IAI Journals.ASM.org

Helicobacter pylori Stores Nickel To Aid Its Host Colonization

Stéphane L. Benoit, Erica F. Miller, Robert J. Maier

Department of Microbiology, The University of Georgia, Athens, Georgia, USA

The transition metal nickel (Ni) is critical for the pathogenicity of *Helicobacter pylori*. Indeed the element is a required component of two enzymes, hydrogenase and urease, that have been shown to be important for *in vivo* colonization of the host gastric mucosa. Urease accounts for up to 10% of the total cellular *H. pylori* protein content, and therefore the bacterial Ni demand is very high. *H. pylori* possess two small and abundant histidine-rich, Ni-binding proteins, Hpn and Hpn-like, whose physiological role in the host have not been investigated. In this study, special husbandry conditions were used to control Ni levels in the host (mouse), including the use of Ni-free versus Ni-supplemented food. The efficacy of each diet was confirmed by measuring the Ni concentrations in sera of mice fed with either diet. Colonization levels (based on rank tests) of the Δ*hpn* Δ*hpn*-like double mutants isolated from the mice provided Ni-deficient chow were statistically lower than those for mice given Ni in their diet. In contrast, *H. pylori* wild-type colonization levels were similar in both host groups (e.g., regardless of Ni levels). Our results indicate that the gastric pathogen *H. pylori* can utilize stored Ni via defined histidine-rich proteins to aid colonization of the host.

Helicobacter pylori is a spiral, Gram-negative, microaerophilic bacterium that has been shown to be the etiological agent of chronic atrophic gastritis and peptic ulcers (1), which can eventually develop into gastric cancers (2). The bacterium can persist in the stomach of humans for a lifetime, in part because of two nickel (Ni^{2+})-containing enzymes, hydrogenase and urease. *H. pylori* has one unique hydrogen-utilizing [NiFe] hydrogenase (3) which permits respiratory (H_2)-based energy production in the mucosa. The enzyme was shown to be required for full colonization of mouse stomachs (4). Urease, one of the most abundant proteins synthesized in *H. pylori*, converts urea into carbon dioxide and ammonia, thereby raising the pH in its environment. The enzyme is required for the colonization of various host animals, such as nude mice, gnotobiotic piglets, and Mongolian gerbils (5–7). Accessory proteins (needed to produce catalytically active urease) are also required for colonization. Indeed, *H. pylori ureG*-negative mutants (which can synthesize apo-urease but are unable to incorporate Ni^{2+} into the active site) are deficient in colonizing the gastric mucosa of nude mice, thereby highlighting a link between Ni activation of urease and host colonization (8).

The importance of Ni^{2+} ions in *H. pylori* physiology is emphasized by the number and diversity of proteins involved in nickel homeostasis. Indeed, *H. pylori* relies on a comprehensive system that includes (i) the TonB-ExbB-ExbD-dependent outer membrane Ni^{2+} transporters FrpB4 and FecA3 (9, 10), (ii) a specific Ni^{2+} permease, NixA (11), (iii) a nickel efflux system, CznABC (12), (iv) an Ni^{2+}-dependent pleiotropic regulator, NikR (13, 14), (v) an Ni^{2+}-binding urease repressor, Mua (15), and (vi) three small Ni^{2+}-binding cytoplasmic proteins: Hpn, Hpn-like, and HspA. The last is a GroES homolog whose histidine (His)-rich C-terminal tail has been shown to be involved in nickel-dependent maturation of hydrogenase (16). Hpn and Hpn-like proteins are the focus of the present study.

Hpn (named to emphasize its origins in *H. pylori* and its affinity for nickel) is an abundant His-rich protein that was originally isolated as an Ni^{2+}-binding protein from *H. pylori* clinical isolates (17). Hpn-like protein is a His- and glutamine (Gln)-rich protein whose gene is induced in the presence of NikR (13). Expression of *hpn* and *hpn*-like genes is acid induced and under the control of the two-component system ArsRS (18, 19). Hpn and Hpn-like can form multimers *in vitro* and are able to bind with micromolar affinity up to five and two Ni^{2+} ions per monomer, respectively; this binding is reversible, as metal can be released from either recombinant protein in the presence of chelating agents or at acidic pH (20, 21). Expression of either Hpn or Hpn-like protein in *E. coli* led to higher intracellular Ni levels and rendered the enterobacteria more resistant to toxic nickel levels, suggesting a role for the proteins in nickel detoxification (20, 21). These results were confirmed by nickel toxicity studies of *H. pylori* mutants: strains lacking *hpn*, *hpn*-like, or both genes were less tolerant to high nickel levels than the wild type (22, 23).

Hpn and Hpn-like proteins play other roles besides nickel detoxification. Indeed, under low nickel concentrations and at neutral pH, mutant strains had higher urease activity and higher intracellular Ni pools than the wild-type strain, suggesting that under these conditions these proteins sequestered limiting nickel reserves at the expense of nickel delivery for urease activation (23). However, chelation of extracellular nickel or growth at acidic pH (the latter condition being one when urease is highly expressed) significantly decreased the urease activity in the mutants but not in the wild type, indicating that urease activity was then much more dependent upon exogenous nickel in the mutants than in the wild type (23).

Since (i) there appears to be a link between both Hpn and Hpn-like proteins and Ni-dependent urease activity and (ii) it is known that *H. pylori* requires Ni-activated urease for colonization of the host (mouse) gastric mucosa, we hypothesized that the *hpn* *hpn*-like double-mutant strain would be attenuated for coloniza-

Received 15 August 2012 **Returned for modification** 9 September 2012
Accepted 2 December 2012
Published ahead of print 10 December 2012
Editor: J. B. Bliska
Address correspondence to Robert J. Maier, rmaier@uga.edu.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/IAI.00858-12.

doi:10.1128/IAI.00858-12

tion in an Ni-deficient environment (e.g., mice kept in an Ni-free environment and given an Ni-depleted diet). Conversely, one might expect colonization levels of wild-type *H. pylori* cells to be unaffected by Ni availability because those cells could rely on Ni storage by Hpn and Hpn-like to generate the active urease needed *in vivo*. Thus, in the present study, stringent conditions were implemented to generate Ni-depleted or Ni-supplemented environments within the host; colonization levels of the wild-type or the *hpn hpn*-like double-mutant strain were then assessed for the two animal groups.

MATERIALS AND METHODS

Bacterial growth conditions. *H. pylori* was routinely grown on brucella agar plates supplemented with 10% defibrinated sheep blood (BA plates) at 37°C under microaerophilic conditions with 5% O_2, 5% CO_2, and 90% N_2. When needed, BA plates were supplemented with kanamycin, chloramphenicol, vancomycin, amphotericin B, and bacitracin to final concentrations of 30, 25, 10, 10, or 50 μg/ml, respectively.

Construction of the Δ*hpn* Δ*hpn*-like double mutant. Each gene was deleted using an overlapping PCR method. Primers used in this study are described in Table S1 in the supplemental material. First, a Δ*hpn*::*cat* mutant was constructed as follows: genomic DNA from strain 26695 and primers Δhpn1 and Δhpn2 were used to amplify a 409 bp-long DNA sequence containing part of the *hp1428* gene (24), the *hp1427* (*hpn*)-*hp1428* intergenic region, and the beginning of a *cat* (Cm^r) cassette; in a separate PCR, primers Δhpn3 and Δhpn4 were used to amplify a 407-bp-long sequence encompassing the end of *hpn*, the *hpn*/*hp1426* intergenic region, and part of *hp1426*, as well as the end of the *cat* cassette. The final amplification step included each PCR product and an 800-bp-long *cat* cassette (with its own promoter and no transcription termination sequence), as well as primers Δhpn1 and Δhpn4. The resulting 1.6-kbp-long PCR product was introduced into *H. pylori* 26695 to generate a Δ*hpn*::*cat* mutant. Mutants were isolated on BA plates supplemented with chloramphenicol. An *hpn*-like::*aphA3* PCR construct was constructed as follows: genomic DNA and primers Δhpn-like1 and Δhpn-like2 were used to amplify a 235-bp-long DNA sequence containing the sequence upstream of *hp1432* (*hpn*-like) and the beginning of an *aphA3* (Kan^r) cassette; in a separate PCR, primers Δhpn-like3 and Δhpn-like4 were used to amplify a 237-bp-long sequence containing the end of *hpn*-like, the *hpn*-like/*hp1433* intergenic region, and the end of the *aphA3* cassette. The final amplification step included each PCR product and an 800-bp-long *aphA3* cassette (with its own promoter and no transcription termination sequence), as well as primers Δhpn-like1 and Δhpn-like4. The resulting 1.25-kbp-long PCR product was introduced within a Δ*hpn*::*cat* mutant to generate a Δ*hpn* Δ*hpn*-like double mutant. Double mutants were isolated on BA plates supplemented with both chloramphenicol and kanamycin. Disruption of both genes was confirmed by PCR using mutant genomic DNA. PCR products were sequenced (Georgia Genomics Facility, University of Georgia) to ensure that no error was introduced by PCR.

Anti-Hpn and anti-Hpn-like antisera and immunoblotting. Two peptides specific for Hpn (HHHYHGGEHHHHHHS and CSTSDSHHQ EEG) and two peptides specific for Hpn-like (MAHHEQQQQQQANS and HNAQQHAEQQAEQQ) were synthesized and conjugated by EZBiolab (Westfield, IN). The mixture of the two specific peptides was used to raise anti-Hpn- or anti-Hpn-like in rabbits, following EZBiolab conventional antiserum production protocols.

Crude cell extracts of *H. pylori* wild-type or Δ*hpn* Δ*hpn*-like double-mutant cells were subjected to SDS-PAGE. The proteins were electrotransferred onto a nitrocellulose membrane. The membrane was then incubated with either anti-Hpn or anti-Hpn-like antiserum (1:500 dilution) followed by incubation with secondary goat anti-rabbit-IgG conjugated with alkaline phosphatase (1:2,000 dilution; Bio-Rad).

Depletion of Ni levels in the host. Depletion of Ni levels in the host was achieved through implementation of the following. (i) Housing: plastic cages (Innocage IVC), food trays, water bottles, and spouts (Innovive Inc., San Diego, CA) were preferred over stainless steel items (which contain traces of Ni). (ii) Food: the composition of the mouse feed is defined, using ultrapure ingredients; the casein used as a source of protein was EDTA treated in our lab before being sent to reconstitute the final product (see below). (iii) Water: deionized Ni-free water was the only source of water given to the mice. The food, water, and mice never contacted metal components.

Preparation of defined mouse feed. The detailed composition of Ni-depleted and Ni-supplemented feed is shown in Table S2 in the supplemental material. Casein is a main mouse chow ingredient (150 g/kg) and contains some Ni (<0.8 ppm, as shown by inductively coupled plasma mass spectrometry [ICP-MS], Harlan Teklad Laboratories). Therefore, to establish an Ni-free medium, Ni was eliminated from casein by following a previously published protocol (25) with slight modification: briefly, 1.25 kg of casein (Harlan Laboratories, Madison, WI) was mixed with 9 liters of deionized water, heated at 50°C, and stirred with 6.2 g of Na-EDTA for 30 min. The slurry was allowed to settle, the supernatant was withdrawn, and the same (EDTA) treatment was repeated four times. Then the slurry was rinsed four times with 10 liters of deionized water (for each rinse) and finally mixed with 2 liters of 95% ethanol before being air dried, as previously described (26). The dried, metal-free casein was processed by Harlan Laboratories with components and metals of the highest purity grade available (Alfa Aesar, Ward Hill, MA) to produce the Ni-depleted mouse feed. The Ni-supplemented feed was made by adding 2.2 ppm of ultrapure nickel carbonate hydroxide tetrahydrate (1.1 ppm Ni) to the Ni-depleted chow (see Table S2).

Determination of Ni levels by atomic absorption. The amount of Ni in defined mouse feed and in mouse sera was determined by atomic absorption using an AA6701 graphite furnace spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD). An atomic absorption-grade Ni solution was used to generate a standard curve (0 to 0.4 μM Ni/0 to 23.5 ppb Ni). All samples, including the standard solution, were diluted in 0.1% HNO_3. A volume of 10 μl was injected into the furnace. For each sample, measurements were done at least 3 times and up to 7 times (until a 15% coefficient of variation [CV] per triplicate was obtained) and values from the best triplicate were used for calculation.

For determination of Ni levels in defined (Ni-free or Ni-supplemented) mouse feed, 100 mg of either chow was incubated in 5 ml of hot 0.1% HNO_3 until completely solubilized. For determination of Ni levels in mouse sera, mice were euthanized and then subsequently bled. Blood was collected and left at 37°C for 1 h and then 4°C for 1 h, and serum was isolated from erythrocytes by centrifugation (2,000 rpm for 2 min). Serum was diluted 10-fold in 0.1% HNO_3 before being analyzed by atomic absorption.

Mouse colonization experiments. *H. pylori* X47 (mouse-adapted, parental strain) or an X47 Δ*hpn* Δ*hpn*-like double-mutant strain were grown on BA plates, harvested, washed thrice in 8 g/liter ultrapure NaCl (99.999%; Sigma-Aldrich), and resuspended to a final optical density at 600 nm (OD_{600}) of 3.0. A group of mice was maintained with an Ni-depleted environment/diet and another group was maintained with an Ni-supplemented environment/diet for 2 weeks before inoculation (0.1 ml of bacterial suspension) by oral gavage. The inoculating needle was filled with 5 mM Na-dimethyl glyoxime (DMG) and left submerged in the DMG solution for 20 min; it was then rinsed by flushing 8 times with double-distilled water. After inoculation, mice were kept in the same Ni-controlled environment for 2 weeks, 6 weeks, or 8 weeks and then sacrificed. Stomachs were quickly removed, weighed, and gently homogenized in 5 ml phosphate-buffered saline (PBS) using a Dounce hand homogenizer. Dilutions were made in PBS, 100 μl of each dilution was plated in duplicate, and plates were incubated for 5 to 7 days at 37°C in a 2% O_2 partial-pressure atmosphere for colony counting. Since mouse-to-mouse variation is common in pathogenesis experiments, a Wilcoxon rank *U* test was used to statistically treat the colonization results (27).

RESULTS

Construction and genetic characterization of the Δ*hpn* Δ*hpn*-like double mutant. Previous *hpn* or *hpn*-like single or double mutants were generated in *H. pylori* strain 43504 by insertion of antibiotic resistance genes within unique restriction sites of each respective gene (23). To rule out the possibility of having truncated proteins still synthesized, deletion mutants were constructed in the mouse colonizing strain X-47 using overlapping PCR. A single Δ*hpn*::*cat* mutant was first generated and then used as the recipient to generate a Δ*hpn*::*cat* Δ*hpn*-like::*aphA3* double-mutant strain. Following homologous recombination, the chromosomal deletion of both genes was confirmed by PCR. Using primers specific for sequences flanking each gene, two unique products with the expected size (corresponding to the concomitant deletion of *hpn* and *hpn*-like and the insertion of *cat* and *aphA3*, respectively) were visualized on an agarose gel (data not shown). PCR products were finally sequenced and compared to DNA sequences from the X47 parental strain. Immunoblotting using anti-Hpn and anti-Hpn-like antisera confirmed that both proteins were synthesized in the X-47 wild-type strain but neither protein was synthesized in the double mutant (data not shown).

Assessment of Ni depletion or Ni supplementation in defined mouse feed. To study the need for nickel or other metals by bacteria, chelating chemicals such as Chelex, EDTA, or dimethyl glyoxime (DMG) are sometimes included in the medium. However, this approach was not used in the present study because of toxicity concerns for the animal. Instead, Ni levels were decreased in the host (mouse) by ensuring that the environment (housing, bedding, food and water supply) contained as little Ni as possible, if any (see Materials and Methods). Defined, Ni-depleted or Ni-supplemented (1.1 μg Ni per g) feed was manufactured, and Ni levels were measured by atomic absorption spectrophotometry. We detected 1.05 ± 0.05 μg of Ni per g of Ni-supplemented chow, in agreement with the expected value. In contrast, nickel levels in the Ni-depleted feed were 30-fold lower (33 ± 25 ng Ni per g) than in the Ni-supplemented feed, and in some cases Ni was even undetectable (detection limit, 14 ng). These results confirmed the efficacy of the Ni depletion from the mouse feed.

Ni-depleted environment lowers Ni serum concentrations in mice. The effects of an Ni-depleted versus Ni-supplemented environment on the host Ni levels were assessed by measuring the amount of Ni in mouse sera from each group. A group of four mice was maintained in an Ni-supplemented environment for 4 weeks while eight other mice were kept in an Ni-depleted environment: four mice for 4 weeks and four mice for 5 weeks. All mice were then euthanized, their blood was collected, and serum Ni levels were determined by using atomic absorption spectrophotometry (Table 1). Nickel concentrations in the sera of the Ni-supplemented group were always detectable and ranged from 58 ± 1 to 122 ± 9 ng Ni per μl serum. In contrast, two mice from the 4-week-Ni-depleted subgroup possessed serum Ni levels that were not detectable. Mice left for 5 weeks in an Ni-depleted environment all possessed serum Ni levels below the limit of detection. Overall, these results confirmed that it is possible to significantly restrict the Ni levels in the host through the selective Ni-poor environment.

Effects of Ni-defined environments on *H. pylori* colonization levels. A group of mice was maintained in an Ni-depleted environment while another group was kept in an Ni-supplemented environment. After 2 weeks, each group was further divided into two subgroups, one inoculated with *H. pylori* wild-type strain X47 and the other group with the Δ*hpn* Δ*hpn*-like double-mutant strain. Mice were kept in their respective Ni-controlled environments for another 6 weeks or 8 weeks, after which they were euthanized, their stomachs were quickly removed and homogenized, and *H. pylori* bacterial counts were assessed. The effect of Ni-defined (depleted versus supplemented) environment on each strain's colonization ability was assessed by (i) comparing the average bacterial count and (ii) ranking the colonization levels from highest to lowest; the correlation between rankings and Ni depletion or Ni supplementation was further analyzed using Wilcoxon's statistical test.

There was a clear correlation between Ni environment and colonization levels for the Δ*hpn* Δ*hpn*-like double-mutant strain; this correlation was observed after 6 weeks and 8 weeks postinoculation (Table 2). Indeed, colonization levels for the double mutant were about 3-fold higher in mice kept in an Ni-supplemented environment than those in mice kept in an Ni-depleted environment. Based on the rank distribution, colonization levels in Ni-supplemented mice were statistically higher ($P < 0.01$) than colonization levels in Ni-depleted mice according to Wilcoxon's U test, for both $t = 6$ weeks and $t = 8$ weeks. The same trend was observed in a shorter-term experiment: when mice were sacrificed only 2 weeks after being inoculated with the double mutant, colonization levels in the Ni-supplemented mice (6.8×10^6 CFU/g of stomach; $n = 5$) were more than 2-fold higher than colonization levels in the Ni-depleted mice (3.1×10^6 CFU/g of stomach; $n = 6$). In contrast, there was no significant difference between wild-type strain colonization levels when comparing mice from Ni-depleted and Ni-supplemented host environments after 6 weeks or 8 weeks postinoculation (Table 3); indeed, average colonization

TABLE 1 Ni levels in mouse sera from mice kept in an Ni-depleted or Ni-supplemented environment[a]

Host environment (no. of weeks) and mouse no.	Serum nickel concn (ppb)[b]
Ni supplemented (4)	
1	122 ± 9
2	58 ± 1
3	85 ± 6
4	73 ± 9
Ni depleted (4)	
5	79 ± 7
6	40 ± 5
7	ND
8	ND
Ni depleted (5)	
9	ND
10	ND
11	ND
12	ND

[a] Nickel was measured by atomic absorption spectrophotometry. Atomic absorption grade nickel standards (0 to 23.6 ppb Ni) were used to generate a standard curve. Measurements were done until a 15% CV per triplicate was obtained (maximum $n = 7$).

[b] Average and standard deviation shown were calculated using triplicate values with best CV. ND, below detection limit (29.5 ppb); no triplicate met the minimum requirement (CV < 15%).

TABLE 2 Colonization levels of *H. pylori* Δ*hpn* Δ*hpn*-like double mutant strain as a function of host Ni environment

Six wk postinoculation						Eight wk postinoculation					
Ni-depleted environment			Ni-supplemented environment			Ni-depleted environment			Ni-supplemented environment		
Mouse	CFU/g	Rank[a]	Mouse	CFU/g	Rank[a]	Mouse	CFU/g	Rank[a]	Mouse	CFU/g	Rank[a]
1	ND[b]	16	9	8.88×10^6	1	1	9.80×10^5	13	9	4.73×10^6	4
2	ND	15	10	1.41×10^6	9	2	3.40×10^5	15	10	1.09×10^7	1
3	7.70×10^5	13	11	4.84×10^6	3	3	8.10×10^5	14	11	5.33×10^6	3
4	1.04×10^6	10	12	2.59×10^6	8	4	8.00×10^4	16	12	1.01×10^7	2
5	8.60×10^5	12	13	5.38×10^6	2	5	4.26×10^6	7	13	4.42×10^6	5
6	6.90×10^5	14	14	1.03×10^6	11	6	1.50×10^6	11	14	1.75×10^6	10
7	3.15×10^6	6	15	2.71×10^6	7	7	2.55×10^6	9	15	2.97×10^6	8
8	3.62×10^6	5	16	3.75×10^6	4	8	4.38×10^6	6	16	1.06×10^6	12
Avg	1.27×10^6		Avg	3.82×10^6		Avg	1.87×10^6		Avg	5.16×10^6	

[a] For each experiment ($n = 16$ mice), colonization levels (CFU per g of stomach) were ranked, 1 being the highest colonization level and 16 the lowest colonization level. Based on the rank distribution, colonization levels of Ni-supplemented mice are statistically higher ($P < 0.01$) than colonization levels of Ni-depleted mice according to Wilcoxon's *U* test.
[b] ND, not detectable; i.e., the number of CFU per g of stomach is below the detection limit (330 CFU/g).

levels were comparable and there was no statistically significant correlation between colonization level rankings and each Ni-defined environment ($P > 0.2$). Taken together, our results indicate that the gastric pathogen *H. pylori* can store nickel (via the nickel storage proteins Hpn and Hpn-like) to aid its colonization of the mouse stomach.

DISCUSSION

Nickel is a key metal for the gastric pathogen *H. pylori*, as it is the cofactor for two important enzymes: hydrogenase and urease. Both enzymes have been shown to be important for colonization of various animal hosts (4, 5, 7), and a previous study linked urease maturation and host colonization (8). Urease is one of the most abundant enzymes in *H. pylori*, which suggests that the demand for nickel is high in this microorganism. Nickel is naturally abundant in all types of food (28); at the same time, based on the fact that there are no known "nickel enzymes" (i.e., enzymes for which nickel is an essential cofactor in catalytic activity), nickel does not appear to be essential for humans and other higher-living organisms. Since there is no "competition" for nickel between the host and the bacterium, most often there is likely to be plenty of nickel available for *H. pylori*. However, the flux of nickel and other nutrients is not continuous: because of its stringent habitat (human stomach), *H. pylori* is likely faced with successive batch-like conditions rather than a constant stream of nutrients and metals. In addition, nickel availability is also a function of pH; the latter fluctuates widely within the gastric compartments. As a consequence, it seems important for *H. pylori* to be able to store nickel at times when (relatively) higher exogenous concentrations are available; this intracellular nickel reservoir would in turn be available for urease or/and hydrogenase maturation when nickel concentrations are limited. Based on previous biochemical characterization of purified proteins, as well as mutant-based studies, two *H. pylori* proteins, Hpn and Hpn-like, were proposed to play such an Ni storage role (17, 20, 21); this role was more specifically linked to urease maturation (23).

In the present study, we aimed at deciphering the physiological role of Hpn and Hpn-like proteins within the host, by determining colonization levels of wild-type and Δ*hpn* Δ*hpn*-like double-mutant cells in mice subjected to Ni-depleted or Ni-supplemented environments. The strategy of modulating Ni levels within mice by controlling the environment was favored over using specific Ni-chelating agents because of health concerns for the animals. While the effects of a depleted or toxic Ni environment have been studied in rats and chicks (28), this is the first time a study has attempted to correlate host Ni levels and colonization of a pathogenic bacterium based on the presence or absence of specific bacterial Ni-binding proteins. In addition, the study herein focuses only on nickel storage or requirement for the bacterium and not on nickel toxicity: the concentration of nickel used for the Ni-supplemented feed was 1.1 ppm Ni (18.7 μM Ni), far below toxic Ni levels reported for the wild-type strain (low mM range) or the

TABLE 3 Colonization levels of *H. pylori* wild-type strain as a function of host Ni environment

Six wk postinoculation						Eight wk postinoculation					
Ni-depleted environment			Ni-supplemented environment			Ni-depleted environment			Ni-supplemented environment		
Mouse	CFU/g	Rank[a]	Mouse	CFU/g	Rank[a]	Mouse	CFU/g	Rank[a]	Mouse	CFU/g	Rank[a]
1	7.05×10^6	1	7	4.64×10^6	2	1	3.00×10^5	7	6	2.00×10^5	8
2	3.46×10^6	3	8	1.50×10^6	9	2	3.10×10^5	6	7	1.70×10^5	10
3	2.19×10^6	6	9	6.25×10^5	10	3	1.78×10^6	2	8	2.77×10^6	1
4	2.50×10^5	11	10	2.50×10^6	5	4	1.70×10^5	9	9	3.90×10^5	5
5	3.44×10^6	4	11	2.14×10^6	7	5	9.00×10^4	11	10	8.50×10^5	3
6	1.53×10^6	8							11	7.50×10^5	4
Avg	2.98×10^6		Avg.	2.28×10^6		Avg.	5.30×10^5		Avg.	8.51×10^5	

[a] For each experiment ($n = 11$ mice), colonization levels (CFU per g of stomach) were ranked, 1 being the highest colonization level and 11 the lowest colonization level. Based on the rank distribution, there is no statistical difference ($P > 0.2$) between colonization levels of Ni-depleted mice and Ni-supplemented mice according to Wilcoxon's *U* test.

Δ*hpn* Δ*hpn*-like double-mutant strain (high μM range) (22, 23). Based on colonization averages and rank distribution, colonization levels of the X-47 parental strain were similar regardless of Ni conditions in the host whereas the Δ*hpn* Δ*hpn*-like double mutant colonized the Ni-supplemented mice at a significantly greater level than the Ni-depleted animals.

Taken together, our results suggest that the presence of Hpn and Hpn-like enables *H. pylori* to successfully colonize the host stomach even when Ni is scarce (Ni-depleted environment). Since previous results indicated a link between Hpn, Hpn-like, and urease Ni maturation, we conclude that this *in vivo* phenotype is likely due to the ability of Hpn and Hpn-like to provide Ni to urease, thereby helping the gastric pathogen to survive and grow in the acidic environment. Since *hpn* and *hpn*-like genes have been found only in *H. pylori* so far (29), they appear to be yet another hallmark of *H. pylori*, and they must certainly contribute to the unique ability of *H. pylori* to persistently colonize the gastric environment.

ACKNOWLEDGMENTS

This work was supported by NIH RO1 DK062852 and by the University of Georgia Foundation.

We thank Susan Maier for helping with the mouse colonization experiments.

REFERENCES

1. **Blaser MJ.** 1995. The role of *Helicobacter pylori* in gastritis and its progression to peptic ulcer disease. Aliment. Pharmacol. Ther. **9**(Suppl 1):27–30.
2. **Sipponen P, Hyvarinen H, Seppala K, Blaser MJ.** 1998. Review article: pathogenesis of the transformation from gastritis to malignancy. Aliment. Pharmacol. Ther. **12**(Suppl 1):61–71.
3. **Maier RJ, Fu C, Gilbert J, Moshiri F, Olson J, Plaut AG.** 1996. Hydrogen uptake hydrogenase in *Helicobacter pylori.* FEMS Microbiol. Lett. **141**: 71–76.
4. **Olson JW, Maier RJ.** 2002. Molecular hydrogen as an energy source for *Helicobacter pylori.* Science **298**:1788–1790.
5. **Tsuda M, Karita M, Morshed MG, Okita K, Nakazawa T.** 1994. A urease-negative mutant of *Helicobacter pylori* constructed by allelic exchange mutagenesis lacks the ability to colonize the nude mouse stomach. Infect. Immun. **62**:3586–3589.
6. **Eaton KA, Brooks CL, Morgan DR, Krakowka S.** 1991. Essential role of urease in pathogenesis of gastritis induced by *Helicobacter pylori* in gnotobiotic piglets. Infect. Immun. **59**:2470–2475.
7. **Wirth HP, Beins MH, Yang M, Tham KT, Blaser MJ.** 1998. Experimental infection of Mongolian gerbils with wild-type and mutant *Helicobacter pylori* strains. Infect. Immun. **66**:4856–4866.
8. **Eaton KA, Krakowka S.** 1994. Effect of gastric pH on urease-dependent colonization of gnotobiotic piglets by *Helicobacter pylori.* Infect. Immun. **62**:3604–3607.
9. **Schauer K, Gouget B, Carriere M, Labigne A, de Reuse H.** 2007. Novel nickel transport mechanism across the bacterial outer membrane energized by the TonB/ExbB/ExbD machinery. Mol. Microbiol. **63**:1054–1068.
10. **Ernst FD, Stoof J, Horrevoets WM, Kuipers EJ, Kusters JG, van Vliet AH.** 2006. NikR mediates nickel-responsive transcriptional repression of the *Helicobacter pylori* outer membrane proteins FecA3 (HP1400) and FrpB4 (HP1512). Infect. Immun. **74**:6821–6828.
11. **Bauerfeind P, Garner RM, Mobley LT.** 1996. Allelic exchange mutagenesis of *nixA* in *Helicobacter pylori* results in reduced nickel transport and urease activity. Infect. Immun. **64**:2877–2880.
12. **Stahler FN, Odenbreit S, Haas R, Wilrich J, Van Vliet AH, Kusters JG, Kist M, Bereswill S.** 2006. The novel *Helicobacter pylori* CznABC metal efflux pump is required for cadmium, zinc, and nickel resistance, urease modulation, and gastric colonization. Infect. Immun. **74**:3845–3852.
13. **Contreras M, Thiberge JM, Mandrand-Berthelot MA, Labigne A.** 2003. Characterization of the roles of NikR, a nickel-responsive pleiotropic autoregulator of *Helicobacter pylori.* Mol. Microbiol. **49**:947–963.
14. **van Vliet AH, Poppelaars SW, Davies BJ, Stoof J, Bereswill S, Kist M, Penn CW, Kuipers EJ, Kusters JG.** 2002. NikR mediates nickel-responsive transcriptional induction of urease expression in *Helicobacter pylori.* Infect. Immun. **70**:2846–2852.
15. **Benoit SL, Maier RJ.** 2011. Mua (HP0868) is a nickel-binding protein that modulates urease activity in *Helicobacter pylori.* mBio **2**:e00039–11. doi: 10.1128/mBio.00039-11.
16. **Schauer K, Muller C, Carriere M, Labigne A, Cavazza C, De Reuse H.** 2010. The *Helicobacter pylori* GroES cochaperonin HspA functions as a specialized nickel chaperone and sequestration protein through its unique C-terminal extension. J. Bacteriol. **192**:1231–1237.
17. **Gilbert JV, Ramakrishna J, Sunderman FW, Jr, Wright A, Plaut AG.** 1995. Protein Hpn: cloning and characterization of a histidine-rich metal-binding polypeptide in *Helicobacter pylori* and *Helicobacter mustelae.* Infect. Immun. **63**:2682–2688.
18. **Bury-Mone S, Thiberge JM, Contreras M, Maitournam A, Labigne A, De Reuse H.** 2004. Responsiveness to acidity via metal ion regulators mediates virulence in the gastric pathogen *Helicobacter pylori.* Mol. Microbiol. **53**:623–638.
19. **Pflock M, Finsterer N, Joseph B, Mollenkopf H, Meyer TF, Beier D.** 2006. Characterization of the ArsRS regulon of *Helicobacter pylori*, involved in acid adaptation. J. Bacteriol. **188**:3449–3462.
20. **Ge R, Watt RM, Sun X, Tanner JA, He QY, Huang JD, Sun H.** 2006. Expression and characterization of a histidine-rich protein, Hpn: potential for Ni^{2+} storage in *Helicobacter pylori.* Biochem. J. **393**:285–293.
21. **Zeng YB, Zhang DM, Li H, Sun H.** 2008. Binding of Ni^{2+} to a histidine- and glutamine-rich protein, Hpn-like. J. Biol. Inorg. Chem. **13**:1121–1131.
22. **Mobley HL, Garner RM, Chippendale GR, Gilbert JV, Kane AV, Plaut AG.** 1999. Role of Hpn and NixA of *Helicobacter pylori* in susceptibility and resistance to bismuth and other metal ions. Helicobacter **4**:162–169.
23. **Seshadri S, Benoit SL, Maier RJ.** 2007. Roles of His-rich Hpn and Hpn-like proteins in *Helicobacter pylori* nickel physiology. J. Bacteriol. **189**: 4120–4126.
24. **Tomb JF, White O, Kerlavage AR, Clayton RA, Sutton GG, Fleischmann RD, Ketchum KA, Klenk HP, Gill S, Dougherty BA, Nelson K, Quackenbush J, Zhou L, Kirkness EF, Peterson S, Loftus B, Richardson D, Dodson R, Khalak HG, Glodek A, McKenney K, Fitzegerald LM, Lee N, Adams MD, Hickey EK, Berg DE, Gocayne JD, Utterback TR, Peterson JD, Kelley JM, Cotton MD, Weidman JM, Fujii C, Bowman C, Watthey L, Wallin E, Hayes WS, Borodovsky M, Karp PD, Smith HO, Fraser CM, Venter JC.** 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori.* Nature **388**:539–547.
25. **Davis PN, Norris LC, Kratzer FH.** 1962. Iron deficiency studies in chicks using treated isolated soybean protein diets. J. Nutr. **78**:445–453.
26. **Alberts JC, Lang JA, Reyes PS, Briggs GM.** 1977. Zinc requirement of the young guinea pig. J. Nutr. **107**:1517–1527.
27. **Wang G, Conover RC, Benoit S, Olczak AA, Olson JW, Johnson MK, Maier RJ.** 2004. Role of a bacterial organic hydroperoxide detoxification system in preventing catalase inactivation. J. Biol. Chem. **279**:51908–51914.
28. **Denkhaus E, Salnikow K.** 2002. Nickel essentiality, toxicity, and carcinogenicity. Crit. Rev. Oncol. Hematol. **42**:35–56.
29. **Benoit SL, Maier RJ.** 2008. Hydrogen and nickel metabolism in helicobacter species. Ann. N. Y. Acad. Sci. **1125**:242–251.