that Hpnl is similar to Hpn, as nickel can be release from Hpnl at acidic pH (pH$_{1/2}$ of 4.6) and in the presence of EDTA. One *in vivo* study by Maier's group indicated *H. pylori* can utilize stored nickel ions *via* Hpn and Hpnl to aid colonization of the host[89].

CONCLUSION

H. pylori is an established agent causing various gastric diseases. The nickel containing urease and hydrogenase are essential for the successful infections of *H. pylori* in the stomach. Nickel is an essential cofactor for urease

and hydrogenase. Various nickel-binding proteins play key roles in microbial nickel homeostasis by shuttling nickel within the cells. In this review we discussed the regulatory, uptake, chaperone and accessory proteins involved in the maturation of urease, especially the proteins NikR, NixA, HypAB, UreEFGH, HspA, Hpn and Hpnl. The proteins function in a coordinated way to maturate the urease in an efficient way for the successful inhabitation of the bacterium in the stomach (Figure 3). The work will deepen our understanding of how this pathogenic bacterium adapts to severe habitant environments in the host.

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