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## Nickel enzyme maturation in *Helicobacter hepaticus*: roles of accessory proteins in hydrogenase and urease activities

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### Abstract

*Helicobacter hepaticus*, a causative agent of chronic hepatitis and hepatocellular carcinoma in mice, possesses a hydrogenase and a urease, both of which are nickel-containing enzymes. Analysis of the genome sequence of *H. hepaticus* revealed a full set of accessory genes which are required for the nickel maturation of each enzyme in other micro-organisms. Erythromycin-resistant mutants were constructed in four of these genes, *hypA*, *hypB*, *ureE* and *ureG*. Controls for polar effect were provided for *hypA* or *hypB* mutants by disrupting each gene located immediately downstream, i.e. *hp0809* or *hypC*, respectively. Urease and hydrogenase activities were determined for each strain with or without supplemented nickel in the medium. As expected, the *ureE* and the *ureG* mutants had negligible urease activity, but they retained normal levels of hydrogenase activity. Urease levels could not be increased by the addition of nickel to the medium. The *H. hepaticus hypA* and *hypB* strains were deficient in both urease and hydrogenase activities, suggesting that both gene products act in a similar fashion as their counterparts in *H. pylori*. However, in contrast with the analogous mutants of *H. pylori*, the addition of nickel into the growth medium failed to restore either urease or hydrogenase enzyme levels in the *H. hepaticus hypA* or *hypB* mutants, indicating a probably unique role for these genes in the mouse liver pathogen.

### INTRODUCTION

*Helicobacter hepaticus* is a spiral-shaped Gram-negative microaerophilic bacterium (Fox *et al.*, 1994). It has been shown to be a causative agent of hepatic and enteric inflammatory diseases and malignancy in mice (Solnick & Schauer, 2001). Therefore, it is an excellent model for studying mechanisms of bacterium-associated liver carcinogenesis. The best known member of the genus is *Helicobacter pylori*, which causes peptic ulcers and gastric cancers in humans (Blaser, 1995; Sipponen *et al.*, 1998). In this well studied bacterium, two nickel-containing enzymes, the hydrogen uptake hydrogenase and urease, have been shown to be required for early colonization and persistence in the stomach (Eaton *et al.*, 1991; Olson & Maier, 2002). *H. hepaticus* also possesses both enzymes (Beckwith *et al.*, 2001; Mehta *et al.*, 2005; Suerbaum *et al.*, 2003). While it is known that *H. hepaticus* hydrogenase mutants are deficient in hydrogen-supported amino acid uptake and cannot cause liver lesions in mice (Mehta *et al.*, 2005), the role of urease in *H. hepaticus* survival in the host remains to be demonstrated.

In every bacterium studied so far, the maturation of hydrogenase or urease is a complex process which requires several accessory proteins. In *H. pylori*, hydrogenase maturation is achieved with the help of Hyp proteins (HypABCDEF) (Benoit *et al.*, 2004; Olson *et al.*, 2001), while urease maturation requires a set of accessory Ure proteins (UreEFGH) (Mehta *et al.*, 2003a; Volland *et al.*, 2003). Interestingly, in the gastric pathogen two hydrogenase accessory proteins, HypA and HypB, have been shown to be involved in urease maturation as well (Olson *et al.*, 2001). *H. pylori* HypA is known to bind nickel (Mehta *et al.*, 2003b), as well as zinc (Kennedy *et al.*, 2007). The involvement of HypA in the nickel activation of urease is mediated through an identified interaction between this accessory protein and UreE, another nickel-binding protein likely to be the preferred donor to the urease apoenzyme (Benoit *et al.*, 2007). Studies on HypB support its role as a GTPase enzyme (Mehta *et al.*, 2003b) and the same role has been assigned to the UreG accessory protein. In both the *H. pylori* *hypA* and *hypB* mutants, addition of excess nickel to the medium can fully or partially restore enzyme activities to wild-type hydrogenase or urease levels, respectively (Olson *et al.*, 2001). In contrast, the urease activity is severely affected in both the *H. pylori* *ureE* and *ureG* mutants and nickel supplementation cannot restore it to wild-type levels (Benoit & Maier, 2003; Benoit *et al.*, 2007; Mehta *et al.*, 2003a); the hydrogenase activity is not affected in these mutants.

Analysis of the *H. hepaticus* genome sequence (Suerbaum *et al.*, 2003) has revealed the presence of *hypA* and *hypB* gene homologues, which prompted us to check whether inactivation of either gene would have a pleiotropic effect on both the urease and hydrogenase activity, as observed in *H. pylori*. Therefore, both genes were disrupted by insertion of an erythromycin resistance cassette and the effect on nickel enzyme activities was determined. The same procedure was followed for the *ureE* and *ureG* genes. To ensure that the observed phenotype was not due to polar effects on the genes transcribed downstream of each targeted gene, genes located immediately downstream of either *hypA* or *hypB* (that is, *hp0809* or *hypC*, respectively) were individually disrupted as controls. The effect of nickel supplementation in the medium on nickel-related enzyme activities (hydrogenase or urease) was investigated in the mutant strains as well as the control strains. Taken together, our results suggest that while *H. hepaticus* *ureE* and *ureG* (and *hypC*) have similar functions as their counterparts in *H. pylori*, *hypA* and *hypB* seem to play different mechanistic roles when compared to their respective homologues in *H. pylori*.

## METHODS

### Bacterial strains and growth conditions

*Helicobacter hepaticus* and *Escherichia coli* strains used in this study are listed in Table 1. *H. hepaticus* strain ATCC 51449 was the parent strain. *H. hepaticus* cells were routinely grown at 37 °C on Brucella agar (Difco) plates supplemented with 10% defibrinated sheep blood (BA plates) under microaerophilic conditions (1–2% O<sub>2</sub>, 5% CO<sub>2</sub>, N<sub>2</sub>). The effect of nickel supplementation on urease and hydrogenase activity was studied by adding 1, 5, 10 or 50 µg NiCl<sub>2</sub> ml<sup>-1</sup> into the medium. *E. coli* was grown aerobically in Luria–Bertani (LB) or Brain–Heart infusion (BHI) medium or on plates at 37 °C. When required, the following antibiotics

were added into the medium: erythromycin (150  $\mu\text{g ml}^{-1}$  in BHI or 5  $\mu\text{g ml}^{-1}$  in BA), ampicillin (100  $\mu\text{g ml}^{-1}$  in LB) or chloramphenicol (30  $\mu\text{g ml}^{-1}$  in LB or 20  $\mu\text{g ml}^{-1}$  in BA).

### Construction of site-directed *H. hepaticus* mutants

Genomic DNA from strain 51449 was used as a template to amplify *hypA* and part of the *hh0809* gene, *hypB*, *hypC*, *ureE* or *ureG*. Primers (Table 2) were designed to amplify each gene together with some flanking sequences in order to have at least 200 bp on each side of the restriction site used for the marker insertion. PCR products of 760, 814, 734 or 904 bp were obtained for *hypA* and part of *hh0809*, *hypB*, *ureE* or *ureG*, respectively, and cloned into either pGEM-T or pBC-KS vector (Table 1), before being transformed into *E. coli*. To generate allelic exchange vectors for construction of *hypA*, *hypB* or *ureE* mutants, an 1140 bp long *ery* cassette conferring resistance to erythromycin (Mehta *et al.*, 2007; Stabb & Ruby, 2002) was inserted into a unique restriction site within each gene (Table 1 and Fig. 1). Since there was no convenient unique site within *ureG*, two different approaches were followed. First, a new *HindIII* restriction site was generated in this gene using the overlapping PCR approach; briefly, two PCR products were obtained by using either primers UpureG with UreGmut2 or RevureG with UreGmut1, gel-purified, mixed together and a final PCR product containing a new *HindIII* site was obtained after amplification with UpureG and RevureG primers. This PCR product was cloned into pGEM-T vector and the new restriction site was used to insert the *ery* gene, generating plasmid pSLB175 (Table 1). For the second approach, a *ureG::ery* vector was constructed by cloning the 904 bp PCR product containing *ureG* into pBC-KS; two *AvaII* sites present within *ureG* were used to insert the *ery* gene, generating plasmid pSLB173 (Table 1).

Site-directed mutants in *hh0809* or *hypC*, the respective controls (for polar effect) of *hypA* or *hypB* mutants, were constructed as follows. Since part of *hh0809* (242 bp) is also present in the pGEM-hypA plasmid, a unique *MluI* restriction site located at the beginning of *hh0809* was used to insert the *ery* cassette. However, prior to use of this site, another *MluI* site (from pGEM-T) had to be removed by digesting pGEM-hypA with *PstI* and *NsiI* and religating. The newly generated plasmid, pSLB290, was then digested with *MluI* and ligated with *ery* to yield plasmid pSLB293 (Table 1). All plasmids were checked by restriction analysis. To construct the *H. hepaticus hypC* mutant, an all-PCR overlapping approach was used. Briefly, two sets of primers, UphypC with hypC-ery1 and RevhypC with hypC-ery2, were used in independent PCR reactions to amplify *hypC* upstream and *hypC* downstream fragments of 280 and 505 bp, respectively. Primers hypC-ery1 and hypC-ery2 were designed to introduce *ery* priming sequences into each PCR product. A final round of PCR was carried out with both (purified) PCR products, along with the whole *ery* cassette as well as primers UphypC and RevhypC. Finally, an 1885 bp PCR product was obtained, purified and directly introduced into wild-type strain 51449 to generate a *hypC::ery* mutant.

Each of the six allelic exchange suicide vectors designed to generate either *hypA::ery*, *hypB::ery*, *hh0809::ery*, *ureE::ery*, *ureG::ery* or *ureG::ery* was introduced into *H. hepaticus* strain 51449 by electroporation (a pulse of 2.5 kV in a Transporator Plus; BTX). Cells were plated on non-selective BA medium and transferred after 24 h onto BA plates supplemented with 5  $\mu\text{g}$  erythromycin  $\text{ml}^{-1}$ . Isolated clones resulting from homologous recombination

appeared after 3–5 days incubation. The disruption of each gene was confirmed by PCR using genomic DNA from each mutant as a template and specific primers for each gene.

### Urease assays

The urease activity of fresh lysates was determined by measuring ammonia production from urea hydrolysis with the phenol/hypochlorite assay as described by Weatherburn (1967). Briefly, cells were grown for 48–72 h on BA plates supplemented with various NiCl<sub>2</sub> concentrations, harvested and resuspended in 50 mM HEPES-NaOH (pH 7.5), and then washed once and resuspended in the same buffer. After sonication, the protein concentration was determined using the BCA assay (Pierce). Lysates were incubated for 20 min in presence of 25 mM urea, and the amount of ammonia released was then assayed with the phenol/hypochlorite assay. A standard ammonium chloride concentration curve was used to convert the absorbance at 625 nm to nmol ammonia. Urease specific activity is measured in units of  $\mu\text{mol ammonia produced min}^{-1} (\text{mg protein})^{-1}$ .

### Hydrogenase assays

Hydrogenase assays were carried out on intact whole cells (grown for 48–72 h) using a previously described amperometric method (Maier *et al.*, 1996). Hydrogenase specific activity is measured in units of nmol H<sub>2</sub> used min<sup>-1</sup> per 10<sup>9</sup> cells.

### Immunoblotting

Whole-cell extracts (10  $\mu\text{g}$  total protein) from cells grown without added nickel in the medium were subjected to SDS-PAGE (12.5 %) with a mini-protean II apparatus (Bio-Rad), according to the method of Laemmli (1970). Prestained SDS-PAGE standards were purchased from Bio-Rad. Proteins were transferred to nitrocellulose as described by Towbin *et al.* (1992) using a Bio-Rad Trans Blotter. Immunoblotting was performed using anti-*H. pylori* UreA or UreB rabbit antisera (a gift from H. L. Mobley, University of Michigan Medical School, USA), diluted 1 : 2000 to 1 : 5000, and goat anti-rabbit immunoglobulin G-alkaline phosphatase (Bio-Rad), diluted 1 : 2000. Bound antibodies were detected by the addition of the chromogenic reagents nitro blue tetrazolium (0.25 mg ml<sup>-1</sup>) and 5'-bromo-4-chloro-3-indolyl phosphate (0.125 mg ml<sup>-1</sup>) (Sigma).

## RESULTS

### Location and homology of the hydrogenase and urease maturation genes

Analysis of the sequenced genome of *H. hepaticus* strain 51449 revealed the presence of homologues of genes needed for the maturation of both the urease (*ure*) and hydrogenase (*hyp*) enzymes in the related species *H. pylori* (Suerbaum *et al.*, 2003). The *H. hepaticus* genes are located and arranged in a similar fashion as their *H. pylori* counterparts: all the *ure* accessory genes, *ureE* (annotated as *hh0410* in the TIGR database), *ureF* (*hh0411*), *ureG* (*hh0412*) and *ureH* (*hh0413*) are clustered in an operon that also contains the urease structural genes *ureA* (*hh0407*) and *ureB* (*hh0408*), as well as a homologue of *ureI* (*hh0409*), encoding the urea trans-porter in *H. pylori* (Fig. 1). The respective location of each *ure* gene within the *ureABIEFGH* operon is the same as in *H. pylori*. The *hyp* genes are located on two different loci. The *hypA* gene (annotated as *hh0808* in the TIGR database) is

part of a four-gene operon which also includes *hh0809*, a homologue of the *hp0868* gene whose role in *H. pylori* is still unknown despite attempts to characterize it (Mehta *et al.*, 2003b), *hh0810*, encoding a putative lipid A disaccharide synthetase, and *hh0811*, which encodes a conserved hypothetical protein. The *hypB* gene (*hh0325*) is part of a seven-gene operon which also includes *hypC* (*hh0324*), *hypD* (*hh0322*), *hypE* (*hh0320*) and *hypF* (*hh0326*) as well as two other genes whose roles have yet to be determined (*hh0321* and *hh0323*).

The corresponding proteins share somewhat significant or even high degrees of similarity or identity with their homologues in *H. pylori*. For the four proteins of interest in this study, the respective percentage of similarity and identity with homologues in the sequenced *H. pylori* strain 26695 are as follows: HypA, 71.9 and 52.6 %; HypB, 83.1 and 62.8 %; UreE, 60.8 and 30.8 %; UreG, 93 and 82.4 %. Further analysis of the protein sequence of *H. hepaticus* HypA reveals the presence of a conserved histidine residue (His2) shown to be involved in nickel binding in *H. pylori* (Mehta *et al.*, 2003b) (Fig. 2); likewise, *H. hepaticus* UreE possesses a His residue (His111), conserved among the UreE proteins and shown to participate in the nickel-binding process in the well studied *Klebsiella aerogenes* (His96) and *Bacillus pasteurii* (His100) (Colpas *et al.*, 1999; Remaut *et al.*, 2001). Both the *H. hepaticus* HypB and UreG proteins possess a nucleotide-binding domain, GSGKT, shown to be essential for the maturation of either hydrogenase or urease in *H. pylori* (Mehta *et al.*, 2003a); indeed, substitution of the conserved lysine residue with an alanine in either HypB or UreG led to inactivation of the urease (in both *hypB* and *ureG* mutants) and hydrogenase (only in the *hypB* mutant). Therefore, the presence of these conserved amino acids and the overall strong protein homology between these four *H. hepaticus* proteins and their *H. pylori* counterparts was in agreement with an anticipated role for HypA, HypB, UreE and UreG in urease and/or hydrogenase maturation.

### Construction of mutant and control strains

Primers were designed to amplify a DNA sequence specific for each gene with at least 200 bp flanking each side of the restriction site used for the marker insertion (Table 1 and Table 2). Erythromycin resistance was used to inactivate each of the four genes, as this marker has proved to be the best available so far to generate mutants in *H. hepaticus* (Mehta *et al.*, 2007). The cassette was introduced within a unique restriction site (for *hypA*, *hypB* or *ureE*) or between two naturally existing *AvaII* restriction sites (for *ureG*) (Fig. 1). Another *ureG* mutant was also created by inserting the same cassette into a unique, PCR-generated, *HindIII* restriction site. After introduction of each suicide vector into *H. hepaticus* by electroporation, cells were allowed to recover for 24 h on non-selective medium plates before being transferred onto selective plates containing erythromycin. For each experiment, dozens of recombinant *H. hepaticus* clones appeared after 3–5 days. Several colonies were picked and their genomic DNA was extracted. The presence of the erythromycin resistance cassette in each gene was confirmed by PCR, using genomic DNA from each mutant as a template and specific primers for each gene (Fig. 3). Indeed, PCR products corresponding to the size of each original PCR product (760 bp for *hypA*, 814 bp for *hypB*, 734 bp for *ureE* and 904 bp for *ureG*) plus the size of the erythromycin resistance cassette (1140 bp) were obtained. A 1669 bp PCR product was obtained when genomic DNA from the *ureG::ery*



strain was used as a template, in good agreement with the expected 375 bp deletion generated within *ureG* in this mutant.

Control strains were provided by disrupting the gene immediately downstream of *hypA* (i.e. *hh0809*) or *hypB* (i.e. *hypC*) (Fig. 1). Both mutants were generated by following the method previously used to generate other mutants (insertion of *ery* into the targeted gene). However, it is worth noting that purified PCR product (instead of plasmid DNA) was used to generate the *hypC::ery* mutant. To our knowledge, it is the first time a linear PCR product has been used for this purpose in *H. hepaticus*. The presence of the 1140 bp erythromycin resistance gene within *hh0809* or *hypC* was verified by PCR using genomic DNA from several individual colonies and primers specific for each gene (data not shown).

### Hydrogenase and urease activity in presence of various amounts of nickel

All five mutant strains (*hypA*, *hypB*, *ureE*, *ureG* and *ureG*), as well as the control *hh0809* and *hypC* strains and the wild-type strain were grown on BA medium without supplemented nickel or with increasing amounts of  $\text{NiCl}_2$  (up to 50 mM). Cells were harvested after 2 days, washed and used the same day for hydrogenase and urease assays.

Hydrogenase activity was amperometrically determined with whole cells (Table 3). The hydrogenase activity in the wild-type strain was enhanced when nickel was added to the medium, as described previously (Mehta *et al.*, 2005). The activity was the same as wild-type in the *H. hepaticus ureE* or the *ureG* mutants, suggesting that neither gene is involved in the maturation of hydrogenase in the mouse pathogen. Similar results have been reported for *H. pylori ureE* and *ureG* mutants (Benoit & Maier, 2003; Mehta *et al.*, 2003a). In contrast, in the *hypA* or *hypB* mutant, the hydrogenase activity was abolished, indicating that both genes are involved in the maturation of the respiratory enzyme, as expected. Addition of up to 10  $\mu\text{M}$  nickel to the medium could not restore the activity, although some hydrogenase activity was detected in both *hypA* and *hypB* mutant strains when 50  $\mu\text{M}$  nickel was added to the medium; this represented only about 2% of the wild-type level (Table 3). This is a significant difference from the results for *H. pylori hypA* or *hypB* mutants, which can be partially complemented even with low amounts of nickel (5  $\mu\text{M}$ ) (Olson *et al.*, 2001). The hydrogenase activity in the control strain *hh0809::ery* was comparable to the wild-type level, suggesting that this gene is not required for hydrogenase activity in *H. hepaticus*, at least under the conditions tested in this study; this result also confirmed that the hydrogenase-negative phenotype observed for the *hypA* mutant can be assigned directly to *hypA* rather than to a polar effect on *hh0809*. Hydrogenase activity was totally abolished in the *hypC::ery* control strain, indicating that the *hypC* gene is required for hydrogenase activity in *H. hepaticus* (Table 3). The result obtained with this control strain was expected, as HypC is known to be required for hydrogenase maturation in various microorganisms, including *H. pylori* and *E. coli* (Benoit *et al.*, 2004; Blokesch & Bock, 2002).

For urease assays, cells were broken, cell-free extracts were isolated and the protein concentration was determined. The ammonia produced from urea hydrolysis was measured by using the phenol/hypochlorite assay (Table 4). Addition of  $\text{NiCl}_2$  to the medium dramatically increased the urease activity in the wild-type (from  $0.06 \pm 0.02$  units of specific activity in unsupplemented medium to  $5.44 \pm 1.42$  units in medium supplemented with 50  $\mu\text{M}$

NiCl<sub>2</sub>), as reported by Belzer *et al.* (2005). Compared to the wild-type, urease activities were severely affected in all four mutants in non-nickel-supplemented medium, suggesting that *hypA*, *hypB*, *ureE* and *ureG* play a role in urease activity, as do their counterparts in *H. pylori* (Table 4). The urease activity was not restored to wild-type level in *H. hepaticus ureE::ery*, *ureG::ery* or the *ureG::ery* mutant, even when the medium was supplemented with up to 50 µM nickel. The same effect has been previously reported for *H. pylori ureE* and *ureG* mutants (Benoit *et al.*, 2007). Interestingly, the urease activity of *H. hepaticus hypA* or *hypB* mutants could not be restored to wild-type level by nickel supplementation either; this is in contrast with the urease activity of *H. pylori hypA* or *hypB* mutants, which has been shown to be complemented by adding 5 µM NiCl<sub>2</sub> to the medium (Olson *et al.*, 2001). Results obtained with the *hh0809::ery* and *hypC::ery* control strains confirmed the non-polarity effect of the *ery* cassette and the definitive involvement of both HypA and HypB in the urease maturation process. These results also suggested a possible role for the *hh0809* gene product and more probably for the HypC protein. Indeed, urease activity levels in the *hh0809::ery* control strain were in general slightly lower than that of wild-type when nickel was added at between 1 and 10 µM to the medium. In contrast, the *H. hepaticus hypC* mutant consistently displayed higher urease activity than the wild-type when grown under the same conditions. Both genes are currently being investigated for their respective roles in nickel enzyme maturation.

#### The amount of urease synthesized is similar in the mutants and the wild-type

To determine whether the decreased urease activity in the various mutants was linked to a decreased amount of urease apoenzyme, immunoblotting was performed on cell-free extracts from each mutant and the wild-type grown on unsupplemented BA medium (Fig. 4). The significant degree of identity between *H. pylori* and *H. hepaticus* UreA and UreB (65 and 69 %, respectively), allowed us to use antisera raised against *H. pylori* UreA or UreB. As shown in Fig. 4, the amount of the structural UreA subunit (expected molecular mass 25.2 kDa) was similar in every lane, indicating that the difference in urease activity between the wild-type and the *hypA*, *hypB*, *ureE* or *ureG* strains was not due to decreased synthesis of UreA. This was also observed in the *ureG::ery* mutant (data not shown). A similar result was also observed with all these mutants and the wild-type when the anti-UreB antiserum was used (data not shown). Therefore, it does not seem that a transcriptional repression mechanism of the urease structural genes accounts for the decreased urease activity in these four mutants. Rather, this phenotype is probably due to a deficient post-translational nickel maturation of the urease enzyme.

## DISCUSSION

Although *H. hepaticus* and *H. pylori* share a similar battery of accessory proteins involved in hydrogenase and/or urease maturation (i.e. UreEFGH and HypABCDEF), our work suggests that HypA, HypB and HypC have a different mechanistic role in each bacterium. While mutants in *hypA* or *hypB* are urease-negative and hydrogenase-negative, respectively, in both *Helicobacter* species, partial or total complementation of both enzyme activities by nickel supplementation only seems to happen in the human gastric pathogen, *H. pylori*. Hence, addition of up to 50 µM NiCl<sub>2</sub> in the growth medium of *H. hepaticus* did not restore

urease activity and could only restore hydrogenase activity up to 2% of the wild-type level in either the *hypA* or the *hypB* mutant. This unexpected absence of complementation by nickel in both mutants indicates that both proteins are required at all times for urease activity as well as hydrogenase activity in this bacterium, independently of the nickel content of the cell (the latter is assumed to increase with increasing amounts of nickel added to the medium). These data further suggest that both proteins could be involved in the final stages of maturation of either of the nickel-containing proteins in the mouse liver pathogen, but they are probably part of early stages of the maturation processes in *H. pylori*.

Although protein sequences of *H. hepaticus* and *H. pylori* HypA share only 52.6% identity, some key residues are well conserved; for instance, *H. hepaticus* HypA possesses a conserved histidine residue (His2) known to be involved in nickel binding and essential for urease activity in *H. pylori* (Mehta *et al.*, 2003b). Likewise, the HypB protein seems well conserved between *H. hepaticus* and *H. pylori*, with the same GTPase P-loop motif (GSGKT) located at the exact same position in the sequence. Nevertheless, despite all these similarities between species, HypA and HypB appear to play a different role in *H. hepaticus* compared to *H. pylori*. Differences in actual protein complexes might account for these discrepancies. Direct interactions between *H. pylori* HypA and HypB have been shown by cross-linking and immunoblotting using antibodies against HypA or HypB (Mehta *et al.*, 2003b); however, interactions between HypA/HypB and either the urease or the hydrogenase apoenzyme complexes have never been reported in *H. pylori*. It is very much anticipated that *H. hepaticus* HypA and HypB proteins will be able to interact with each other, although it remains to be demonstrated; most of all, future studies will be aimed at isolating complexes between HypA/HypB and HydA-HydB or UreA-UreB heterodimers. The HypC protein is also likely to play an interesting role with respect to these putative protein complexes, if one looks at the phenotype of the *H. hepaticus* *hypC* mutant (hydrogenase-negative and urease hyperpositive). It is not clear whether the HH0809 hypothetical protein plays a role in the urease maturation process in *H. hepaticus*; indeed, the *hh0809* mutant showed slightly or more pronounced decreased urease activity when the cells when grown on medium supplemented with 1–10 mM nickel. We do not have any explanation for this phenotype. HH0809 is a homologue of the HP0868 hypothetical protein in *H. pylori*, and an *H. pylori* *hp0868* mutant has been shown to have a wild-type-like phenotype for both urease and hydrogenase activities.

Our study also showed that *H. hepaticus* *ureE* and *ureG* mutants are totally devoid of urease activity. These results were expected, because UreE and UreG have been shown to be required for urease activity in several micro-organisms, including *H. pylori* (Mehta *et al.*, 2003a; Volland *et al.*, 2003), *Proteus mirabilis* (Island & Mobley, 1995) and *K. aerogenes* (Mulrooney & Hausinger, 1990). As shown previously for *H. pylori* (Benoit *et al.*, 2007), nickel supplementation of the growth medium did not restore urease activity in either *H. hepaticus* mutant. This seems to favour UreE and UreG being involved in the later, irreversible steps of insertion of nickel within the urease apoenzyme.

A recent study in our lab showed interactions between *H. pylori* HypA and UreE; these interactions are believed to be needed when nickel is scarce in the medium and the bacterial cell (Benoit *et al.*, 2007). Under these conditions, it seems that *H. pylori* cannot rely on UreE



alone to provide urease with nickel because the UreE protein has a poor nickel-binding capacity, especially when compared to other histidine-rich UreE proteins, like that of *P. mirabilis* (Jones & Mobley, 1989) or *K. aerogenes* (Mulrooney & Hausinger, 1990). In *H. hepaticus*, given that UreE does not possess histidine-rich sequences either, and HypA appears to be required for urease activity, it is likely that a HypA-UreE transient association is also needed for transfer of nickel from HypA to the apourease.

While the addition of nickel did not restore urease activity in any of the four *H. hepaticus* mutants of interest in this study (i.e. *hypA*, *hypB*, *ureE*, *ureG*), it increased the activity in the wild-type strain 51449. Analysis of the genome sequence of the mouse liver pathogen reveals the presence of a gene (*hh0352*) encoding a homologue of NikR, a nickel-responsive regulatory protein involved in urease regulation in *H. pylori*. However, Belzer *et al.* (2005) have shown that the surge in urease activity in the wild-type was the consequence of a post-translational nickel activation of the apourease rather than a transcriptional mechanism. This result is in good agreement with the finding that the four nickel-related mutants have similar amounts of urease, as shown by immunoblotting against UreA or UreB. This confirms that the dramatic decrease of urease activity in these mutants is due to deficient post-translational nickel maturation, not to transcriptional repression of the urease structural genes.

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## REFERENCES

- Beckwith CS, McGee DJ, Mobley HL, Riley LK. Cloning, expression, and catalytic activity of *Helicobacter hepaticus* urease. *Infect Immun.* 2001; 69:5914–5920. [PubMed: 11500473]
- Belzer C, Stoof J, Beckwith CS, Kuipers EJ, Kusters JG, van Vliet AH. Differential regulation of urease activity in *Helicobacter hepaticus* and *Helicobacter pylori*. *Microbiology.* 2005; 151:3989–3995. [PubMed: 16339943]
- Benoit S, Maier RJ. Dependence of *Helicobacter pylori* urease activity on the nickel-sequestering ability of the UreE accessory protein. *J Bacteriol.* 2003; 185:4787–4795. [PubMed: 12896998]
- Benoit S, Mehta N, Wang G, Gatlin M, Maier RJ. Requirement of *hydD*, *hydE*, *hypC* and *hypE* genes for hydrogenase activity in *Helicobacter pylori*. *Microb Pathog.* 2004; 36:153–157. [PubMed: 14726233]
- Benoit SL, Mehta N, Weinberg MV, Maier C, Maier RJ. Interaction between the *Helicobacter pylori* accessory proteins HypA and UreE is needed for urease maturation. *Microbiology.* 2007; 153:1474–1482. [PubMed: 17464061]
- Blaser MJ. The role of *Helicobacter pylori* in gastritis and its progression to peptic ulcer disease. *Aliment Pharmacol Ther.* 1995; 9(Suppl 1):27–30. [PubMed: 7495938]
- Blokesch M, Bock A. Maturation of [NiFe]-hydrogenases in *Escherichia coli*: the HypC cycle. *J Mol Biol.* 2002; 324:287–296. [PubMed: 12441107]
- Colpas GJ, Brayman TG, Ming LJ, Hausinger RP. Identification of metal-binding residues in the *Klebsiella aerogenes* urease nickel metallochaperone, UreE. *Biochemistry.* 1999; 38:4078–4088. [PubMed: 10194322]
- Eaton KA, Brooks CL, Morgan DR, Krakowka S. Essential role of urease in pathogenesis of gastritis induced by *Helicobacter pylori* in gnotobiotic piglets. *Infect Immun.* 1991; 59:2470–2475. [PubMed: 2050411]

- Fox JG, Dewhirst FE, Tully JG, Paster BJ, Yan L, Taylor NS, Collins MJ Jr, Gorelick PL, Ward JM. *Helicobacter hepaticus* sp. nov., a microaerophilic bacterium isolated from livers and intestinal mucosal scrapings from mice. *J Clin Microbiol.* 1994; 32:1238–1245. [PubMed: 8051250]
- Island MD, Mobley HL. *Proteus mirabilis* urease: operon fusion and linker insertion analysis of *ure* gene organization, regulation, and function. *J Bacteriol.* 1995; 177:5653–5660. [PubMed: 7559355]
- Jones BD, Mobley HL. *Proteus mirabilis* urease: nucleotide sequence determination and comparison with jack bean urease. *J Bacteriol.* 1989; 171:6414–6422. [PubMed: 2687233]
- Kennedy DC, Herbst RW, Iwig JS, Chivers PT, Maroney MJ. A dynamic Zn site in *Helicobacter pylori* HypA: a potential mechanism for metal-specific protein activity. *J Am Chem Soc.* 2007; 129:16–17. [PubMed: 17199266]
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 1970; 227:680–685. [PubMed: 5432063]
- Maier RJ, Fu C, Gilbert J, Moshiri F, Olson J, Plaut AG. Hydrogen uptake hydrogenase in *Helicobacter pylori*. *FEMS Microbiol Lett.* 1996; 141:71–76. [PubMed: 8764511]
- Mehta N, Benoit S, Maier RJ. Roles of conserved nucleotide-binding domains in accessory proteins, HypB and UreG, in the maturation of nickel-enzymes required for efficient *Helicobacter pylori* colonization. *Microb Pathog.* 2003a; 35:229–234. [PubMed: 14521881]
- Mehta N, Olson JW, Maier RJ. Characterization of *Helicobacter pylori* nickel metabolism accessory proteins needed for maturation of both urease and hydrogenase. *J Bacteriol.* 2003b; 185:726–734. [PubMed: 12533448]
- Mehta NS, Benoit S, Mysore JV, Sousa RS, Maier RJ. *Helicobacter hepaticus* hydrogenase mutants are deficient in hydrogen-supported amino acid uptake and in causing liver lesions in A/J mice. *Infect Immun.* 2005; 73:5311–5318. [PubMed: 16113246]
- Mehta NS, Benoit SL, Mysore J, Maier RJ. *In vitro* and *in vivo* characterization of alkyl hydroperoxide reductase mutant strains of *Helicobacter hepaticus*. *Biochim Biophys Acta.* 2007; 1770:257–265. [PubMed: 17098365]
- Mulrooney SB, Hausinger RP. Sequence of the *Klebsiella aerogenes* urease genes and evidence for accessory proteins facilitating nickel incorporation. *J Bacteriol.* 1990; 172:5837–5843. [PubMed: 2211515]
- Olson JW, Maier RJ. Molecular hydrogen as an energy source for *Helicobacter pylori*. *Science.* 2002; 298:1788–1790. [PubMed: 12459589]
- Olson JW, Mehta NS, Maier RJ. Requirement of nickel metabolism proteins HypA and HypB for full activity of both hydrogenase and urease in *Helicobacter pylori*. *Mol Microbiol.* 2001; 39:176–182. [PubMed: 11123699]
- Remaut H, Safarov N, Ciurli S, Van Beeumen J. Structural basis for Ni<sup>2+</sup> transport and assembly of the urease active site by the metallochaperone UreE from *Bacillus pasteurii*. *J Biol Chem.* 2001; 276:49365–49370. [PubMed: 11602602]
- Sipponen P, Hyvarinen H, Seppala K, Blaser MJ. Review article: pathogenesis of the transformation from gastritis to malignancy. *Aliment Pharmacol Ther.* 1998; 12(Suppl. 1):61–71. [PubMed: 9701004]
- Solnick JV, Schauer DB. Emergence of diverse *Helicobacter species* in the pathogenesis of gastric and enterohepatic diseases. *Clin Microbiol Rev.* 2001; 14:59–97. [PubMed: 11148003]
- Stabb EV, Ruby EG. RP4-based plasmids for conjugation between *Escherichia coli* and members of the Vibrionaceae. *Methods Enzymol.* 2002; 358:413–426. [PubMed: 12474404]
- Suerbaum S, Josenhans C, Sterzenbach T, Drescher B, Brandt P, Bell M, Droge M, Fartmann B, Fischer HP, et al. The complete genome sequence of the carcinogenic bacterium *Helicobacter hepaticus*. *Proc Natl Acad Sci U S A.* 2003; 100:7901–7906. [PubMed: 12810954]
- Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. 1979. *Biotechnology.* 1992; 24:145–149. [PubMed: 1422008]
- Voland P, Weeks DL, Marcus EA, Prinz C, Sachs G, Scott D. Interactions among the seven *Helicobacter pylori* proteins encoded by the urease gene cluster. *Am J Physiol Gastrointest Liver Physiol.* 2003; 284:G96–G106. [PubMed: 12388207]

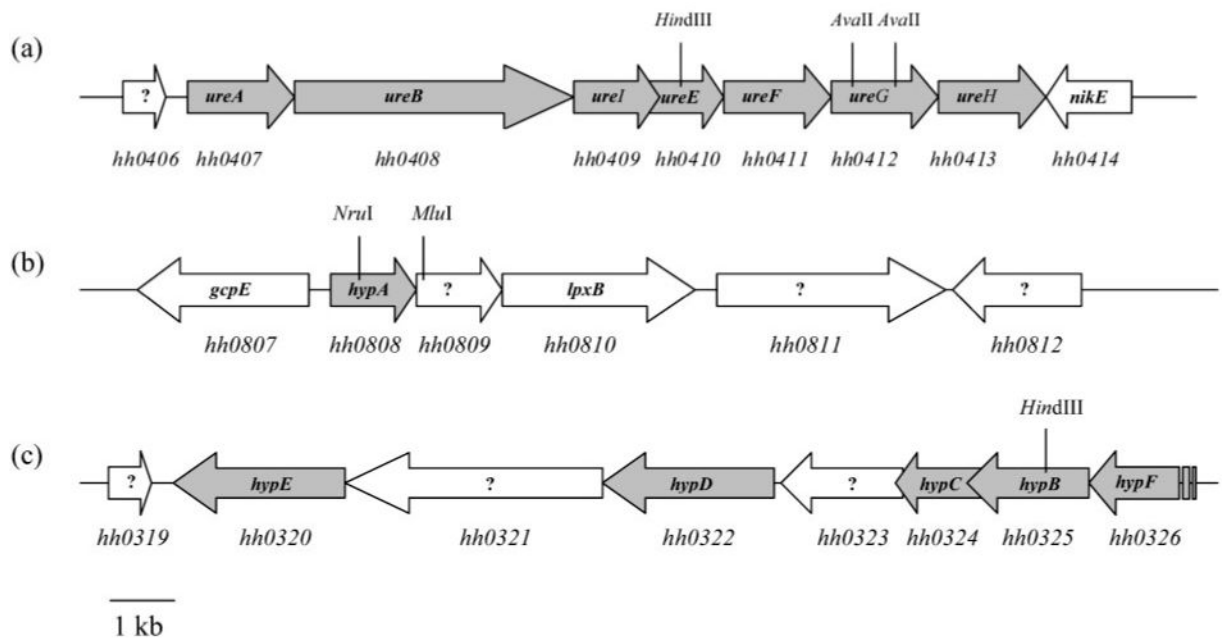
Weatherburn MW. Phenol-hypochlorite reaction for determination of ammonia. *Anal Chem.* 1967; 39:971–974.

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**Fig. 1.**

Genome location and organization of the *ure* and *hyp* genes in *H. hepaticus*. Gene annotations are according to the TIGR website ([www.tigr.org](http://www.tigr.org)). The *ure* and *hyp* genes are shown in grey. Restriction sites used to disrupt each of the genes studied are indicated. An approximate scale is shown bottom left.

```

HypA      1      10      20      30      40
           MHEYSIVASLIQMCESHAKEHNAASIAKVCIAVGERSGVD...

HypB      30      40      50      60      70
           ...NDLKAQELREKYKQLGVYVINLMSSPGSGKTTLLESLSAF...

UreE      80      90      100     110     120
           ...IIIISILPTWVLCMKPTHWHTMARLCYEIGNLHIPLFYNKD...

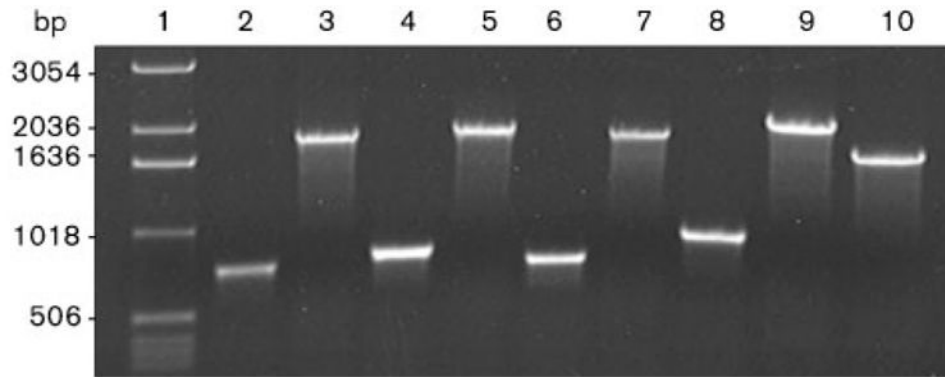
UreG      1      10      20      30      40
           MVKIGICGPVGSGKTALIESLTRKMNEQYSLAVITNDIYT...

```

**Fig. 2.**

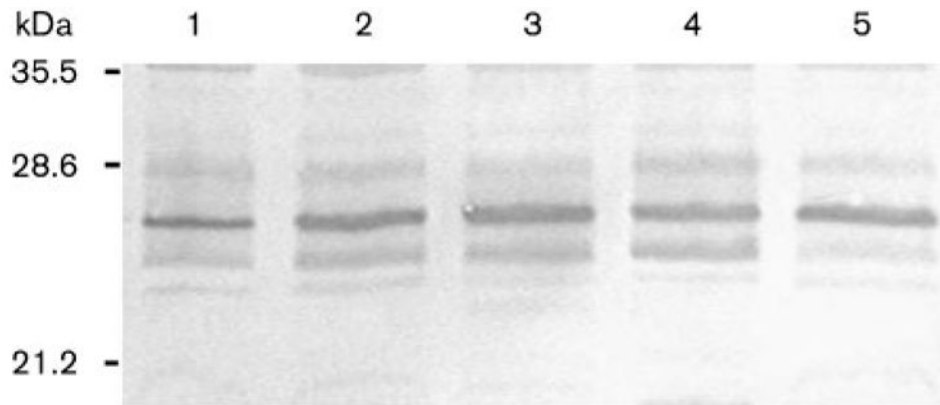
Partial protein sequences of *H. hepaticus* HypA, HypB, UreE and UreG. Conserved residues whose roles have been demonstrated in homologous proteins from *H. pylori* (HypA, HypB, UreG), *K. aerogenes* (UreE) or *B. pasteurii* (UreE) are shown in bold. A conserved p-loop involved in GTPase activity is highlighted for HypB or UreG.





**Fig. 3.**

Agarose gel of PCR products used to verify cassette insertions. Lane 1 contains a 1 kb DNA ladder, with sizes indicated on the left. Lanes 2–10 contain fragments amplified from *H. hepaticus* genomic DNA from the following strains: 2, 4, 6 or 8, wild-type strain 51449; 3, *hypA::ery*; 5, *hypB::ery*; 7, *ureE::ery*; 9, *ureG::ery*; 10: *ureG::ery*. The following primers were used: lanes 1–2, UphypA and RevhypA; 3–4, UphypB and RevhypB; 5–6, UpureE and RevureE; 7–10, UpureG and RevureG.



**Fig. 4.** Immunoblotting analysis of *H. hepaticus* parental and mutant strain cell extracts with anti-*H. pylori* UreA. Ten micrograms of cell-free extract from each strain was subjected to SDS-PAGE, transferred to nitrocellulose and blotted with anti-UreA antibodies. Lanes: 1, wild-type strain 51449; 2, *hypA::ery*; 3, *hypB::ery*; 4, *ureE::ery*; 5, *ureG::ery*. Sizes corresponding to prestained molecular mass markers are indicated on the left.

Table 1

Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics*	Source or reference
<b><i>H. hepaticus</i></b>		
51449	Parent strain for all <i>H. hepaticus</i> strains	ATCC <sup>†</sup>
<i>hypA</i> mutant	<i>hypA::ery</i> ; Er <sup>r</sup>	This study
<i>hypB</i> mutant	<i>hypB::ery</i> ; Er <sup>r</sup>	This study
<i>hypC</i> mutant	<i>hypC::ery</i> ; Er <sup>r</sup>	This study
<i>ureE</i> mutant	<i>ureE::ery</i> ; Er <sup>r</sup>	This study
<i>ureG</i> mutant	<i>ureG::ery</i> ; Er <sup>r</sup>	This study
<i>ureG</i> mutant	<i>DureG::ery</i> ; Er <sup>r</sup>	This study
<i>hh0809</i> mutant	<i>hh0809::ery</i> ; Er <sup>r</sup>	This study
<b><i>E. coli</i></b>		
TOP10	Cloning strain	Invitrogen
<b>Plasmids</b>		
pGEM-T	Cloning vector; Ap <sup>r</sup>	Promega
pBC-KS	Cloning vector; Cm <sup>r</sup>	Stratagene
pBluescript-KS	Cloning vector; Ap <sup>r</sup>	Stratagene
pSLB167	pBC-KS with <i>ery</i> ( <i>EcoRV</i> ); Cm <sup>r</sup> Err; source of <i>ery</i> cassette	Mehta <i>et al.</i> (2007)
pGEM- <i>hypA</i>	pGEM with a 760 bp sequence containing <i>H. hepaticus hypA</i> and part of <i>hh0809</i>	This study
pSLB164	pGEM- <i>hypA</i> with <i>ery</i> gene inserted within <i>NruI</i> of <i>hypA</i>	This study
pGEM- <i>hypB</i>	pGEM with a 814 bp sequence containing <i>H. hepaticus hypB</i>	This study
pSLB165	pGEM- <i>hypB</i> with <i>ery</i> gene inserted within <i>HindIII</i> of <i>hypB</i>	This study
pGEM- <i>ureE</i>	pGEM with a 734 bp sequence containing <i>H. hepaticus ureE</i>	This study
pSLB166	pGEM- <i>ureE</i> with <i>ery</i> gene inserted within <i>HindIII</i> of <i>ureE</i>	This study
pBC- <i>ureG</i>	pBC-KS with a 904 bp sequence containing <i>H. hepaticus ureG</i>	This study
pSLB173	pBC- <i>ureG</i> with <i>ery</i> gene inserted within <i>AvaI</i> of <i>ureG</i>	This study
pGEM- <i>ureG</i>	pGEM with a 904 bp <i>H. hepaticus ureG</i> with an engineered <i>HindIII</i>	This study
pSLB175	pGEM- <i>ureG</i> with <i>ery</i> gene inserted within <i>HindIII</i> of <i>ureG</i>	This study
pSLB290	pGEM- <i>hypA</i> deleted for <i>MluI</i> ( <i>PstI/NsiI</i> -digested; religated)	This study
pSLB293	pSLB290 with <i>ery</i> inserted within <i>MluI</i> of <i>hh0809</i>	This study

\* Ap<sup>r</sup>, Ampicillin resistance; Cm<sup>r</sup>, chloramphenicol resistance; Er<sup>r</sup>, erythromycin resistance.

<sup>†</sup> ATCC, American Type Culture Collection, Manassas, VA, USA.

**Table 2**

Primers used in this study

Name	Sequence (5'-3')*	Usage
UphypA	ataggtgcatcaccgctactg	Construction of <i>hypA</i> and <i>hh0809</i> mutant
RevhypA	tttgctcaatggcgcaagg	Construction of <i>hypA</i> and <i>hh0809</i> mutant
UphypB	gccaacacattcttgcctcc	Construction of <i>hypB</i> mutant
Revhyp B	cgcgagtattagtttgccc	Construction of <i>hypB</i> mutant
UphypC	ttgtgagcaaggcagatatg	Construction of <i>hypC</i> mutant
Revhyp C	cttcaagcactataatagg	Construction of <i>hypC</i> mutant
HypC-ery1	<u>GCGATACCGTCGAGAT</u> tacaactgcatattattg	Construction of <i>hypC</i> mutant
HypC-ery2	<u>CTAGCGATAAGCTT</u> gatacattagcggtgag	Construction of <i>hypC</i> mutant
UpureE	ggcttacaggctgattgaaag	Construction of <i>ureE</i> mutant
RevureE	tgcacatagctttctagccc	Construction of <i>ureE</i> mutant
UpureG	ctcaaagcagtgaggcaagag	Construction of <i>ureG</i> mutant
RevureG	atcctcattcttagtgggc	Construction of <i>ureG</i> mutant
UreGmut1	AGAGAAGA <b>Agctt</b> ctatgaacttg	Creation of <i>HindIII</i> within <i>ureG</i>
UreGmut2	TCATAGA <b>AGCT</b> tcttctgattgctg	Creation of <i>HindIII</i> within <i>ureG</i>

\* Lower-case letters indicate *H. hepaticus*-derived sequences and bold letters indicate newly generated restriction sites. *ery*-specific sequences are underlined. All primers were purchased from Integrated DNA Technology (IDT).

Hydrogenase activity of *H. hepaticus* parental and mutant strains as a function of nickel added to the growth medium

**Table 3**

Strain	Hydrogenase activity (nmol H <sub>2</sub> min <sup>-1</sup> per 10 <sup>9</sup> cells)					
	Nickel added (μM):					
	0	1	5	10	50	
WT 51449	0.77±0.04	1.15±0.08	1.90±0.15	3.64±0.50	9.18±1.20	
<i>hypA</i>	<0.01	<0.01	<0.01	<0.01	0.20±0.12	
<i>hh0809</i>	0.83±0.10	0.95±0.20	1.33±0.23	2.50±0.18	4.09±0.85	
<i>hypB</i>	<0.01	<0.01	<0.01	<0.01	0.16±0.05	
<i>hypC</i>	<0.01	<0.01	<0.01	<0.01	<0.01	
<i>ureE</i>	0.91±0.15	1.12±0.06	1.50±0.22	3.01±0.21	5.32±0.90	
<i>ureG</i>	0.87±0.19	0.92±0.25	1.24±0.10	5.64±0.07	9.81±1.05	
<i>ureG</i>	0.67±0.05	0.85±0.12	ND	ND	8.21±1.32	

Cells were harvested from Brucella agar plates supplemented with various amounts of NiCl<sub>2</sub>. Hydrogen uptake activity was determined amperometrically from whole cells, with O<sub>2</sub> provided as the final electron acceptor. Results shown are means of two to four independent cultures±SD, with each assay made in duplicate or triplicate. ND, Not determined.



Urease activity of *H. hepaticus* parental and mutant strains as a function of nickel added to the growth media

**Table 4**

Strain	Urease activity [ $\mu\text{mol NH}_3 \text{ min}^{-1} (\text{mg total protein})^{-1}$ ]						
	Nickel added ( $\mu\text{M}$ ):		0	1	5	10	50
WT 51449			0.06 $\pm$ 0.02	0.26 $\pm$ 0.05	0.54 $\pm$ 0.12	1.97 $\pm$ 0.23	5.44 $\pm$ 1.42
<i>hypA</i>			<0.01	<0.01	<0.01	<0.01	<0.01
<i>hh0809</i>			0.08 $\pm$ 0.03	0.13 $\pm$ 0.04	0.34 $\pm$ 0.08	0.58 $\pm$ 0.13	3.30 $\pm$ 0.54
<i>hypB</i>			<0.01	<0.01	<0.01	<0.01	<0.01
<i>hypC</i>			0.16 $\pm$ 0.01	0.57 $\pm$ 0.03	1.52 $\pm$ 0.12	2.85 $\pm$ 0.34	14.96 $\pm$ 1.83
<i>ureE</i>			<0.01	<0.01	<0.01	<0.01	<0.01
<i>ureG</i>			<0.01	<0.01	<0.01	<0.01	<0.01
<i>ureG</i>			<0.01	<0.01	<0.01	<0.01	<0.01

Cells were harvested from Brucella agar plates supplemented with various amounts of nickel chloride, washed and lysed by sonication. Protein concentration was determined by using the BCA method (Pierce). Urease activity was determined by measuring ammonia production from urea hydrolysis by using the phenol/hypochlorite assay. Results shown are means of two to five independent cultures $\pm$ SD, with each assay made in duplicate or triplicate.