Nickel homeostasis in *Helicobacter* species

Jeroen Stoof

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Nikkel Homeostase in *Helicobacter* Species

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Chapter 1

Aims & Outline

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Aims & Outline

Gastric *Helicobacter* species are adapted to colonize the acidic environment of the stomach. Colonization with *H. pylori* is life long if untreated, and can lead to gastritis, peptic ulcer disease and eventually to gastric cancer. Although *H. pylori* is sensitive to many antibiotics in vitro, only a limited number of antibiotics can be used in vivo while increasing resistance against these therapeutics significantly impairs the treatment of *H. pylori* infection.
Metals play an essential role in the metabolism of all living organisms, including gastric *Helicobacter* species, but can also be deleterious when metal availability is either too low or too high. Therefore cells need to maintain homeostasis of intracellular metal concentrations to allow survival and growth. A better understanding of metal homeostasis in gastric *Helicobacter* species may allow for the knowledge-led development of therapeutics which are based on disturbing the balance of the intracellular metal concentrations to either toxicity or restriction. The focus of this PhD-thesis is on nickel metabolism, since this metal is the cofactor of the urease enzyme and hydrogenase enzyme, both essential for colonization of gastric *Helicobacter* species. The high expression level of the urease enzyme mediates acid resistance in the presence of urea, but also necessitates the import of relatively high concentrations of nickel. Although transcriptional regulation by the nickel-dependent regulator NikR has been studied, relative little is known about which proteins are involved in actual transport of nickel. The genome era has opened the possibility of functional genomics investigations, using the information from the genomes of different *Helicobacter* species. Many of the genes of *Helicobacter* species do not yet have a predicted function, or have been assigned a putative function only based on homology with genes from other bacterial species. Comparison of the genomic content of different *Helicobacter* species and transcriptional and functional characterization of the genes putatively involved in nickel homeostasis, as presented in this thesis, will provide more insight in how these bacteria are able to acquire sufficient concentrations of nickel.

Chapter 2 Provides a general introduction on the knowledge of iron and nickel metabolism in *Helicobacter* species at the start of this thesis. The completed genome sequences of the human gastric pathogen *H. pylori*, the ferret pathogen *H. mustelae,* and the murine enterohepatic *Helicobacter*, *H. hepaticus* allowed comparison of putative iron and nickel uptake storage and regulatory systems. The differences in metal metabolism and their putative contribution in adaptation to a gastric or enterohepatic niche are discussed.

Chapter 3 describes the discovery of a second urease operon, *ureA2B2,* in the feline gastric pathogen *H. felis*. The structural subunits UreA2 and UreB2 encoded by this urease operon display high homology with the structural urease subunits from other *Helicobacter* species, like *H. pylori* and the amino acids involved in nickel binding are completely conserved. However the *ureA2B2* operon differs from the standard urease operon *ureAB* since the *ureA2B2* operon does not encode accessory proteins, normally essential for nickel incorporation and enzyme activation. Although inactivation of this second urease cluster

resulted in a reduced urease activity, an UreAB mutant did not demonstrate any residual urease activity.

In **Chapter 4** it is demonstrated that the UreA2B2 urease cluster described in chapter 3 is not unique for *Helicobacter felis,* but is also present in two other carnivore-colonizing gastric *Helicobacter* species, *Helicobacter acinonychis* (large felines) and *Helicobacter mustelae* (ferrets). The experiments in this chapter describe that the second urease in *H. mustelae* has several remarkable features. In contrast to the nickel induced expression of UreAB, expression of UreA2B2 is repressed by nickel. Moreover, in contrast to all other ureases described so far UreA2B2 is inactivated upon cell lyses and does not require the standard accessory proteins for activation. We hypothesize that UreA2B2 may not require nickel as cofactor and therefore may be an adaptation to the nickel-limited diet of carnivore-colonizing *Helicobacter* species.

In **chapter 5** the *H. mustelae ureA* and *ureA2* promoters have been further characterized. The identification of the NikR binding site in the promoter regions of *H. mustelae ureA* and *ureA2*, and alignment with identified NikR binding sites of *H. pylori*, allowed the definition of an improved consensus sequence for prediction of NikR operators. Since many nickel acquisition genes in other bacteria are repressed by nickel and NikR, this novel consensus sequence was subsequently used to predict which genes are putatively regulated and thus might be involved in nickel homeostasis in *H. pylori*, *H. acinonychis*, *H. hepaticus* and *H. mustelae*. As proof of principle, one of the *H. mustelae* genes with such a predicted NikR binding site, *nikH,* was demonstrated to be nickel regulated, while inactivation resulted in a decrease of the nickel cofactored urease activity, suggestive of a role in nickel acquisition.

The *H. pylori* genome encodes many genes annotated as putative outer membrane proteins involved in iron transport, but nickel was thought to pass through the outer membrane without the need for active transport. However transcriptional analysis demonstrated that some of the outer membrane transporters were not iron responsive. In **chapter 6** it has been demonstrated that two of these genes, encoding the putative outer membrane receptors for iron, FecA3 and FrpB4, are nickel-repressed by the nickel-dependent transcriptional regulator NikR. We hypothesized that these genes play a role in nickel transport across the outer membrane.

Chapter 7 relates to the work presented in Chapter 5, and focuses on other genes putatively involved in nickel acquisition of *H. mustelae*. Using urease activity and cellular nickel content as readout systems, combined with the bioinformatics predictions of NikR binding sites, two novel genes involved in nickel acquisition and urease activity in *H. mustelae* have been identified. Inactivation of these genes in combination with inactivation of known nickel transport genes has allowed identification of two different nickel acquisition pathways in *H. mustelae*.

In **Chapter 8** data provided in this thesis is summarized and placed in the context of results, published in peer reviewed publications. We conclude that some *Helicobacter* species, naturally encountering nickel limited conditions, have adapted to this situation by expression of a second urease. This may suggest that *Helicobacter* species lacking such an adaptation, like *H. pylori*, may become more acid sensitive under nickel limited conditions. The identification of transport mechanisms of nickel, as described in this thesis, may result in elucidation of possible strategies to inhibit nickel acquisition. Finally some suggestions are made for further research.

Chapter 2

Introduction:

Metal-responsive gene regulation and metal acquisition in *Helicobacter* species

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INTRODUCTION

The genus *Helicobacter* belongs to the epsilon subdivision of the proteobacteria, order Campylobacterales, family of the *Helicobacteraceae*. The best known related species is *Campylobacter jejuni* (27). Members of the genus *Helicobacter* are all microaerophilic organisms, and in most cases catalase- and oxidase-positive, and many are also urease-positive. *Helicobacter* species colonize the mucosal surfaces of the gastrointestinal and hepatobiliary tract of mammals, including humans. These mucosal surfaces provide a challenging environment with continuous changes in environmental conditions, including the availability of metal ions (75).

Colonization is usually lifelong, and often leads to the development of a wide range of inflammatory diseases (15). Infection with *Helicobacter* species induces a strong proinflammatory response which includes the production of reactive oxygen species by the host immune system, and this necessitates the expression of systems detoxifying reactive oxygen species. Furthermore, the formation of reactive oxygen species is connected with iron metabolism, as oxygen radicals can be produced via the Fenton and Haber-Weiss reactions ($Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^-$ and $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$). *Helicobacter* species are microaerophilic bacteria tolerating oxygen concentrations ranging from 2-10%. To combat oxidative stress, *Helicobacter* species expresses several key components of bacterial oxidative stress resistance, which are often metal-cofactored and controlled by metal-responsive regulatory systems (3, 28, 36, 41, 56, 63, 86). Many of the components of oxidative stress defense are essential for gastric colonization by *H. pylori* in animal models (4, 37, 57), and are likely to be of similar importance to colonization of other *Helicobacter* species.

Helicobacter species can be subdivided into two major lineages (32, 65). The first lineage consists of the gastric *Helicobacter* species, which colonize the mucus layer overlaying the gastric epithelium. The best known example of a gastric *Helicobacter* species is the human pathogen *Helicobacter pylori*, the causative agent of gastritis, peptic ulcer disease and gastric adenocarcinoma (15). However, many mammals are infected with gastric *Helicobacter* species, and they have been isolated from a wide variety of hosts, including dolphins, big cats and ferrets (32, 65).

The second lineage consists of the enteric or enterohepatic *Helicobacter* species, which colonize the mammalian intestinal and hepatobiliary tract, and have so far mostly been identified in rodents. The best characterized enteric *Helicobacter* species is *Helicobacter hepaticus*, which infects the intestines, bile ducts and liver of mice, and can cause hepatitis, hepatocellular carcinoma and possibly induce the formation of gallstones (32, 48, 65).

The chronicity of infection suggests that *Helicobacter* species are well adapted to the conditions in their specific niches, and are more than capable of handling changes in these conditions (15). *Helicobacter* species have a relatively small genome of 1.5-2.0 Mbp. The complete genome sequences of *H. pylori* (1, 71) and *H. hepaticus* (69) have been published previously, whereas the almost finished, but unannotated genome sequence of the ferret gastric pathogen *Helicobacter mustelae* is available online (at http://www.sanger.ac.uk/Projects/H_mustelae). Together with the availability of the complete genome sequence of *C. jejuni* (60) this has allowed for comparative genomics (27). In this

chapter we will review the knowledge on metal metabolism and metal-responsive gene regulation of *Helicobacter* species, focussing on the role of the metals iron, nickel and copper, as known at the start of this project.

METAL-TRANSPORT AND STORAGE OF *HELICOBACTER* SPECIES

Metals play an important role in the metabolism of all organisms, and this is reflected by the wide variety of chemical reactions in which they are involved. Metals can be cofactors of enzymes, catalyzing basic functions like electron transport, redox reactions and energy metabolism, and are essential for maintaining the osmotic pressure of the cell (75). Since both metal limitation and metal overload delay growth and can cause cell death, metal homeostasis is of critical importance to all living organisms. In bacteria, metal homeostasis is achieved by balancing import, efflux, metabolism and storage (Figure 1).

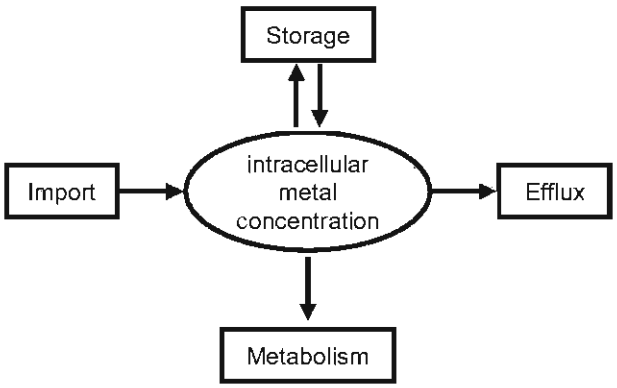

Figure 1. Schematic representation of the mechanisms involved in maintaining homeostatic control of the intracellular metal concentration via concerted action of import, efflux, storage and metabolism.

The gastric and enteric mucosa are niches with continuous changes in environmental conditions. For instance, the pH in the gastric mucosa is thought to range between 4 and 6.5, but acid shocks occasionally occur. Bioavailability of metals like iron and nickel is linked to the environmental pH, and thus *Helicobacter* species have to cope with both conditions of metal-restriction and metal-overload. Furthermore, the intestinal tract contains many bacterial species and thus enterohepatic *Helicobacter* species have to compete for metals and other nutrients. Metal metabolism has been studied intensively in *H. pylori*, but relatively little is known about metal metabolism of *H. mustelae* and *H. hepaticus*, and much of the information in this section is inferred from comparative genomics using the *H. pylori* and *C. jejuni* genome sequences. In this section we will review the metal-transporter and metal-storage systems of the three selected *Helicobacter* species, of which an overview is given in Fig. 2.

Iron

Iron availability in the host tissues is mostly too low to support bacterial growth, as most iron is complexed into hemoglobin, or chelated by lactoferrin at mucosal surfaces (2). Due to the insolubility of ferric iron (Fe^{3+}), ferric iron transport requires an outer membrane receptor to transport the iron over the outer membrane, as well as an ABC transporter which transports the iron from the periplasm to the cytoplasm. In contrast, ferrous iron (Fe^{2+}) is soluble and only requires an inner membrane receptor.

(i) *Helicobacter pylori.* Iron sources available in the gastric mucosa are lactoferrin, heme compounds, and iron derived from pepsin-degraded food. Iron is predicted to have increased

solubility in the acidic conditions in the gastric mucosa, and eukaryotic iron-complexing proteins display lowered binding affinity at these conditions. *H. pylori* has been reported to be able to utilize heme compounds and ferric citrate as sole iron source (26, 82), and it may be able to also use human lactoferrin, although this is still under debate (13). The *H. pylori* genome encodes eleven proteins predicted to be involved in iron transport and two proteins thought to function as iron storage proteins (Fig. 2).

H. pylori has three copies of the putative ferric citrate outer membrane receptor FecA, and three copies of the FrpB outer membrane receptor, which resembles a low-affinity enterochelin transporter in *Neisseria* species (18), for which in *H. pylori* the substrate is still unknown. There are two copies of the periplasmic binding protein CeuE, and finally a single inner membrane permease (FecD) and ATP-binding protein (FecE) (Fig. 2). Currently, mutants have only been described in the *fecDE* system and in one of the *fecA* genes (82). Rather surprisingly, this did not affect iron transport. Thus the contribution of ferric citrate to iron acquisition in *H. pylori* remains to be clarified. In the acidic, microaerobic gastric environment, ferrous iron (Fe^{2+}) is thought to constitute the main form of free iron, and this is transported by *H. pylori* via the FeoB protein (82).

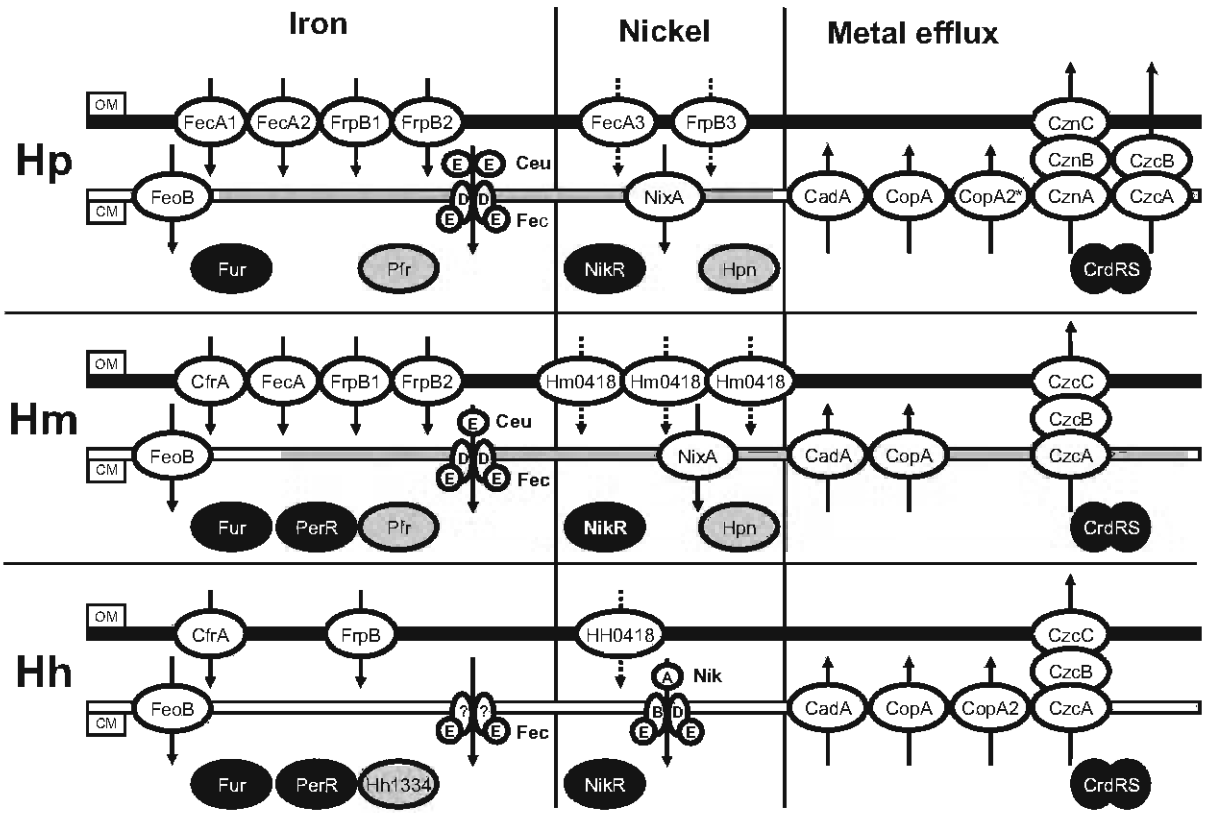

Figure 2. Comparison of iron-transporters, nickel-transporters, metal efflux systems, metal storage and metal-responsive regulatory systems of *Helicobacter pylori* (Hp, top), *Helicobacter mustelae* (Hm, middle) and *Helicobacter hepaticus* (Hh, bottom). The ion transported and the direction of transport are indicated. OM, outer membrane; CM, cytoplasmic membrane. Ion transporters are grouped depending on the metal (putatively) transported. Arrows denotes the predicted direction of transport, dashed arrows indicate that this transport function is speculative. Note that the subdivision of outer membrane transporters for nickel is also tentative and speculative. Regulatory proteins are given in black, storage proteins in grey.

FeoB-mediated iron acquisition is of major importance to *H. pylori*, as isogenic *feoB* mutants were unable to colonize the gastric mucosa of mice (82).

Two iron-storage proteins have been characterized in *H. pylori*, the Pfr ferritin and the HP-NAP (Dps) bacterioferritin (Fig. 2) (14, 72). The Pfr ferritin serves as an intracellular iron deposit and protects *H. pylori* against intracellular iron toxicity and free iron-mediated oxidative stress (83). Iron stored in Pfr can be released and reused to support growth under iron-limited conditions (83). The HP-NAP protein is both homologous to bacterioferritins as well as to the DNA-binding proteins of the Dps family (72). A role of HP-NAP in *H. pylori* iron storage, although suggested, is yet to be demonstrated.

(ii) *Helicobacter mustelae.* *H. mustelae* has been reported to be able to utilize heme compounds and iron chloride as sole iron source (26). In addition, it is also capable of using ferric citrate (68). This is partially reflected in the putative outer membrane iron-uptake proteins identified in *H. mustelae* (Fig. 2). There is a single ortholog of the outer membrane ferric citrate transporter FecA. Furthermore *H. mustelae* contains two genes in tandem encoding orthologs of the FrpB outer membrane receptor, and an ortholog of the *C. jejuni* CfrA outer membrane enterochelin receptor. Finally, there are three consecutive, >80% identical genes encoding orthologs of the *H. hepaticus* HH0418 putative outer membrane protein, tentatively named Hm0418-1, Hm0418-2 and Hm0418-3 (Fig. 2). The function of the CfrA, FrpB and Hm0418 orthologs is currently unclear. There is one ortholog of the periplasmic binding protein CeuE, and a single inner membrane FecDE ABC transporter system. Furthermore, there is an ortholog of the ferrous iron transporter FeoB present (Fig. 2). Finally, *H. mustelae* contains orthologs of the Pfr ferritin and HP-NAP (Dps) bacterioferritin, and it is likely that these have similar functions as the *H. pylori* counterparts.

(iii) *Helicobacter hepaticus.* *H. hepaticus* has been reported to be able to utilize lactoferrin, transferrin, heme compounds and iron chloride (26). *H. hepaticus* colonizes the intestinal and hepatobiliary tract, and this is reflected in differences in its repertoire of putative iron-acquisition systems when compared to *H. pylori* and *H. mustelae* (Fig. 2). The *H. hepaticus* genome contains a single ortholog of the FrpB outer membrane receptor, for which it is tempting to speculate that this functions as heme receptor. Interestingly, despite the relatively close phylogenetic relationship and similar ecological niche as *C. jejuni*, there are suprising differences in the iron acquisition systems present. *H. hepaticus* and *C. jejuni* share a CfrA ortholog, which in *C. jejuni* functions as enterochelin uptake system (58). However, it is not known whether *H. hepaticus* is able to use enterochelin as iron source. An ortholog of the *C. jejuni* hemin transporter ChuA is absent in the *H. hepaticus* genome sequence (81). Next to predicted outer membrane iron-transporters, *H. hepaticus* also contains a gene annotated as *fecE*, the ATPase component of the Fec ABC transporter. However, an ortholog of the permease protein FecD is not present in the *H. hepaticus* genome (Fig. 2), although this function may be fulfilled by a protein lacking sufficient homology to FecD. There is also an ortholog of the ferrous iron transporter FeoB present. Finally, there is also a ferritin present in *H. hepaticus*, encoded by the HH1334 gene. The bacterioferritin (Dps) ortholog of *H. hepaticus* also binds iron, but is reported to function in DNA-protection against oxidative stress (41).

Nickel

Nickel plays an important role in metabolism of *Helicobacter* species and in pathogenesis of infection. This is mostly due to the role of nickel as cofactor for the urease and hydrogenase enzymes, which are both involved in acid resistance, mucosal colonization and metabolism. Nickel availability in human serum is very low (2-11 nM) (20), and the nickel concentration in ingested food varies significantly depending on the diet and on food sources (70). Therefore *Helicobacter* species require efficient high-affinity transport of nickel to cater for the urease and hydrogenase enzymes.

(i) *Helicobacter pylori.* The adaptation of *H. pylori* to life in the gastric mucosa seems to have led to a more pronounced role of nickel in its metabolism. The major nickel-transporter of *H. pylori* is the NixA protein, a 37 kDa protein of the HoxN-type of nickel/cobalt transporters (52). Mutation of the *nixA* gene results in clear reduction of nickel transport and lowered urease activity (5), and mutation of *nixA* complemented nickel-sensitivity of a *H. pylori nikR* mutant (30). Absence of *nixA* also affected colonization efficiency in a mouse model of *H. pylori* infection, presumably due to the reduced urease activity (55). A second putative nickel transport system is the *abcCD* locus, since *abcC nixA* double mutants showed only residual urease activity (38). However, nickel transport by the AbcCD system is yet to be demonstrated. A third system thought to be involved in nickel transport is the Dpp dipeptide permease, but mutation of this gene did not affect overall urease activity (21).

Several urease- and hydrogenase-associated systems have adapted to the central role of nickel. The accessory proteins involved in hydrogenase biogenesis also affect urease activity, and the HspA (GroES) chaperone contains a nickel-binding motif (44). Next to the nickel-transporters and urease/hydrogenase accessory proteins, *H. pylori* also expresses one or two small, very histidine-rich proteins (Hpn) which show strong binding to nickel (33, 34). Mutation of the *hpn* gene rendered *H. pylori* sensitive to nickel (53), which is suggestive of a role of Hpn in nickel storage or nickel sequestration, but this remains to be proven experimentally.

(ii) *Helicobacter mustelae.* Nickel transport in *H. mustelae* looks very similar to *H. pylori*. Both organisms have highly active urease and hydrogenase enzymes, and thus require efficient transport of nickel to cater for the high demand of nickel. *H. mustelae* contains a NixA ortholog, which probably represents the major nickel transporter. Interestingly, there are no clear orthologs of the FrpB3 and FecA3 proteins implicated in nickel transport of *H. pylori* (29), but *H. mustelae* contains three tandem copies of the putative outer membrane receptor Hm0418, which may fulfill a similar role as FecA3/FrpB3 in *H. pylori*. However, this awaits experimental validation. Finally, *H. mustelae* was suggested to also expresses the histidine-rich Hpn protein (34), which is thought to function in nickel storage, although the corresponding gene is absent from the *H. mustelae* genome sequence.

(iii) *Helicobacter hepaticus.* *H. hepaticus* expresses two nickel-cofactored enzymes, urease and Ni/Fe hydrogenase (46, 49). This has necessitated the efficient transport of nickel, and this is likely to be mediated by the NikABDE ABC transporter system, which displays homology with the *E. coli* NikABCDE nickel transport system (22). NikA is an ortholog of the *E. coli* nickel-binding periplasmic protein, and NikB is the predicted cytoplasmic membrane permease. NikD and NikE are predicted to function as ATPases which energize the

transport (54). There is no NixA ortholog present in *H. hepaticus*. The *H. hepaticus* genome contains a gene encoding a putative siderophore outer membrane receptor (HH0418), which is divergently orientated to the *nikABDE* operon encoding the predicted nickel-uptake ABC transporter system (7). It is therefore tempting to speculate that the Hm0418 protein may function in nickel uptake.

Copper

Copper is a cofactor for several proteins involved in electron transport, oxidases, and hydroxylases, but may also contribute to the formation of reactive oxygen species.

(i) *Helicobacter pylori.* *H. pylori* expresses several proteins which are either involved in either copper transport or may act as copper chaperones. It is currently unclear whether there is a specific import system for copper, or whether it is transported by other metal transporters like FeoB or NixA (53). However, *H. pylori* expresses several proteins involved in copper export, including the CopA and CopA2 P-type ATPases (Table 1), and the Crd copper resistance determinant (50, 84). Furthermore, *H. pylori* also expresses a small protein, CopP, that may function as copper chaperone (6, 8).

(ii) *Helicobacter mustelae and* (iii) *Helicobacter hepaticus.* Very little is known about copper metabolism of both *H. mustelae* and *H. hepaticus*. Both genomes contain orthologs of the CrdRS two-component regulatory system mediating copper-responsive induction of a Czc-like metal efflux system (84, 85), but a role in copper metabolism is not yet established.

Magnesium

Magnesium is the most abundant divalent cation in living cells, and is often found bound to cellular polyanions like nucleic acids. Furthermore it regularly functions as cofactor in conjunction with ATP in many enzymatic reactions, like those catalyzing modification, replication and transcription of nucleic acids. Because it is continuously complexed, magnesium does not contribute much to osmotic processes (64). Many *H. pylori* enzymes probably use magnesium as cofactor, like its restriction-modifications systems, but this has not been investigated experimentally. Magnesium uptake has been extensively studied in *S. typhimurium*, which contains four magnesium transport systems, the CorA, MgtA, MgtB and MgtE transporters (64).

(i) *Helicobacter pylori. H. pylori* contains only a CorA homolog (HP1344), which is thought to mediate both import and export of magnesium, cobalt and nickel (62, 64). A *H. pylori* CorA mutant was only capable of growing in medium supplemented with Mg^{2+} concentrations exceeding 20 mM (62), suggesting that there are no other high-affinity Mg^{2+} transporters in *H. pylori*.

(ii) *Helicobacter mustelae and* (iii) *Helicobacter hepaticus.* Both *H. mustelae* and *H. hepaticus* genomes contain an ortholog of CorA, but no experimental data is available to confirm their function in magnesium transport.

Metal efflux systems

(i) *Helicobacter pylori*. Metal efflux is of great importance to *H. pylori*. The urease enzyme of *H. pylori* is sensitive to transition metals (40, 61), and thus effective homeostasis of transition metals is necessary to prevent acid-sensitivity. *H. pylori* expresses three P-type ATPases, which can function in efflux of toxic metals. Two of these P-type ATPases are suggested to be CopA homologs which are involved in copper metabolism (see previous section). The third P-type ATPase is the *H. pylori* CadA protein, which is homologous to bacterial cadmium and copper P-type ATPases, and contains eight transmembrane domains (51). Mutational studies demonstrated that CadA is involved in resistance to cadmium, zinc and cobalt, but not to copper or nickel. This indicates that the CadA exporter is not specific for a single metal. Mutation of *cadA* also reduced urease activity and nickel accumulation in some but not all *cadA* mutants (40). The other type of metal-efflux systems in *H. pylori* consists of the Czc system. These are cation-proton antiporters, and usually contain a cytoplasmic membrane transporter (CzcA), a periplasm-spanning protein (CzcB) and an OM protein thought to be involved in transport over the OM (CzcC). The *H. pylori* genome contains two Czc-like systems, the Crd copper resistance determinant (see previous section), and a cobalt-zinc-nickel (Czn) resistance determinant (Table 1) (66). The latter system is also implicated in acid-resistance (possibly via nickel efflux) (66) and efflux of metronidazole (73).

(ii) *Helicobacter mustelae* and (iii) *Helicobacter hepaticus*. The *H. mustelae* and *H. hepaticus* genomes contains respectively two and three genes encoding P-type ATPase orthologs, which are tentatively named CadA, CopA and CopA2, like in *H. pylori*. Similarly, there is a single CzcABC metal efflux system present. The function of these systems has not been investigated yet.

METAL-RESPONSIVE REGULATORY SYSTEMS OF *HELICOBACTER* SPECIES

Rapid responses to stressful changes in environmental conditions are often mediated via changes in transcription of sets of genes, that encode some factor involved in the dealing with these stresses. Examples of this are the expression of oxidative stress defense genes in response to oxidative stress. In many bacteria, such stress-responsive systems are often encoded by genes organized in an operon, and the transcription is regulated by one or two regulatory proteins.

The ion-responsive regulatory systems of bacteria usually consist of a single regulatory protein, that combines sensor and effector functions in one molecule (31). It senses the cytoplasmic ion concentration and, when activated, can induce or repress transcription of the genes encoding the corresponding uptake, efflux and/or storage systems (2). Four types of metal-responsive proteins have been identified in *Helicobacter* species: the iron-responsive regulator Fur (12), the peroxide-regulatory protein PerR (10, 69), the nickel-responsive regulator NikR (78) and the copper-responsive two-component regulatory system CrdRS (85). Comparison of the three *Helicobacter* genome sequences with that of *C. jejuni* has indicated an interesting distribution of metal-responsive regulators (Fig. 3A). The Fur regulator is present in all *Helicobacter* species and in *C. jejuni*, although its functions and regulons differ (see below). The PerR regulator is present in *H. mustelae*, *H. hepaticus* and *C. jejuni*, but

absent in *H. pylori* (Fig. 3A). This is rather surprising since the main proteins regulated by PerR are catalase and AhpC (16, 74), and these are present in all four species. The NikR regulator is present in all three *Helicobacter* species, but is absent in *C. jejuni*. This is likely to be linked to the presence of the nickel-cofactored urease system in the investigated *Helicobacter* species, and the absence of this enzyme in *C. jejuni*. Finally, the distribution of the CrdRS is difficult to predict, since two-component regulatory systems share significant sequence homology both for the response regulator and the sensory histidine kinase, and this makes predictions based on homology searches risky.

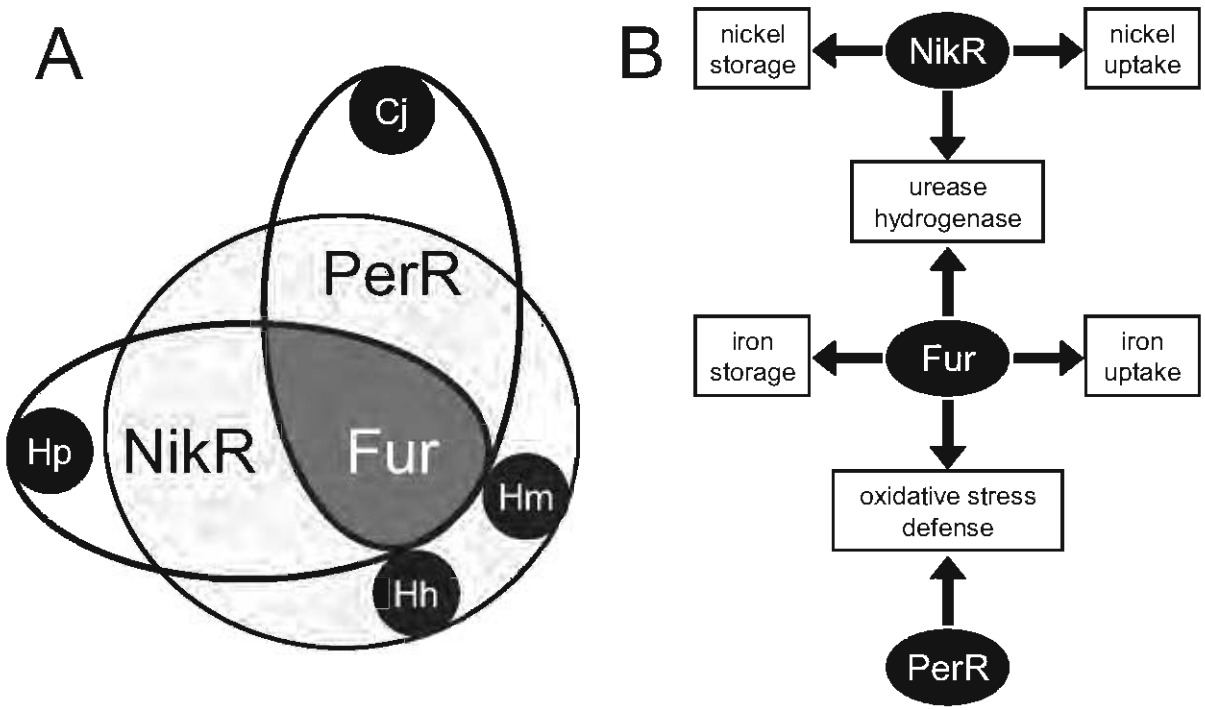

Figure 3. Distribution and overlapping functions of the Fur, NikR and PerR regulatory systems in *Helicobacter* and *Campylobacter* species. (A) Distribution of the regulators in *Helicobacter pylori* (Hp), *Helicobacter mustelae* (Hm), *Helicobacter hepaticus* (Hh) and *Campylobacter jejuni* (Cj). The Fur protein is ubiquitous in all four species (indicated by the dark grey area), whereas the PerR and NikR proteins are only present in 3/4 species (indicated in light grey). (B) Overlapping functions of the Fur, NikR and PerR regulatory systems in *Helicobacter*.

(i) Fur. The Fur protein is primarily known for its role as repressor of iron acquisition systems (35). It fullfils this classical function in all three *Helicobacter* species as well, as the *H. pylori* Fur mediates regulation of iron acquisition outer membrane proteins (24, 80). However, the Fur protein also regulates part of the nitrogen metabolism of *H. pylori*, via iron-responsive repression of the AmiE aliphatic amidase (79). Interestingly, the *H. pylori* Fur protein is bifunctional, since it also functions in its iron-free (apo)form. Iron-free Fur represses the expression of the Pfr ferritin and the iron-cofactored superoxide dismutase SodB (25, 28). This form of regulation may compensate for the absence of iron-responsive regulation via small RNAs like RyhB (47), which is of great importance in several pathogenic bacteria, but has so far not been reported for *H. pylori*.

In *H. hepaticus*, Fur co-regulates expression of the peroxide stress defense genes AhpC and KatA with PerR (see below) (10), and also mediates iron-responsive regulation of the urease system (11). The rationale behind Fur-regulation of urease expression is not directly apparent, but may be linked to Fur functioning as a sensory system for iron-restricted conditions encountered in the natural niche of *H. hepaticus*.

(ii) PerR. The peroxide stress regulator PerR is a Fur homolog and is responsible for iron- and manganese-dependent regulation of *ahpC* and *katA* in *Bacillus subtilis* and *C. jejuni* (16, 74). The *H. hepaticus* and *H. mustelae* genomes contain a gene encoding a PerR homolog, and this may serve as a replacement system for the OxyR regulator, which is absent in *Helicobacter* species and *C. jejuni*. However, PerR is not present in *H. pylori*, and this raises the interesting question whether the PerR system was either lost by *H. pylori* or gained by the other species.

In *C. jejuni*, *perR* mutants were shown to express very high levels of both catalase and alkyl hydroperoxide reductase and to be highly resistant to peroxide stress (74). Like Fur repressors, PerR repressors also recognise a specific sequence upstream of their target genes although the consensus sequence has only been confirmed in *B. subtilis* (39). Identification of PerR-binding sites is complicated by their similarity to Fur-binding sequences. Although PerR responds to iron, it has been suggested that PerR may use manganese as its metal cofactor (39). Preliminary analysis of Fur and PerR expression in *H. hepaticus* suggests that Fur and PerR co-regulate expression of catalase and AhpC, in a similar level to what was described for *C. jejuni* (10).

(iii) NikR. The NikR regulator belongs to the class of ribbon-helix-helix (RHH) proteins. It was first described in *E. coli* as repressor of the nickel uptake operon *nikABCDE* (22). Binding of nickel-cofactored NikR to a 5'-GTATGA-N_{16}-TCATAC-3' sequence in the *nikA* promoter region results in repression of transcription (19), and this allows NikR to maintain intracellular nickel homeostasis in a similar manner as Fur and iron. NikR orthologs are present in all the *Helicobacter* genomes sequenced thusfar, but has only been studied in *H. pylori* (76). NikR was shown to function as a repressor as well as an inducer of transcription, since nickel-dependent binding of NikR to 5'-TATWATT-N_{11}-AATWATA-3' sequences in the *ureA* and *nixA* promoters resulted in induction and repression of transcription, respectively (23, 30). Furthermore, NikR and Fur display overlapping regulons and each regulates the expression of the other protein and the corresponding regulon (17, 77), allowing intricate regulation of gene expression in different environmental conditions.

In *H. pylori* NikR controls utilization of nickel, by mediating nickel- responsive regulation of urease expression (30, 78). Interestingly, the urease system of *H. hepaticus* is not nickel-responsive at the transcriptional level, but only at the post-translational level (9). This indicates that NikR may not be involved in controlling urease expression in *H. hepaticus*, and thus this suggests that NikR-controlled nickel-responsive regulation of urease expression represents a specific adaptation to the gastric lifestyle.

(iv) CrdRS. The CrdRS system was identified in *H. pylori* as the two-component regulatory system required for copper-responsive induction of the copper-resistance determinant CrdAB-CzcBA (84, 85). It is not yet known how the CrdRS system senses copper, but absence of

either CrdR or CrdS disturbs regulation of Crd (85). The CrdRS system plays an important role in gastric colonization in a mouse infection model for *H. pylori* (59) and is also implicated in acid resistance of *H. pylori* (45). Whether *H. mustelae* and *H. hepaticus* contain functional orthologs of CrdRS is not known, although both genomes contain two-component regulatory systems with sequence homology to CrdRS.

CONCLUSIONS

Although they are considered to be fastidious, *Helicobacter* species are well adapted to their ecological niche. From genomic comparisons it is apparent that while clustered in a single species, there are considerable differences in genomic content (27) which are also reflected in the components of metal-transport systems as reviewed here. There seems to be no specific evolution of metal-transporters equipped for either the gastric or enteric environment, with possibly the exception of the NixA vs the NikABDE nickel transporters, the Hpn nickel-binding protein and possibly the presence of a ferric citrate outer membrane transporter, which are only present in the gastric *Helicobacter* species. Therefore differences in niche adaptation may well be more at the level of gene regulation and biochemical properties of the regulated proteins.

The considerable differences between *H. pylori* and *H. mustelae* also need to be taken into account when using the *H. mustelae* ferret infection model, which has been proposed as suitable animal model for *H. pylori* infection. Although pathogenesis of *H. mustelae* infection does mimic that of *H. pylori* infection of humans, there are still many differences between *H. pylori* and *H. mustelae* as well as between man and ferret that need to be taken into account, and will make direct application of this model difficult. Similarly, the differences between host, niche and genetics of *H. hepaticus* and *H. pylori* make direct comparisons very difficult.

In conclusion, the availability of genome sequences of different *Helicobacter* species has allowed for comparative genomic analyses of metal transport systems, and this shows the significant diversity in these systems within a single genus. It also displays the versatility of these bacteria to adapt to the diversity of conditions encountered in their natural gastric or enteric niches in mammals.

Table 1. Metal-transport and metabolism-associated genes of selected *Helicobacter* species

Gene	*H. pylori* [a]	*H. hepaticus* [b]	*H. mustelae*	Proposed function
fecA1	0686	-	present	Outer membrane receptor
fecA2	0807	-	-	Outer membrane receptor
fecA3	1400	-	-	Outer membrane receptor
frpB1	0876	1847	present	Outer membrane receptor
frpB2	0916/0915	-	present	Outer membrane receptor
frpB3 [c]	1512	-	-	Outer membrane receptor
cfrA	-	0721	present	Outer membrane receptor
hh0418	-	0418	3 copies	Outer membrane receptor
fecD	0889	?	present	Inner membrane permease
fecE	0888	1759	present	ATPase
ceuE1	1562	?	present	Periplasmic Binding protein
ceuE2	1561	-	-	Periplasmic Binding protein
feoB	0687	0033	present	Ferrous iron transporter
Pfr	0653	1334	present	Ferritin
dps [d]	0243	0210	present	Bacterioferritin/Dps ortholog
nixA	1077	-	present	Inner membrane nickel transporter
nikABDE	-	0417-0414	-	Inner membrane nickel ABC transporter
cadA	0791	0586	present	P-type ATPase
copA	1072	0682	present	P-type ATPase
copA2	1502	1022	-	P-type ATPase
cznABC	0969-0971	0625-0623	present	Cobalt-zinc-nickel resistance determinant
crdAB	1326-1327	-	present	Copper resistance determinant
Fur	1027	0893	present	Ferric Uptake Regulator
perR	-	0942	present	Peroxide stress regulator
nikR	1338	0352	present	Nickel-responsive regulator
crdRS	1365/1364	1656-1657?	?	two-component copper-regulatory system

a. Gene numbers from the genome sequence of *H. pylori* strain 26695 (71).

b. Gene numbers from the genome sequence of *H. hepaticus* strain ATCC51449 (69).

c. Alternative designation *frpB4*.

d. The *H. pylori* Dps protein is also known as Neutrophil Activating Protein (HP-NAP).

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Chapter 3

UreA2B2: a second urease system in the gastric pathogen *Helicobacter felis*

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ABSTRACT

Urease activity is vital for gastric colonization by *Helicobacter* species, such as the animal pathogen *Helicobacter felis*. Here it is demonstrated that *H. felis* expresses two independent, and distinct urease systems. *H. felis* isolate CS1 expressed two proteins of 67 and 70 kDa reacting with antibodies to *H. pylori* urease. The 67 kDa protein was identified as the UreB urease subunit, whereas the N-terminal amino acid sequence of the 70 kDa protein displayed 58% identity with the UreB protein and was tentatively named UreB2. The gene encoding the UreB2 protein was identified and located in a gene cluster named *ureA2B2*. Inactivation of *ureB* led to complete absence of urease activity, whereas inactivation of *ureB2* resulted in decreased urease activity. Although the exact function of the UreA2B2 system is still unknown, it is conceivable that UreA2B2 may contribute to pathogenesis of *H. felis* infection through a yet unknown mechanism.

INTRODUCTION

Members of the genus *Helicobacter* are gram-negative microaerophilic bacteria that chronically colonize the gastrointestinal and hepatobiliary tract of both human and animals. Colonization with *Helicobacter* species results in chronic inflammation, which can progress into ulceration, atrophy, metaplasia and gastrointestinal and hepatic malignancies (reviewed in Solnick & Schauer, 2001; Kusters *et al.*, 2006). *Helicobacter felis* is a gastric pathogen of cats and dogs (Lee *et al.*, 1988; Paster *et al.*, 1991; Eaton *et al.*, 1996), but it can also infect mice, where it is used as animal model for *H. pylori* infection in humans (Lee *et al.*, 1990; Enno *et al.*, 1995; Nedrud, 1999).

Urease is a major virulence factor of gastric *Helicobacter* species. This enzyme hydrolyses urea into bicarbonate and ammonia, a reaction thought to be crucial in buffering the periplasm and/or cytoplasm of gastric *Helicobacter* species when colonizing their acidic gastric habitat (Stingl *et al.*, 2002; Marcus *et al.*, 2005). Ammonia is probably also used as a nitrogen source (Williams *et al.*, 1996), and is thought to assist in damaging the mucosal barrier, thereby releasing nutrients for the bacterium and maintaining the inflammation process (Smoot *et al.*, 1990). The vital role of urease in colonization by gastric *Helicobacter* species is exemplified by the finding that urease-negative mutants are unable to colonize the stomach in different animal models (Eaton & Krakowka, 1994; Tsuda *et al.*, 1994; Andrutis *et al.*, 1995).

The *Helicobacter* urease enzyme consists of two subunit proteins, UreA (27 kDa) and UreB (62 kDa) (Dunn *et al.*, 1990; Hu & Mobley, 1990). In *H. pylori* the *ureA* and *ureB* genes are organized in an operon transcribed from a promoter upstream of the *ureA* gene (Labigne *et al.*, 1991). Downstream of the *ureAB* genes, the urease operon consists of genes encoding the UreEFGH accessory proteins, which are required for the activation of the urease enzyme (Cussac *et al.*, 1992), and the UreI protein which is an inner membrane protein that functions as a H^+ gated urea channel. This channel allows access of the urea substrate from the surrounding environment to the cytoplasm of the bacterium (Weeks *et al.*, 2000).

Although *H. felis* has been extensively used for studies on gastritis and vaccination against *Helicobacter* (reviewed in Solnick & Schauer, 2001; Kusters *et al.*, 2006), surprisingly little is known about function and regulation of its virulence factors such as urease. The *ureAB* gene cluster of *H. felis* was previously identified and showed high homology to the *H. pylori* urease system (Ferrero & Labigne, 1993), but further information is not available. Therefore we have set out to characterize the urease system of *H. felis*.

MATERIALS AND METHODS

Bacterial isolates and growth conditions

H. felis isolate CS1 (CCUG28539) was grown on Jalava plates [Brain Heart Infusion (BHI) plates (Oxoid) supplemented with 1 g L^{-1} Yeast extract (Becton Dickinson), 20 g L^{-1} Bacto agar (Becton Dickinson), 7% defibrinated horseblood (BioTRADING), amphotericin B (5 mg L^{-1}) and Skirrow antibiotic supplement (Oxoid)]. *H. felis* is highly motile, and requires slightly moist plates for growth. Consequently *H. felis* grows as a lawn (Gottwein *et al.*, 2001). Therefore mutants were isolated by repeated subculturing limiting dilution series on

chloramphenicol-containing agar plates. For broth cultures *H. felis* strains were grown in BHI broth supplemented with 10% newborn calf serum (Invitrogen). Bacteria were incubated at 37°C in a microaerophilic atmosphere of 5% O_2, 10% CO_2 and 85% N_2. *Escherichia coli* strain DH5α was cultured in LB media. When appropriate chloramphenicol was added to growth media to a final concentration of 10 mg L^{-1}.

General genetic manipulations

Plasmid DNA was isolated and purified with the Wizard® Plus SV minipreps DNA purification system (Promega). All DNA modifying enzymes were purchased from Promega, and were used according to manufacturers descriptions. PCR amplification reactions were performed in an automated thermal cycler (I-Cycler; Bio-Rad) using Taq polymerase (Promega).

Construction of *H. felis* urease mutants

Part of the *ureAB* and *ureA2B2* gene clusters of *H. felis* isolate CS1 was amplified using the primer combinations HfUreABF / HfUreABR and HfUreA2B2F / HfUreA2B2R, respectively (Table 1). The resulting amplicons were subsequently cloned in pGEM-Teasy vector (Promega) and transformed into *E. coli* strain DH5α using standard techniques (Sambrook *et al.*, 1989). A single random colony was selected and correct insertion was confirmed by sequence analysis of the insert.

Table 1. Oligonucleotide primers used in this study

Primer	Sequence (5' → 3')
HfUreABF	CGGAAAGAAGCCATTAGC
HfUreABR	CTTCAGCCGCGATAGTTTG
HfUreABF2	ACTATCCACACTCCGGTAGA
HfUreABR2	TTGTCTGCTGTCTGCCAAGT
HfUreA2B2F	TGATCCTGTGTTGTGGGTGGTA
HfUreA2B2R	CACGATCGCACACGCAAGAC
HfUreA2B2R2	GAGGCGATGGGTTTCAATAC
HfUreA2B2F2	GGCGAAGTGGCTAGAA
CatoutF	TAGTGGTCGAAATTACTCTTTTCGTG
CatoutR	CCCTTATCGATTCAAGTGCATCATG
HfureIprobeF	ATTGTATGTTGCGGTCGTGCTG
HfureIprobeR [a]	ctaatacgactcactatagggagaGGCCGCTGGGATAGTGTTG

a.Primer contains a 5'-extension consisting of the T7 promoter sequence (lowercase letters) for the creation of an antisense RNA probe.

Plasmid pAV35 was the source of the chloramphenicol resistance cassette (Cm^R-cassette) (van Vliet *et al.*, 1998). After cutting with *CfoI*, the Cm^R cassette was introduced into the cloned *ureAB* and *ureA2B2* fragments cut with *BsmI*, resulting in constructs pUreabCm and pUrea2b2Cm. The constructs were then transformed to *H. felis* isolate CS1 by electroporation using a minor modification of a previously described protocol (Josenhans *et al.*, 1999), since recovery after electroporation was performed for 16 hours on Jalava plates. Subsequently

bacteria were transferred to Jalava plates containing 10 or 20 mg/L chloramphenicol and were incubated for 8-10 days. Mutant strains resistant to chloramphenicol were selected by repeated subculturing limiting dilution series on chloramphenicol-containing agar plates. Correct replacement of the wild-type urease genes by the interrupted copies was confirmed by PCR using combinations of the primers HfUreABF2, HfUreABR2, HfUreA2B2F2, HfUreA2B2R2, CatoutF, and CatOutR (Table 1).

Urease assay

The enzymatic activity of urease was determined by measuring ammonia production from hydrolysis of urea, by using the Berthelot reaction (van Vliet *et al.*, 2001). The concentration of ammonia in the samples was calculated from a standard NH_4Cl concentration curve. Enzyme activity was expressed in micromoles of urea hydrolyzed per minute per milligram of protein.

Protein analysis

Broth cultures were centrifuged for 10 min at 13000 × *g* and resuspended in phosphate-buffered saline to a final OD_{600} of 10. Bacteria were diluted 1:10 and subsequently lysed by sonication for 15 sec with an MSE Soniprep 150 set at amplitude 6. Protein concentrations were determined with the BCA protein Assay kit (Pierce). Total protein was separated by SDS-PAGE, followed by staining with Coomassie brilliant blue or immunoblotting. Expression of urease subunits was monitored on immunoblots using 1:1000 diluted *H. pylori* urease-specific antiserum (Phadnis *et al.*, 1996). The N-terminal amino acid sequence of the 67 and 70 kDa proteins was determined by Edman degradation.

RNA analysis

Total RNA was isolated from approximately $5x10^8$ *H. felis* cells using TriZOL (Invitrogen). RNA was visualized by methylene blue staining, and RNA concentrations were normalized based on 16S and 23S rRNA band intensities (van Vliet *et al.*, 2001). An internal fragment of the *ureI* gene was amplified with primers HfureIprobeF and HfureIprobeR (Table 1). The PCR fragment contained a T7 promoter sequence and was used for the production of an antisense RNA probe labeled with digoxigenin by transcription with T7 RNA polymerase (Roche Diagnostics). Northern hybridization and stringency washes were performed at 68°C, and bound probe was visualized with the DIG-Detection Kit (Roche Diagnostics) and chemiluminescent substrate CPD-Star (Amersham Pharmacia) (van Vliet *et al.*, 2001). The sizes of the hybridizing RNA were calculated from comparison with a digoxigenin-labeled RNA size marker (RNA Marker I; Roche Diagnostics).

Accession numbers

The sequences of the *H. felis* CS1 *ureABIEFGH* and *ureA2B2* urease gene clusters have been deposited in the GenBank sequence database under accession numbers DQ865138 and DQ865139, respectively.

Phylogenetic analysis

Protein sequences of the large urease subunits of *E. coli* O157:H7 EDL933 (UreC, accession number AAG55290), *Campylobacter lari* CF89-12 (UreB, accession number BAD89502) *Helicobacter hepaticus* ATCC51449 (UreB, accession number AAP77005) and *H. pylori* 26695 (UreB, accession number AAD07143) were retrieved from the GenBank database. Multiple sequence alignments were made using the ClustalW program (Thompson *et al.*, 1994), and a phylogenetic tree was constructed using the Protdist and Neighbor programs of the Phylip 3.66 software package (Felsenstein, 1989).

Statistical analysis

Statistical analyses were performed using the Wilcoxon Signed Ranks test. Two-sided p values <0.05 were considered significant.

RESULTS

***H. felis* expresses multiple proteins reacting with urease antibodies**

To monitor urease expression, lysates from *H. felis* isolate CS1 and *H. pylori* strain 26695 were analyzed by immunoblotting using an antiserum raised to purified *H. pylori* urease enzyme (Phadnis *et al.*, 1996). In the *H. pylori* lane, two immunoreactive bands of approximately 27 kDa and 62 kDa were detected, which correspond to the UreA and UreB proteins (Fig. 1). The urease antiserum showed only mild binding in the *H. felis* CS1 lane in the area corresponding to the UreA protein, but displayed strong antibody-binding towards two separate proteins of 67 and 70 kDa, respectively (Fig. 1).

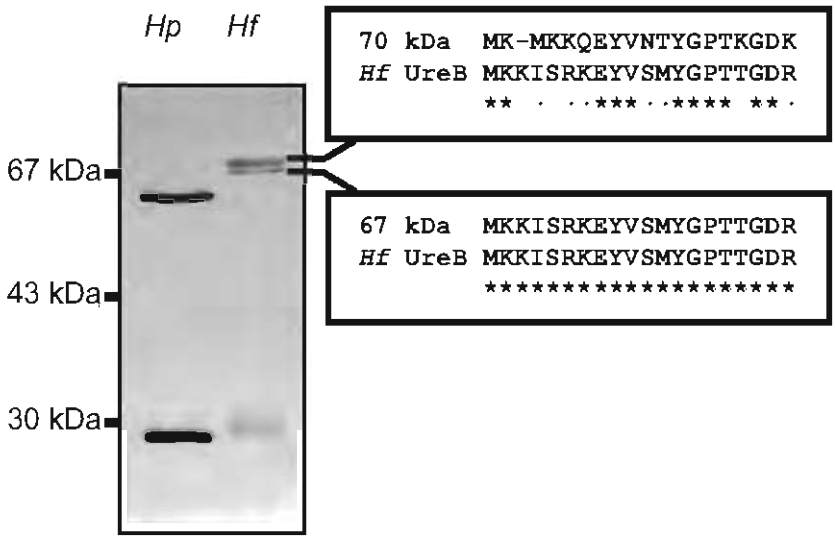

Figure 1. *H. felis* expresses two UreB-like proteins. Immunoblot incubated with polyclonal antibodies raised to *H. pylori* urease. Lane 1, *H. pylori* (*Hp*); lane 2, *H. felis* CS1 (*Hf*). The 67 and 70 kDa bands reacting with the anti-*H. pylori* urease antibodies are indicated on the right, relevant marker sizes on the left. The N-terminal amino acid sequences of the 67 and 70 kDa proteins are given, aligned with the *H. felis* UreB sequence. Asterisks indicate identical residues, dots indicate conservative substitutions.

The N-terminal amino acid sequences of the 67 and 70 kDa *H. felis* proteins were subsequently determined. The N-terminal amino acid sequence of the 67 kDa protein was

identical (20/20 residues) to that of the *H. felis* UreB protein (Ferrero & Labigne, 1993), and was therefore designated UreB. The N-terminal amino acid sequence of the 70 kDa protein was different from the 67 kDa protein, but displayed homology to the UreB protein (Ferrero & Labigne, 1993), with 11/19 residues identical, and was tentatively named UreB2 (Fig. 1).

***H. felis* contains two independent gene clusters encoding putative urease systems**

The regions containing the *ureB* and *ureB2* genes were subsequently cloned from *H. felis* isolate CS1 by PCR using primers based on conserved regions in the protein sequences. As predicted, the *H. felis ureB* gene was preceded by the *ureA* gene (Ferrero & Labigne, 1993), and contained an operon downstream containing orthologs of the *H. pylori ureIEFGH* genes (Fig. 2). Upstream of *ureB2* a gene (*ureA2*) was present putatively encoding a protein homologous to the *H. felis* UreA protein. The *ureA2* and *ureB2* genes were present in six independent *H. felis* isolates, as tested by PCR using a primer located in the *ureA2* and a primer located in the *ureB2* gene (data not shown). We have subsequently tried to identify the genes downstream of *ureB2*, but repeated attempts with either inverse PCR and degenerate primers based on *ureI* and *ureE* sequences were unsuccessful (data not shown), suggesting that accessory genes are absent downstream of *ureB2* (Fig. 2), although further investigation is required to confirm this. Analysis of the protein sequences encoded by the *ureAB* and *ureA2B2* operons showed that the UreA and UreA2 proteins display 46% sequence identity, whereas the UreB and UreB2 proteins display 73% sequence identity. When compared to other *Helicobacter* species the UreA2 protein displays 48-54% sequence identity and the UreB2 protein display 68-74% sequence identity. The UreB and UreB2 proteins both contain the predicted active site of urease (Hulo *et al.*, 2004) as well as the predicted nickel binding site (Mobley *et al.*, 1995).

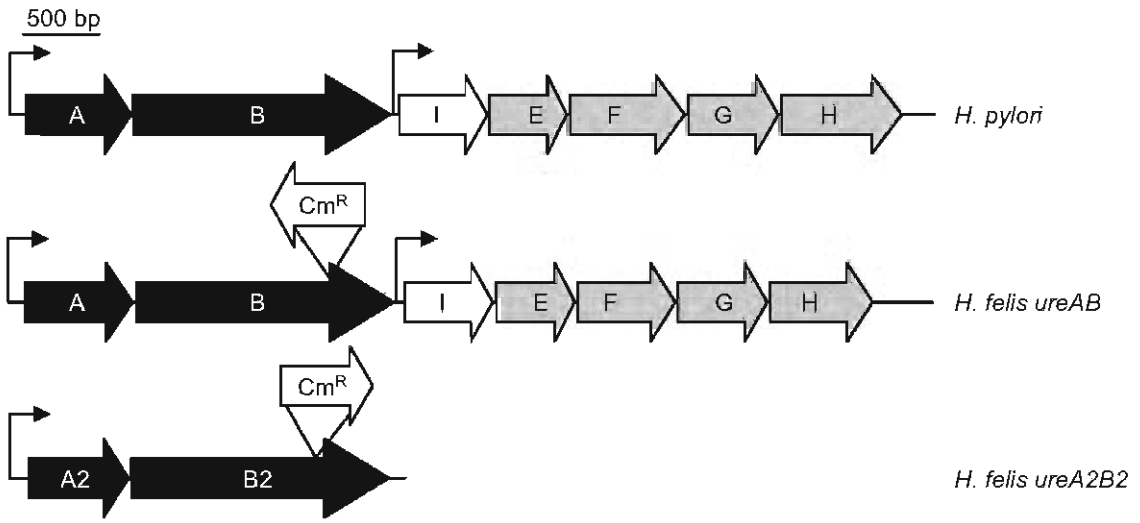

Figure 2. Genetic organisation of the *H. pylori* and two *H. felis* urease gene clusters and the orientation and insertion site of the chloramphenicol cassette (Cm^R) in the *H. felis* mutants. Small arrow symbols indicate the putative promoters upstream of *ureA*, *ureI* and *ureA2* predicted to drive transcription of the urease operons.

Mutation of the *ureB2* gene affects overall urease activity of *H. felis*

To determine whether the UreA2B2 system encodes a functional urease enzyme, we inactivated the *ureB* and *ureB2* genes of *H. felis* CS1 by insertional mutagenesis (Fig. 2).

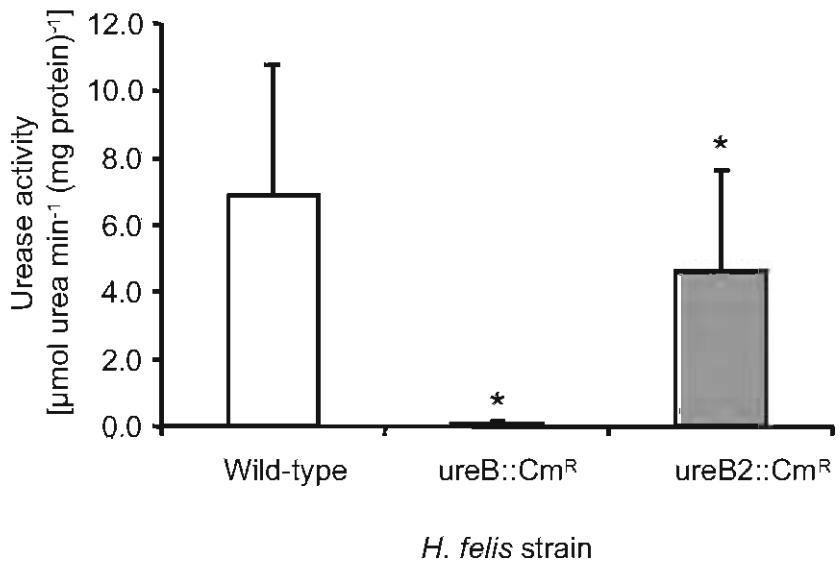

Figure 3. Effect of *ureB* and *ureB2* inactivation on *H. felis* urease activity. Urease activity measurements of *H. felis* CS1 (white bar), *ureB* mutant (black bar) and *ureB2* mutant (grey bar), grown in broth culture. Results show the average of 5-7 independent urease activity measurements. The error bars denote standard deviations, the asterisks indicate a significant difference in urease activity of the mutants when compared to the wild-type strain ($P < 0.05$, Wilcoxons Signed Ranks test).

Unfortunately despite repeated attempts the kanamycin resistance cassette that has been frequently used to generate mutants in *H. pylori* did not generate any kanamycin resistant colonies in *H. felis* (data not shown). As a consequence only the chloramphenicol resistance cassette could be used for the generation of mutants in *H. felis*, and thus it was not possible to construct a double mutant with both genes, *ureB* and *ureB2*, inactivated. Urease activity of *H. felis* isolate CS1 and isogenic mutants was compared to determine the relative contribution of the UreAB and UreA2B2 systems in *H. felis* urease activity (Fig. 3). Mutation of the *ureB* gene resulted in a complete absence of urease activity, suggesting that the UreA2B2 urease is inactive under *in vitro* growth conditions. However, mutation of the *ureB2* gene also resulted in a modest but significant decrease in urease activity (Fig. 3), suggesting that the UreA2B2 urease does contribute to overall urease activity of *H. felis*.

The urease-negative phenotype of the *ureB* mutant is not due to effects on transcription of urease accessory genes

To determine whether the discrepancy between the effects of the inactivation of *ureB* and *ureB2* on urease activity of these mutants was caused by polar/downstream effects of insertion of the chloramphenicol cassette in the *ureB* gene, the effect on the transcription of the downstream *ureIEFGH* operon was determined by Northern hybridization. Based on the situation in *H. pylori*, it was anticipated that a *ureI*-specific probe should allow us to evaluate the transcription originating from the predicted *ureA* and *ureI* promoters in *H. felis* (Akada *et*

al., 2000; van Vliet *et al.*, 2001). In the wild-type *H. felis* isolate, the *ureI* probe reacted with two mRNA species of approximately 1 and 3 kb, predicted to represent the *ureABIE* and *ureIE* mRNAs, respectively (Akada *et al.*, 2000; van Vliet *et al.*, 2001). In the *ureB* mutant the *ureABIE* mRNA is missing, but the *ureIE* mRNA is still present, albeit in lower amounts (Fig. 4). In the *ureB2* mutant both the *ureABIE* and the *ureIE* mRNAs are still present, and therefore the absence of UreA2B2-associated urease-activity in the *ureB* mutant is probably not caused by lack of transcription of the *ureIEFGH* operon including the accessory genes.

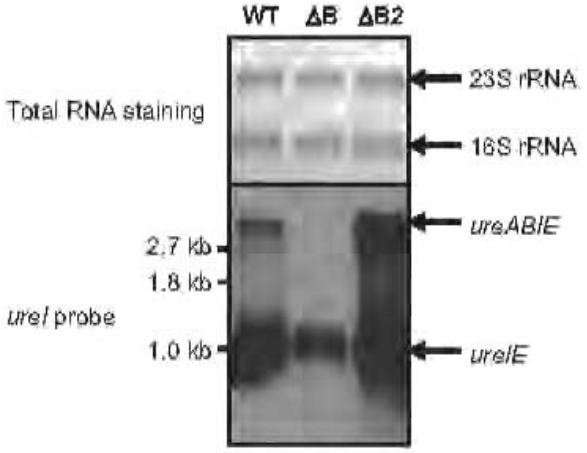

Figure 4. Absence of urease activity in the *H. felis ureB* mutant is not caused by a polar effect on transcription of the downstream urease accessory genes. The figure shows a Northern hybridization with a *ureI* probe. WT: *H. felis* CS1 (wild-type strain); ΔB: *H. felis* CS1 *ureB* mutant (*ureB*::Cm^R); ΔB2: *H. felis* CS1 *ureB2* mutant (*ureB2*::Cm^R). Staining of transferred RNA by methylene blue is included for comparison of RNA amounts (top panel). The position of the predicted *ureABIE* and *ureIE* transcripts is indicated on the right side, whereas the probe used and relevant marker sizes are included on the left side.

DISCUSSION

The urease enzyme is important in pathogenesis of *Helicobacter* infection, as shown by the inability of urease-negative mutants of different *Helicobacter* species to colonize their respective hosts (Eaton & Krakowka, 1994; Tsuda *et al.*, 1994; Andrutis *et al.*, 1995) While the urease system of *H. pylori* has been extensively studied, relatively little is known about the urease systems of other *Helicobacter* species.

Part of the *H. felis* urease gene cluster was previously identified in part, as the sequence of the *ureA* and *ureB* genes was published (Ferrero & Labigne, 1993). When we tested whether the urease of *H. felis* shared cross-reactive epitopes with *H. pylori* urease, we noticed that antiserum to *H. pylori* urease recognized two proteins bands of approximately 70 kDa in *H. felis* (Fig. 1). The identity of both proteins was established by N-terminal amino acid sequencing, and the 67 kDa band was identical to the UreB protein, and the 70 kDa band contained a different N-terminal amino acid sequence which shared homology with the *H. felis* UreB protein (Fig. 1) (Ferrero & Labigne, 1993). Therefore we set out to characterize the gene encoding this putative second urease subunit, and we have also extended the characterization of the gene cluster downstream of the *ureB* gene. The *ureB* and *ureB2* genes

are located in two separate gene clusters, one cluster containing both structural and accessory genes (*ureAB* and *ureIEFGH*), and one cluster only containing the *ureA2B2* genes (Fig. 2). Both the sequence and organization of the complete urease gene cluster is similar to that of *H. pylori* (Mobley *et al.*, 1995) and other *Helicobacter* species (Zhu *et al.*, 2002). Also, the *ureA2B2* gene cluster was present in all five additionally tested *H. felis* isolates (strains CS7, 2301, 144, 1538 and 390) (data not shown).

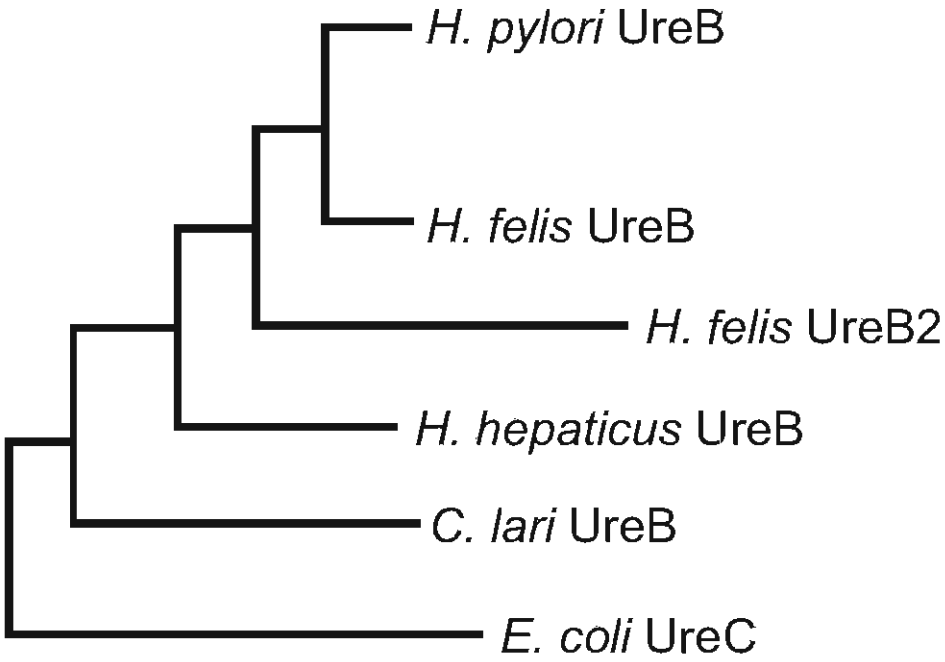

Figure 5. Phylogenetic tree displaying the evolutionary relationship between the *H. felis* UreB2 protein and other urease large subunits (UreB/UreC). The tree was constructed from a distance matrix, calculated from a multiple sequence alignment of the respective sequences.

The UreA2 and UreB2 proteins are 46% and 73% identical to the UreA and UreB proteins, respectively, and are phylogenetically most closely related to the *Helicobacter* UreA and UreB proteins (Fig. 5). In view of the conservation of the active site and nickel-binding site present in the UreB2 protein, this makes it likely that the UreA2B2 complex represents a urease enzyme. However, the data obtained using insertional mutagenesis of the *ureB* and *ureB2* genes contradict each other. In view of the absence of urease activity in the *ureB* mutant, it can be concluded that UreA2B2 does not contribute to total urease activity, whereas the reduction in urease activity in the *ureB2* mutant suggests differently (Fig. 3). The phenotype of the *ureB* mutant is probably not caused by lack of enzyme activation via absence of urease accessory proteins, since the presence of the *ureIE* mRNA suggests that transcription from the *ureI* promoter still occurs (Fig. 4). Although unlikely, there may also be polar effects of the introduction of the chloramphenicol resistance cassette in the *ureB* and *ureB2* genes. This issue can however only be addressed by genetic complementation, which is currently not feasible in *H. felis*.

The presence of two urease gene clusters is not unique in bacteria, as it has been described in the genome sequences of *Brucella suis* and enterohaemorrhagic *E. coli* (Perna *et al.*, 2001;

Paulsen *et al.*, 2002). However, in both *B. suis* and enterohaemorrhagic *E. coli* the two urease gene clusters include accessory genes, while our data thusfar indicate that in *H. felis* only a single set of accessory genes is present, directly downstream the *ureAB* genes (Fig. 2). The UreA2 and UreB2 proteins cluster together with the other *Helicobacter* UreA and UreB proteins (Fig. 5), and the *H. felis* UreA and UreA2 proteins are both fusions of the ancestral bacterial alpha and gamma subunits (Mobley *et al.*, 1995; Burne & Chen, 2000), suggesting that the UreA2B2 cluster has not been recently acquired by horizontal gene transfer.

The function and regulation of the UreA2B2 system is currently unknown. It is tempting to speculate that the presence of two separate urease systems may allow differential expression of urease systems depending on the environmental conditions. The presence of the second urease should also be taken into account with both infection and vaccination studies using the *H. felis* mouse model, since cross-neutralization or cross-protection between both antigens is likely, but may not result in full protection. Further investigations are needed to determine the possible role of the second urease in gastrointestinal and hepatic malignancies.

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Chapter 4

Inverse nickel-responsive regulation of two urease enzymes in the gastric pathogen *Helicobacter mustelae*

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ABSTRACT

The acidic gastric environment of mammals can be chronically colonized by pathogenic *Helicobacter* species, which use the nickel-dependent urea-degrading enzyme urease to confer acid resistance. Nickel availability in the mammal host is low, being mostly restricted to vegetarian dietary sources, and thus *Helicobacter* species colonizing carnivores may be subjected to episodes of nickel-deficiency and associated acid-sensitivity. The aim of this study was to investigate how these *Helicobacter* species have adapted to the nickel-restricted diet of their carnivorous host. Three carnivore-colonizing *Helicobacter* species express a second functional urea-degrading urease enzyme (UreA2B2), which functions as adaptation to nickel-deficiency. UreA2B2 was not detected in seven other *Helicobacter* species, and is in *H. mustelae* only expressed in nickel-restricted conditions, and its expression was higher in iron-rich conditions. In contrast to the standard urease UreAB, UreA2B2 does not require activation by urease or hydrogenase accessory proteins, which mediate nickel incorporation into these enzymes. Activity of either UreAB or UreA2B2 urease allowed survival of severe acid shocks in the presence of urea, demonstrating a functional role for UreA2B2 in acid-resistance. Pathogens often express colonization factors which are adapted to their host. The UreA2B2 urease could thus represent an example of pathogen adaptation to the specifics of the diet of their carnivorous host, rather than to the host itself.

INTRODUCTION

Bacterial pathogens colonizing mammals typically face many stresses, as the host uses many non-specific and specific defense mechanisms to obstruct pathogenic microorganisms from persistent colonization. One of these host defense mechanisms present at mucosal surfaces is acidic pH, which should prevent bacterial colonization. However, the human pathogen *Helicobacter pylori* is able to colonize the mucus layer overlaying the gastric epithelial cells, and the resulting gastritis predisposes to peptic ulceration and gastric cancer (Kusters *et al.*, 2006). Since the discovery of *H. pylori*, many other *Helicobacter* species have been identified, and it now generally acknowledged that the gastric mucosa of most mammals can be colonized by gastric *Helicobacter* species (Solnick and Schauer, 2001). These non-*pylori* *Helicobacter* species may provide good animal models to study *Helicobacter* infection in an endogenous host (O'Rourke and Lee, 2003) and comparative genomics may contribute in our understanding of host specificity (Eppinger *et al.*, 2006).

One of the major factors allowing gastric colonization by *Helicobacter* species is the urease enzyme, which catalyzes the conversion of urea into ammonia and carbon dioxide, thus leading to an increase in pH (Burne and Chen, 2000). Both ammonia and carbon dioxide play important roles in acid resistance of gastric *Helicobacter* species (Stingl et al., 2002; Marcus et al., 2005), and urease-negative mutants of different *Helicobacter* species are unable to colonize the gastric environment (Tsuda et al., 1994; Andrutis et al., 1995). Urease is a multimeric enzyme, that requires activation by incorporation of the metal cofactor nickel (Burne and Chen, 2000). The urease enzyme of *Helicobacter* species consists of two subunits named UreA and UreB, and in *H. pylori* exists in a hexadodecamer which may improve enzyme stability in acidic conditions (Ha *et al.*, 2001). The urease operons of ureolytic *Helicobacter* species characterized to date also encode the UreI and UreEFGH proteins. UreI functions as acid-activated urea channel (Weeks et al., 2000; Bury-Mone et al., 2001), whereas the UreEFGH accessory proteins incorporate the nickel cofactor and activate the enzyme (Burne and Chen, 2000). The urease system of *H. pylori* is positively regulated by the availability of nickel as cofactor via the NikR regulatory protein (van Vliet et al., 2001; van Vliet et al., 2002; Delany et al., 2005; Ernst et al., 2005) and by acidic pH via the two component regulatory system ArsRS (Akada et al., 2000; Bury-Mone et al., 2004; Pflock et al., 2005).

Acquisition of nickel is of prime importance to gastric *Helicobacter* species, as urease is a highly expressed enzyme (up to 10% of the soluble protein of *H. pylori*) (Hu and Mobley, 1990), and reduced urease activity leads to acid-sensitivity (Nolan *et al.*, 2002). However, as nickel is neither used nor stored by the mammal host (Denkhaus and Salnikow, 2002), the primary source of nickel will probably be dietary. The major dietary sources of nickel are vegetarian, and include roots, and nuts and vegetables (Sunderman, 1993). This poses a potential problem for *Helicobacter* species colonizing the gastric environment of obligate carnivores, since meat is unlikely to contain sufficient amounts of nickel to cater for urease, and nickel will probably only be available when the intestinal contents of the prey is consumed (Denkhaus and Salnikow, 2002). However, the gastric environment of carnivores

can be colonized lifelong by *Helicobacter* species (Solnick and Schauer, 2001), and hence these *Helicobacter* species must have adapted to the scarcity of nickel in their niche.
There are three gastric *Helicobacter* species known to colonize the gastric environment of such carnivores: *Helicobacter acinonychis* (big cats), *Helicobacter felis* (cat) and *Helicobacter mustelae* (ferret) (Solnick and Schauer, 2001; Kusters et al., 2006). We recently reported the presence of a gene cluster (*ureA2B2*) in *H. felis* which potentially encode the subunit proteins of a second urease enzyme, but were unable to demonstrate any function in urea degradation in *H. felis* (Pot *et al.*, 2007). In this study we report that the UreA2B2 urease system is also present in *H. mustelae* and *H. acinonychis*, and report on the function of the UreA2B2 urease system in *H. mustelae*. Since the UreA2B2 system seems only present in *Helicobacter* species colonizing obligate carnivores and is only expressed in nickel-restricted but iron-rich conditions, it may represent an adaptation of *Helicobacter* species to the (nickel-limited and iron-rich) diet of their carnivorous hosts.

RESULTS

UreA2B2 is only detected in three carnivore-colonizing *Helicobacter* species

A study on the prevalence of multiple urease systems in members of the genus *Helicobacter* was initiated by using Southern hybridization, PCR with degenerate primers and immunoblotting with UreB-specific antibodies (Pot et al., 2001). These preliminary experiments suggested the presence of multiple *ureB* genes or UreB protein in two other *Helicobacter* species, *H. mustelae* and *H. acinonychis*, whereas other tested ureolytic *Helicobacter* species did not seem to contain multiple *ureB* genes or UreB proteins (data not shown). Searches of the complete but unannotated *H. mustelae* genome sequence (http://www.sanger.ac.uk/Projects/H_mustelae) and the *H. acinonychis* genome sequence (Eppinger et al., 2006) allowed the identification of two urease operons in each of these species, and their surrounding genes. Both *H. mustelae* and *H. acinonychis* contained an operon similar to the *H. pylori* urease operon, consisting of the *ureAB* genes encoding the structural subunits and the *ureIEFGH* genes encoding the urea channel and urease accessory proteins (Fig. 1*A*). The second urease operon contained only orthologs of the *ureA* and *ureB* genes, designated *ureA2* and *ureB2*, analogous to the situation in *H. felis* (Pot et al., 2007). Both urease operons were approximately 500 kb apart on both the *H. mustelae* and *H. acinonychis* genomes. The *H. mustelae* UreA2 and UreB2 proteins are 57.4% and 69.5% identical and 86.5% and 92.3% similar to the *H. mustelae* UreA and UreB proteins, respectively. The *H. acinonychis* UreA2 and UreB2 proteins are 55.1% / 86.2% and 71.9% / 94.7% identical / similar to the *H. acinonychis* UreA and UreB proteins, respectively.
Comparison of the predicted UreB2 protein sequences with those of the corresponding *Helicobacter* UreB subunits and with the UreC urease subunits of *Klebsiella pneumoniae*, *Bacillus subtilis* and *Brucella suis* indicated that the amino acid motifs involved in metal-binding and the active site of the enzyme are conserved (Supplementary Fig. 1). Even though the *Helicobacter* UreB2 sequences cluster separately from the other *Helicobacter* UreB sequences, they are still more closely related to the *Helicobacter* UreB sequences than to the

B. subtilis, B. suis and K. pneumoniae UreC sequences included in the alignment (Supplementary Fig. 1). Thus we hypothesized that the UreA2B2 system encodes an active urease, and have further characterized the UreA2B2 system in *H. mustelae*, due to the availability of techniques for genetic manipulation in this *Helicobacter* species (O Croinin *et al.*, 2007).

Inverse nickel-responsive regulation of UreA2B2 and UreAB expression and activity

There was no detectable expression of the UreA2B2 system when *H. mustelae* was grown in Brucella media (data not shown). To test whether this was due to the growth conditions, we also grew *H. mustelae* in Ham's F-12 (Kaighn's modification) (Testerman *et al.*, 2006), a tissue culture medium that does not contain any added nickel. In this medium the UreA2B2 urease was expressed at high levels, while the UreAB urease was only expressed at relatively low levels (Fig 1*B*). Addition of $NiCl_2$ to Ham's F-12 medium at final concentrations of 100 nM or higher resulted in the absence of UreA2B2 expression, and an increase in UreAB expression (Fig. 1*B*, upper panel). The UreAB and UreA2B2 urease systems of *H. acinonychis* and *H. felis* displayed the same nickel-responsive induction and repression, respectively (Fig. 1*C*), indicating that *H. mustelae* is a good representative of the group of ureolytic carnivore-colonizing gastric *Helicobacter* species. The inverse nickel-responsive regulation of UreAB and UreA2B2 expression in *H. mustelae* was mediated at the transcriptional level, as was shown by Northern hybridization (Fig. 1*B*, lower panels), and was consistent with the absence of UreA2B2 expression in standard laboratory media like Brucella broth, as the nickel content of this medium equals or exceeds 200 nM (Bereswill *et al.*, 2000). In *H. pylori*, nickel-responsive transcriptional regulation of urease expression is controlled by the NikR nickel-responsive regulator (Ernst et al., 2005), and we hypothesized that the *H. mustelae* NikR ortholog would mediate this regulation in *H. mustelae* (Belzer *et al.*, 2007a). Therefore the *H. mustelae nikR* gene was inactivated by insertion of an antibiotic resistance cassette, and the effect of this mutation on urease expression and activity was assessed (Fig. 1*B*). Absence of NikR resulted in locking of both the expression of UreAB and of UreA2B2 in the state detected in unsupplemented (i.e. nickel-restricted) Ham's F-12 medium: low level expression of UreAB, high level expression of UreA2B2 (Fig. 1*B*).

Expression of UreA2B2 is iron-induced and nickel-repressed

We hypothesized that the inverse regulation of UreA2B2 and UreAB expression could be due to these ureases fulfilling a role in different environmental conditions. Therefore the effect of varying iron (a major constituent of meat) and nickel (from vegetarian dietary sources) concentrations in the Ham's F-12 growth medium on the expression of the UreA2B2 and UreAB ureases was assessed. Growth of wild-type *H. mustelae* in medium lacking nickel and iron resulted in low levels of UreB2 and UreB expression and transcription, as measured using SDS-PAGE/immunoblot (Fig. 2*A*) and qRT-PCR (Fig. 2*B*). The presence of iron did not significantly affect UreB expression, but resulted in a significant increase in UreB2 expression. In contrast, the presence of nickel resulted in a significant increase in UreB expression, and a significant decrease in UreB2 expression (Fig. 2). This demonstrates that

UreA2B2 expression is both nickel- and iron-responsive, whereas UreAB expression is only nickel-responsive.

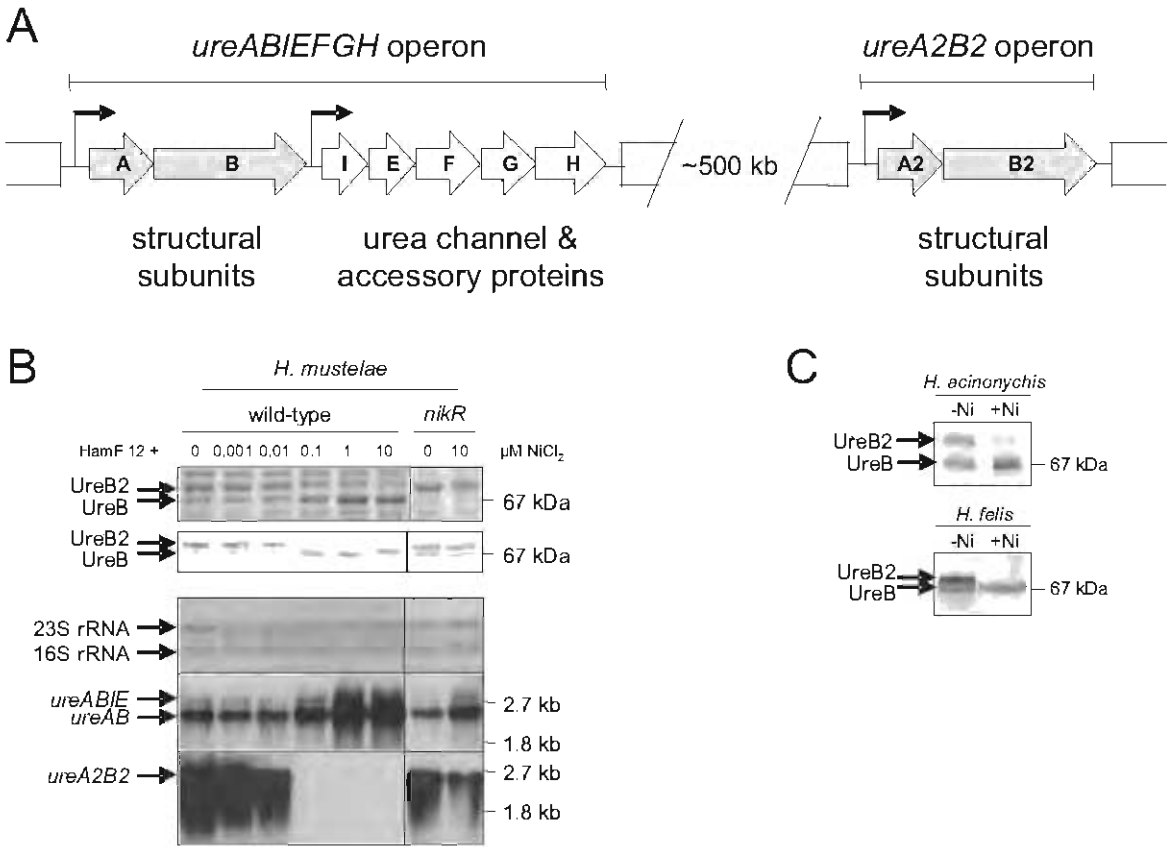

Figure 1. Expression of the *ureA2B2* operon of *Helicobacter mustelae* is nickel- and NikR-repressed. A) Genomic organization of the two urease operons of *H. mustelae* and *H. acinonychis*, obtained from the complete genome sequences http://www.sanger.ac.uk/Projects/H_mustelae and (Eppinger et al., 2006), respectively). B) The two urease systems of *H. mustelae* are inversely nickel-regulated via the NikR regulatory system, as shown by SDS-PAGE and immunoblot with an antibody recognizing both the UreB and UreB2 subunits (top panel), and by Northern hybridization using *H. mustelae ureB*- and *ureB2*-specific probes (bottom panels). Strains and nickel-supplementation of Ham's F-12 medium is shown above the panels, and a methylene blue staining of transferred rRNA is included to show that equal amounts of RNA were loaded. Only the relevant parts of the gels are shown. C) The UreAB and UreA2B2 systems of *Helicobacter acinonychis* and *Helicobacter felis* are subject to nickel-responsive regulation similar to *H. mustelae*, as demonstrated by immunoblot using an antibody recognizing both the UreB and UreB2 subunits.

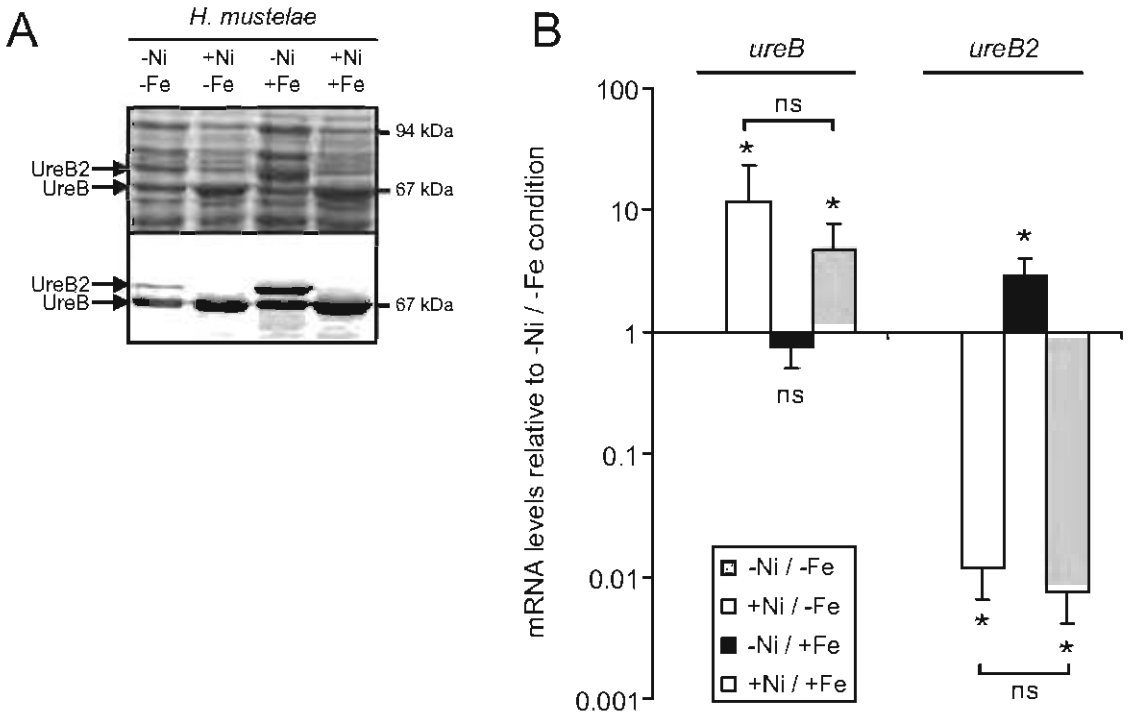

Figure 2. Expression of the UreAB urease in *H. mustelae* is nickel-induced but iron-independent, whereas UreA2B2 expression is iron-induced and nickel-repressed, as shown by SDS-PAGE/immunoblot with urease-specific antibody (A) and quantitative reverse transcriptase PCR (B). The levels of mRNA observed in the qRT-PCR were normalized to the levels of 16S rRNA (Livak and Schmittgen, 2001) and the -Ni/-Fe condition was set to 1. All other mRNA levels are expressed as compared to the -Ni/-Fe condition. Error bars represent standard deviation, an asterisk indicates a significant difference ($P<0.05$, Mann-Whitney U test) in transcription when compared to the -Ni/-Fe condition, ns=not significant. Results shown are the average of three to five independent experiments.

Both urease homologs encode an active urease enzyme

Several combinations of *H. mustelae* mutants were created by inactivation of the *nikR*, *ureB1* and *ureB2* genes to study their effect on urease activity. Interestingly, initial measurements of urease activity using standard protocols used for *H. pylori* (van Vliet et al., 2001) still did not reveal any UreA2B2-associated urease activity (Fig. 3*A*), not even in *nikR* mutant derivatives which displayed high-level, constitutive expression of the UreA2B2 urease subunits. This led us to investigate whether the lysis step included in the standard protocols for urease detection could ablate UreA2B2 urease activity. This was indeed the case: the *nikR ureB* mutant displayed urease activity, and UreA2B2 urease activity was absent upon cell lysis, and was also absent in a *nikR ureB ureB2* mutant (Fig. 3*B*). The absence of detectable UreA2B2 urease activity in lysates was not due to the presence of EDTA in the assay buffer, as omission of EDTA did not result in detectable UreA2B2 urease activity in lysates, and did not affect UreA2B2 urease activity in whole cells (data not shown). Activity of the UreA2B2 urease (approximately 0.5 U/mg total protein, with 1 unit representing 1 µmol urea hydrolyzed per minute) was rather low when compared to the UreAB urease, which was already 3-5 U/mg protein in unsupplemented Ham's F-12 medium, and could reach up to 50 U/mg protein in nickel-supplemented Brucella medium (data not shown).

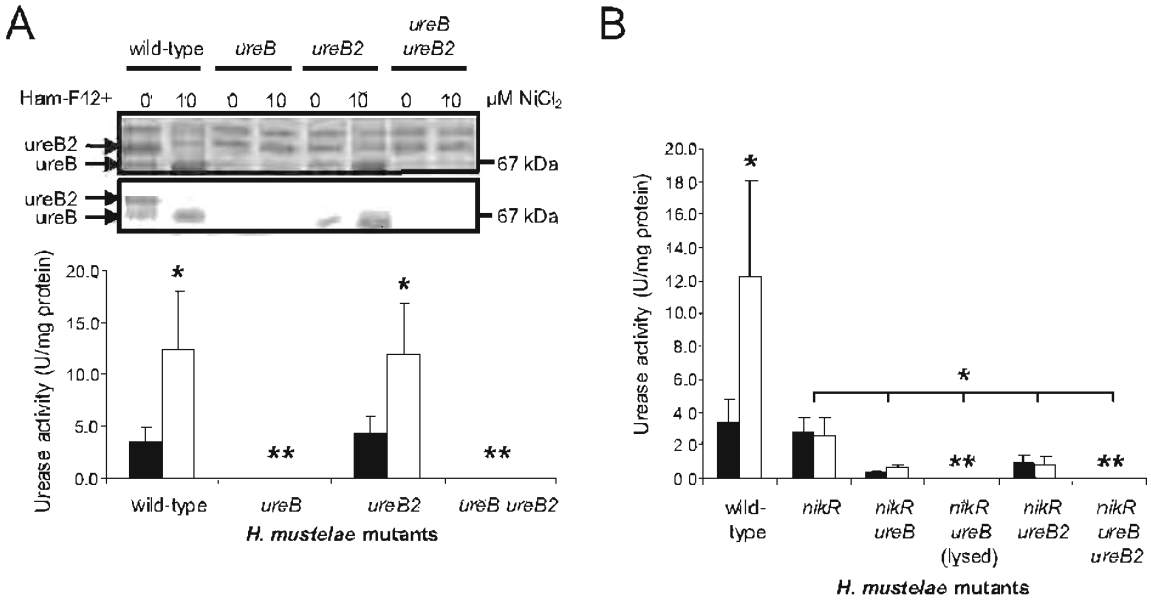

Figure 3. Both UreAB and UreA2B2 of *H. mustelae* are urea-degrading enzymes, but UreA2B2 activity is lost after cell lysis. A) Mutation of the *H. mustelae ureB* gene results in absence of expression and activity of both the UreAB and UreA2B2 ureases, whereas mutation of *ureB2* does not affect UreAB expression or activity. Expression of the UreB and UreB2 proteins was monitored using SDS-PAGE and immunoblot with antibodies recognizing both UreB and UreB2 (top panel), and by measuring enzyme activity in lysates. B) Mutation of *nikR* results in constitutive expression and activity of UreAB and UreA2B2. Strains used with their respective mutations are shown on the X-axis, urease activity is shown on the Y-axis. One unit (U) of urease activity represents 1 µmol of urea hydrolyzed per minute, and is expressed as U per milligram total protein. Strains were grown in Ham's F-12 medium (black bars) or Ham's F-12 medium supplemented with 10 µM $NiCl_2$ (white bars). Error bars represent standard deviation, an asterisk indicates a significant difference ($P<0.05$, Mann-Whitney U test) in urease activity between unsupplemented medium and nickel-supplemented medium (in the wild-type strain) or between the *nikR* mutant and its derivatives lacking either UreB or UreB2. ** = no activity detected. Results shown are the average of three to five independent experiments.

UreA2B2 is not activated by the urease or hydrogenase accessory proteins

Urease is a nickel-containing enzyme that requires activation/maturation by the accessory proteins UreEFGH (Burne and Chen, 2000). The *ureAB* gene cluster of both *H. mustelae* and *H. acinonychis* contains genes encoding the UreEFGH accessory proteins, but the *ureA2B2* gene cluster of both species does not have sequences potentially encoding such accessory proteins downstream or upstream of the operon (Fig. 1*A*). To test whether the accessory genes of the UreAB system also activate the UreA2B2 urease, we inactivated the *ureG* gene in *H. mustelae nikR* mutant strains, and tested the effect on UreAB (*nikR ureB2* mutant) and UreA2B2 (*nikR ureB1* mutant) enzyme activity. Inactivation of *ureG* completely abolished UreAB activity, similar to what has been described for *H. pylori* (Eaton and Krakowka, 1994), but absence of UreG did not affect UreA2B2 activity (Fig. 4). The other nickel-containing enzyme of *Helicobacter* species is the NiFe-hydrogenase, and maturation of this enzyme is mediated by the Hyp accessory proteins (Olson et al., 2001; Mehta et al., 2003). The hydrogenase maturation protein HypB has been reported to be also involved in urease maturation in *H. pylori* (Olson et al., 2001; Mehta et al., 2003), but inactivation of the *hypB* gene in the *nikR ureG* mutant background did not result in any significant change in UreA2B2

activity (Fig. 4). This suggests that neither the urease accessory proteins nor the hydrogenase accessory proteins are required for activation of UreA2B2, and that UreA2B2 may not utilize nickel as cofactor.

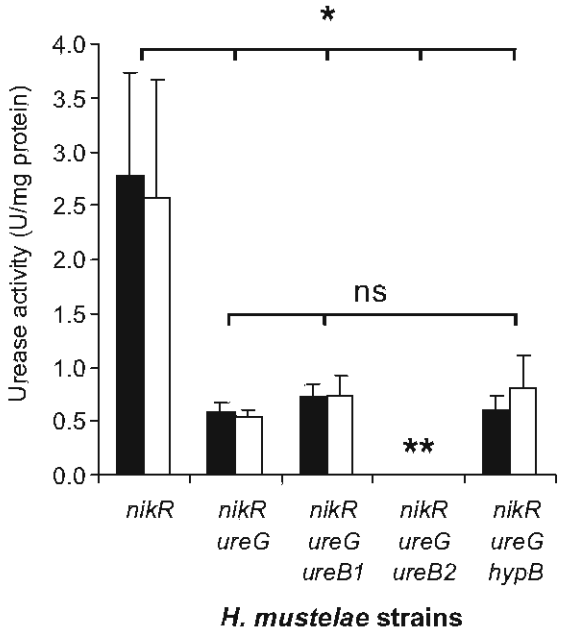

Figure 4. The *H. mustelae* UreA2B2 urease does not require activation via the standard urease or hydrogenase maturation systems, whereas the UreAB urease requires the urease maturation system. Strains were grown in Ham's F-12 medium (black bars) or Ham's F-12 medium supplemented with 10 μM $NiCl_2$ (white bars). The graph shows the average of 3-4 independent urease assays. One unit (U) of urease activity represents 1 μmol of urea hydrolyzed per minute, and is expressed as U per milligram total protein. The *nikR* mutant functions in this experiment as parental strain, as it expresses both ureases. Error bars represent standard deviation, an asterisk indicates a significant difference ($P<0.05$, Mann-Whitney U test) in urease activity between the *nikR* parental strain and its isogenic mutants; ns = non-significant, ** = no activity detected.

Both ureases mediate urea-dependent acid-resistance in *H. mustelae*

Urease is the major acid-resistance determinant of gastric *Helicobacter* species (Eaton and Krakowka, 1994; Tsuda et al., 1994; Andrutis et al., 1995), and lowered urease activity reduces colonization rates of *H. pylori* in a mouse model of infection (Nolan et al., 2002). We therefore determined whether the *H. mustelae* UreA2B2 system is involved in resistance to acidic pH (Dunn et al., 1991; Andrutis et al., 1995; Solnick et al., 1995). Wild-type *H. mustelae* was able to survive acid shock at pH 1.5 only in the presence of 5 mM urea, whereas urease-deficient mutants (*ureB1 ureB2* and *nikR ureB1 ureB2*) showed a signficant reduction in cfu counts at pH 1.5 (Fig. 5). Absence of urea resulted in a 4-log reduction in survival of the wild-type strain at pH 1.5, indicating the importance of urease in survival of severe acid shocks. Although the *nikR* mutant is unable to regulate its urease expression, it did not show any reduction in the capacity to survive acid shocks at pH 1.5 in the presence of urea, when compared to the wild-type strain (Fig. 5). Inactivation of either one of the two urease systems in the *nikR* mutant background did not significantly affect survival at pH 1.5 in the presence of urea (Fig. 5), suggesting that, despite the difference in activity level between the two urease

systems, expression of a single urease system is already sufficient for acid-resistance of *H. mustelae*.

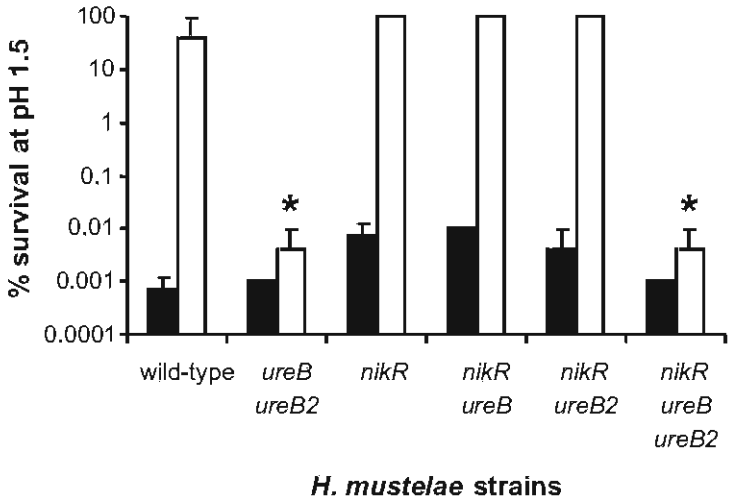

Figure 5. Both the UreAB and UreA2B2 ureases contribute to acid resistance of *H. mustelae* by allowing survival of acid shock at pH 1.5. Percentage of survival of *H. mustelae* strains after acid shock of pH 1.5 in the absence of urea (black bars) or presence of 5 mM urea (white bars). *H. mustelae* strains used are shown on the X-axis, percentage survival (Y-axis) is calculated using survival of the same strain in phosphate buffered saline pH 7.4 without urea. The error bars represent standard deviations calculated from the three independent experiments performed. The absence of an error bar means that identical measurements were made in all three experiments, an asterisk indicates a significant difference ($P<0.05$, Mann-Whitney U test) in acid-shock survival in the presence of urea when compared to wild-type *H. mustelae*.

DISCUSSION

The different members of the genus *Helicobacter* are all capable of causing chronic infections of the gastrointestinal and hepatobiliary tract (Solnick and Schauer, 2001; Kusters et al., 2006), and many of these *Helicobacter* species are associated with the development of disorders related to chronic inflammation. *H. pylori* has long been the model organism for investigating the group of *Helicobacter* species colonizing the gastric mucosa. This environment is regarded as hostile, due to the presence of several host defense mechanisms present at mucosal surfaces, which include an active immune response, nutrient restriction via compounds like lactoferrin, and also an acidic pH. The pH in the lumen of the stomach is often below 2, and it was therefore long thought that the healthy human stomach was sterile, but this paradigm was refuted by the discovery of *H. pylori* (Marshall and Warren, 1984). Since then many new *Helicobacter* species have been identified (Solnick et al., 1995; Fox, 2002), and most of these species have a relatively limited host range. *Helicobacter* species display exquisite adaptations to specific hosts, which is exemplified in for instance the molecular mimicry of Lewis-bloodgroup antigens (Appelmelk et al., 1996; O Croinin et al., 1998) which is involved in evasion of the host immune response. The blood group antigens mimicked by *H. pylori* match the human Lewis X and Y blood group antigens, whereas *H. mustelae* expresses Lewis A antigens as found in the ferret host. Specificity in adhesins and their cognate ligands is also thought to contribute to host specificity (Odenbreit, 2005), and

differences in additional ammonia-producing enzymes have previously been reported for gastric *Helicobacter* species colonizing different animal species (Bury-Mone *et al.*, 2003). Pathogen adaptation to specifics of the host's diet as we propose here has however not been reported to our knowledge.

The urease enzyme is one of the major colonization factors of many *Helicobacter* species, and all known gastric colonizers in the genus *Helicobacter* express an active urease which is thought to mediate acid resistance (Solnick and Schauer, 2001; Fox, 2002; Gueneau and Loiseaux-De Goer, 2002). However, an active urease is insufficient to confer acid-resistance for gastric colonization, since several enterohepatic *Helicobacter* species are urease-positive but acid-sensitive (e.g. *Helicobacter hepaticus*) (Belzer *et al.*, 2005) and unable to colonize the gastric environment (Solnick and Schauer, 2001). The urease system of *H. pylori* is a key example of adaptation to gastric colonization, as is apparent at the level of structure, regulation of expression and regulation of enzyme activity (Akada et al., 2000; Ha et al., 2001; Stingl et al., 2002; van Vliet et al., 2004; Pflock et al., 2005). The regulation of UreAB urease expression observed in this study for the three gastric *Helicobacter* species investigated (*H. mustelae*, *H. acinonychis* and *H. felis*) mimics that of the *H. pylori* urease, since expression and activity of the UreAB system is nickel-induced (Fig. 1). This contrasts with the regulation of urease expression of the sole enterohepatic *Helicobacter* species studied to date (*H. hepaticus*), which displays nickel-independent but iron-responsive repression of expression (Belzer et al., 2005; Belzer et al., 2007b), and with the nickel-repression of UreA2B2 expression (this study).

In this study we have described the role and regulation of the UreA2B2 in three carnivore-colonizing *Helicobacter* species. Expression of UreA2B2 was nickel-repressed in all three UreA2B2-positive *Helicobacter* species (Fig. 1), and expression of UreA2B2 was also iron-induced in *H. mustelae* (Fig. 2). The observed nickel-repression of expression of UreA2B2 may also explain why the UreA2B2 enzyme was not noticed in a previous study on *H. mustelae* urease, since *H. mustelae* in that study was grown in nickel-replete conditions at which expression of the UreA2B2 system is completely repressed (Dunn et al., 1991). The absence of a role for the urease and hydrogenase accessory proteins in activating UreA2B2 (Fig. 4) is consistent with our hypothesis that the UreA2B2 urease is an adaptation to a nickel-restricted diet of the carnivorous host, and mediates acid-resistance (Fig. 5) during periods of nickel-restriction where the UreAB urease would be unable to function. We have summarized this hypothesis in Figure 6.

This is the first report on the presence of two active, inversely regulated ureases in bacteria. In *Brucella* species two urease gene clusters have been identified (Bandara et al., 2007; Sangari et al., 2007), but in contrast to *H. mustelae* both gene clusters contain genes encoding accessory proteins. In addition, one of the urease clusters of *B. suis* and *Brucella abortus* (*ure2*) has been reported to be inactive (Bandara et al., 2007; Sangari et al., 2007), but this conclusion may be preliminary as this is based on analyses of cell lysates only, and expression was not investigated at the protein level. We hypothesize that the *Brucella* urease *ure2* system may have properties similar to *H. mustelae* UreA2B2, and that its activity is only present in intact *Brucella* cells, or may only be expressed in specific conditions.

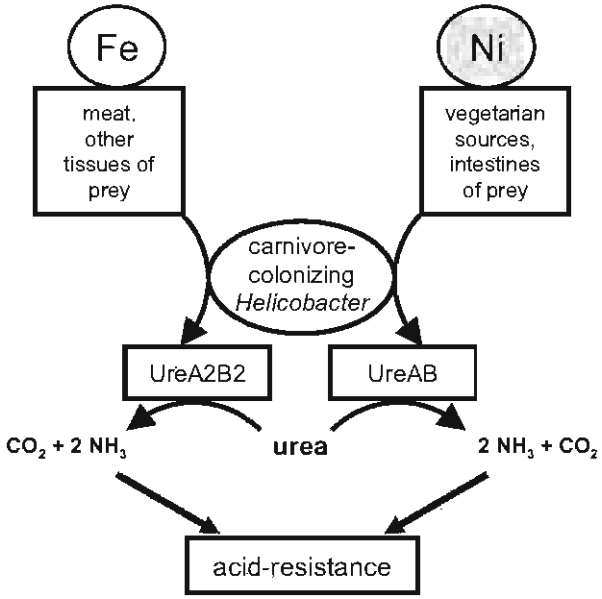

Figure 6. Speculative model representing the roles and regulation of the UreAB and UreA2B2 of carnivore colonizing gastric *Helicobacter* species.

Based on the regulatory pattern observed in *H. mustelae, H. felis* and *H. acinonychis* (showing UreA2B2 expression only at severe nickel-restriction) and the independence of the UreA2B2 system from the urease and hydrogenase maturation systems, it is tempting to speculate that the UreA2B2 system does not use nickel as cofactor, but requires an alternative (metal ion) cofactor, which would be a paradigm shift for urease. Expression of urease activity in the absence of accessory proteins has so far only been reported for *B. subtilis*, which contains only a single urease cluster (Kim *et al.*, 2005). The *B. subtilis* genome sequence lacks genes encoding known nickel-specific transporters, but metal cofactor analysis of recombinant *B. subtilis* urease purified from *Escherichia coli* revealed the presence of small amounts of both nickel and zinc in the recombinant enzyme (Kim et al., 2005).

Unfortunately the absence of UreA2B2 enzyme activity upon cell lysis has to date prevented the purification of active UreA2B2 protein for determination of its metal content. Expression of the *H. mustelae* UreA2B2 system in *E. coli* did not result in urease activity (data not shown). We can only speculate as to why the UreA2B2 activity loses activity after cell lysis. Perhaps the proton motive force is required for the function of the enzyme, or alternatively the enzyme could be using a oxidation-sensitive metal cofactor, like ferrous iron. A role of iron is supported by the regulatory pattern (Fig. 2), where iron induces expression of the UreA2B2 system, and the metal-binding motif in UreA2B2 may support iron-binding (Liaw et al., 2003; Vincent et al., 2004). Additional support for a non-nickel metal cofactor for UreA2B2 comes from comparisons with other metal-cofactored dinuclear hydrolase enzymes, which contain metal-binding domains and active sites closely resembling that of urease. Several of these enzymes contain a metal binding domain which includes the HxH-metal binding motif (see also Supplementary Figure 1), but have zinc or iron cofactors. Examples of such enzymes are the *Alcaligenes faecalis D*-aminoacylase enzyme, which has a bi-zinc center (Liaw et al.,

2003), and the *B. subtilis* *N*-acetylglucosamine-6-phosphate deacetylase (NagA) which contains a bi-iron center (Vincent et al., 2004). These enzymes are not known to require accessory proteins for insertion of their metal cofactor, similar to the *B. subtilis* urease (Kim et al., 2005) and the UreA2B2 urease (this study). We have also considered that the UreA2B2 system may represent an alternative pathway for urea degradation, but this is unlikely since the UreA2B2 system does not have any significant sequence homology to allophanate hydrolase, and also does not have any signature sequence for the required biotin cofactor (Hausinger, 2004).

Taken together, we describe the presence of two independent functional urea-degrading enzymes in *Helicobacter* species colonizing carnivores. These carnivores are predicted to have nickel-restricted conditions in their gastric mucosa, due to the low concentration of this metal in the majority of the food sources consumed, which could result in insufficient availability of nickel to cater for the urease enzyme of colonizing *Helicobacter* species. The *Helicobacter* species colonizing such carnivorous hosts have adapted to this potential limitation via the expression of a second urease system active during nickel-restricted, iron-rich conditions, as are predicted to occur after digestion of meat sources. In view of the prevalence, expression pattern and the specifics of both enzymes, we speculate that the expression of the UreA2B2 urease system is an adaptation of these *Helicobacter* species to the nickel-restriction imposed by the diet of the host. We propose that the important influences of host dietary factors on the genetics of its pathogenic microorganisms should be further evaluated.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

Helicobacter isolates used in this study were *H. mustelae* strain NCTC12198 (National Collection of Type Cultures), *H. acinonychis* strain NCTC12686 (Eaton *et al.*, 1993) and *H. felis* strain 2301 (Jalava *et al.*, 1998). *Helicobacter* isolates were cultured at 37°C in a microaerophilic atmosphere of 5% O_2, 7.5% CO_2, 7.5% H_2 and 80% N_2. *H. mustelae* and *H. acinonychis* were routinely grown on Dent agar plates consisting of Columbia agar (Oxoid) supplemented with 7% saponin-lysed defibrinated horse blood (BioTrading), 0.004% triphenyltetrazolium chloride (Sigma) and Dent Selective Supplement (Oxoid). *H. felis* was grown on Jalava plates, consisting of Brain Heart Infusion broth (Oxoid) supplemented with 1 mg ml^{-1} yeast extract (Becton Dickinson), 20 mg ml^{-1} bacto agar (Becton Dickinson), 7% defibrinated horse blood, 5 μg ml^{-1} amphotericin B and Skirrow antibiotic supplement (Oxoid) (Pot et al., 2007). Liquid cultures of *H. mustelae* and *H. acinonychis* were grown in either Brucella broth (Difco) supplemented with 3% Newborn Calf Serum (Difco) and Dent supplement or in Ham's F-12 tissue culture medium (Kaighn's modification, Invitrogen) supplemented with 0.2% ß-cyclodextrin (Fluka) and Dent supplement. Broth cultures of *H. felis* were grown in Ham's F-12 (Kaighn's modification) supplemented with 10% newborn calf serum (Invitrogen) and Dent supplement. Broth cultures were shaken at 70 rpm and incubated at 37°C for a maximum of 24 h. Iron-restriction of Ham's F-12 medium (Kaighn's

modification) was achieved by addition of deferoxamine to a final concentration of 4 μM, whereas iron-replete medium was obtained by supplementing iron-restricted Ham's F-12 medium (Kaighn's modification) with $FeCl_3$ to a final concentration of 100 μM.
E. coli strains DH5α and M147 were cultured in Luria-Bertani media (Biotrading) (Sambrook *et al.*, 1989). When appropriate growth media were supplemented with chloramphenicol (20 μg ml^{-1}), kanamycin (20 μg ml^{-1}) or erythromycin (250 μg ml^{-1}) for selection of *E. coli* transformants. *H. mustelae* mutants were selected by growth on media containing chloramphenicol (10 μg ml^{-1}), kanamycin (10 μg ml^{-1}) or erythromycin (10 μg ml^{-1}). Concentrations of antibiotics given represent their final concentration in the growth medium.

General genetic manipulations
All standard DNA and protein manipulations were performed as described previously (Sambrook et al., 1989). Plasmid DNA was isolated and purified with the Miniprep kit from Promega. All DNA modifying enzymes were purchased from Promega and were used according to manufacturers descriptions. All polymerase chain reaction (PCR) amplification reactions were performed in an automated thermal cycler (I-Cycler; BioRad) using the PCR-core system I (Promega).

Construction of *H. mustelae* mutants
The *ureB*, *ureB2*, *ureG*, *hypB* and *nikR* genes of *H. mustelae* strain NCTC12198 were PCR amplified as ~1 kb fragments using primers listed in Table 1, and the amplicons were cloned in pGEM-T_{easy} vector (Promega). The sources of the chloramphenicol, kanamycin and erythromycin resistance cassettes were plasmids pAV35 (van Vliet *et al.*, 1998), pJMK30 (van Vliet et al., 1998) and pDH20 (Haas *et al.*, 1993), and these were inserted in unique restriction sites of the corresponding genes. The constructs were then introduced into *H. mustelae* strain NCTC12198 or its isogenic mutants strains by natural transformation (O Croinin et al., 2007) and correct replacement by homologous recombination of the genes was confirmed by PCR using primers located outside of the recombination region.

RNA analysis
Total RNA was isolated from approximately 5×10^8 *H. mustelae* cells using TriZOL (Invitrogen) (van Vliet et al., 2001). RNA was visualized by methylene blue staining, and RNA concentrations were normalized based on 16S and 23S rRNA band intensities (van Vliet et al., 2001). Probes were amplified with primers listed in Table 1. The PCR fragments contained a T7 promoter sequence and were used for the production of an antisense RNA probe labeled with digoxigenin by transcription with T7 RNA polymerase (Roche Diagnostics). Northern hybridization and stringency washes were performed at 68°C, and bound probe was visualized with the DIG-Detection Kit (Roche Diagnostics) and chemiluminescent substrate CPD-Star (Amersham Pharmacia) (van Vliet et al., 2001). The sizes of the hybridizing RNA were calculated from comparison with a digoxigenin-labeled RNA size marker (RNA Marker I, Roche Applied Science).

Table 1. Oligonucleotide primers used in this study

Primer	Sequence (5' → 3')
Hmus-ureB-KO-F1	CTTGGAGACACAGAACTCATC
Hmus-ureB-KO-R1	GCTATCTGCAAACGCAACG
Hmus-ureB2-KO-F1	ACTGGGGTACAACACCAAG
Hmus-ureB2-KO-R1	CAGGTTTGGAGGTGCAGAG
Hmus-nikr-KO-F1	GGAGATTGCTGGACTCAAAC
Hmus-nikr-KO-R1	AGCTTCTCGCTGAAGATACAG
Hmus-ureG-KO-F1	TTGTGGACCCGTTGGAAGTG
Hmus-ureG-KO-R1	TCGCCCTTCATCAACCCAG
Hmus-hypB-KO-F	AACAGGGCCAAGGGTTACAG
Hmus-hypB-KO-R	CGCCTAGAGTCTGGATGGTC
Hmus-ureB-F3	CAAAGAAGACGTTGCGTTTG
Hmus-ureB-R1-T7 [a]	ctaatacgactcactatagggagaCGCCTTTGATGATTGTCTCAG
Hmus-ureB2-F3	TTGGTGATACGGACCTTTGG
Hmus-ureB2-R1-T7 [a]	ctaatacgactcactatagggagaACATGGTTGTAACGCCATTG
Hmus-ureB1-qpcr-F	GCGCTGCTGAGGAATACAC
Hmus-ureB1-qpcr-R	TACCACCGATTGCTGCTAAC
Hmus-ureB2-qpcr-F	GCAGCTGAGGATACGCTTC
Hmus-ureB2-qpcr-R	GAGCCGATGTATTCGCTCAC
Hmus-16S-qpcr-F	ATTGCGAAGGCGACCTAC
Hmus-16S-qpcr-R	CAGGCGGAATGCTTAATGTG

[a] Primer contains a 5'-extension with T7 promoter sequence (in lowercase letters) for the creation of an antisense RNA probe.

Quantitative Reverse Transcriptase PCR analysis

cDNA was produced by reverse transcription by incubating 100 ng of RNA with 0.5 µg random hexamers (Promega) for 10 min at 70°C and rapid cooldown to 4°C. Then 20 U RNasin (Promega), 10 mM dNTP and 5 units of avian myeloblastosis virus reverse transcriptase (Promega) were added. Elongation was performed for 10min at room temperature followed by 1 h at 42°C. The reverse transcriptase enzyme was inactivated by 5 min incubation at 95°C followed by 5 min at 4°C. The cDNA was used directly for Quantitative Reverse Transcriptase-PCR (qRT-PCR) using the Bio-Rad IQ5 Real-Time PCR Detection System. qRT-PCR reactions contained 0.5 µl of cDNA template, 25 pM gene specific primers (Table 1, combinations used were Hmus-ureB1-qpcr-F/Hmus-ureB1-qpcr-R, Hmus-ureB2-qpcr-F/Hmus-ureB2-qpcr-R and Hmus-16S-qpcr-F/Hmus-16S-qpcr-R), 10 nM dNTP, 1 unit Taq polymerase (Promega), Taq polymerase buffer and dH_20 to a final volume of 25 µl. SYBR green was added in a 10,000 fold dilution as instructed by the manufacturer (Bio-Rad, IQ5 manual). qRT-PCR assays were performed using RNA isolated from at least three independent growth experiments. Transcript levels were normalized against the levels of 16S rRNA in each sample [2(ΔΔC(T)) analysis] (Livak and Schmittgen, 2001), and expressed as ratio to the mRNA level in cells grown in nickel- and iron-restricted Ham's F-12 medium (Kaighn's modification).

Urease assay

The enzymatic activity of urease was determined by measuring ammonia production from hydrolysis of urea, by using the Berthelot reaction as described previously (van Vliet et al., 2001). Briefly, intact cells or cells lysed by sonication were incubated for 30 min at 37°C in buffer consisting of 100 mM sodium phosphate pH 7.5, 10 mM EDTA, and 50 mM urea, and the ammonia produced was measured after addition of phenol nitroprusside and alkaline hypochlorite (Sigma Diagnostics). The optical density (OD) of the samples was determined at 570 nm, and compared to a standard NH_4Cl concentration curve. Protein concentrations were determined with the bicinchoninic acid method (Pierce) using bovine serum albumin as standard. Urease enzyme activity was expressed as units representing micromoles (μmoles) of urea hydrolyzed per min, and is expressed as U/mg of total protein.

Protein analysis

Liquid cultures were centrifuged for 10 min at 4000 × g and resuspended in phosphate-buffered saline to a final OD_{600} of 10. Bacteria were lysed by sonication for 15 sec with an MSE Soniprep 150 set at amplitude 6. Whole-cell proteins (OD10) were separated on a 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) (Sambrook et al., 1989). The gel was subsequently stained with Coomassie brilliant blue or transferred to a nitrocellulose membrane. Expression of urease subunits was monitored on immunoblots by using a 1:10,000 dilution of antibodies raised against H. felis urease (Belzer et al., 2005; Pot et al., 2007).

Acid-shock survival

Survival of acid shock of pH 1.5 was determined using a modification of the assay previously described for *H. mustelae* (Solnick et al., 1995). *H. mustelae* strains were grown overnight (24 hours) in serum-supplemented Brucella broth, and cells were resuspended in phosphate-buffered saline pH 7.4 to an OD_{600} of approximately 10 (representing approximately 5×10^9 cells/ml). Cells were tenfold diluted in phosphate-buffered saline of pH 1.5 or pH 7.4 in either the presence or absence of 5 mM urea, and incubated for 30 minutes at 37°C in microaerophilic conditions. Serial 10-fold dilutions of the cell suspension were made in a 96 well microplate in phosphate-buffered saline pH 7.4. Five μl of each dilution was then spotted on Dent plates, allowed to absorb for a few minutes, after which the plates were incubated for 4 days at 37°C in microaerophilic conditions. The concentration of surviving bacterial cells in the original suspension was calculated back using the highest dilution still showing colonies on the spotted surface. Acid-survival was expressed as percentage of cells surviving compared to the number of cells surviving in phosphate-buffered saline of pH 7.4 without added urea.

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sequence, and Robert Hausinger for helpful discussions and annotations for Supplementary Figure 1. Urease antibodies were donated by the Department of Bacteriological R&D of Intervet International BV (Boxmeer, The Netherlands).

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Supplementary Figure 1

Alignment of the UreB and UreB2 protein sequences of the carnivore-colonizing *Helicobacter* species *H. mustelae* NCTC12198 (H.muste) (Solnick et al., 1995), *H. acinonychis* (H.acino) (Eppinger et al., 2006) and *H. felis* (H.felis) (Ferrero and Labigne, 1993; Pot et al., 2007) with the UreB protein of *H. pylori* (H.pylor) (Tomb et al., 1997), *H. hepaticus* (H.hepat) (Beckwith et al., 2001), *Bacillus subtilis* (B.subti) (Cruz-Ramos et al., 1997), the two urease systems of *Brucella suis* (Br.suis) (Bandara et al., 2007) and the UreC protein of *Klebsiella pneumoniae* (K.pneum) (Mulrooney and Hausinger, 1990). Alignments were made with the ClustalW program, and the urease active site and the nickel-binding residues were identified using signatures present in the Prosite database. Asterisks represent residues conserved in all sequences, colons represent conserved substitutions, and dots indicate semi-conserved substitutions. The residues listed in blue represent the dinuclear Ni-active site of urease (H_{134}-H_{136}-K_{217}-H_{246}-H_{272}-D_{360} in the *K. pneumoniae* UreC sequence (Mulrooney and Hausinger, 2003; Pot et al., 2007)). Residues listed in yellow represent the nickel-ligand consensus pattern and the urease active site as listed in the ProSite database at http://www.expasy.org, with the consensus sequence given in green (Takishima et al., 1988; Jabri et al., 1995; Mobley et al., 1995). Capital letters in the consensus sequence indicate residues conserved throughout all known ureases, x represents any amino acid. The bold residues in the UreB2 sequences indicate the substitutions of the non-polar amino acids valine/isoleucine in the UreB/UreC sequences by the polar amino acid threonine in the UreB2 sequences. Protein sequences were aligned with the ClustalW program (version 1.83) (Chenna et al., 2003) using the BLOSUM matrix. The urease active site and nickel-binding site were identified using the Prosite database at http://www.expasy.org. Protein sequences of the large urease subunits of *Klebsiella pneumoniae*, *Helicobacter mustelae*, *H. acinonychis*, *H. felis* and *H. pylori* were retrieved from the GenBank database or deduced from genome sequences and aligned with the UreB2 sequences.

Alignment of urease alpha-subunits

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K.pneum UreC    MSN-ISRQAYADMFGPTVGDKVRLADTELWIEVEDDL-TTYGEEVKFGGGKVIRDGMGQ-
H.muste UreB2   M--KMKRQEYVNTYGPTTGDKVRLGDTDLWAEVEHDY-TVYGEELKFGAGKTIREGMGQS
H.acino UreB2   M--KMKKLDYVNTYGPTKGDKVRLGDTELWAEVEHDY-TIYGEELKFGAGKTIREGMGQS
H.felis UreB2   M--KMKKQEYVNTYGPTKGDKVRLGDTDLWAEVEHDY-TTYGEELKFGAGKTIREGMGQS
H.muste UreB    MI-KISRKEYVSMYGPTTGDKVRLGDTELIAEIEKDY-TVYGEEIKFGGGKTIRDGMSQS
H.felis UreB    MK-KISRKEYVSMYGPTTGDRVRLGDTDLILEVEHDC-TTYGEEIKFGGGKTIRDGMSQT
H.acino UreB    MK-KINRKEYASMYGPTTGDRVRLGDTDLIAEVEHDY-TIYGEELKFGGGKTLREGMSQS
H.pylor UreB    MK-KISRKEYVSMYGPTTGDKVRLGDTDLIAEVEHDY-TIYGEELKFGGGKTLREGMSQS
H.hepat UreB    MI-KISRKQYASMYGPTTGDKVRLGDTNLFAEIEKDY-TLYGEEIKFGGGKTIRDGMAQS
Br.suis UreC    MPARISRATYAQMFGPTVGDKVRLADTDLIIEVERDL-TTYGEEVKFGGGKVIRDGMGQ-
Br.suis UreC2   MTQ-ISRQQYADLYGPTIGDKIRLGDSDLYVEIEKDLRATYGDELQYGGGKTLRDGMGSE
B.subti UreC    M--KMSREEYAELFGPTTGDKIRLGDTDLWIEVEKDF-TVYGEEMIFGGGKTIRDGMGQN
                *   . .  *   .*** **..**.*..*  *.* *  . **.*. .*.** .*.**.

K.pneum UreC    GQMLAAD-CVDLVLTNALIVDHW-GIVKADIGVKDGRIFAIGKAGNPDIQPNVT--IPIG
H.muste UreB2   NSPDENT--LDLVITNALIIDYT-GIYKADIGIKNGKIHGIGKAGNKDMQDGVTPHMVVG
H.acino UreB2   NSHDENT--LDLVITNALIIDYT-GIYKADIGIKNGKIAGIGKAGNKDMQDGVSPNLVVG
H.felis UreB2   NSPDENT--LDLVITNAMIIDYT-GIYKADIGIKNGKIHGIGKAGNKDMQDGVSPHMVVG
H.muste UreB    VSPDVNE--LDAVITNAMIIDYT-GIYKADIGIKDGKIAGIGKAGNRDTQDGVGMDLVVG
H.acino UreB    NNPSKEE--LDLIITNALIIDYT-GIYKADIGIKDGKIAGIGKGGNKDMQDGVKNNLSVG
H.felis UreB    NSPSSYE--LDLVLTNALIVDYT-GIYKADIGIKDGKIAGIGKAGNKDMQDGVDNNLCVG
H.pylor UreB    NNPSKEE--LDLIITNALIVDYT-GIYKADIGIKDGKIAGIGKGGNKDMQDGVKNNLSVG
H.hepat UreB    ASTYTNE--LDAVITNAMIIDYT-GIYKADIGIKGGKIVGIGKAGNPDTQDSVNEAMVVG
Br.suis UreC    SQLSRAEGAMDTVITNALILDHS-GIYKADIGLLDGRIALIGKAGNPDTQPGIS--IIIG
Br.suis UreC2   NFLTQEAGCLDLVITNVTVIDAIQGVVKADVGIRNGRIVGLGKAGNPSTMDGVTRGLVTG
B.subti UreC    GRITGKDGALDLVITNVVLLDYT-GIVKADVGVKDGRIVGVGKSGNPDIMDGVDPHMVIG
                         .* ..**  ..*   *. ***.*.  *.*  .**.**      .   .  *
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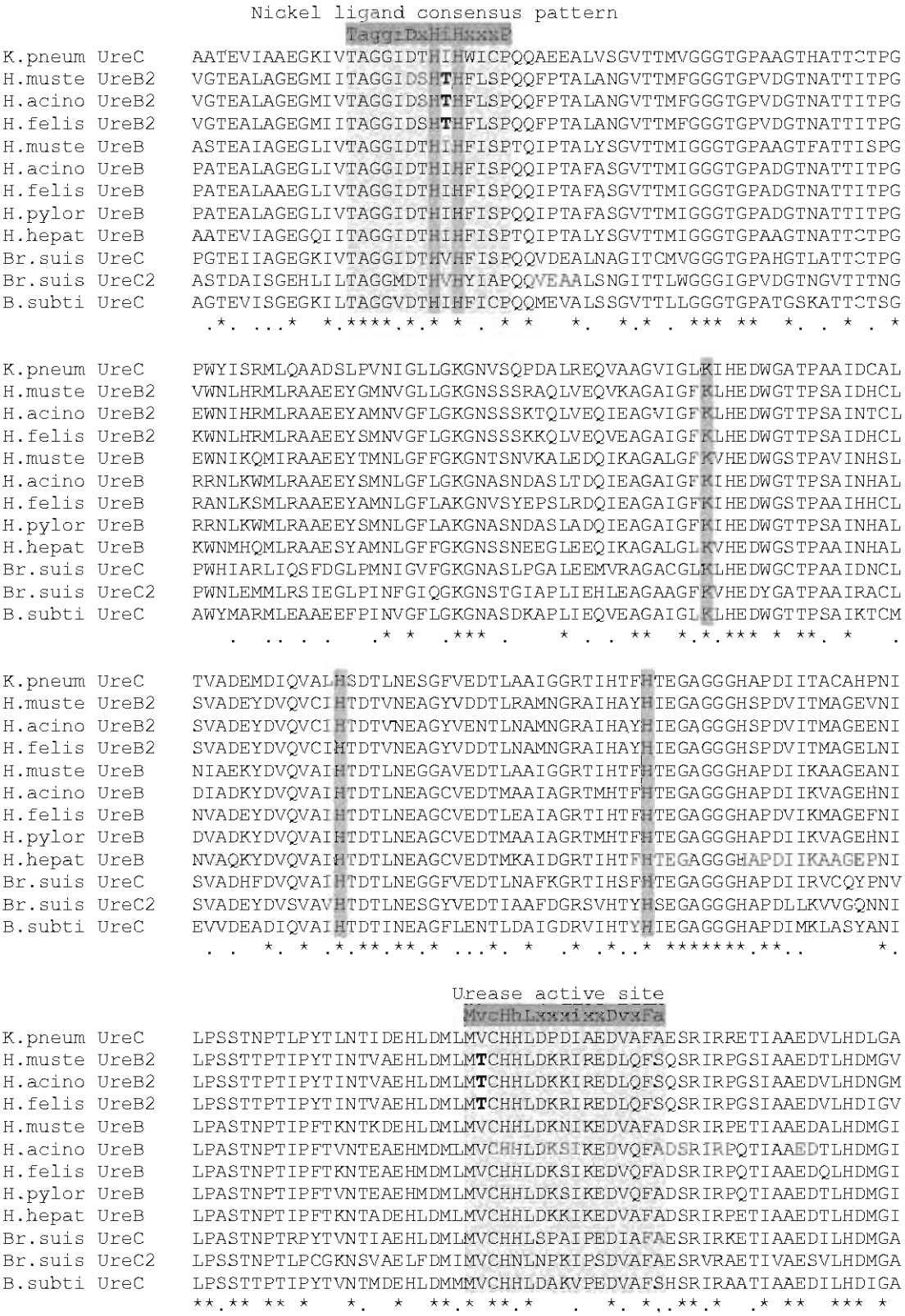
Nickel ligand consensus pattern
TaggiDxHiHxxxP
K.pneum UreC AATEVIAAEGKIVTAGGIDTHIHWICPQQAEEALVSGVTTMVGGGTGPAAGTHATTCTPG
H.muste UreB2 VGTEALAGEGMIITAGGIDSHTHFLSPQQFPTALANGVTTMFGGGTGPVDGTNATTITPG
H.acino UreB2 VGTEALAGEGMIVTAGGIDSHTHFLSPQQFPTALANGVTTMFGGGTGPVDGTNATTITPG
H.felis UreB2 VGTEALAGEGMIITAGGIDSHTHFLSPQQFPTALANGVTTMFGGGTGPVDGTNATTITPG
H.muste UreB ASTEAIAGEGLIVTAGGIDTHIHFISPTQIPTALYSGVTTMIGGGTGPAAGTFATTISPG
H.acino UreB PATEALAGEGLIVTAGGIDTHIHFISPQQIPTAFASGVTTMIGGGTGPADGTNATTITPG
H.felis UreB PATEALAAEGLIVTAGGIDTHIHFISPQQIPTAFASGVTTMIGGGTGPADGTNATTITPG
H.pylor UreB PATEALAGEGLIVTAGGIDTHIHFISPQQIPTAFASGVTTMIGGGTGPADGTNATTITPG
H.hepat UreB AATEVIAGEGQIITAGGIDTHIHFISPTQIPTALYSGVTTMIGGGTGPAAGTNATTCTPG
Br.suis UreC PGTEIIAGEGKIVTAGGIDTHVHFISPQQVDEALNAGITCMVGGGTGPAHGTLATTCTPG
Br.suis UreC2 ASTDAISGEHLILTAGGMDTHVHYIAPQQVEAALSNGITTLWGGGIGPVDGTNGVTITNG
B.subti UreC AGTEVISGEGKILTAGGVDTHIHFICPQQMEVALSSGVTTLLGGGTGPATGSKATTCTSG
PWYISRMLQAADSLPVNIGLLGKGNVSQPDALREQVAAGVIGLKIHEDWGATPAAIDCAL
VWNLHRMLRAAEEYGMNVGLLGKGNSSSRAQLVEQVKAGAIGFKLHEDWGTTPSAIDHCL
EWNIHRMLRAAEEYAMNVGFLGKGNSSSKTQLVEQIEAGVIGFKLHEDWGTTPSAINTCL
KWNLHRMLRAAEEYSMNVGFLGKGNSSSKKQLVEQVEAGAIGFKLHEDWGTTPSAIDHCL
EWNIKQMIRAAEEYTMNLGFFGKGNTSNVKALEDQIKAGALGFKVHEDWGSTPAVINHSL
RRNLKWMLRAAEEYSMNLGFLGKGNASNDASLTDQIEAGAIGFKIHEDWGTTPSAINHAL
RANLKSMLRAAEEYAMNLGFLAKGNVSYEPSLRDQIEAGAIGFKIHEDWGSTPAAIHHCL
RRNLKWMLRAAEEYSMNLGFLAKGNASNDASLADQIEAGAIGFKIHEDWGTTPSAINHAL
KWNMHQMLRAAESYAMNLGFFGKGNSSNEEGLEEQIKAGALGLKVHEDWGSTPAAINHAL
PWHIARLIQSFDGLPMNIGVFGKGNASLPGALEEMVRAGACGLKLHEDWGCTPAAIDNCL
PWNLEMMLRSIEGLPINFGIQGKGNSTGIAPLIEHLEAGAAGFKVHEDYGATPAAIRACL
AWYMARMLEAAEEFPINVGFLGKGNASDKAPLIEQVEAGAIGLKLHEDWGTTPSAIKTCM
TVADEMDIQVALHSDTLNESGFVEDTLAAIGGRTIHTFHTEGAGGGHAPDIITACAHPNI
SVADEYDVQVCIHTDTVNEAGYVDDTLRAMNGRAIHAYHIEGAGGGHSPDVITMAGEVNI
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SVADEYDVQVCIHTDTVNEAGYVDDTLNAMNGRAIHAYHIEGAGGGHSPDVITMAGELNI
NIAEKYDVQVAIHTDTLNEGGAVEDTLAAIGGRTIHTFHTEGAGGGHAPDIIKAAGEANI
DIADKYDVQVAIHTDTLNEAGCVEDTMAAIAGRTMHTFHTEGAGGGHAPDIIKVAGEHNI
NVADEYDVQVAIHTDTLNEAGCVEDTLEAIAGRTIHTFHTEGAGGGHAPDVIKMAGEFNI
DVADKYDVQVAIHTDTLNEAGCVEDTMAAIAGRTMHTFHTEGAGGGHAPDIIKVAGEHNI
NVAQKYDVQVAIHTDTLNEAGCVEDTMKAIDGRTIHTFHTEGAGGGHAPDIIKAAGEPNI
SVADHFDVQVAIHTDTLNEGGFVEDTLNAFKGRTIHSFHTEGAGGGHAPDIIRVCQYPNV
SVADEYDVSVAVHTDTLNESGYVEDTIAAFDGRSVHTYHSEGAGGGHAPDLLKVVGQNNI
EVVDEADIQVAIHTDTINEAGFLENTLDAIGDRVIHTYHIEGAGGGHAPDIMKLASYANI
Urease active site
MvcHhLxxxixxDvxFa
LPSSTNPTLPYTLNTIDEHLDMLMVCHHLDPDIAEDVAFAESRIRRETIAAEDVLHDLGA
LPSSTTPTIPYTINTVAEHLDMLMTCHHLDKRIREDLQFSQSRIRPGSIAAEDTLHDMGV
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LPSSTTPTIPYTINTVAEHLDMLMTCHHLDKRIREDLQFSQSRIRPGSIAAEDVLHDIGV
LPASTNPTIPFTKNTKDEHLDMLMVCHHLDKNIKEDVAFADSRIRPETIAAEDALHDMGI
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LPASTNPTIPFTVNTEAEHMDMLMVCHHLDKSIKEDVQFADSRIRPQTIAAEDTLHDMGI
LPASTNPTIPFTKNTADEHLDMLMVCHHLDKKIKEDVAFADSRIRPETIAAEDTLHDMGI
LPASTNPTRPYTVNTIAEHLDMLMVCHHLSPAIPEDIAFAESRIRKETIAAEDILHDMGA
LPSSTNPTLPCGKNSVAELFDMIMVCHNLNPKIPSDVAFAESRVRAETIVAESVLHDMGA
LPSSTTPTIPYTVNTMDEHLDMMMVCHHLDAKVPEDVAFSHSRIRAATIAAEDILHDIGA

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K.pneum UreC    FSLTSSDSQAMGRVGEVILRTWQVAHRMKVQRGALAEE-TGDNDNFRVKRYIAKYTINPA
H.muste UreB2   IAMTSSDSQAMGRAGEVIPRTWQTADKNKKEFGRLTEE-KGDNDNFRIKRYISKYTINPA
H.acino UreB2   IAMTSSDSQAMGRAGEVVPRTWQTADKNKKEFGPLKEDAQNGNDNFRIKRYISKYTINPA
H.felis UreB2   IAMTSSDSQAMGRAGEVIPRTWQTADKNKKEFGRLKEE-KGDNDNFRIKRYISKYTINPA
H.muste UreB    FSITSSDSQAMGRVGEVITRTWQVADKSKKEFGRLKEE-TGDNDNFRIKRYVSKYTINPA
H.acino UreB    FSITSSDSQAMGRVGEVITRTWQTADKNKKEFGRLKEE-KGDNDNFRIKRYLSKYTINPA
H.felis UreB    FSITSSDSQAMGRVGEVITRTWQTADKNKKEFGRLKEE-KGDNDNFRIKRYISKYTINPA
H.pylor UreB    FSITSSDSQAMGRVGEVITRTWQTADKNKKEFGRLKEE-KGDNDNFRIKRYLSKYTINPA
H.hepat UreB    FSITSSDSQAMGRVGEVITRTWQTADKCKNEFGALKEE-CGENDNFRIKRYISKYTINPA
Br.suis UreC    FSIISSDSQAMGRVGEMIIRCWQTADKMKKQRGSLPDD-RPGNDNYRARRYIAKYTINPA
Br.suis UreC2   ISMIGSDSQAMGRIGETFLRAIQTADAMKKARGPLPEDAPGN-DNFRVLRYIAKVTINPA
B.subti UreC    ISMTSSDSQAMGRVGEVIIRTWQVADKMKKQRGALAGE-N-GNDNVRAKRYIAKYTINPA
                ... .******** **   *  * *.  *   * *  .     ** *  **..* *****

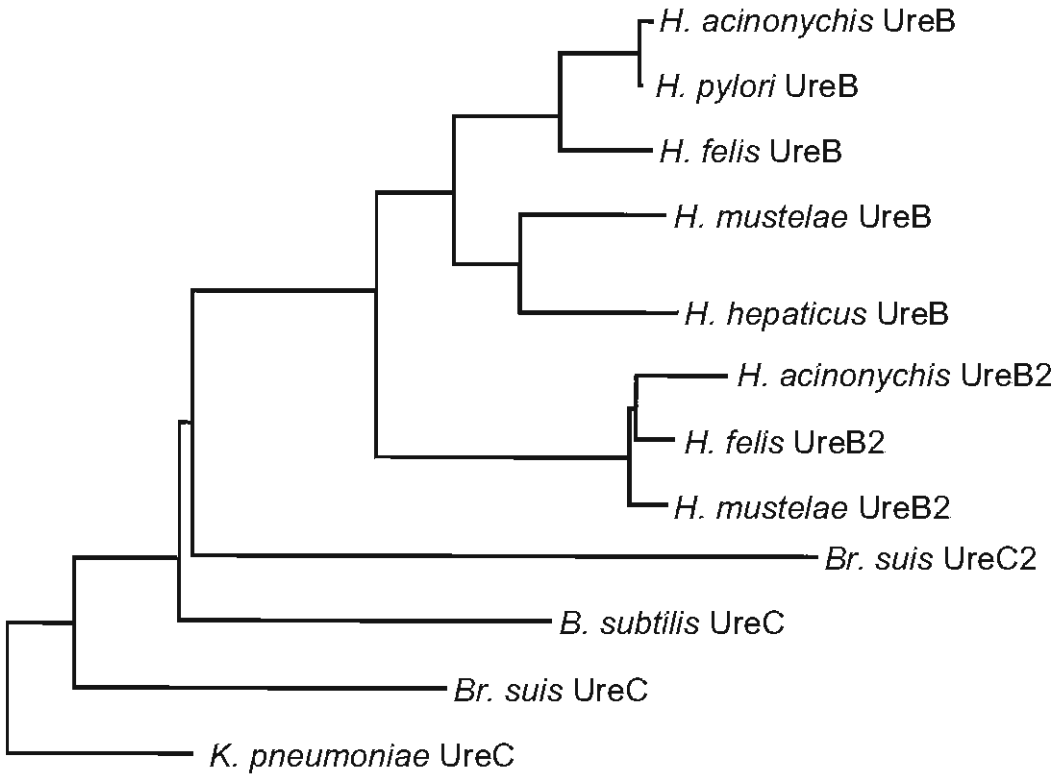
K.pneum UreC    LTHGIAHEVGSIEVGKLADLVVWSPAFFGVKPATVIKGGMIAIAPMGDINASIPTPQPVH
H.muste UreB2   ITHGVSEYIGSVEEGKIADLVVWNPAFFGVKPKIIIKGGMVVFSEMGDSNASVPTPQPVY
H.acino UreB2   ITHGVSEYIGSVEAGKIADLVVWNPAFFGVKPKIIIKGGLVVFSEMGDSNASVPTPQPVY
H.felis UreB2   LTHGVSEYIGSVEEGKIADLVVWNPAFFGVKPKIVIKGGMVVFSGMGDSNASVPTPQPVY
H.muste UreB    IAHGISDYIGSVEKGKIADLVLWDPGLFGIKPETIIKGGMIVASKMGDSNASIPTPEPVV
H.acino UreB    IAHGISEYVGSVEVGKVADLVLWSPAFFGVKPNMIIKGGFIALSQMGDANASIPTPQPVY
H.felis UreB    IAHGISDYVGSVEVGKYADLVLWSPAFFGIKPNMIIKGGFIALSQMGDANASIPTPQPVY
H.pylor UreB    IAHGISEYVGSVEVGKVADLVLWSPAFFGVKPNMIIKGGFIALSQMGDANASIPTPQPVY
H.hepat UreB    IAHGISEYVGSVEVGKFADLVLWKPSMFGIKPEMILKNGMIVAAKIGDSNASIPTPEPVV
Br.suis UreC    IAHGMAHEIGSVEVGKRADLVLWNPAFFGVKPDMVLLGGWIATAPMGDANGSIPTPQPMH
Br.suis UreC2   LTAGVGDVIGSIESGKFADLVLWEPAFFGVKPKLVLKGGLVAWANMGDPNASLPTPQPMY
B.subti UreC    ITHGLSHEVGSVEKGKLADLVLWDPVFFGVKPELVLKGGMIARAQMGDPNASIPTPEPVF
                .. *... .**.* ** ****.* *  **.**  ..  * .  . .** *.*.***.*.

K.pneum UreC    YRPMFGALGSARHHCRLTFLSQAAAANGVAERLNL-RSAIAVVKGCRTVQKADMVHNSLQ
H.muste UreB2   YREMFGHHGKAKFDTSITFVSKVAYENGIKEKLGLERKVL-PVKNCRNVTKKDFKFNNTT
H.acino UreB2   YREMFGHHGKAKFDTSITFVNKLAYEKGIKEKLGLERKVL-PIKNVRNITKKDFKFNNTT
H.felis UreB2   YREMFGHHGKAKFDTSITFVSKVAYENGVKEKLGLERQVL-PVKNCRNITKKDFKFNDKT
H.muste UreB    YKDMFGAHGKAKYKCNVTFVSQASLDLGIKEELGLERTLL-PVKNCRNIGKRDMKFNDVV
H.acino UreB    YREMFAHHGKAKYDANITFVSQAAYDKGIKEELGLERQVL-PVKNCRNITKKDMQFNDTT
H.felis UreB    YREMFGHHGKNKFDTNITFVSQAAYKAGIKEELGLDRVVL-PVKNCRNITKKDLKFNDVT
H.pylor UreB    YREMFAHHGKAKYDANITFVSQAAYDKGIKEELGLERQVL-PVKNCRNITKKDMQFNDTT
H.hepat UreB    YAPMFGSYGKAKYNCAITFVSKIAYDCHIKEELGLERILL-PVKNCRNITKKDMKFNDVI
Br.suis UreC    TRPMFGSFGKARTNTSITFVSQAAMDEGLREKIGVDKQLVAVVNTRGGIGKHSMILNNAM
Br.suis UreC2   YRPMFAAYGSALQKTSITFVSRAAYDKGVADRFGLQRLVM-PVSGTRVIGKAHMVRNSYL
B.subti UreC    MRQMYASYGKANRSTSITFMSQASIERGVAESLGLEKR-ISPVKNIRKLSKLDMKLNSAL
                   *..  *       .**... .    . . . . . .  .     . *     *

K.pneum UreC    PNITVDAQTYEVRVDGELITSEPADVLPMAQRYFLF
H.muste UreB2   AKITVNPETFEVFVNGKLCTSKPATEVALASRYTFF
H.acino UreB2   GKLTVDPKSFEVFLEGKLCTSKPASELPLAQRYTFF
H.felis UreB2   AKITVDPKTFEVFVDGKLCTSKPTSEVPLAQRYTFF
H.muste UreB    AHIDVNPETYKVKVNGKEVTSKSADSVPLSQLYNLF
H.acino UreB    AHIEVNPETYHVFVDGKEVTSKPANKVTLAQLFSIF
H.felis UreB    AHIDVNPETYKVKVDGKEVTSKAADELSLAQLYNLF
H.pylor UreB    AHIEVNPETYHVFVDGKEVTSKPANKVSLAQLFSIF
H.hepat UreB    TPIEVNPETYEVRVNNTKITSKPVEKVSLGQLYCLF
Br.suis UreC    PQMEVDPETYEVRA-GELLTCEPVDVVPMAQRYFLF
Br.suis UreC2   PNIEVDPQTFAVKVDGVHATVKPPQSISLNQLYFFS
B.subti UreC    PKIEIDPKTYQVFADGEELSCQPVDYVPLGQRYFLF
                  . ... .. *       .  .   . .   . .
```

Phylogenetic tree of urease alpha subunits

The phylogenetic tree shown below was constructed using the Neighbor-Joining method with *K. pneumoniae* as outgroup, using a protein distance table built by the Protdist program based on the alignment shown above. All programs are part of the Phylip program suite, version 3.66 (Felsenstein, 1989).



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Chapter 5

Characterization of NikR-responsive promoters of urease and metal transport genes of *Helicobacter mustelae*

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ABSTRACT

The NikR protein is a nickel-responsive regulator, which in the gastric pathogen *Helicobacter pylori* controls expression of nickel-transporters and the nickel-cofactored urease acid resistance determinant. Although NikR–DNA interaction has been well studied, the *Helicobacter* NikR operator site remains poorly defined. In this study we have identified the NikR operators in the promoters of two inversely nickel-regulated urease operons (*ureAB* and *ureA2B2*) in the ferret pathogen *Helicobacter mustelae*, and have used bioinformatic approaches for the prediction of putative NikR operators in the genomes of four urease-positive *Helicobacter* species. *Helicobacter mustelae* NikR bound to the *ureA2* promoter to a sequence overlapping with the -35 promoter region, leading to repression. In contrast, NikR binding to a site far upstream of the canonical σ^{80} promoter in the *H. mustelae ureA* promoter resulted in transcriptional induction, similar to the situation in *H. pylori*. Using *H. pylori* NikR operators and the newly identified *H. mustelae* NikR operators a new consensus sequence was generated (TRWYA-N_{15}-TRWYA), which was used to screen the genomes of four urease-positive *Helicobacter* species (*H. mustelae*, *H. pylori*, *H. acinonychis* and *H. hepaticus*) for putative NikR-regulated promoters. One of these novel putative NikR-regulated promoters in *H. mustelae* was upstream of a putative TonB-dependent outer membrane protein designated NikH, which displayed nickel-responsive expression. Insertional inactivation of the *nikH* gene in *H. mustelae* resulted in a significant decrease in urease activity, and this phenotype was complemented by nickel-supplementation of the growth medium, suggesting a function for NikH in nickel transport accross the outer membrane. In conclusion, the *H. mustelae* NikR regulator directly controls nickel-responsive regulation of ureases and metal transporters. The improved consensus NikR operator sequence allows the prediction of additional NikR targets in *Helicobacter* genomes, as demonstrated by the identification of a new nickel-repressed outer membrane protein in *H. mustelae*.

INTRODUCTION

The mammalian stomach is an inhospitable environment for bacteria, and therefore was long thought to be sterile, but this dogma was refuted by the discovery of the human pathogen *Helicobacter pylori* (Marshall & Warren 1984). This bacterium is able to colonize the environment of the mucus layer overlaying the gastric epithelial cells, and the resulting gastritis predisposes to peptic ulceration and gastric cancer (Kusters *et al.* 2006). Since the discovery of *H. pylori*, many other *Helicobacter* species have been identified, and it is now generally acknowledged that the gastric mucosa of most, if not all, mammals can be colonized by gastric *Helicobacter* species (Solnick & Schauer 2001). These non-*pylori Helicobacter* species may provide good animal models to study *Helicobacter* infection in their natural hosts (O'Rourke & Lee 2003) and comparative genomics may contribute in our understanding of host specificity (Eppinger *et al.* 2006).

The divalent metal nickel plays a central part in the gastric lifestyle of *Helicobacter* species. Nickel is the cofactor of the urease enzyme (Burne & Chen 2000), which is the key component that enables gastric *Helicobacter* species to survive the stressful acidic conditions in the gastric mucosa. Urease converts urea into ammonia and carbon dioxide, which results in a net increase in the local pH (Burne & Chen 2000), and urease-negative mutants of different *Helicobacter* species are unable to colonize the gastric environment (Tsuda *et al.* 1994; Andrutis *et al.* 1995; Stingl *et al.* 2002).

Metal ions like nickel pose a problem for bacteria. While they are essential for metabolism, they are also capable of generating toxic compounds like reactive oxygen species, and hence bacteria have developed finely tuned systems to control intracellular availability of metals (Mulrooney & Hausinger 2003). For nickel, a nickel-responsive regulatory protein represents the common mechanism of control in bacteria. This nickel-responsive regulatory protein is capable of repressing nickel import mechanisms once cytoplasmic nickel concentrations exceed a certain threshold. The most common nickel-responsive regulator in bacteria is the ribbon-helix-helix regulatory protein NikR (Chivers & Sauer 2000). The NikR protein was previously identified as a key regulator of urease expression in *H. pylori* (van Vliet *et al.* 2002). In addition to urease, NikR regulates expression of other genes involved in nickel homeostasis by binding to NikR operators in, or upstream of, the promoter regions (Delany *et al.* 2005; Ernst *et al.* 2005b; Davis *et al.* 2006; Ernst *et al.* 2006; Danielli *et al.* 2009). *H. pylori* NikR binds with different affinities to NikR operators *in vitro* (Abraham *et al.* 2006; Benanti & Chivers 2007; Dosanjh *et al.* 2009), and the sequence variation in the NikR operators has made it difficult to define a consensus sequence which correctly predicts NikR-regulated genes.

Recently we described the presence (Pot *et al.* 2007) and characterization (Stoof *et al.* 2008) of two urease gene clusters (*ureABIEFGH* and *ureA2B2*) in three *Helicobacter* species (*H. mustelae*, *H. acinonychis* and *H. felis*) colonizing obligate carnivores (ferrets, big cats and cats, respectively). As with the *H. pylori* urease gene cluster, *H. mustelae* UreAB is positively regulated by the availability of nickel (van Vliet *et al.* 2001; van Vliet *et al.* 2002). In sharp contrast to the UreAB urease system, the expression of the *H. mustelae* UreA2B2 urease system is repressed upon nickel supplementation, but induced upon iron supplementation. Due to the regulatory pattern, independency of accessory proteins and the inactivation of the enzyme upon lyses, we speculated that this gene cluster may encode an iron cofactored urease. UreA2B2 may therefore be an evolutionary adaptation of carnivore colonizing

Helicobacter species to the nickel-limited but iron-rich diet of their host (Stoof *et al.* 2008). Although nickel-dependent regulation of both ureases was absent in an *H. mustelae nikR* mutant, direct interaction of NikR with the *ureA* and *ureA2* promoters was not tested.
In this study we have investigated the role of NikR in nickel-responsive regulation of the UreAB and UreA2B2 urease systems of *H. mustelae*. We demonstrate that NikR directly interacts with both urease promoters of *H. mustelae*. Using the newly identified NikR binding sites of the *H. mustelae ureA* and *ureA2* promoters, a new NikR operator consensus sequence has been proposed, and this new consensus sequence has been used for the prediction of new NikR operators in complete genome sequences of *Helicobacter* species. All previously confirmed high-affinity NikR operators in *H. pylori* were recognized, and searching of the *H. mustelae* genome sequence allowed the identification of a new nickel-regulated outer membrane protein in *H. mustelae*, which contributes to urease activity.

MATERIALS AND METHODS

Bacterial strains and growth conditions

H. mustelae strain NCTC 12198 (ATCC 43772) was used for all experiments in this study and was cultured at 37°C in a microaerobic atmosphere of 5% O_2, 7.5% CO_2, 7.5% H_2 and 80% N_2. Dent agar plates consisting of Columbia agar (Oxoid) supplemented with 7% saponin-lysed defibrinated horse blood (BioTrading), 0.004% triphenyltetrazolium chloride (Sigma) and Dent Selective Supplement (Oxoid) were used for routine growth. Broth cultures of *H. mustelae* were grown in Ham's F-12 tissue culture medium (Kaighn's modification, Invitrogen) supplemented with 0.2% ß-cyclodextrin (Fluka) and Dent supplement. Since this medium may not contain ligands needed for nickel transport across the outer membrane, the urease assay was also performed with *H. mustelae* grown in Brucella broth supplemented with 3% heat inactivated Newborn Calf Serum (Difco) and Dent supplement.
Broth cultures were shaken at 70 rpm and incubated at 37°C for a maximum of 24 h. Iron-restriction of Ham's F-12 medium (Kaighn's modification) was achieved by addition of deferoxamine (Sigma) to a final concentration of 4 µM, whereas iron-replete medium was obtained by supplementing iron-restricted Ham's F-12 medium (Kaighn's modification) with $FeCl_3$ to a final concentration of 10 µM (Stoof *et al.* 2008).
E. coli strains DH5α and M147 were cultured in Luria-Bertani media (Biotrading) (Sambrook *et al.* 1989). When appropriate, growth media were supplemented with ampicillin (100 µg ml^{-1}), chloramphenicol (20 µg ml^{-1}), kanamycin (20 µg ml^{-1}) or erythromycin (250 µg ml^{-1}) for selection of *E. coli* transformants. *H. mustelae* mutants were selected on media containing chloramphenicol (10 µg ml^{-1}), kanamycin (10 µg ml^{-1}) or erythromycin (10 µg ml^{-1}). Concentrations of antibiotics given represent their final concentration in the growth medium.

Construction of *H. mustelae fur*, *hm0418-1*, *hm0418-2* and *hm0418-3* mutants

Construction of the *H. mustelae nikR* mutant was described previously (Stoof *et al.* 2008). The *fur*, *hm0418-1*, *hm0418-2* and *hm0418-3* genes of *H. mustelae* strain NCTC 12198 were PCR amplified using the KO primers described (Table 1), and the amplicons were cloned in pGEM-T_{easy} vector (Promega). The sources of the chloramphenicol, kanamycin and erythromycin resistance cassettes were plasmids pAV35 (van Vliet *et al.* 1998), pJMK30 (van Vliet *et al.* 1998) and pDH20 (Haas *et al.* 1993), and these were inserted in the unique

restriction sites *Bcl*I (*fur*), *Bam*HI (*hm0418-1*), *Eco47*III (*hm0418-2*) and *Eco47*III (*hm0418-3*) of the corresponding genes, in the same transcriptional orientation. The interrupted genes were subsequently introduced into *H. mustelae* strain NCTC 12198 by natural transformation (Croinin *et al.* 2007; Stoof *et al.* 2008) and correct replacement by homologous recombination of the genes with the interrupted version was confirmed by PCR using ORS primers described (Table 1) which are located outside of the recombination region.

Table 1. Oligonucleotide primers used in this study

Primer	Sequence (5' → 3')
Hmus_fur_KO_F	AGGCCATCTCCCTGTATTGC
Hmus_fur_KO_R	TGCACGCAGTCTCTTTGTG
Hmus_fur_ORS_F	GCAAAAGCACAATCCCCTCTG
Hmus_fur_ORS_R	AGAATTGAGGGGAAAACGTG
Hmus_0418-1_KO_F	GAGGACGTGGATGGAGTTTG
Hmus_0418-1_KO_R	TGATAAGGAAGCGCGATGTG
Hmus_0418-1_ORS_F	GCAGAGCAAGGGCGAAATTC
Hmus_0418-1_ORS_R	TATGCCCACCACCTTATCCC
Hmus_0418-2_KO_F	GGATTTCAGGGCTGCATGTG
Hmus_0418-2_KO_R	CCCAGTGGCTCACCAAATTC
Hmus_0418-2_ORS_F	GGCGTGCCCACACCTATAAG
Hmus_0418-2_ORS_R	GAATGCCGCCAGCGTATAGG
Hmus_0418-3_KO_F	CCAAGAAGGCGATTCTTTGC
Hmus_0418-3_KO_R	CCCACTGGCTATTTAGAGTC
Hmus_0418-3_ORS_F	GCGCAATTCATGACGAATCC
Hmus_0418-3_ORS_R	TTATCCTGCCCATCACAACC
Hmus_NikR_BamHI_F	GGATCCATGCGGACTATGGAAAAGGA
Hmus_NikR_PvuII_R	CAGCTGTCAAAGATCTTTGGGGAAATGAC
Hmus_ureA1_prom_F	CAATCCAAGCGCGTTTTTCAT
Hmus_ureA1_prom_R-dig	CGGCATAATGCAACATCATC
Hmus_ureA2_prom_F	AGTTAAGACTTTTGCCGTGTAG
Hmus_ureA2_prom_R-dig	CTCTCCTGCATAATACAACAAGAAT
ureA2_GS_NikR [a]	AATTATTACTAAATAATACTTTTTTAAAAAAGTTAATACAAAGTATCAAGA
ureA2_GS_NikRc [a]	CTTGATACTTTGTATTAACTTTTTTAAAAAAGTATTATTTAGTAATAATTA
ureA2_GS_NikR_pal12 [a]	AATTATTACTAAACCCCCCCTTTTTAAAAAACCCCCCCCAAAGTATCAAGA
ureA2_GS_NikR_pal12_c [a]	CTTGATACTTTGGGGGGGGTTTTTTAAAAAGGGGGGGTTTAGTAATAATTA
pGEM_F	ACGCCAAGCTATTTAGGTGAC
pGEM_R-Dig	AAACGACGGCCAGTGAATTG

a. Primer contains an additional 3' A residue to facilitate cloning into the pGEM-T_{easy} plasmid

Protein analysis

Liquid cultures were centrifuged for 10 min at 4000 × g and resuspended in phosphate-buffered saline to a final OD_{600} of 10. Bacteria were lysed by sonication for 15 sec with an MSE Soniprep 150 set at amplitude 6. Whole-cell proteins (OD10) were separated on 6% (Hm0418 detection) or 8% (UreB detection) sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) (Sambrook et al., 1989). For Western immunoblot, proteins were subsequently

transferred to a nitrocellulose membrane. Expression of urease subunits was monitored on immunoblots by using a 1:10,000 dilution of antibodies raised against H. felis urease (Belzer et al. 2005; Pot et al. 2007).

Urease assay

The enzymatic activity of urease was determined by measuring ammonia production from hydrolysis of urea, by using the Berthelot reaction as described previously (van Vliet *et al.* 2001). Briefly, cells lysed by sonication were incubated for 30 min at 37°C in buffer consisting of 100 mM sodium phosphate pH 7.5, 10 mM EDTA and 50 mM urea, and the ammonia produced was measured after addition of phenol nitroprusside and alkaline hypochlorite (Sigma Diagnostics). The absorbance of the samples was determined at 570 nm, and compared with a standard NH_4Cl concentration curve. Protein concentrations were determined with the bicinchoninic acid method (Pierce) using bovine serum albumin as standard. Urease enzyme activity was expressed as units representing µmol of urea hydrolysed per min, and is expressed as U mg^{-1} of total protein.

Electrophoretic mobility shift assay

Recombinant *H. mustelae* NikR protein was produced in *E. coli* using the StrepTag system, essentially as described for *H. pylori* NikR (Ernst *et al.* 2005b). Briefly, the *H. mustelae nikR* gene was amplified using primers HmusNikR_BamHI F and HmusNikR_PvuII R (Table 1), cloned into the pASK-IBA7 vector (IBA, Gottingen, Germany), and recombinant NikR protein was purified as previously described (Ernst *et al.* 2005b). The promoter region of UreA and UreA2 was PCR amplified with primers UreA F1 and DIG-labeled UreA_R1 and UreA2_R1 (Table1). Two complementary 50 nt oligonucleotides (ureA2_GS_NikR and ureA2_GS_NikR_c, Table 1), containing the putative NikR binding sites of the *ureA2* promoter, were mixed in equimolar ratio and heated to 80°C, and were slowly cooled to room temperature. As negative control the same oligonucleotides were synthesized with replacement of both halves of the NikR bindingsite by a stretch of C-residues (ureA2_GS_NikR_pal12 and ureA2_GS_NikR_pal12_c, Table 1). Subsequently the double stranded products were cloned into pGEM-T_{easy} (Promega) and checked for sequence integrity. A 250 bp fragment was amplified from these pGEM-T_{easy} clones using the primers pGEM_F and pGEM_R-dig (Table 1). Electrophoretic mobility shift assays were performed with recombinant NikR protein as described previously (Ernst *et al.* 2005b). Briefly, 32.5 pM of digoxygenin-labeled *ureA* of *ureA2* promoter fragment was mixed with recombinant NikR protein at concentrations ranging from 0 to 122 pM. Protein and DNA were mixed in binding buffer (24% glycerol, 40 mM Tris-Cl, pH 8.0, 150 mM KCl, 2 mM DTT, 600 µg/ml bovine serum albumin, 2.5 ng/µl herring sperm DNA, 200 µM $NiCl_2$) in a 20 µl (final volume) mixture and incubated at 37°C for 30 min. Samples were subsequently separated on a 5% polyacrylamide gel in running buffer (25 mM Tris, 190 mM glycine) for 30 min at 200 V. The gel was then blotted onto a nylon membrane (Roche Molecular Biochemicals), and this was followed by chemiluminescent detection of DIG-labeled DNA.

Purification and analysis of RNA

Total RNA was isolated using Trizol (Gibco) according to the manufacturer's instructions. The amount of RNA was determined spectrophotometrically using the Qubit Quantitation platform according to the manufacturers' instructions (Invitrogen). The transcription start site of both the *ureA* and the *ureA2* gene of *H. mustelae* strain NCTC12198 ware determined by primer extension analysis (Ernst *et al.* 2005a). Briefly, approximately 5 to 7 µg of total RNA isolated from *H. mustelae* NCTC 12198 was incubated with 50 pmol of 5'-DIG-labeled primer UreA_R1_dig and UreA2_R1_dig (Table 1) and avian myeloblastosis virus reverse transcriptase (Promega). The sequence reactions were performed using the fmol sequencing kit (Promega) with 5'-DIG-labeled primer UreA2_R2_dig. Primer extension products and sequence reactions were separated on an 8% polyacrylamide-8 M urea gel and blotted onto a nylon membrane (Roche), and this was followed by chemiluminescent DIG detection (van Vliet *et al.* 2001). Quantitative reverse transcriptase-PCR (qRT-PCR) was essentially performed as described previously (Stoof *et al.* 2008), using primer combinations Hmus_ureB1_qpcr_F/Hmus_ureB1_qpcr_R, Hmus_ureB2_qpcr-F/Hmus_ureB2_qpcr_R and Hmus_16S_qpcr_F/Hmus_16S_qpcr_R (Table 1), with the IQ5 system (Bio-Rad) and SYBR-green. qRT-PCR assays were performed using RNA isolated from at least three independent growth experiments. Transcript levels were normalized against the levels of 16S rRNA in each sample [2(ΔΔC(T)) analysis] (Livak & Schmittgen 2001), and expressed as ratio to the mRNA level in cells grown in nickel- and iron-restricted Ham's F-12 medium (Kaighn's modification).

Bioinformatic prediction of NikR operators in *Helicobacter* genome sequences

The intergenic regions from -198 to +2 (relative to the first nucleotide of the annotated translation initiation codon, excluding overlaps with upstream coding sequences) of *H. pylori*, *H. acinonychis* and *H. hepaticus* were extracted using the Regulatory Sequence Analysis Tools (RSAT) website (http//:rsat.ulb.ac.be/rsat/) (Thomas-Chollier *et al.* 2008). The *H. mustelae* genome sequence was obtained from the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/Projects/H_mustelae/) and first interrogated using *H. pylori* protein sequences from metal metabolism genes. Subsequently the upstream sequences of the corresponding coding sequences were extracted. The DNA-pattern module of the RSAT website was used to search for NikR operators using the TRWYA-N_{15}-TRWYA consensus sequence, after extraction of the -198 to +2 sequences of intergenic regions relative to the first nucleotide of the translation initiation codon. The Weblogo algorithm (Crooks *et al.* 2004) was used to represent sequence conservation in predicted NikR operators.

RESULTS

Role of Fur and NikR in nickel-responsive expression of *H. mustelae* urease genes.

We previously demonstrated that inactivation of the *H. mustelae nikR* gene results in nickel-independent expression of both ureases of *H. mustelae*, but did not further investigate the mechanism governing iron-responsive induction of *ureA2B2* transcription (Stoof *et al.* 2008).

The *H. mustelae* genome sequence contains a gene encoding an ortholog of the iron-responsive regulatory protein Fur (Bereswill *et al.* 1998), and to further define the role of the NikR and Fur metal-responsive regulators in nickel- and iron-responsive expression of *ureA2B2* transcription, an *H. mustelae fur* mutant was created using insertional mutagenesis. UreB2 expression and *ureB2* transcription were very high in the *nikR* mutant (Stoof *et al.* 2008), and independent of the iron and nickel concentration, whereas mutation of *fur* did not significantly affect the nickel-responsive pattern UreB2 *and ureB2* expression (Figure 1A, B). The expression pattern of the UreB protein and *ureB* gene was similar to that of *H. pylori* (van Vliet *et al.* 2001; van Vliet *et al.* 2002), as mutation of *nikR* resulted in absence of nickel-responsive induction, whereas mutation of *fur* did not affect UreB expression or *ureB* transcription.

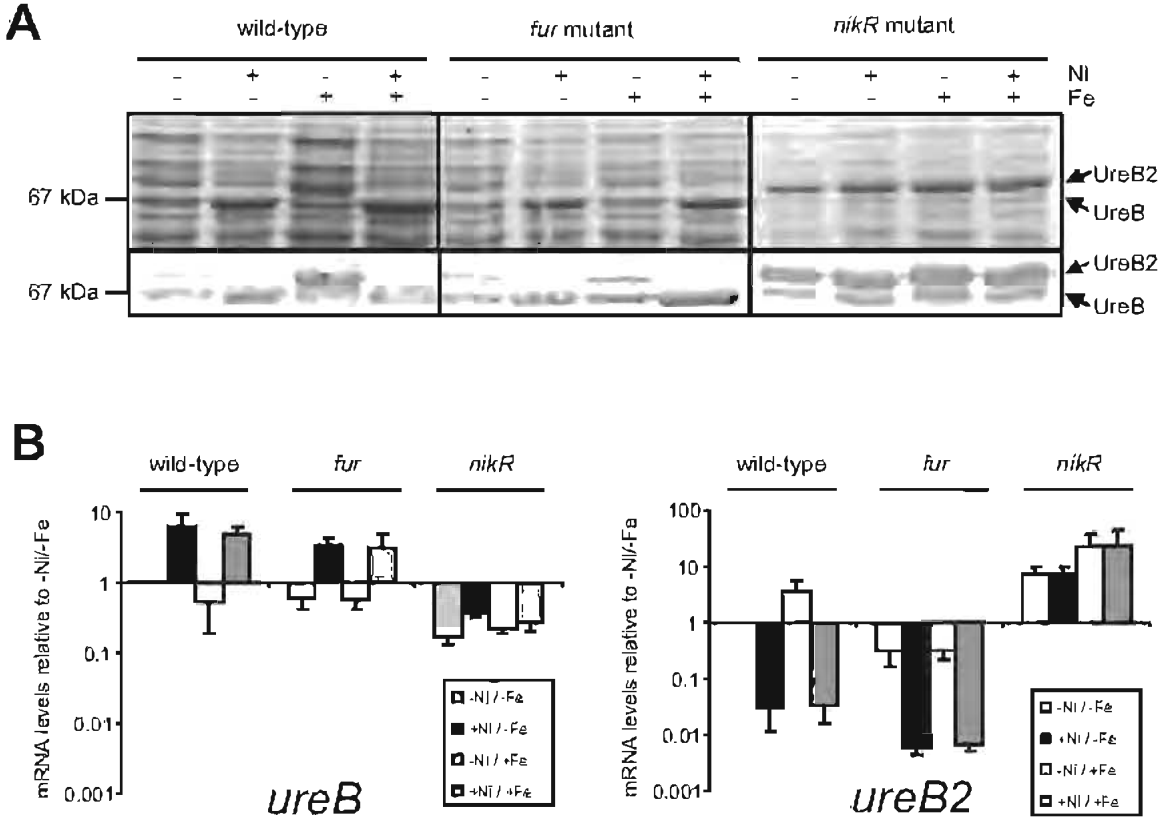

Figure 1. NikR but not Fur controls nickel-responsive expression of the UreAB and UreA2B2 ureases of *Helicobacter mustelae*. (A) Regulation of the UreB and UreB2 protein in the *H. mustelae* wild-type strain and its isogenic *fur* and *nikR* mutants, grown in different combinations of nickel-restricted, nickel-replete, iron-restricted and iron-replete conditions (see top panel), as demonstrated by SDS-PAGE (top panel) and immunoblot using an antibody recognizing both the UreB and UreB2 subunits (bottom panel). The relevant marker size is indicated on the left, the position of the UreB and UreB2 proteins on the right. (B) Regulation of *ureB* and *ureB2* mRNA levels in the *H. mustelae* wild-type strain and its isogenic *fur* and *nikR* mutants, grown in the same combinations of nickel- and iron-conditions as shown in panel A, as determined using quantitative reverse transcriptase PCR. The levels of mRNA observed in the qRT-PCR were normalized (Livak & Schmittgen 2001) to the levels of 16S rRNA, and the -Ni/-Fe condition in the wild-type strain was set to 1. Normalization to the *ppk* gene gave similar results (data not shown) All other mRNA levels are expressed as compared to the -Ni/-Fe condition in the wild-type strain. Error bars represent standard deviation. Results shown are the average of three independent experiments.

Identification of the *ureAB* and *ureA2B2* promoters.

The transcription start site of both urease gene clusters of *H. mustelae* was identified using primer extension analysis (Figure 2A). Primers specific for *ureA* and *ureA2* were used in equimolar concentrations or added separately (identical results, data not shown) for primer extension analysis with RNA isolated from *H. mustelae* NCTC12198 grown in media with either nickel- and iron-restricted or -replete conditions. The transcription start site of *ureA* is located at the G residue 52 nt upstream of the *ureA* ATG start codon. The transcription start site of *ureA2 is* located at the G residue 52 nt upstream of the *ureA2* ATG start codon. Both transcription start sites are preceded by a σ^{80} -10 sequence (Figure 2B) at the correct distance from the transcription initiation site (Petersen *et al.* 2003), similar to what has been observed for the *H. pylori* urease promoter (Davies *et al.* 2002; van Vliet *et al.* 2002). Nickel- and iron-responsive regulation of the transcript start site cDNA of both urease clusters is in accordance with the immunoblot and qRT-PCR data (Figure 1A, B).

In *H. pylori*, NikR binds to palindromic sequence with as consensus sequence 5'-TATWATT-N_{11}-AATWATA, with one of the two half sites usually being less conserved (Delany *et al.* 2005; Ernst *et al.* 2006; Dosanjh *et al.* 2009). The *ureA* and *ureA2* promoters of *H. mustelae* and *H. acinonychis* were searched for such putative binding sites (adapted to 5'-TATWA-N_{15}-TWATA, Figure 2B). A putative NikR box was detected from 140 to 164 nt upstream of the ATG start codon of *H. mustelae ureA* (Figure 2C). Similar searches in the *ureA2* promoter region of *H. mustelae* allowed the identification of a putative NikR-binding site 88 to 112 nt (Figure 2B) upstream of the ATG start codon of *ureA2*. In *H. mustelae* this binding sequence overlaps with the -35 region of the σ^{80} promoter upstream of the *ureA2* gene, whereas the binding sequence upstream of the *ureA* gene is located from -112 to -88, upstream of the canonical σ^{80} promoter (Figure 2C).

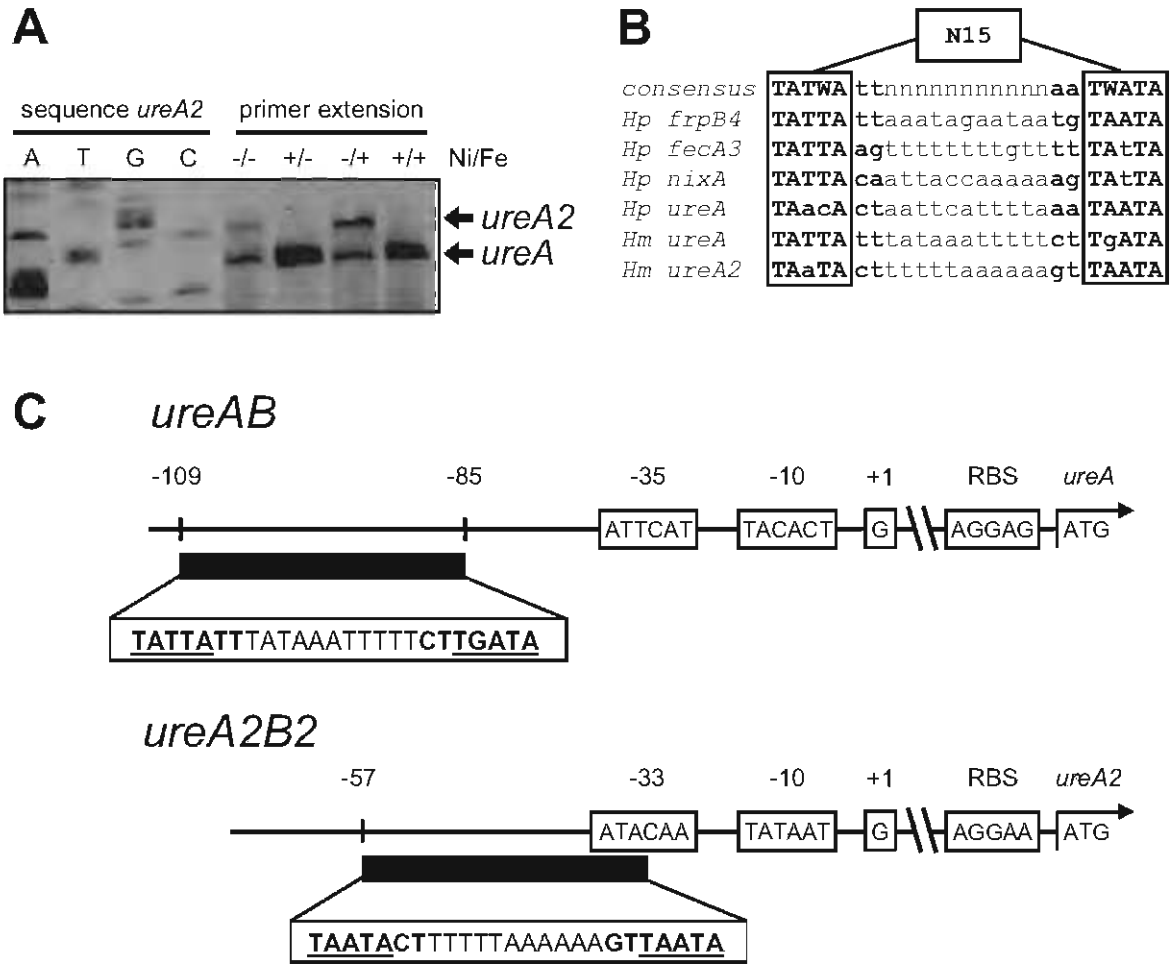

Figure 2. Characterization of the *H. mustelae ureA* and *ureA2* promoter regions. (A) Determination of the transcriptional start site (TSS) of the *ureA* and *ureA2* genes by semi-quantitative primer extension analysis, with RNA from *H. mustelae* NCTC 12198 wild-type cells grown in different combinations of nickel-restricted, nickel-replete, iron-restricted and iron-replete conditions. The primer extension products representing the *ureA* and *ureA2* TSS are indicated on the right. The position of the TSS was determined using a sequencing reaction of the *ureA2* promoter as displayed on the left (with lanes A, T, G, C). (B) Prediction of the NikR operator sequence in the *H. mustelae* (Hm), based on the previously described consensus sequence (TATWA-N_{15}-TWATA) of *H. pylori* NikR (Delany *et al.* 2005; Ernst *et al.* 2006; Dosanjh *et al.* 2009). (C) Graphical representation of the *H. mustelae ureA* and *ureA2* promoter regions with the TSS, –10 and -35 regions, ribosomal binding site and ATG start codon of the *ureA* and *ureA2* genes. The predicted location and sequence of the NikR operators are indicated with a black bar and the sequence underneath.

NikR binds with different affinities to the *ureA* and *ureA2* promoter.

To investigate whether the nickel-responsive regulation of *ureAB* and *ureA2B2* transcription is mediated by NikR binding to the *ureA* and *ureA2* promoters, an electrophoretic mobility shift assay was performed using both the *ureA* and *ureA2* promoter regions and recombinant *H. mustelae* NikR protein. In the absence of nickel, NikR neither bound the *ureA* promoter nor the *ureA2* promoter (data not shown). In the presence of nickel, NikR was able to bind to both promoters (Figure 3A) albeit with different affinities. Incubation of NikR with the *ureA2* promoter region led to two bands in the electrophoretic mobility shift assay (Figure 3A), which is suggestive for the presence of multiple NikR binding sites. When the *ureA* and

ureA2 promoters were mixed in equimolar concentrations, NikR first shifted the *ureA2* promoter and subsequently the *ureA* promoter (Figure 3A), which is consistent with the regulatory patterns of *ureAB* and *ureA2B2* transcription, where *ureA2B2* transcription is repressed at lower nickel concentrations than those where *ureAB* transcription is induced (Stoof *et al.* 2008)To confirm the predicted NikR-binding site in the *H. mustelae ureA2* promoter (Figure 2B, 2C), we performed electrophoretic mobility shift assays with cloned 50 nt fragments of the *H. mustelae ureA2* promoter region, one version representing the wild-type promoter including the putative NikR binding sequence, and one version where the putative NikR binding was replaced with a C stretch, as described previously (Dosanjh *et al.* 2009). Recombinant NikR did bind when the *ureA2* promoter with the putative NikR-binding site was present (Figure 3B, first and second panel), but replacement of the NikR-binding site by a C-stretch resulted in absence of binding (Fig 3B, third panel).

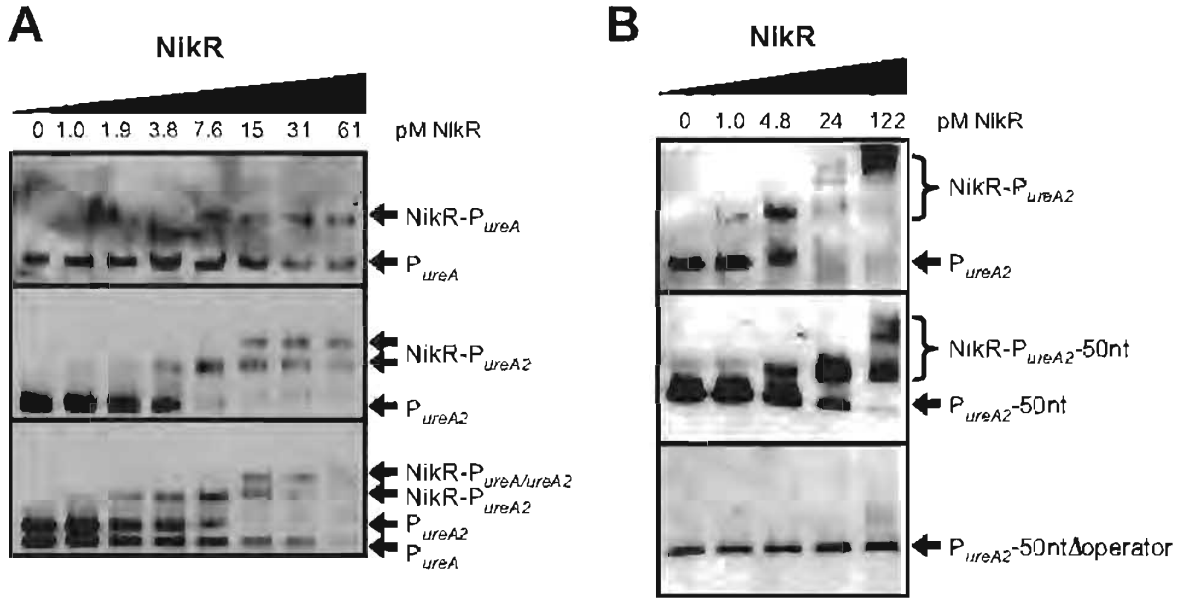

Figure 3. NikR controls *ureAB* and *ureA2B2* transcription by sequence-specific direct binding to the *H. mustelae ureA* and *ureA2* promoter regions. (A) Electrophoretic mobility shift assays with recombinant *H. mustelae* NikR protein and the *ureA* (P_{ureA}) and *ureA2* promoters (P_{ureA2}) in the presence of $NiCl_2$. The NikR-complexed *ureA* and *ureA2* promoters are indicated as NikR-P_{ureA} and NikR-P_{ureA2}. The first panel shows NikR binding to the *ureA* promoter, the second panel to the *ureA2* promoter. The third panel shows that when the *ureA* and *ureA2* promoters are mixed at equimolar ratios, the *ureA2* promoter is shifted at lower concentrations of NikR and hence has a higher affinity for NikR. (B) NikR binds to the predicted operator in the *ureA2* promoter. The predicted operator in the *ureA2* promoter was reconstructed as a 50 nt fragment (P_{ureA2}-50nt) with the TAATACT-N_{11}-GTTAATA operator replaced by CCCCCCC-N_{11}-CCCCCCC (P_{ureA2}-50ntΔoperator). The three panels show electrophoretic mobility shift assays with recombinant *H. mustelae* NikR protein in the presence of $NiCl_2$. Top panel: full length *ureA2* promoter, middle panel: P_{ureA2}-50nt, bottom panel: P_{ureA2}-50ntΔoperator. The unbound and NikR-complexed versions are indicated on the right. NikR concentrations used (in pM) are indicated above the lanes; the DNA concentration was 32.5 pM of each promoter region.

Prediction of NikR operators in *Helicobacter* complete genome sequences.

The previously suggested consensus sequence for NikR operators (TATWA-N_{15}-TWATA) does not allow identification of several of the confirmed high-affinity NikR operators in *H. pylori*, due to mismatches with C or G residues. Using the previously described *H. pylori* high

affinity NikR binding sites (Delany *et al.* 2005; Ernst *et al.* 2006; Dosanjh *et al.* 2009) and the *H. mustelae* NikR operators in the *ureA* and *ureA2* promoters, we redefined the consensus sequence to TRWYA-N_{15}-TRWYA.

Table 2. Prediction of NikR-operators within intergenic regions of *Helicobacter* genomes, upstream of genes putatively involved in metal homeostasis

Gene	Position[a]	Box sequence[b]	Notes
H.pylori	**26695**		
ureA (hp0073)	-143 -119	ata**TAACA**ctaattcattttaaa**TAATA**att	
nixA (hp1077)	-48 -24	ata**TATTA**caattaccaaaaaag**TATTA**ttt	
fecA3 (hp1400)	-112 -88	cat**TATTA**agtttttttgtttt**TATTA**ctt	
frpB4 (hp1512)	-91 -67	agg**TATTA**ttaaatagaataatg**TAATA**ata	
frpB4 (hp1512)	-88 -64	tat**TATTA**aatagaataatgtaa**TAATA**acc	
frpB2 (hp0916)	-75 -51	aaa**TAATA**ctttttagttataa**TAACA**att	
fecDE (hp0890)	-125 -101	gaa**TATTA**gaggaatttaaaaa**TAATA**aga	c
hopV (omp31)	-49 -25	ata**TATTA**ttatttctttataag**TAATA**ctt	
hopW (omp32)	-99 -75	cta**TAATA**aaataattaaaaaag**TAACA**ctt	
copAP (hp1067)	-99 -75	tgc**TATTA**tttggaacgatttat**TATTA**taa	c
copA2 (hp1502)	-160 -136	aag**TGTTA**ctttttaattattt**TATTA**tag	
H.acinonychis	**Sheeba**		
ureA (Hac_1532)	-140 -116	aga**TAACA**ctaatttgttacaaa**TAACA**ttc	
ureA2 (Hac_0448)	-81 -57	aag**TATTA**ctttcttaaaaaagt**TAATA**aca	
frpB (Hac_0072)	-89 -65	agg**TATTA**ttaaatagaatagtg**TAATA**ata	
frpB (Hac_0072)	-86 -62	tat**TATTA**aatagaatagtgtaa**TAATA**atc	
fecA (Hac_0865)	-112 -88	cgt**TATTA**aatttctttagtttt**TATTA**cct	
omp31 (Hac_0095)	-54 -30	tta**TAATA**aaataattcagaaag**TAATA**ctg	
omp32 (Hac_1718)	-77 -53	ata**TATTA**ttattccactataag**TAATA**ctt	
H.hepaticus	**ATCC 51449**		
nikA (hh0417)	-145 -121	cat**TATTA**ctctttaatatttta**TGTTA**tta	d
hh0418	-28 -4	taa**TAACA**taaaatattaaagag**TAATA**atg	d
arsRS (hh1608)	-77 -53	ttt**TATTA**tacaatcctcattttt**TAATA**cct	e
H. mustelae	**NCTC 12198**		
ureA	-164 -140	ttt**TATTA**tttataaatttttct**TGATA**aat	
ureA2	-142 -118	atc**TAATA**attttgatataaat**TATTA**gtt	
ureA2	-132 -108	ttc**TGATA**taaattattactaaa**TAATA**ttt	
ureA2	-112 -88	aaa**TAATA**ctttttaaaaaagt**TAATA**caa	
0418-1 (nikH)	-73 -49	tag**TAATA**ttgcatcgcaaaaaa**TATTA**cat	

a) Position of the TRWYA-N_{15}-TRWYA sequence relative to annotated or predicted translation start codon

b) Residues in gray background are mismatches to the previously suggested TATWA-N_{15}-TWATA consensus sequence (Delany *et al.* 2005; Ernst *et al.* 2006; Dosanjh *et al.* 2009).

c) Predicted to be in an operon. The predicted operator is present in the intergenic region upstream of the first gene of the predicted operon.

d) Overlaps with startcodon of *hh0418* gene, shared NikR operator in divergent *nikA-hh0418* promoter region

e) The *hh1608-1607* genes encode a two-component regulatory system homologous to the *H. pylori* ArsRS acid-responsive regulatory system (Pflock *et al.* 2006).

This consensus sequence was used to search the intergenic regions from -198 to +2 (relative to the first nucleotide of the annotated translational startcodon) of the *H. pylori*, *H. acinonychis* and *H. hepaticus* genome sequences (Tomb *et al.* 1997; Suerbaum *et al.* 2003; Eppinger *et al.* 2006). We also searched the unannotated *H. mustelae* genome sequence for orthologs of metal transport and metal-regulatory genes, and included their predicted promoters in the search. Table 2 shows genes of all four *Helicobacter* species, which fulfilled two criteria: 1) a putative NikR operator in predicted promoter region 2) either a predicted function in metal homeostasis or previously shown to be controlled by NikR (Contreras *et al.* 2003; Delany *et al.* 2005; Ernst *et al.* 2005b; Abraham *et al.* 2006; Ernst *et al.* 2006; Benanti & Chivers 2007; Dosanjh *et al.* 2009). Interestingly, many genes previously suggested to be NikR-regulated in *H. pylori* (Contreras *et al.* 2003; Ernst *et al.* 2005b; Abraham *et al.* 2006; Ernst *et al.* 2006; Danielli *et al.* 2009) were independently identified in our predictive search (Table 2). The absence of a NikR operator in the urease promoter region of *H. hepaticus* is consistent with the lack of nickel-responsive regulation of urease transcription (Belzer *et al.* 2005).

Identification of a nickel-regulated outer membrane protein of *H. mustelae*.

One of the potential NikR operators in *H. mustelae* is located upstream of a gene encoding a putative TonB-dependent outer membrane protein homologous to the HH0418 protein of *H. hepaticus* (Suerbaum *et al.* 2003; Belzer *et al.* 2007). In *H. hepaticus* the *hh0418* gene is located divergently to the *nikABDE* nickel-specific ABC transporter sytem (Beckwith *et al.* 2001; Suerbaum *et al.* 2003). In the unpublished *H. mustelae* genome, the *hm0418-1* gene is preceded upstream by two paralogs tentatively named *hm0418-2* and *hm0418-3*, and the three Hm0418 paralogs are 64% identical over the whole length of the proteins, with the Hm0418-2 and Hm0418-3 proteins showing higher identity. The *hm0418-1* gene is followed downstream by a *ceuE* gene encoding a putative periplasmic iron-binding protein (Figure 4A). The *H. hepaticus* HH0418 protein was previously suggested to be involved in nickel-transport (Belzer *et al.* 2007), similar to the *H. pylori* FecA3 and FrpB4 proteins (Davis *et al.* 2006; Ernst *et al.* 2006; Schauer *et al.* 2007). To test whether the presence of a NikR operator upstream of the *hm0418-1* gene was indicative of NikR- and nickel-responsive regulation, we compared protein profiles of *H. mustelae* grown in nickel-restricted and nickel-replete conditions. One band corresponding to the predicted molecular weight (92 kDa) of the Hm0418 proteins was repressed in nickel-replete conditions, but present in nickel-restricted conditions (Figure 4B). The protein was absent in a *hm0418-1* negative mutant, but was present in *hm0418-2* and *hm0418-3* mutants of *H. mustelae*. This confirmed that *hm0418-1* is nickel-regulated.

To test whether Hm0418-1 could be contributing to nickel transport, analogous to the *H. pylori* FrpB4 protein (Schauer *et al.* 2007), we assessed the effect of the *hm0418* mutations on activity of the UreAB nickel-cofactored urease, since reduced nickel import will reduce urease activity (Schauer *et al.* 2007). To exclude any contribution of the UreA2B2 urease, cells were lysed by sonication which abolishes all UreA2B2 activity (Stoof *et al.* 2008). Inactivation of the *hm0418-1* gene significantly reduced urease activity, while inactivation of

the *hm0418-2* and *hm0418-3* genes did not affect urease activity (Figure 4C). Supplementation of Brucella media with nickel, which may enter the periplasm via outer membrane porins, restored urease activity of the *hm0418-1* mutant to wild-type levels (Figure 4D), suggesting that the reduced urease activity in the *hm0418-1* mutant is caused by reduced nickel transport accross the outer membrane. Since the presence of the NikR operator, nickel-responsive regulatory pattern and the effect on urease activity all suggest a function of *hm0418-1* in nickel uptake by *H. mustelae*, and we propose the name of NikH for Hm0418-1.

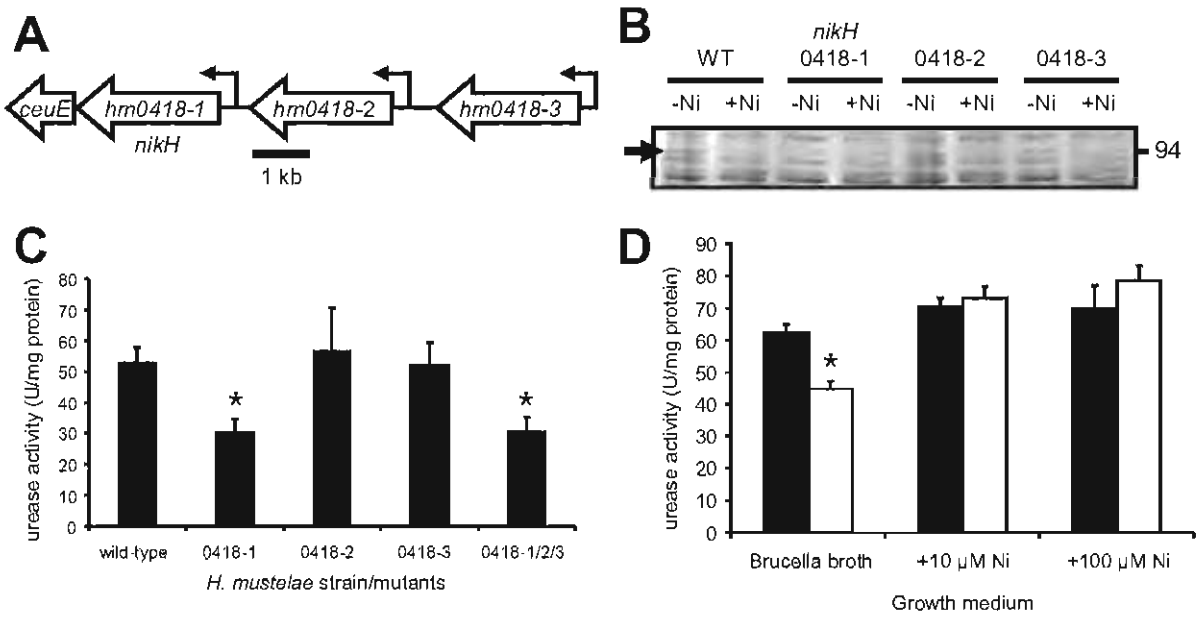

Figure 4. Nickel-responsive expression of the *H. mustelae* Hm0418-1 (NikH) protein and contribution of NikH to urease activity. (A) Schematic representation of the *H. mustelae* genomic region containing the *hm0418-1* (*nikH*), *hm0418-2*, *hm0418-3* and *ceuE* genes. (B) SDS-PAGE analysis of protein profiles of wild-type *H. mustelae* NCTC 12198 and its isogenic *hm0418-1*, *hm0418-2* and *hm0418-3* mutants, grown under either nickel-restricted (-Ni) or nickel-replete (+Ni) conditions. The wild-type strain shows one protein of approximately 92 kDa which displays expression under nickel-restricted conditions only, and is absent in the *hm0418-1* mutant but not in the *hm0418-2* and *hm0418-3* mutants. The arrow on the left indicates the Hm0418-1 (NikH) protein, the closest marker size (in kDa) is indicated on the right. (C) Urease activity of *H. mustelae* wild-type and isogenic *hm0418* mutants. Insertional mutagenesis of the nickel regulated *hm0418-1* (*nikH*) gene resulted in a significant decreased urease activity. Insertional mutagenesis of the *hm0418-2* and *hm0418-3* genes did not affect urease activity, whereas a triple mutant lacking all three *hm0418* genes gave the same phenotype as the *hm0418-1* mutant. (D) Supplementation of Brucella media with nickel restores urease activity in the *H. mustelae hm0418-1* mutant to levels comparable to the wild-type strain. Black bars represent the wild-type strain, white bars the *hm0418-1* (*nikH*) mutant strain. Results shown are the average of three independent growth experiments. Error bars represent standard deviation, an asterisk represents a significant difference in urease activity ($p \leq 0.05$, Mann-Whitney U test) when compared to the wild-type strain (Panel C) or unsupplemented Brucella media (Panel D).

DISCUSSION

The nickel-cofactored urease enzyme is widespread among bacterial species, where it often has a role in the production of ammonia for cellular nitrogen metabolism. However, in several bacterial pathogens urease also contributes to resistance to low pH and pathogenesis of

infection (Burne & Chen 2000). This is especially apparent in *Helicobacter* species colonizing the gastric mucosa of many mammals, as all gastric *Helicobacter* species display high-level expression of either one or two urease enzymes (Solnick & Schauer 2001; Kusters *et al.* 2006; Stoof *et al.* 2008). This high level of urease expression in gastric *Helicobacter* species has not only necessitated the development of high-affinity acquisition systems of the nickel cofactor (Eitinger & Mandrand-Berthelot 2000; Schauer *et al.* 2007), but handling of such potentially toxic metals also requires mechanisms controlling all aspects of nickel metabolism (Mulrooney & Hausinger 2003; Belzer *et al.* 2007; Maier *et al.* 2007).

We recently demonstrated that the three carnivore-colonizing *Helicobacter* species, *H. mustelae*, *H. acinonychis* and *H. felis* have a second, independent urease system designated UreA2B2 (Pot *et al.* 2007; Stoof *et al.* 2008), which in *H. mustelae* allows survival of acid shocks at pH 1.5 (Stoof *et al.* 2008). Expression of UreA2B2 and UreAB was inversely regulated in response to nickel, with UreA2B2 being nickel-repressed, and UreAB being nickel-induced (Stoof *et al.* 2008). Although nickel dependent regulation was absent in a *nikR* mutant, direct interaction of NikR with the urease promoters was not demonstrated. In this study we have further characterized this regulatory mechanism, by studying the role of NikR and Fur in nickel-responsive regulation of *H. mustelae* urease expression. From the studies with the *H. mustelae nikR* and *fur* mutants (Figure 1), it was apparent that the NikR regulator has a dominant phenotype, as inactivation of the *nikR* gene resulted in constitutive, nickel- and iron-independent expression of both the UreAB and UreA2B2 ureases, whereas mutation of *fur* had no effect on nickel dependent regulation of the ureases (Figure 1).

Further characterization of the promoters driving transcription of the *ureAB* and *ureA2B2* genes by primer extension showed that both promoters are transcribed from promoters recognised by the σ^{80}-cofactored RNA polymerase (Petersen *et al.* 2003), upstream of the *ureA* and *ureA2* genes. Bioinformatic searches for potential NikR-binding sites were based on those identified in *H. pylori* (van Vliet *et al.* 2001; Delany *et al.* 2005; Ernst *et al.* 2005b; Benanti & Chivers 2007; Zambelli *et al.* 2008; Dosanjh *et al.* 2009), and allowed the prediction of a binding sites in both the *H. mustelae ureA* and *ureA2* promoters, albeit in different locations. The *H. mustelae ureA* promoter was similar to the *H. pylori ureA* promoter (Delany *et al.* 2005; Ernst *et al.* 2005b), having a NikR-binding site far upstream of the canonical σ^{80} promoter, and shows the same nickel-induced expression pattern (van Vliet *et al.* 2001). The *ureA2* promoter was more similar to the *H. pylori nixA* promoter (Ernst *et al.* 2005b), although one of the predicted binding sites overlaps with the -35 sequence of the promoter (Figure 2C). A secondary *ureA2* NikR operator is located directly upstream of the tested NikR operator (Table 2), and could also be involved in regulation, through multimerisation at the promoter analogous to what has been described for Fur in *E. coli* (Escolar *et al.* 1998). In fact the second shift at higher nickel concentrations (middle panel figure 3A) may represent NikR binding to a second (lower affinity) NikR binding site. Direct binding of NikR to the *ureA* and *ureA2* promoters was confirmed by gel-shift assays (Figure 3A), and using site-directed mutagenesis we showed that the initial prediction of a NikR binding site in the *ureA2* promoter was correct, as replacement of these residues with a stretch of C residues abolished binding of NikR to this binding site (Figure 3B).

When the *ureA* and *ureA2* promoters were mixed at equimolar concentrations, NikR first shifted the *ureA2* promoter (Figure 3A). This suggests that NikR has a higher affinity for the *ureA2* promoter, and is consistent with the observed regulatory pattern of UreAB and UreA2B2 expression. In nickel-restricted conditions, UreA2B2 expression is high, and UreAB expression relatively low (Figure 1) (Stoof *et al.* 2008). At increasing nickel concentrations, UreA2B2 expression is first switched off, allowing pre-produced UreAB enzyme to be activated (van Vliet *et al.* 2002). When nickel concentrations increase further, preproduced UreAB is saturated with nickel, and *de novo* expression of the UreAB urease is increased (van Vliet *et al.* 2002; van Vliet *et al.* 2004; Stingl & De Reuse 2005; Dosanjh *et al.* 2009). These results suggest that the different promoters compete for available NikR protein, and that promoter affinity determines the order of regulation at increasing nickel concentrations. Interestingly, comparison of the NikR operators in the *ureA* and *ureA2* promoters did suggest differences in binding sequence (Figure 2B) which may lead to differences in affinity. The *ureA2* promoter does contain a perfect palindromic repeat of the left and right arms of the operator sequence whereas the *ureA* promoter is an imperfect repeat which may lead to reduced binding affinity (Table 2). This *ureA/ureA2* promoter configuration is mirrored in *H. acinonychis* (Table 2). However, the exact role of these differences in binding sites remains to be elucidated, since also the presence of multiple binding sites and the sequences surrounding the binding site, may play a role in determining affinity of NikR for its operator sequences (Benanti & Chivers 2007; Zambelli *et al.* 2008; Dosanjh *et al.* 2009).

The NikR binding sites found in the *ureA* and *ureA2* promoter of *H. mustelae* were used to define a new consensus sequence (TRWYA-N_{15}-TRWYA) which was able to identify all confirmed high affinity binding sites in *H. pylori* (Dosanjh *et al.* 2009). Next to all the confirmed high affinity operators in the promoters of the *H. pylori fecA3*, *frpB4*, *ureA* and *nixA* genes, we also identified novel putative NikR operators (Table 2). Amongst these were the genes encoding the *H. pylori* outer membrane porins HopV (Omp31) and HopW (Omp32) which were previously reported as NikR-regulated by microarray analysis (Contreras *et al.* 2003). Surprisingly a perfect NikR operator was also found before an operon starting with the *hp0890* gene, which includes the genes encoding for the putative ferric citrate ABC transporter system (Tomb *et al.* 1997; Velayudhan *et al.* 2000). As for *frpB4* and *fecA3*, the *fecDE* genes were reported to be iron-independent (van Vliet *et al.* 2002), and inactivation of *fecD* gene did not affect iron transport in *H. pylori* (Velayudhan *et al.* 2000). The *fecDE* and upstream *hp0890* gene of *H. pylori* are acid-regulated by the ArsRS two-component regulatory system (Pflock *et al.* 2006), supporting a role of *fecDE* in acid-resistance of *H. pylori*, and a putative role for the *fecDE* genes in nickel metabolism of *H. mustelae* is currently under investigation.

Screening the genome sequences of other non- pylori *Helicobacter* species with the revised consensus sequence confirmed the presence of putative NikR operators in front of the nickel-responsive urease systems in *H. acinonychis* (Stoof *et al.* 2008), while such an operator is absent in the *H. hepaticus* urease promoter (Belzer *et al.* 2005). Similar to *H. pylori*, its close relative *H. acinonychis* also encodes homologs of *fecA3*, *frpB4*, *hopV* and *hopW* which all

contain putative NikR boxes in their putative promoter region (Table 2). The *H. mustelae* and *H. hepaticus* genomes do not contain orthologs of the *H. pylori frpB4* gene, which mediates TonB-dependent nickel transport (Davis *et al.* 2006; Ernst *et al.* 2006; Schauer *et al.* 2007). However both genomes do encode a different TonB-dependent outer membrane ortholog with no known homologs in other bacterial genera (HH0418 in *H. hepaticus* (Suerbaum *et al.* 2003; Belzer *et al.* 2007)). In *H. mustelae* there are three orthologs of *hh0418*, arranged in tandem (Figure 4A). The *hh0418* and *hm0418-1* genes contain a putative NikR operator in their promoter sequence, whereas the *hm0418-2* and *hm0418-3* gene do not. In *Helicobacter hepaticus* this gene could potentially be involved in nickel transport since the location of the gene is divergent to NikABDE, the periplasmic and inner membrane transporters for nickel (Beckwith *et al.* 2001; Belzer *et al.* 2007). In *H. mustelae*, expression of Hm0418 is nickel-repressed (Figure 4B), and inactivation of the *hm0418-1* gene but not *hm0418-2* or *hm0418-3* results in reduced urease activity (Fig 4C). The decreased urease activity in the *hm0418-1* mutant could be restored to wild-type levels by nickel-supplementation of the growth medium (Figure 4D). Taken together, this strongly suggests that Hm0418-1 functions as an outer membrane transporter for nickel, although further experiments are required to confirm this.

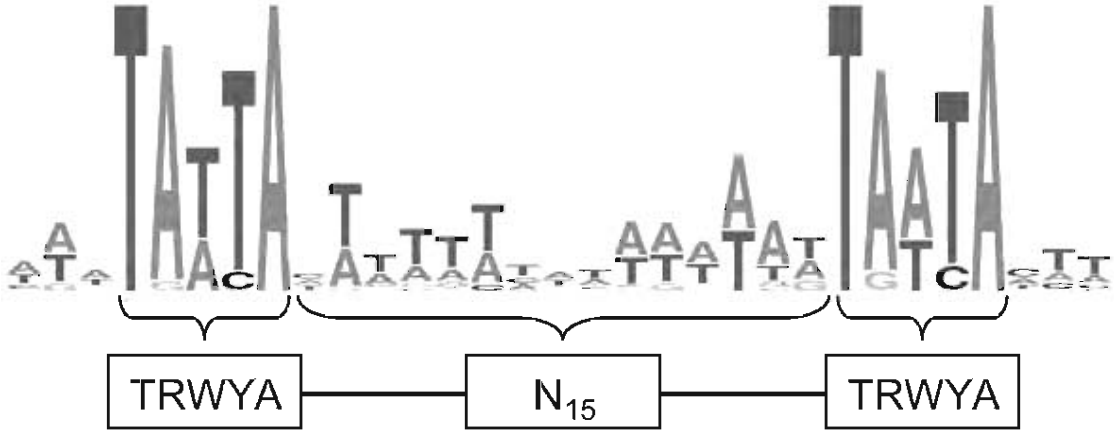

Figure 5. Weblogo representation of *Helicobacter* NikR operators identified in the *H. pylori, H. mustelae, H. acinonychis* and *H. hepaticus* genomes. The Weblogo algorithm (http://weblogo.berkeley.edu/logo.cgi (Crooks *et al.* 2004)) was used to represent nucleotide conservation within the *Helicobacter* NikR operator sequences identified in the upstream regions of *Helicobacter* genes predicted to be involved in metal metabolism (Table 2), using the TRWYA-N_{15}-TRWYA consensus sequence.

Comparison of all predicted NikR operators in promoters of *Helicobacter* metal metabolism genes using the Weblogo program suggests that the NikR operator in *Helicobacter* is surprisingly well conserved (Figure 5). To reduce the number of false-positive hits in genomes it may be sufficient to use TRWTA-N_{15}-TRWTA as consensus sequence if both DNA strands are included in the search. The binding site TRWYA-N_{15}-TRWYA is quite similar to the *E. coli* NikR binding site *GTATGA*-N_{16}-*TCATAG* and the predicted pseudo-NikR box TATTAC-N_{14}-GTAATA, present in front of urea carboxylase genes in α- and β-proteobacteria, where these represent an alternative, nickel independent urea degradation pathway (Rodionov *et al.* 2006). This supports our approach for the prediction of NikR operators in *Helicobacter* species, and this can potentially be extended to NikR regulators from other bacterial species.

When the genome sequences of the first *Helicobacter* species were originally published (Berg *et al.* 1997; Tomb *et al.* 1997; Suerbaum *et al.* 2003), it was suggested that these bacterial species have a relatively limited capacity for gene regulation, which is consistent with their restricted ecological niches. However ongoing studies on the nickel-regulatory protein NikR revealed more and more genes regulated by this protein, but lack of apparent sequence homology in the NikR operator, made it difficult to predict new targets. Here we present a study which demonstrates that the *ureA* and *ureA2* promoters of *H. mustelae* are directly regulated by NikR and using previously and newly identified NikR operators, we have been able to refine the NikR consensus sequence to one predicting new NikR targets in different *Helicobacter* species. Our prediction was validated by the identification of the *H. mustelae nikH* (*hm0418-1*) gene, a nickel-regulated outer membrane protein contributing to urease activity.

ACKNOWLEDGEMENTS

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Chapter 6

NikR mediates nickel-responsive transcriptional repression of the *Helicobacter pylori* outer membrane proteins FecA3 (HP1400) and FrpB4 (HP1512)

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ABSTRACT

The transition metal nickel plays an important role in gastric colonization and persistence of the important human pathogen *Helicobacter pylori*, as it is the cofactor of its abundantly produced acid-resistance factor urease. Nickel-uptake over the inner membrane is mediated by the NixA protein, and the expression of NixA is controlled by the NikR regulatory protein. Here we describe that NikR also controls nickel-responsive expression of the FecA3 (HP1400) and FrpB4 (HP1512) outer membrane proteins (OMPs), as well as of an ExbB-ExbD-TonB system which may function in energization of outer membrane transport. Transcription and expression of the *frpB4* and *fecA3* genes was nickel-repressed in wild-type *H. pylori* 26695, but nickel-independent and derepressed in an isogenic *nikR* mutant. Both the *frpB4* and *fecA3* genes were transcribed from a promoter directly upstream of their start codon. Regulation by NikR was mediated via nickel-dependent binding to specific operators overlapping either the +1 or -10 sequences in the *frpB4* and *fecA3* promoters, respectively, and these operators contained sequences resembling the proposed *H. pylori* NikR recognition sequence (TATWATT-N_{11}-AATWATA). Transcription of the *hp1339-1340-1341* operon encoding the ExbB2-ExbD2-TonB2 complex was also regulated by nickel and NikR, but not by Fur and iron. In conclusion, *H. pylori* NikR controls nickel-responsive expression of the HP1400 (FecA3) and HP1512 (FrpB4) OMPs. We hypothesize that these two NikR-regulated OMPs may participate in the uptake of complexed nickel ions, and that this process is energized by the NikR-regulated ExbB2-ExbD2-TonB2 system, another example of the specific adaptation of *H. pylori* to the gastric lifestyle.

INTRODUCTION

The human gastric pathogen *Helicobacter pylori* colonizes the mucus layer overlaying the gastric epithelial cells in the human stomach. If not removed by antibiotic treatment, the infection usually remains lifelong and may progress to peptic ulcer disease or the development of adenocarcinoma of the distal stomach (22). In its niche *H. pylori* is exposed to hostile environmental conditions, caused by acid and changes in nutrient availability. Since about half of the world population is infected with *H. pylori* (22), the bacterium is clearly well adapted to the hostile conditions occuring in the gastric mucosa.

The nickel-cofactored urease and hydrogenase enzymes are major factors in gastric colonization by *H. pylori* (14, 26). When cytoplasmic nickel availability is insufficient, the urease and hydrogenase systems cannot be fully activated (37), leading to acid-sensitivity and decreased survival and colonization of *H. pylori* in the gastric mucosa (14, 26). However, the cell also needs to prevent toxicity from high intracellular concentrations of nickel (24, 37). The intracellular concentration of nickel is therefore carefully controlled, by regulation of nickel uptake and usage. In *H. pylori*, nickel homeostasis is controlled by the NikR (HP1338) protein, which mediates transcriptional regulation of expression of the NixA nickel uptake system and the urease operon (12, 17, 40, 46).

Nickel uptake in gram-negative bacteria is complicated by the two membrane barriers. Soluble nickel compounds can enter the periplasm via the outer membrane porins, and are subsequently transported by the NixA cytoplasmic membrane protein (4, 19, 23, 45). However, it is conceivable that nickel can be complexed to eukaryotic proteins, or may be present in poorly soluble complexes, and thus these complexes cannot reach the periplasm via the porins. Thus is situation similar to iron transport (2), where the soluble ferrous iron is transported by the cytoplasmic membrane FeoB transporter, but insoluble ferric iron complexes require specific, high-affinity iron uptake outer membrane transporters (2). The *H. pylori* genome contains six genes encoding such outer membrane proteins (1, 36), three annotated as orthologs of the *Escherichia coli* ferric citrate receptor FecA (47), and three annotated as orthologs of the *Neisseria meningitidis* FrpB protein (27). While the exact function of the *H. pylori* FecA and FrpB proteins is currently unknown, analysis of their expression has shown that two out of three copies of both FecA and FrpB orthologs (HP0686, HP0807, HP0876 and HP0916/0915) display iron- and Fur regulated expression (15, 41) as expected for iron-uptake systems (2), whereas expression of the HP1400 (FecA3) and HP1512 (FrpB4) copies was iron- and Fur-independent (41), suggesting that these proteins may not be involved in iron-transport.

Transport of complexes over the outer membrane is an energy-consuming process, and this energy is generated from the proton motive force, and transduced to the outer membrane protein via the TonB-ExbB-ExbD protein complex (20, 30). *H. pylori* contains two genes encoding TonB orthologs, of which the *tonB2* (*hp1341*) gene is located in an operon with genes encoding ExbB (*exbB2, hp1339*) and ExbD orthologs (*exbD2, hp1340*) (1, 9, 12, 36). In transcriptome studies on NikR-responsive gene regulation in *H. pylori* it was reported that transcription of *frpB4* and *fecA3* was altered in a *nikR* mutant (9), but these array-results were

not independently confirmed and neither was the molecular mechanism further investigated, and thus it remained possible that regulation of *fecA3* and *frpB4* was indirect via a regulatory cascade (7, 37, 38). In the present study, it is demonstrated that expression of the FrpB4 and FecA3 proteins is nickel-repressed, and that this regulation is mediated at the transcriptional level via binding of NikR to the *frpB4* and *fecA*3 promoter regions.

MATERIALS AND METHODS

Bacterial strains, plasmids, media and growth conditions

H. pylori strains 26695 (36) and its isogenic *nikR* (40) and *fur* (41) mutants were routinely cultured on Dent agar (39) at 37°C under microaerophilic conditions (10% CO_2, 5% O_2 and 85% N_2). Broth cultures were grown in Brucella broth (Difco, Sparks, MD) supplemented with 0.2% ß-cyclodextrin (Fluka) (BBC) and shaken at 37°C with 40 rpm for a maximum of 24 h. $NiCl_2$ (Sigma) was used to supplement BBC medium to achieve various nickel concentrations. Iron-restriction was achieved by supplementing Brucella broth with desferal (deferoxamine mesylate, Sigma) to a final concentration of 20 μM (41) before adding ß-cyclodextrins. Iron-replete conditions were achieved by supplementing desferal-treated BBC with ferric chloride (Sigma) to a final concentration of 100 μM (41). *E. coli* strains were grown aerobically at 37°C in Luria-Bertani medium (33). When needed, growth media were supplemented with ampicillin, kanamycin, or chloramphenicol to final concentrations of 100 μg/ml, 20 μg/ml and 20 μg/ml, respectively.

Membrane fractionation and protein analysis

Approximately 4×10^9 *H. pylori* cells resuspended in 10 mM Tris-HCl, pH 8.0 were sonicated and the supernatant was cleared from non-disrupted cells by centrifugation. The membranes present in the supernatant were subsequently pelleted in an ultracentrifuge (Beckman Optima L-080, rotor type 42.2 Ti, 155000 × g), and subsequently the pellet was resuspended in 40 μl solubilisation buffer (10 mM Tris-HCl pH 7.5, 7 mM EDTA, 0.6% sarcosyl) (42). After a second ultracentrifugation step, the pellet containing the outer membrane fraction was resuspended in 25 μl 10 mM Tris-HCl, pH 8.0 and was separated by sodium dodecyl sulfate-6% polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue. The two nickel-regulated outer membrane proteins from wild-type *H. pylori* 26695 were subsequently cut out from the SDS-PAGE gel after Coomassie staining and used for protein identification. The proteins were trypsin digested and analyzed by Matrix Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) mass spectrometry as described previously (41).

Purification and analysis of RNA

Total RNA was isolated from 4×10^9 *H. pylori* cells using Trizol (Gibco), according to the manufacturer's instructions. For Northern hybridization experiments RNA was separated on 2% formaldehyde-1.5% agarose gels in 20 mM sodium phosphate buffer (pH 7), transferred

to positively charged nylon membranes (Roche), and covalently bound to the membrane by cross-linking with 0.120 J/cm^2 of UV-light of 254 nm wavelength (39). Directly after transfer, the membranes were stained with methylene blue to confirm integrity of the RNA samples, and to confirm loading of equal amounts of RNA based on the relative intensities of the 16S and 23S rRNA. The sizes of the hybridizing RNA species were calculated from comparison with a digoxigenin-labeled marker (RNA marker I, Roche). The DIG-labeled specific RNA probes were synthesized by *in vitro* transcription using T7 RNA polymerase (Roche) and PCR products amplified using primers listed in Table 1. Detection of RNA was carried out as described previously (15, 17, 39).

Recombinant DNA techniques

Restriction enzymes and DNA-modifying enzymes were used according to the manufacturer's instructions (Promega). Plasmid DNA was prepared using the Wizard system (Promega) and PCR was carried out using Taq polymerase (Promega).

Primer extension

To map the transcriptional start site of the *H. pylori frpB4* and *fecA3* genes, primer extension was carried out as described previously (16). The digoxygenin-labeled primers frpB4-DFP-rev or fecA3-DFP-rev were annealed stepwise to 10 µg of total RNA from strain 26695, and cDNA was synthesized after addition of 5 U of AMV-reverse transcriptase (Promega) and incubation for 1 h at 42˚C. Nucleotide sequencing reactions were carried out with the f-mol® DNA Cycle Sequencing System (Promega) using primer frpB4-DFP-rev on a fragment created with primers frpB4-DFP-for and frpB4-DFP-rev, as well as using primer fecA3-DFP-rev on a fragment created with primers fecA3-DFP-for and fecA3-DFP-rev (Table 1). Sequence reactions were separated on a 7% acrylamide-8 M urea sequencing gel, and then blotted onto a nylon membrane (Roche), followed by chemiluminescent DIG-detection (16).

Table 1. Oligonucleotide primers used in this study

Primer	Sequence (5' → 3')
FrpB4-F4	AGCCGTCTCTTAAGGGTAAC
FrpB4-R-T7[a]	ctaatacgactcactatagggagaTCGCTATTGCTTGGATCTTG
FrpB4-DFP-for	TGCTTGATTCAGCCGCTCAG
FrpB4-DFP-rev[b]	TGCTAGCGACAATACAAGAG
FecA3-F3	GATTACCGCGCCTAAGAGTT
FecA3-R4-T7[a]	ctaatacgactcactatagggagaCTGCCTCCACCCTTGATCAC
FecA3-DFP-for	GCGTCAAAGAGTGTCTTGTG
FecA3-DFP-rev[b]	TCCTTAGCGAACAAAGACTC
Hp1339-F	AGCTTTGTGGTTTGCGATTG
Hp1339-R-T7[a]	ctaatacgactcactatagggagaGTGGGAATCGCCACAGCAAG
Hp1340-F	AGCATCAGAAGAGGCGATGG
Hp1340-R-T7[a]	ctaatacgactcactatagggagaCTGAGCTTGCGTGGAGATGG
Hp1341-F	AATGCTGAGTCGGCTAAACC
Hp1341-R-T7[a]	ctaatacgactcactatagggagaGTCCGTAACGCTCCCATCAG

a. Primer contains a 5'-extension with T7 promoter sequence (in lowercase letters) for the creation of an antisense RNA probe.

b. primer is digoxigenin labeled

Expression and purification of *H. pylori* NikR

The recombinant NikR protein was overexpressed as a fusion protein with an N-terminal Strep-tag and purified using streptactin columns as described previously (17). The recombinant protein (designated Strep-NikR (17)) was over 90% pure as determined by staining with Coomassie Blue following electrophoresis on 12% polyacrylamide-SDS gels (8). The strep-NikR protein preparation was stored at -80°C in small aliquots which were not refrozen after use, and used without further purification in DNase I footprinting assays.

DNase I footprinting

Primers frpB4-DFP-for and frpB4 DFP-rev (Table 1) were used to amplify a 228 bp digoxigenin-labeled fragment of the promoter region of the *frpB4* gene (P*frpB4*), and primers fecA3-DFP-for and fecA3 DFP-rev (Table 1) were used to create a 351 bp digoxigenin-labeled fragment of the promoter region of the *fecA3* gene (P*fecA3*). DNase I footprinting was performed using 721 pM and 469 pM of P*frpB4* and P*fecA3*, respectively. DNA fragments were incubated without or with 2.86 μM of Strep-NikR protein in the presence or absence of 100 μM $NiCl_2$ in binding buffer (10 mM HEPES (pH 7.6), 100 mM KCl, 3 mM $MgCl_2$ and 1.5 mM $CaCl_2$) for 30 min at 37°C. Subsequently the DNA was digested with 0.25 U DNase I (Promega) for 1 min, and the reaction was stopped as described previously (13). Fragments were separated on a 7% acrylamide-8 M urea sequencing gel (Bio-Rad). Gels were blotted onto a positively charged nylon membrane (Roche), followed by chemiluminescent DIG-detection (17).

RESULTS

NikR regulates transcription and expression of the *fecA3* (HP1400) and *frpB4* (HP1512) genes in *H. pylori*

To examine the role of NikR and nickel in the regulation of both *frpB4* and *fecA3*, RNA from *H. pylori* wild-type and *nikR* mutant strains was isolated, and hybridized to probes specific for the *frpB4* and *fecA3* genes (Fig. 1A). Transcription of both genes was nickel-repressed in the wild-type strain, since the transcript was not detected when the wild-type strain was grown in nickel-supplemented medium (Fig. 1A). In contrast, in the *nikR* mutant transcription of both genes was constitutively high and independent of $NiCl_2$ supplementation (Fig. 1A). The transcriptional pattern is identical to that of the NikR-regulated *nixA* gene (17), and this suggests that NikR may directly mediate regulation of both the *fecA3* and *frpB4* genes. To prove that FecA3 and FrpB4 are indeed nickel- and NikR-regulated outer membrane proteins, we isolated and compared the outer membrane fraction of *H. pylori* wild-type and the *nikR* mutant. The outer membrane fraction of wild-type *H. pylori* 26695 contained two nickel-repressed proteins of approximately 95 kDa, and expression of these two proteins no longer nickel-repressed in the *nikR* mutant (Fig. 1B). The two nickel-regulated proteins of wild-type *H. pylori* 26695 were subsequently positively identified as FrpB4 and FecA3 by MALDI-TOF mass spectometry (Fig. 1B).

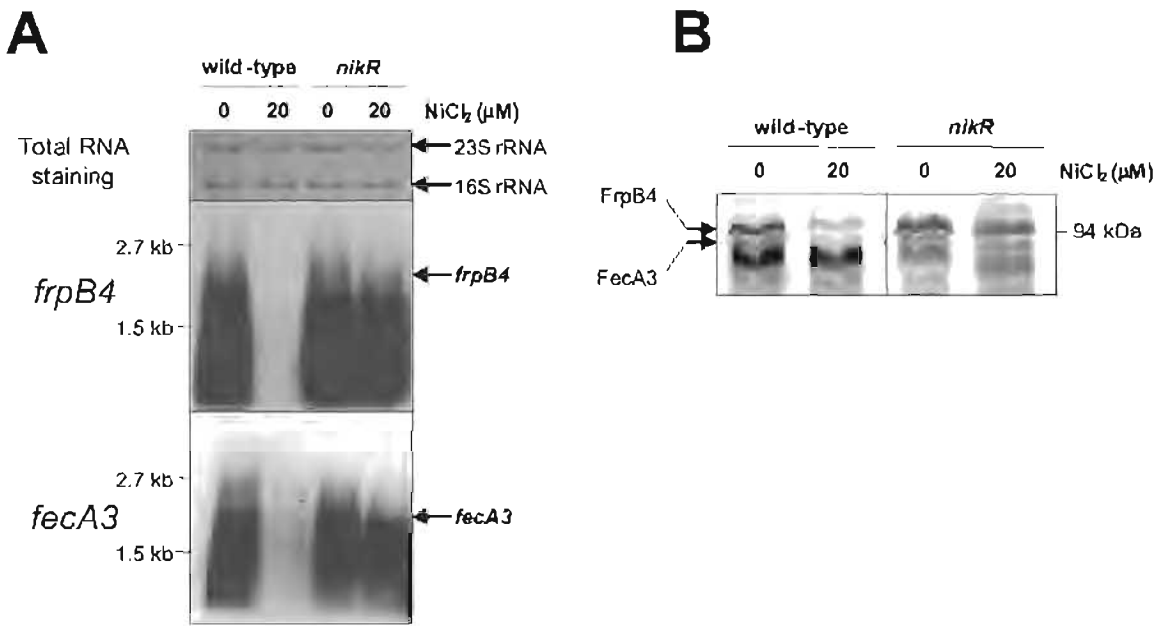

Figure 1. Transcription and expression of the *H. pylori fecA3* and *frpB4* genes is nickel- and NikR-repressed. (A) Northern hybridization of RNA from *H. pylori* 26695 wild-type strain and its isogenic *nikR* mutant, grown in BBC medium supplemented with 0 and 20 μM $NiCl_2$. The *frpB4* and *fecA3* transcripts are indicated on the right side, whereas probes used and relevant marker sizes are included on the left side. (B) Comparison of expression of the FrpB4 and FecA3 proteins using SDS-PAGE of the outer membrane protein fraction from *H. pylori* 26695 wild-type and *nikR* mutant, grown in BBC medium supplemented with 0 and 20 μM $NiCl_2$. Proteins are stained with Coomassie blue, and the FrpB4 and FecA3 proteins were identified using MALDI-TOF from *H. pylori* strain 26695 grown without nickel supplementation.

NikR mediates repression of *hp1512* (*frpB4*) transcription by nickel-dependent binding to the *hp1512* promoter region

The transcription start site (TSS) of the *frpB4* gene was identified with the help of primer extension (Fig. 2A). Transcription of *frpB4* started at the A-residue 54 bp upstream of the ATG start codon of the *frpB4* gene, and the primer extension product was only detected when *H. pylori* 26695 was grown without nickel-supplementation (Fig. 2A). The +1 position is preceded by a possible -10 promoter sequence (AATAAT) and an extended -10 region (TG at position -14) (6), whereas the -35 sequence does not resemble the *E. coli* consensus sequence (Fig. 2C). Direct nickel-dependent binding of the NikR protein to the *frpB4* promoter was demonstrated using DNase I footprinting (Fig. 2B). In the presence of nickel, recombinant Strep-NikR protein blocked DNase I degradation of a single 36 bp sequence (AAATTTAAGGTATTATTAAATAGAATAATGTAATAA). This sequence is located from -43 to -8 relative to the transcription start site (Fig. 2B, C) and overlaps with the putative -10 promoter region (Fig. 2B, C). The -43 to -8 region was not protected against DNase I degradation by Strep-NikR in the absence of nickel (Fig. 2B).

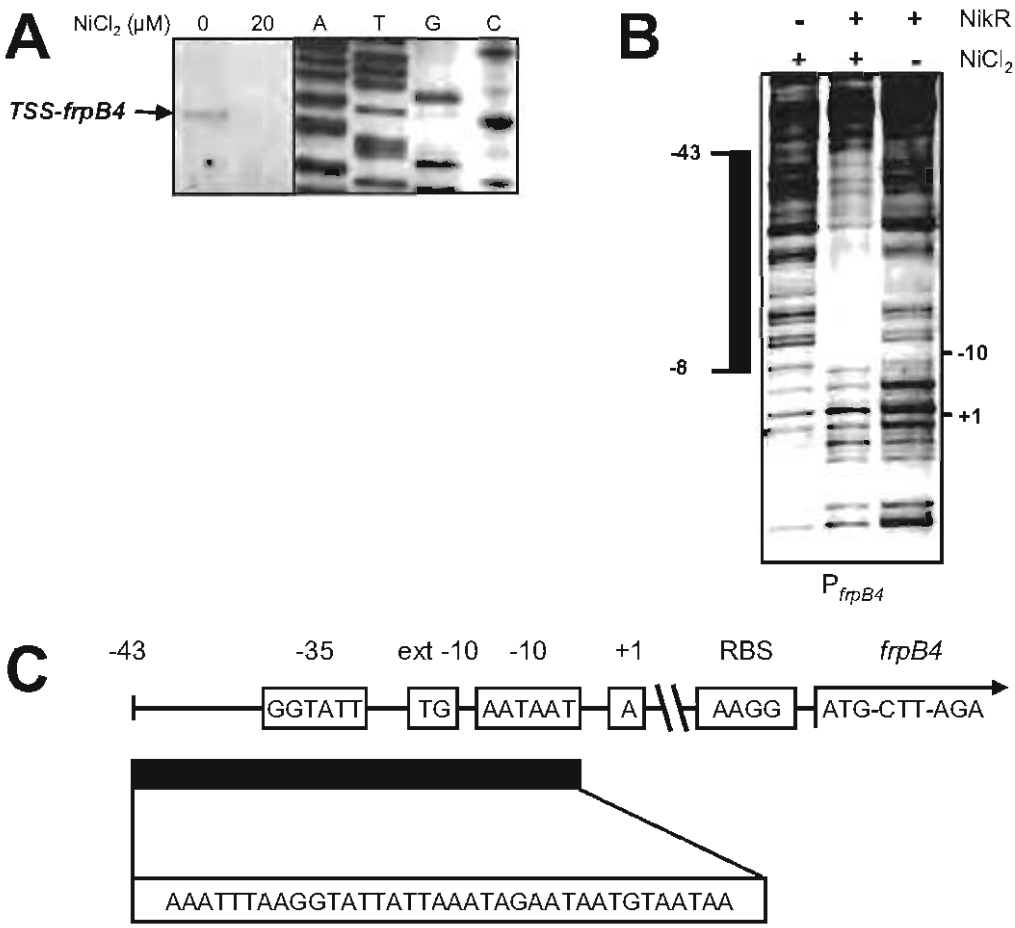

Figure 2. NikR directly represses *frpB4* transcription by nickel-dependent direct binding to a specific operator in the *H. pylori frpB4* promoter region. (A) Determination of the transcriptional start site (TSS) of *frpB4* by primer extension, with RNA from *H. pylori* 26695 wild-type cells grown in medium supplemented with 0 and 20 µM $NiCl_2$. The primer extension fragment of *frpB4* is marked with an arrow, the sequence reaction is displayed in lanes A, T, G, C. (B) Identification of the NikR operator sequence in the *frpB4* (P*frpB4*) promoter by DNase footprinting in the absence (-) and presence (+) of recombinant Strep-NikR protein in the absence (-) or presence (+) of $NiCl_2$. The protected region is indicated by a black bar on the left side of the panel. The location of the TSS and -10 residue is also indicated. (C) Graphical representation of the *frpB4* promoter region with the TSS, –10, extended -10 and -35 box, ribosomal binding site and ATG start codon of the *frpB4* gene. The location and sequence of the NikR binding site indicated with a black bar and the sequence underneath. -43 indicates the boundary of the NikR binding site.

NikR mediates repression of *hp1400* (*fecA3*) transcription by nickel-dependent binding to the *hp1400* promoter region

The transcription start site (TSS) of the *fecA3* gene was identified with the help of primer extension (Fig. 3A). Transcription of *fecA3* started at the A-residue 113 bp upstream of the GTG start codon of the *fecA3* gene (Fig. 3A), and the primer extension product was only detected when *H. pylori* 26695 was grown without nickel-supplementation (Fig. 3A). Again the +1 position is preceded by a suitable –10 promoter region (TAAAAT, Fig. 3C) and an extended -10 region (TG at position -14), but no discernable -35 sequence.

Direct nickel-dependent binding of the NikR protein to the *fecA3* promoter was demonstrated using DNase I footprinting. In the presence of nickel, Strep-NikR protein blocked DNase I

degradation of a single 38 bp sequence (ATTCCGCACATTATTAAGTTTTTTTTGT-TTTTATTACT) in the promoter of the *fecA3* gene (Fig. 3B). This sequence is located from -7 to +31 relative to the transcription start site (Fig. 3B, C), and thus overlaps with the +1 sequence. Strep-NikR did not bind to the *fecA3* promoter in the absence of nickel (Fig. 3B).

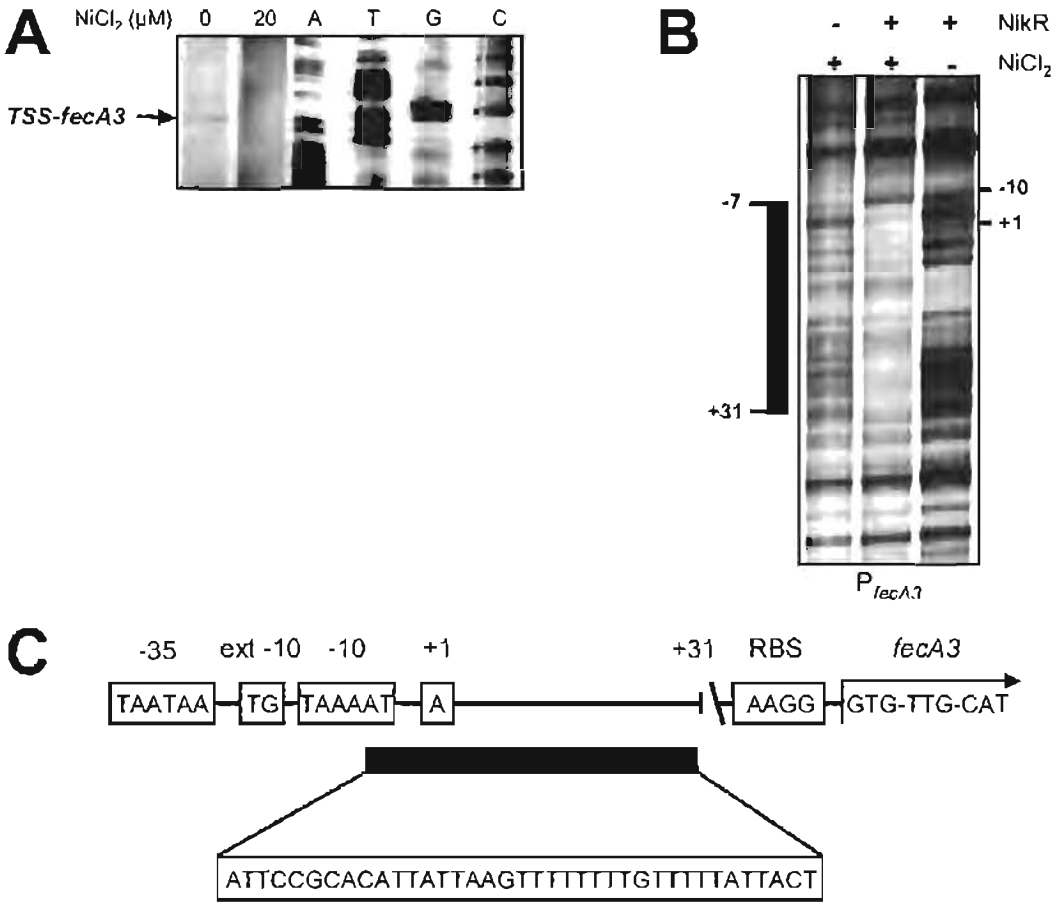

Figure 3. NikR directly represses *fecA3* transcription by nickel-dependent direct binding to a specific operator in the *H. pylori fecA3* promoter region. (A) Transcriptional start site (TSS) of *fecA3* determined by primer extension with RNA from *H. pylori* 26695 wild-type cells grown in medium supplemented with 0 and 20 µM $NiCl_2$. The primer extension fragment of *fecA3* is marked with an arrow, the sequence reaction is displayed in lanes A, T, G, C. (B) Identification of the NikR operator sequence in the *fecA3* (P*fecA3*) promoter by DNase footprinting in the absence (-) and presence (+) of recombinant Strep-NikR protein in the absence (-) or presence (+) of $NiCl_2$. The protected region is indicated by a black bar on the left side of the panel, the location of the TSS and the -10 residue is indicated on the right side. (C) Graphical representation of the *fecA3* promoter region with the TSS, -10, extended -10 and -35 box, ribosomal binding site and GTG start codon of the *fecA3* gene. The location and sequence of the NikR binding site indicated with a black bar and the sequence underneath. +31 indicates the boundary of the NikR binding site.

The *exbB2-exbD2-tonB2* operon is nickel- and NikR-, but not iron- and Fur-dependent regulated

Based on their homology with TonB-dependent receptor proteins of other bacteria, and the presence of a plug and a TonB-dependent receptor domain as revealed by a search in the Pfam database, we predicted both FrpB4 and FecA3 proteins to be TonB-dependent transporter proteins (3). Therefore the link between NikR and transcription of the *H. pylori exbB2-exbD2-*

tonB2 operon (*hp1339-hp1340-hp1341*) was also investigated. The role of nickel and iron on transcription of the *tonB2* gene as well as the linked *exbB2* and *exbD2* genes was analyzed at the transcriptional level in *H. pylori* 26695 and its isogenic *nikR* and *fur* mutants. Transcription of the *exbB2-exbD2-tonB2* gene cluster displayed NikR-dependent nickel-responsive repression (Fig. 4A) (9, 12), similar to the *frpB4* and *fecA3* genes (Fig. 1A). In contrast, all three genes were constitutively transcribed in the wild-type strain and the *fur* mutant, independent of iron availability (Fig. 4B).

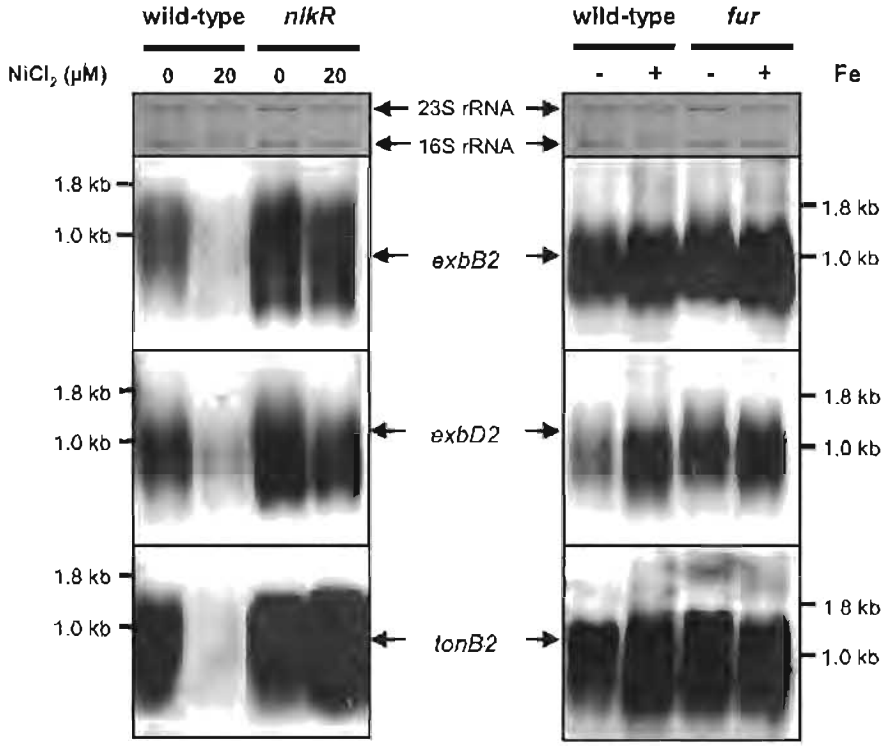

Figure 4. Transcription of the *tonB2* operon is nickel-and NikR-dependent repressed, but not iron- and Fur regulated. Northern hybridization of RNA from *H. pylori* 26695 wild-type and *nikR* mutant cells grown in medium supplemented with 0 and 20 μM $NiCl_2$ (left panel) and with RNA from *H. pylori* 26695 wild-type and *fur* mutant cells grown in medium in the absence or presence of iron (right panel). Staining of transferred RNA by methylene blue is included for comparison of RNA amounts. The *exbB2*, *exbD2* and *tonB2* transcripts are indicated between the left and right panels.

DISCUSSION

One of the adaptations of *H. pylori* to its gastric lifestyle has been the high-level expression of the nickel-dependent urease enzyme, which allows *H. pylori* to survive the acidic pH in the gastric environment, both during initial infection and chronic colonization (32). This has resulted in making scavenging and acquisition of sufficient levels of nickel a very important activity for *H. pylori*, and it can therefore be predicted that *H. pylori* has multiple mechanisms for the transport of nickel. However, uncontrolled acquisition of transition metals like nickel may lead to toxicity, as they may participate in the generation of toxic oxygen radicals or block incorporation of cofactors into enzymes (24). Therefore acquisition, utilization and storage of transition metals needs to be carefully monitored and actively controlled, and this

function is usually mediated by metal-responsive regulatory proteins. Three such proteins have been identified in *H. pylori*: the iron-responsive regulatory protein Fur (13, 41), the copper-responsive two-component regulatory system CrdRS (43), and the nickel-responsive regulator NikR (12, 17, 40).

The NikR protein belongs to the Ribbon-Helix-Helix family of transcriptional regulators, which bind to the DNA as tetramers (8, 12). It was recently demonstrated that *H. pylori* NikR can function both as a nickel-dependent repressor and activator of gene transcription by binding to the promoter region of its target genes (12, 17). Activation of transcription occurs when NikR binds upstream of the *ureA* promoter at position -50 to -90 (12, 17), whereas repression occurs when NikR binds to the promoter region overlapping the -10 and +1 region of the *nixA* (17), *fur* and *exbB2* (12) promoters. Binding to this region is believed to prevent transcription due to competition of the regulator with RNA polymerase (8, 11). The nickel- and NikR-dependent regulation of *frpB4* and *fecA3* is similar to that of the *nixA* and *exbB2-exbD2-tonB2* genes, since NikR binds at position -43 to -8 in the promoter region of the *frpB4* gene (Fig. 2) and at position -7 to +31 in the promoter region of the *fecA3* gene (Fig. 3). Therefore, in both genes either the -10 or the +1 site is protected by NikR, and thus blocked from binding of RNA polymerase (17). The NikR-protected operators in the *frpB4* and *fecA3* promoters sequences resembling the *H. pylori* NikR consensus sequence (TATWATT-N_{11}-AATWATA) (Fig. 5) (12).

Consensus				TATWATT	----N11----	AATWATA		
fecA3	(HP1400)	-7	ATTCCGCA	CATTATT	AAGTTTTTTTT	GTTTTTA	TTACT	+31
frpB4	(HP1512)	-43	AAATTTAAGG	TATTATT	AAATAGAATAA	TGTAATA	A	-8
exbB2	(HP1339)	-37	ATTGACTTGT	TATTATT	AAAACAATATA	ATCAACA	AAC	+1
nixA	(HP1077)	-13	AAATATAT	TACAATT	ACCAAAAAAGT	ATTATTT	TTTC	+21
nikR	(HP1338)	-27	ATCCAGTTTG	TATTATA	ATTGTTCATTT	TAAATTA	AT	+10
fur	(HP1027)	-24	TC	TATGTTT	CATCGCATTAT	TATTGTA	TAATAATATTC	+1
ureA	(HP0073)	-91	CAAAGATA	TAACACT	AATTTATTTTA	AATAATA	AT	-56

Figure 5. Alignment of the putative NikR operator sites, based on the consensus sequence proposed in (12). The two parts of the palindrome are boxed, black background indicates conserved bases compared to the consensus sequence. The numbers surrounding the sequences indicate the position of the NikR binding site with respect to the +1 transcriptional start site. The W in the consensus sequence represents an A or T residue.

In the annotation of the *H. pylori* genome sequences, the three *fecA* genes are annotated as ferric iron dicitrate transporters, whereas the *frpB* genes are annotated as predicted iron-regulated outer membrane proteins (1, 5, 36). However, only two copies of each displayed the typical Fur-mediated iron-responsive regulatory pattern usually associated with iron-acquisition systems (13, 15, 41). Since uncontrolled uptake of iron by the FrpB4 (HP1512) and FecA3 (HP1400) proteins would probably lead to iron toxicity, it could therefore be envisaged that these proteins may function in the transport of other compounds, as has been shown for TonB-dependent outer membrane proteins in other bacteria (20, 35). Indeed, in the present work it is demonstrated that both FecA3 and FrpB4 are nickel- and NikR-regulated outer membrane proteins with the help of fractionation (Fig. 1B).

The *H. pylori fecA3* gene shares homology with metal-citrate uptake genes, and in *Bacillus subtilis* it was demonstrated that the metal-citrate transporter CitM imports Ni^{2+}-citrate, but also Ni^{2+}-isocitrate complexes (21, 44). Consistent with earlier reports (9, 12), it is also demonstrated in the present study that the *exbB2*, *exbD2* and *tonB2* genes are transcribed as a nickel- and NikR-dependent regulated operon (Fig. 4A), but not by Fur and iron (Fig. 4B). This complements the description of NikR binding to the *exbB2* promoter (12), thus demonstrating that the *exbB2-exbD2-tonB2* operon is nickel- and NikR-regulated. The *tonB2* operon therefore may to be specific for the nickel-and NikR-dependent regulated genes. A similar system with regard to receptor specificity of multiple TonB orthologs has been described for *Vibrio cholerae* (25, 34). So far known, the TonB complexes are mostly of importance for the transport of iron complexes over the outer membrane into the periplasm, which is an energy-dependent process (2, 30). As the outer membrane does not have a proton motive force, due to its permeability through pores, the proton motive force of the inner membrane is used to power many outer membrane transporters (30). It is thought that TonB is responding to the proton motive force by binding a proton and thereby changes conformation in an energized form. Energized TonB subsequently transduces the energy to the outer membrane transporter by binding to a TonB box (30).

In view of the concerted regulation observed for FrpB4, FecA3 and the ExbB-ExbD-TonB system, we currently favour the model where FecA3 and FrpB4 function in nickel acquisition, as is outlined in in Figure 6. This model is hypothetical, and partially based on assumptions for which we currently lack direct experimental support, but it is consistent with the data from an independent, concurrent study of Davis and co-workers (10). They have independently identified the nickel- and NikR-responsive regulation of the *frpB4* (HP1512) gene, and showed that mutation of the *hp1512* gene in *H. pylori* strain 26695 resulted in a significant decrease in urease activity, and an increase in transcription of other nickel-responsive genes. Taken together, this suggests a decrease in cytoplasmic nickel availability in the *hp1512* mutant when compared to the wild-type strain (10). Overall, this supports our working model where FecA3 and FrpB4 are involved in transport of nickel compounds over the outer membrane (Fig. 6), but additional data are required to further establish the link between TonB-energized outer membrane transport of nickel compounds.

Transcription of *frpB1*, *frpB2*, *fecA1* and *fecA2* was previously demonstrated to be iron- and Fur-dependent regulated, whereas *frpB4* and *fecA3* were not regulated by Fur, and suggested to be constitutively transcribed (41). In a subsequent transcriptome analysis study, it was suggested that both *frpB4* and *fecA3* were NikR-regulated (9), but these findings were not further investigated, and thus it remained possible that the observed role of NikR was indirect, via regulation of the *fur* gene (7, 37, 38). In this study we have elucidated the molecular mechanism of NikR-mediated regulation of the *fecA3* and *frpB4* genes, and have shown that NikR binds to regions overlapping either the +1 or -10 regions of the respective promoters (Figs. 2, 3). In addition, we demonstrate on the level of transcription and protein expression that NikR-regulation results in nickel-responsive transcription and nickel-responsive expression (Fig. 1A,B), as was predicted but not investigated. Taken together, this not only confirms, but significantly extends the previous report (9).

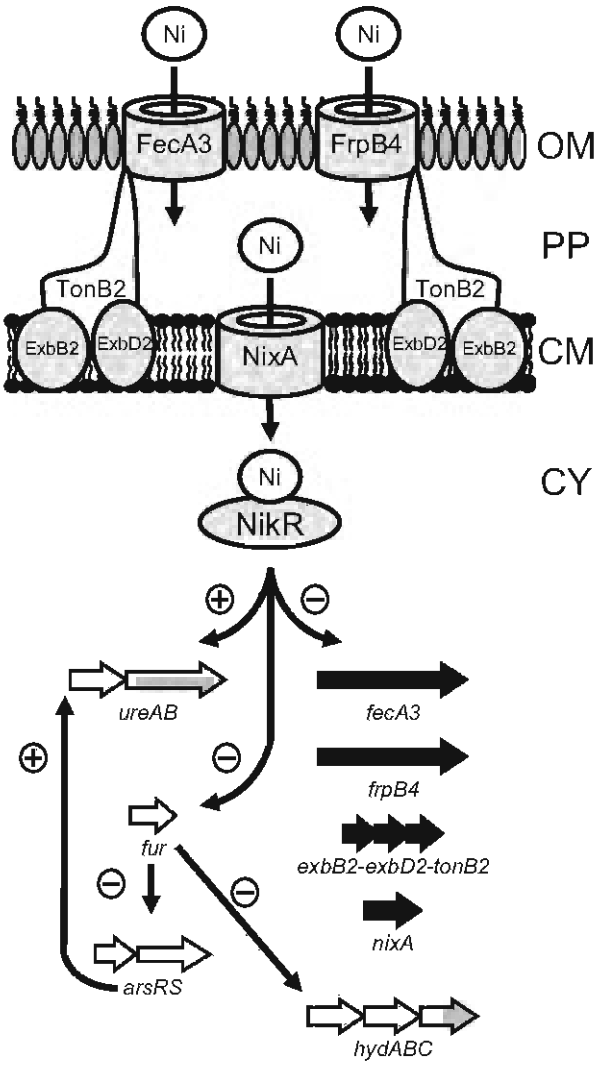

Figure 6. Graphical representation of our hypothetical model illustrating the potential links between NikR and the different NikR-regulated genes in nickel-uptake and nickel utilization in *H. pylori*. OM = outer membrane, CM = cytoplasmic membrane, PP = periplasm, CY = cytoplasm. Grey arrows represent genes involved in nickel utilization, black arrows represent genes involved in nickel transport, and white arrows represent genes involved in regulation of transcription.

In conclusion, *H. pylori* NikR regulates transcription of the genes encoding the FecA3 and FrpB4 outer membrane proteins. So far, all genes for which NikR-regulation has been confirmed at the molecular level, have been demonstrated or have been implicated to play a role in nickel metabolism (summarized in Fig. 6). The urease system uses the majority of transported nickel, whereas the NixA protein is the major cytoplasmic membrane transporter for nickel. Furthermore, the FecA3 and FrpB4 proteins may transport complexed nickel compounds over the outer membrane, a process energized by the TonB2-ExbB2-ExbD2 complex. Finally, the nickel- and NikR-regulated *fur* gene (7, 12, 38) controls expression of the hydrogenase system, and is involved in regulation of the HP0166-0165 regulatory system, which participates in regulation of urease and the Hpn nickel-storage proteins (18, 28, 29). This allows NikR to control nickel metabolism both directly and indirectly, and displays the extended reportoire of the NikR regulator when compared to its *E. coli* counterpart, where its

main function is regulation of nickel uptake in anaerobic conditions (8, 31). This adaptation may have an important function in long-term colonization by *H. pylori* of hostile environmental niches like the human stomach.

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Chapter 7

An ABC transporter and a TonB ortholog contribute to *Helicobacter mustelae* nickel and cobalt acquisition

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ABSTRACT

The genomes of *Helicobacter* species colonizing the mammalian gastric mucosa (like *Helicobacter pylori*) contain a large number of genes annotated as iron acquisition genes, but only few nickel acquisition genes, which contrasts with the central position of nickel in urease-mediated acid resistance of these gastric pathogens. In this study we have investigated the predicted iron and nickel acquisition systems of the ferret pathogen *Helicobacter mustelae*. Expression of the outer membrane protein-encoding *frpB2* gene was iron- and Fur-repressed, whereas expression of the ABC transporter genes *fecD* and *ceuE* was iron- and Fur-independent. Inactivation of the two *tonB* genes showed that TonB1 is required for heme utilization, whereas absence of TonB2 only marginally affected iron-dependent growth but led to reduced cellular nickel content and urease activity. Inactivation of the *fecD* and *ceuE* ABC-transporter genes did not affect iron levels, but resulted in a significant reduced urease activity and cellular nickel content. Surprisingly, inactivation of the *nixA* nickel transporter gene only affected cellular nickel content and urease activity when combined with inactivation of other nickel acquisition genes, like *fecD* or *ceuE*. The FecDE ABC transporter is not specific for nickel, since a *fecD* mutant also showed reduced cellular cobalt levels and increased cobalt resistance. We conclude that the *H. mustelae fecDE* and *ceuE* genes encode an ABC transporter involved in nickel and cobalt acquisition, which works independently of the nickel transporter NixA, while TonB2 is primarily required for nickel acquisition, with TonB1 being required for heme utilization.

INTRODUCTION

The genus *Helicobacter* comprises bacterial pathogens that colonize the alimentary tract of mammals, with the best known example being the human gastric pathogen *Helicobacter pylori* (17). Other examples include *Helicobacter* species colonizing the gastric mucosa of big cats (*Helicobacter acinonychis*), cats and dogs (*Helicobacter felis*) and ferrets (*Helicobacter mustelae*) (20, 31). Gastric colonization by *Helicobacter* species can illicit a strong immune response, which may develop into pathologies like peptic ulcer disease and precancerous lesions (17). The lifelong colonization of the gastric mucosa suggests that these *Helicobacter* species are well adapted to this harsh environment, and are able to combat the diverse antimicrobial activities employed by the host within the gastric mucosa, such as iron-restriction and acidity (43).

Transition metals like iron and nickel are both essential for gastric *Helicobacter* species. Iron is involved in redox reactions and functions as a cofactor of many enzymes, whereas nickel is the cofactor of two important enzymes in gastric *Helicobacter* species: urease and hydrogenase. The urease enzyme is the main factor allowing acid resistance, whereas hydrogenase is important for energy production, and both enzymes are essential for gastric colonization by *Helicobacter* species (2, 22, 32, 37). Analysis of complete genome sequences of gastric *Helicobacter* species allowed prediction of many genes putatively involved in iron acquisition, but surprisingly few predicted nickel acquisition genes (12, 22, 35).

Ferric iron acquisition in Gram-negative bacteria is usually mediated by a TonB-dependent outer membrane receptor, coupled to an ABC transporter for transport in the periplasm and over the inner membrane. Ferrous iron acquisition only requires a FeoB-like single component system for inner membrane transport (1, 41). As for ferrous iron, transport of nickel was until recently thought not to require an outer membrane component but only an ABC transporter or single component NiCoT transporter (18, 19). However, it has become apparent that there is much more variation in these themes, and that TonB-dependent outer membrane proteins, annotated as putative iron acquisition systems, may be involved in transport of range of other metals or compounds (10, 14, 28, 29, 34). Examples of these new insights stem from recent work in *H. pylori*, where two out of three FecA and FrpB orthologs were shown to be regulated by iron and Fur, whereas the third FecA and FrpB orthologs are NikR and nickel-repressed (9, 14, 39) and have been proposed to function in nickel acquisition (10, 28). Similarly, the *H. mustelae* putative TonB-dependent outer membrane protein NikH contributes to urease activity, probably by mediating nickel acquisition (34). Caution also needs to be taken when annotating ABC transporters, as the proposed ferric citrate permease and ATPase FecD and FecE, respectively, were shown not to contribute to iron acquisition in *H. pylori* (41), suggesting a role for these genes in *H. pylori* acquisition of other metals or compounds. Hence the currently available genome annotations are potentially unreliable, and functional and mutational data are required to validate or correct the annotation of genome sequences.

Here we present a study of some of the putative iron and nickel acquisition systems identified in the *H. mustelae* genome sequence (21) (listed in Table S1). We have determined their contribution to iron and nickel acquisition by using regulatory studies, growth promotion

assays, urease activity and intracellular metal content, and demonstrate that the two TonB systems play differential roles in iron and nickel acquisition. We also show that the CeuE and the FecDE ABC transporter components are likely to be misannotated, as they function in nickel and cobalt acquisition.

MATERIALS AND METHODS

Bacterial strains and growth conditions

H. mustelae strain NCTC 12198 (ATCC 43772) and its isogenic mutants (Tables S1 and S2) were routinely grown at 37°C in a microaerobic atmosphere of 5% O_2, 7.5% CO_2, 7.5% H_2 and 80% N_2, on Dent agar plates consisting of BBL Columbia agar (Becton Dickinson) supplemented with 7% saponin-lysed defibrinated horse blood (BioTrading), 0.004% triphenyltetrazolium chloride (Sigma) and Dent Selective Supplement (Oxoid). Broth cultures of *H. mustelae* for urease assays, Northern hybridization and metal content determination were grown in brucella broth (Becton Dickinson) supplemented with 3% newborn calf serum (Biotrading) and Dent supplement. Iron-restriction of brucella broth was achieved by addition of Desferal (deferoxamine B, Sigma) to a final concentration of 20 µM, whereas iron-replete medium was obtained by supplementing Desferal-treated medium with $FeCl_3$ to a final concentration of 100 µM. The effect of metals on *H. mustelae* growth was assessed using $CoCl_2$ and $NiCl_2$ supplementation of defined Ham's F-12 tissue culture medium (Kaighn's modification, Invitrogen) with 0.2% ß-cyclodextrin (Fluka) and Dent supplement. Broth cultures were shaken at 70 rpm and incubated at 37°C for a maximum of 24 h. *E. coli* strains DH5α and M147 were cultured in Luria-Bertani media (Biotrading) (Sambrook *et al.*, 1989). When appropriate, growth media was supplemented with chloramphenicol (20 µg ml^{-1}), kanamycin (20 µg ml^{-1}) or erythromycin (250 µg ml^{-1}) for selection of *E. coli* transformants. *H. mustelae* mutants were selected by growth on media containing chloramphenicol (10 µg ml^{-1}), kanamycin (10 µg ml^{-1}) or erythromycin (10 µg ml^{-1}). Concentrations of antibiotics given represent their final concentration in the growth medium.

Construction of isogenic *H. mustelae* mutants

The *H. mustelae nikR* and *fur* mutants were described previously (33, 34). For insertional mutagenesis of the other genes studied here (Tables S1 and S2), a ~1 kb fragment of the gene of interest was PCR amplified from DNA from *H. mustelae* strain NCTC12198 using Knock Out (KO) primers (Table S2). The amplicons were cloned in pGEM-T_{easy} vector (Promega), and the cloned sequences were interrupted by insertion of an antibiotic resistance cassette obtained from plasmids pJMK30 (kanamycin), pAV35 (chloroamphenicol) and pDH20 (erythromycin) (16, 40). The antibiotic resistance cassettes were inserted in unique restriction sites in the cloned fragments: *Bgl*II for *tonB1*, *tonB2*, *nixA* and *ceuE*; *Bcl*I for *frpB1*; *Eco*RV for *frpB2* and *fecA*; *Kpn*I for *cfrA*; *Hin*dIII for *feoB*; and *Eco*47III for *fecD*. The constructs were then introduced into *H. mustelae* strain NCTC 12198 and selected mutant strains by natural transformation (8) and correct replacement by homologous recombination of the gene

was confirmed by PCR using primers (Table S2) located Outside of the Recombination Side (ORS).

Protein analysis

Cells obtained from overnight cultures were centrifuged for 10 min at 4000 × g and resuspended in phosphate-buffered saline to a final OD_{600} of 10. Bacteria were lysed by sonication for 15 sec with an MSE Soniprep 150 set at amplitude 6. Whole-cell proteins were separated on a 6% or 15% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) (27). Assessment of UreB and UreB2 urease subunit expression was based on immunoblots incubated with a 1:10,000 dilution of antibodies raised against H. felis urease (3, 25, 33).

Purification and analysis of RNA

Total RNA was isolated using Trizol (Gibco) according to the manufacturer's instructions. RNA was separated on 2% formaldehyde–1.5% agarose gels in 20 mM sodium phosphate buffer (pH 7), transferred to positively charged nylon membranes (Roche Diagnostics), and covalently bound to the membrane by UV cross-linking (33, 34). RNA was visualized by methylene blue staining, and RNA concentrations were normalized based on 16S and 23S rRNA band intensities. Probes were amplified with primers listed in Table S3. The PCR fragments contained a T7 promoter sequence and were used for the production of an antisense RNA probe labelled with digoxigenin by transcription with T7 RNA polymerase (Roche Diagnostics). Northern hybridization and stringency washes were performed at 68°C, and bound probe was visualized with the DIG-Detection Kit (Roche Diagnostics) and chemiluminescent substrate CPD-Star (Amersham Pharmacia) as described previously (33, 34).

Growth promotion assay

Medium was prepared by mixing 7 gram brucella broth (Becton Dickinson) and 3.75 gram Bacto Agar (Becton Dickinson) in 250 ml water, followed by sterilization by autoclaving. Medium was cooled down to 56° C, and 7.5 ml heat inactivated newborn calf serum (Biotrading), 250 µl 20 mM Desferal (sigma) and 250 µl Dent Selective Supplement (Oxoid) were added, together with triphenyl tetrazolium chloride (Sigma) to a final concentration of 0.004%. Medium was distributed over 9 cm Petri dishes, at 17.5 ml per dish. *H. mustelae* strain NCTC12198 and isogenic mutants (Tables S1 and S2) were grown for 24 hours at 37°C on Dent agar, harvested by centrifugation and diluted in PBS to an OD_{600} of 0.2. This suspension was spread equally across the agar plate using a cotton swab. Blanc paper discs (Becton Dickinson) were saturated with 30 µl of either 1% heme (Sigma) solution in 0.1N NaOH, or 0.5 mM $FeCl_3$. PBS was used as negative control. The discs were placed on the agar plate and incubated for 48 hours at 37°C in a microaerobic atmosphere. The diameter of the zone of growth was measured, and each experiment was performed at least three times independently.

Urease assay

The enzymatic activity of the *H. mustelae* UreAB urease was determined by measuring ammonia production from hydrolysis of urea, by using the Berthelot reaction as described previously (33, 34). Cells were lysed by sonication and incubated for 30 min at 37°C in buffer consisting of 100 mM sodium phosphate pH 7.5, 10 mM EDTA and 50 mM urea, and the ammonia produced was measured after addition of phenol nitroprusside and alkaline hypochlorite (Sigma Diagnostics) by measuring the OD at 570 nm. Protein concentrations were determined with the bicinchoninic acid method (Pierce) using bovine serum albumin as standard. Urease enzyme activity was expressed as units representing mmol of urea hydrolysed per min, and is expressed as U mg^{-1} of total protein. The average is shown of three independent experiments. Only activity of the *H. mustelae* UreAB urease was measured, since sonication inactivates the UreA2B2 urease of *H. mustelae* (33).

Determination of cellular metal content

H. mustelae strains were grown as broth cultures for 24 hours, and cells were harvested by centrifugation. The cell pellet was washed three times with 1 ml PBSE solution, consisting of PBS supplemented with EDTA to a final concentration of 1 mM. Cell density was assessed by OD_{600}, and cells were subsequently lysed using 500 µl HNO_3 (65% suprapur, Merck) and 250 µl of a 30% H_2O_2 solution (suprapur, Merck). Metal concentrations were determined by High Resolution Induction Coupled Plasma Mass Spectrometry (HR-ICPMS) (23, 36). Levels of iron, cobalt and nickel in the wild-type strain were 4.31×10^6 ($\pm$ 1.21×10^6), 2.08×10^3 ($\pm$ 3.19×10^3) and 5.94×10^6 ($\pm$ 0.98×10^6) atoms/cell, respectively, and values for the respective mutants are expressed as a percentage of the levels found in the wild-type strain. The number of cells was calculated using a conversion formula of $OD_{600} = 1$ equals 5×10^8 cells/ml. Results shown are the average of three independent experiments.

The HR-ICPMS equipment used for the quantification was an Element XR (Thermo Scientific) equipped with a PC^3 Peltier Cooled inlet system (Elemental Scientific, ESI) and Nickel interface cones. The following operating conditions for the HR-ICPMS were used: Radio Frequency (RF) power 12.5 kW; gas flow rates, 16 l min^{-1}, 0.7 l min^{-1} (auxiliary) and 1.1 l min^{-1} (nebulizer). The PC3 consists of a Peltier Cooled inlet system which incorporate the ESI cyclonic spray chamber, the ESI PFA-ST nebulizer and the ESI Fast introduction system. On the Fast introduction system a 0.25 ml loop was used and the PFA-ST nebulizer was fed by an ESI syringe system at a constant flow rate of 112 µl/min. The analytical masses (m/z) used for the analysis were: Fe 56, Co, 59 and Ni 60. The medium resolution (MR, greater than 4000 m/Δm) was used because these elements were heavily interfered by polyatomic ion species produced by a combination of isotopes coming from plasma, matrix and reagents. Samples were diluted ten times before analysis and only ultra pure acids and Ultra High Quality (UHQ) water (18.2 MΩ) were used. The calibration range was adapted to the element concentrations in the unknown samples. In order to correct for eventual instrumental drifts and matrix effects Ge 73 was used as an internal standard. In order to check the quality of the analysis, the certified reference samples SPS-SW1 and SPS-SW2 and TMDA-52.3 were analyzed inbetween the samples and were within their certified values.

RESULTS

The *fecD*, *ceuE* and *tonB2* genes are unlikely to encode iron acquisition proteins

We first assessed whether transcription of the proposed ferric citrate and enterochelin-uptake genes *fecD* and *ceuE* is regulated by Fur and/or iron, as is commonly observed for iron acquisition systems (1). RNA was isolated from *H. mustelae* wild-type and *fur* mutant strains, cultured in iron-restricted and iron-replete conditions. As in *H. pylori* (13, 15, 39), transcription of the *ceuE* and *fecD* genes was not affected by iron or the absence of Fur (Fig. 1A), suggesting a role in acquisition of metals other than iron. As a control we included the *frpB2* gene, which displayed the characteristic iron-repressed transcription in the wild-type strain, but derepressed and iron-independent transcription in the *fur* mutant (Fig. 1A).

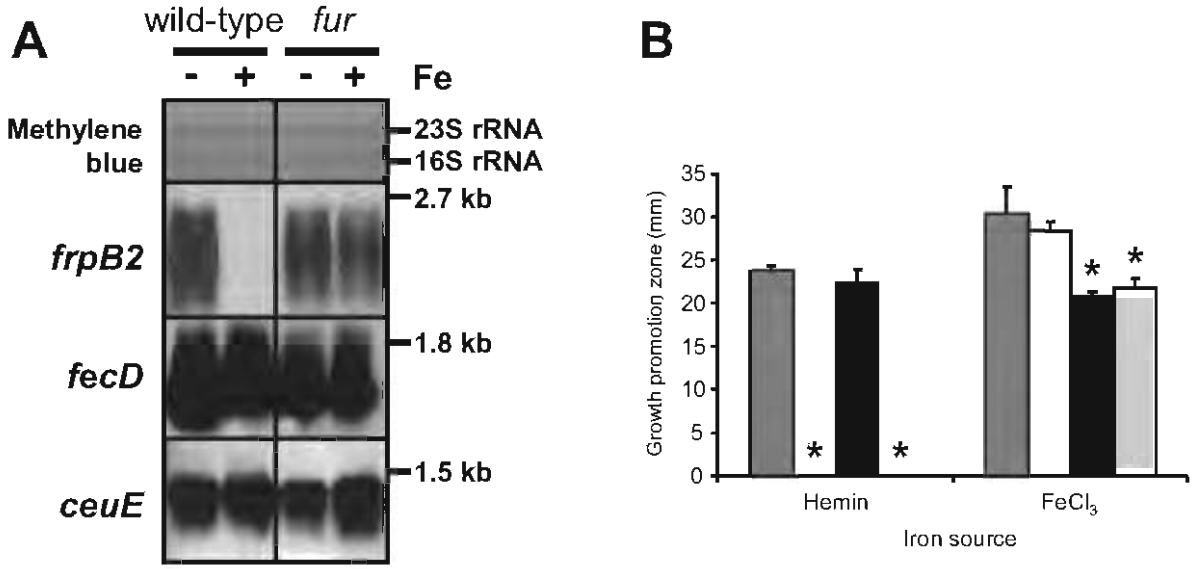

Figure 1. The *tonB2*, *fecD* and *ceuE* genes are likely to lack a role in iron acquisition. (A) Fur controls iron-responsive expression of the putative iron acquisition gene *frpB2*, but does not control transcription of the *ceuE* and *fecD* genes. Northern hybridization using RNA isolated from *H. mustelae* wild-type NCTC 12198 and its isogenic *fur* mutant, grown in iron-restricted (-) and iron-replete (+) conditions. Genes detected are listed on the left, relevant marker sizes on the right. A methylene blue staining of 16S and 23S rRNA is provided to demonstrate that similar amounts of RNA were loaded. (B) The *H. mustelae tonB1* gene is involved in utilization of heme as sole iron source, whereas the *tonB2* gene does not have a clearly defined role in iron acquisition. The graph represents growth promotion zones measured on iron-restricted brucella media supplemented with a sole iron-source on a filter disc. Dark grey bars represent wild-type *H. mustelae* NCTC 12198, whereas white, black and light grey bars represent the isogenic *tonB1*, *tonB2* and *tonB1 tonB2* double mutants, respectively. Results shown are the average of three independent growth experiments. Error bars represent standard deviation, an asterisk represents a significant difference in growth promotion zone ($p \leq 0.05$, Mann-Whitney U test) when compared to the wild-type strain.

We also investigated whether the two *H. mustelae* TonB orthologs are involved in iron acquisition by growth promotion assays with heme and ferric chloride as sole iron source. Iron was chelated from serum-supplemented brucella medium using desferal (39), resulting in absence of growth without an external iron source, whereas addition of ferric chloride restored growth of wild-type *H. mustelae* (Fig. 1B). *H. mustelae* was capable to use heme as sole iron source, however an inhibition zone around the heme-supplemented disc indicated heme toxicity at higher concentrations, as reported recently (30). Inactivation of the *tonB1*

gene prevented the utilization of heme as sole iron source (Fig. 1B), but did not affect growth using iron chloride. In contrast, inactivation of the *tonB2* did not affect utilization of heme as single iron source (Fig. 1B). Absence of both TonB orthologs gave the same results as with the *tonB1* mutant for heme, and the *tonB2* mutant for iron chloride (Fig. 1B). Inactivation of any of the seven putative TonB dependent outer membrane transporter genes (Table S1) did not affect the ability to use in heme or iron chloride as sole iron source (data not shown). Overall this suggests that the *tonB2*, *fecD* and *ceuE* genes are not involved in iron acquisition, but may function in acquisition of other metals or compounds.

Inactivation of the *nikH*, *tonB2*, *fecDE* and *ceuE* genes results in reduced urease activity and a reduced cellular nickel content

We subsequently investigated the possible role of the two *tonB* genes and the *fecDE* and *ceuE* genes in nickel acquisition, similar to what was shown previously for the *H. pylori* TonB2 system (28) and the *H. mustelae nikH* gene (34). Urease enzyme activity was initially used as reporter system for the intracellular nickel concentration (34, 45). As positive control we included the *H. mustelae nikH* gene, since inactivation of *nikH* results in ~50% reduction of urease activity in unsupplemented brucella medium (34). Inactivation of *tonB2*, *fecD* and *ceuE* resulted in a ~50% reduction of urease activity when compared to the wild-type strain, whereas inactivation of *tonB1* and *feoB* did not affect urease activity (Fig. 2A). Surprisingly, inactivation of the *nixA* gene, encoding the *H. mustelae* ortholog of the *H. pylori* inner membrane transporter for nickel (19, 46), did not result in a reduction of urease activity, suggesting the presence of an alternative pathway for nickel transport across the inner membrane. Inactivation of the *fecD* gene in combination with *nixA*, *nikH* or *tonB2* or inactivation of the *ceuE* gene in combination with *nixA*, *nikH* resulted in a virtual absence of urease activity (Fig. 2A). In contrast, inactivation of either *tonB2* or *nikH* in combination with a *nixA* mutation did not show a further reduction of urease activity. This suggests that NikH and NixA are involved in the same nickel acquisition pathway. Finally, nickel supplementation of brucella broth with nickel to a final concentration of 100 μM fully restored urease activity in the *fecD* mutant, similar to what was described for an *H. mustelae nikH* mutant (34), but only partially restored urease activity in the *nixA fecD* double mutant (Fig. S1).

High resolution-induction coupled mass spectrometry (HR-ICPMS) (23, 36) was used to determine the cellular levels of nickel and cobalt in the wild-type strain and eight of the *H. mustelae* mutants previously tested for urease activity (Fig. 2B). There was a very good match between cellular nickel content and urease activity, since reduced urease activity in the *nikH*, *tonB2*, *fecD*, *ceuE* and *fecD*/*nixA* mutants was reflected in a similar decrease in nickel content (Fig. 2B). Iron levels were not significantly different between tested strains, ranging from 95%-128% of the levels in the wild-type strain (Fig. S2). We also used the regulation of expression of the two *H. mustelae* urease systems to confirm the effect of mutations on nickel levels in the cell, since the UreAB urease is nickel-induced, and the UreA2B2 urease is nickel-repressed, both via the NikR regulator (33, 34). When grown in nickel-supplemented Ham-F12 tissue culture medium, only the UreB subunit was detected in the wild-type strain,

consistent with an absence of UreA2B2 expression (Fig. 2C) (33). Inactivation of *nixA* did not alter this expression profile, whereas inactivation of *fecD* in both the wild-type strain and the *nixA* mutant resulted in expression of both UreB and UreB2 (Fig. 2C), consistent with nickel-limited intracellular conditions in these mutants, leading to simultaneous expression of both the UreAB and UreA2B2 ureases.

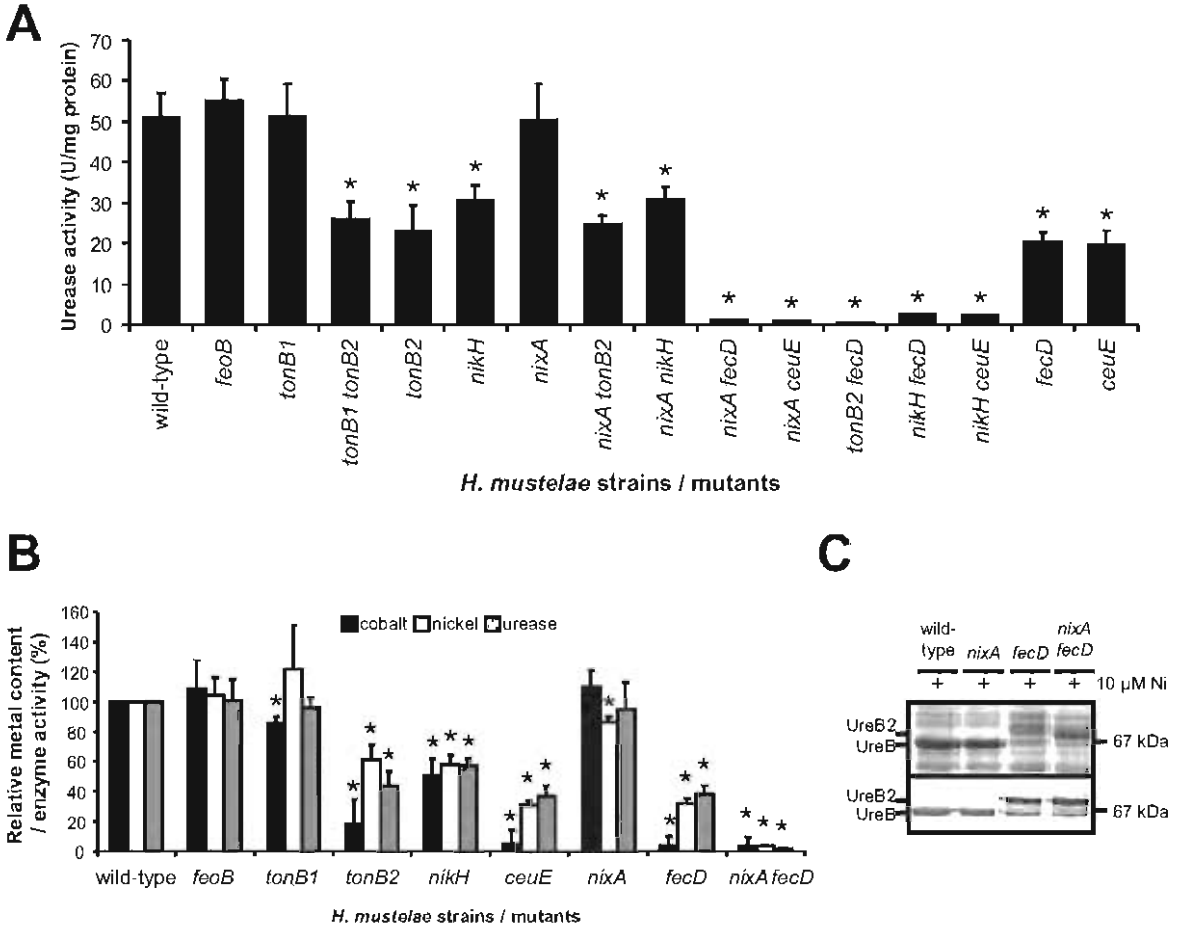

Figure 2. Inactivation of the putative nickel acquisition genes *nikH*, *tonB2*, *ceuE* and *fecD* results in decreased urease activity, decreased cellular nickel and cobalt content, and deregulation of urease expression in *H. mustelae*. A) UreAB-mediated urease activity after growth in brucella broth. Genes inactivated are shown on the X-axis, with wild-type representing *H. mustelae* NCTC 12198. Please note that a *tonB2 ceuE* mutant is lacking as it was not generated for this study. B) Urease activity, and cellular levels of nickel and cobalt as determined by using HR-ICPMS, expressed as percentage of the levels observed in *H. mustelae* wild-type NCTC 12198. Normalization to percentages is required due to the different levels of metal atoms and urease enzyme activity, as otherwise direct comparison of data would not be possible. The 100% values for the wild-type strain of cobalt and nickel are 2.08×10^3 and 5.94×10^6 atoms/cell, respectively. Levels of metal are also compared to urease activity using data from Fig. 3A. Black bars represent relative cellular cobalt levels, white bars represent relative cellular nickel levels, and grey bars represent relative UreAB urease activity. C) Inactivation of the *fecD* gene results in deregulation of expression of the nickel-responsive UreB2 and UreB proteins. The top panel shows the relevant part of an SDS-PAGE gel, the bottom panel shows an immunoblot using an antibody recognizing both the UreB and UreB2 subunits. Relevant marker sizes are indicated on the left, the positions of the UreB2 and UreB proteins are indicated on the right. Results in A) and B) are the average of three independent experiments. Error bars represent standard deviation, an asterisk represents a significant difference in urease activity ($p \leq 0.05$, Mann-Whitney U test) when compared to the wild-type strain.

Overlap between nickel and cobalt acquisition pathways in *H. mustelae*

To assess whether the putative nickel acquisition systems identified here are specific for nickel, we also determined cellular cobalt levels by HR-ICPMS. Relative cobalt levels matched the trend in cellular nickel levels, since inactivation of *fecD*, *ceuE*, *tonB2* and *nikH* resulted in a significant decrease in cobalt levels, whereas inactivation of *nixA* did not affect cobalt levels (Fig. 2B). We also noticed that cobalt supplementation of Ham-F12 medium resulted in growth inhibition, already at low concentrations (Fig. 3A), and hence also assessed cobalt resistance of the *nixA*, *fecD* and *nixA fecD* double mutant. Inactivation of the *fecD* gene resulted in a significant increase in cobalt resistance, with growth unaffected at 10 µM cobalt, where the wild-type and *nixA* mutant showed virtually no increase in OD_{600} when compared to the inoculum at 10 µM cobalt (Fig. 3A). The overall growth rate of the *nixA* mutants was reduced, but the pattern of cobalt-resistance was not altered by the *nixA* mutation when compared to the wild-type strain and the *fecD* mutant background (Fig. 3A). In contrast, there was no difference between the mutants in nickel sensitivity, as a decrease in final OD_{600} was only observed at 1000 µM nickel, but not at 10 and 100 µM nickel (Fig. S3).

Cobalt also affected expression of the two *H. mustelae* ureases (Fig. 3B), since growth in Ham-F12 medium supplemented with 1 µM cobalt resulted in repression of UreB2 expression, similar to the effect of nickel supplementation (33). Inactivation of the *fecD* gene abolished the cobalt-dependent repression of UreB2 expression (Fig. 3B), similar to what was observed in a *H. mustelae nikR* mutant (data not shown), supporting a role for the FecD protein in cobalt acquisition.

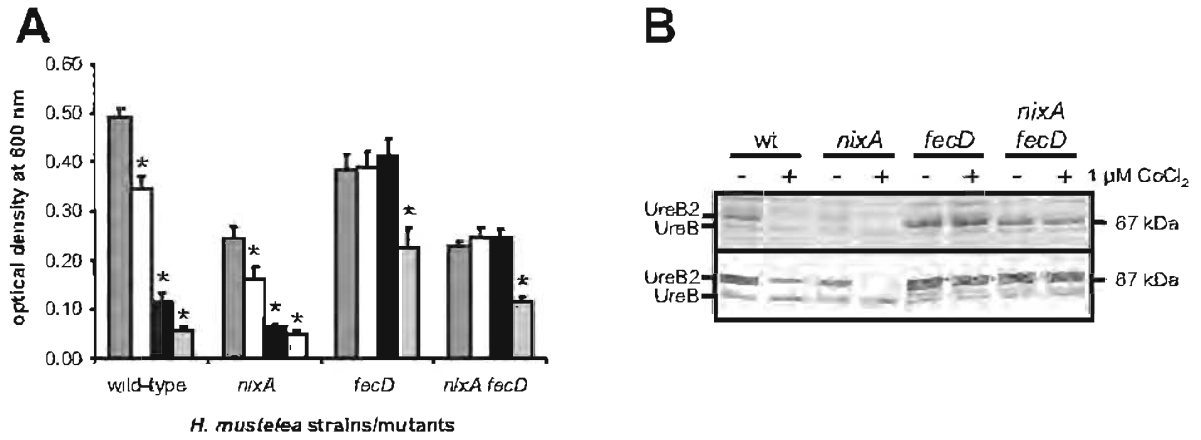

Figure 3. Inactivation of the *fecD* gene increases cobalt resistance of *H. mustelae*, and both inactivation of *fecD* and cobalt deregulate expression of urease subunits. A) Cobalt-sensitivity of wild-type *H. mustelae* NCTC 12198 and its isogenic *nixA*, *fecD* and *nixA fecD* double mutant, grown in Ham-F12 medium, as assessed by the optical density at 600 nm after 24 h of growth. Results shown are the average of three independent experiments. Error bars represent standard deviation, an asterisk represents a significant difference in OD_{600} ($p \leq 0.05$, Mann-Whitney U test) when compared to the same strain grown without addition of cobalt. Mutants are shown on the X-axis; dark grey bars represent no addition of cobalt, whereas white, black and light grey bars represent a final concentration of 1, 10 and 100 µM cobalt in the medium, respectively. B) Cobalt supplementation at a final concentration of 1 µM deregulates urease expression in wild-type *H. mustelae* NCTC 12198 and its isogenic *nixA* mutant, but does not affect urease expression patterns in the isogenic *fecD* and *fecD nixA* mutants. The top panel shows the relevant part of an SDS-PAGE gel, the bottom panel shows an immunoblot using an antibody recognizing both the UreB and UreB2 subunits. Relevant marker sizes are indicated on the left, the positions of the UreB2 and UreB proteins are indicated on the right.

DISCUSSION

Iron and nickel are two essential metal ions for gastric *Helicobacter* species, since iron participates in many redox reactions and in respiration, whereas nickel functions as cofactor for the urease and hydrogenase enzymes, responsible for acid resistance, nitrogen metabolism and energy production (22, 32). Many studies on the role of iron and nickel in *Helicobacter* species have focused on the regulation of metal acquisition systems (24, 38, 44), while relatively few studies have investigated the putative metal acquisition systems themselves (10, 11, 28, 41). In this study we have inactivated genes annotated as iron and nickel acquisition systems of *H. mustelae*, and demonstrate that several of the proposed iron acquisition genes have been misannotated, and should be reannotated as probable nickel/cobalt transporters. Furthermore our data support a role for *H. mustelae* TonB2 in facilitating transport of other metals than iron, like nickel (28, 29).

Since heme is a predominant iron source in the host, it was not surprisingly that both *H. pylori* and *H. mustelae* are able to utilize heme as sole iron source (11). We were able to confirm these results with our growth promotion assay (Fig. 1B). Interestingly, the genome sequences of *H. pylori* and *H. mustelae* lack genes encoding clear candidates for heme transporters (21, 35), and although our studies did not allow us to identify a heme transporter, we were able to demonstrate that a *tonB1* mutant was unable to grow on heme as sole iron source (Fig. 1B) suggesting a role for a TonB-dependent outer membrane receptor.

Two of the annotated *H. mustelae* iron acquisition genes (*fecD* and *ceuE*) were not regulated by either Fur or iron (Fig. 1A), which mirrored similar findings in *H. pylori* (14, 28, 39, 41). Also, the lack of a clear role for the *tonB2* gene in iron-dependent growth (Fig. 1B) supports our hypothesis that the *tonB2*, *fecD* and *ceuE* genes may be involved in acquisition of other metals or compounds. In this study we focused on the role of the *fecD*, *fecE*, *ceuE* and *tonB2* genes in nickel utilization, by insertional inactivation of single genes and creation of double mutants, followed by measuring the effect of the inactivation on urease activity, cellular nickel content and regulation of expression and activity of the UreAB and UreA2B2 ureases (Fig. 2). The observation that inactivation of *tonB2* resulted in a reduction in urease activity similar to the *H. mustelae nikH* mutant (34) was therefore not surprising. These results are also consistent with observations in *H. pylori*, where inactivation of *tonB2* and *frpB4* also resulted in a partial reduction in nickel acquisition (28), suggesting the presence of an alternative nickel acquisition system.

Comparison of complete genome sequences of *Helicobacter* species previously suggested that only the *H. hepaticus* genome contains a complete ABC transporter system for nickel (4), while the *H. pylori, H. acinonychis* and *H. mustelae* genome sequences lack a clear candidate for such an ABC transporter (12, 21, 35),with only the FecDE predicted ferric citrate permease and ATPase, and a CeuE periplasmic binding protein for enterochelin annotated (12, 21, 35). Our finding that the FecD and CeuE are involved in nickel acquisition are in agreement with the absence of Fur- and iron responsive regulation of these genes in *H. pylori* (39) and *H. mustelae* (Fig. 1A), the location of the *H. mustelae ceuE* gene directly downstream of the *nikH* gene (22, 34), nickel responsive regulation of *ceuE* in *H. pylori* (7) and predicted NikR operators in the *H. pylori fecDE* promoter (34).

The mutational studies also suggest that *H. mustelae* contains two separate acquisition pathways for nickel, one based on the TonB2-energized NikH outer membrane transporter and the NixA inner membrane transporter, whereas the CeuE/FecDE ABC transporter functions independently (Fig. 4). However, some overlap between these two systems may exist, in view of the absence of any effect of the *nixA* mutation on urease activity or cellular nickel content which contrasts to the decreased nickel content of the *tonB2* and *nikH* mutants (Fig. 2). This suggests that FecDE is able to satisfy nickel-transport requirements in brucella broth without a need for NixA. While our studies clearly support a role for the FecDE and CeuE systems in nickel acquisition, it is still unclear what nickel substrate is recognized by the outer membrane receptor(s) for nickel, or whether a nickelophore is involved (28). Also, we have not proven actual transport of nickel, although this is likely based on analogy with nickel transport as demonstrated for the FecA3 protein in *H. pylori* (28).

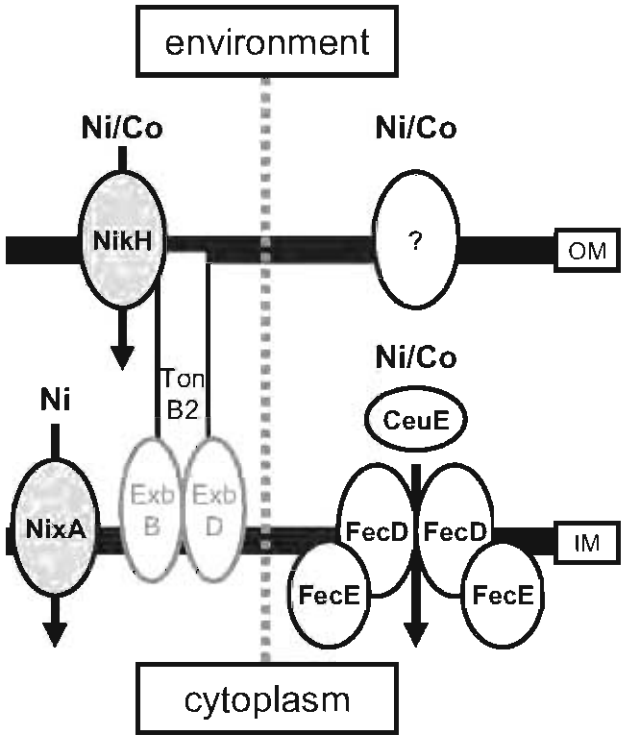

Figure 4. Schematic overview of the proposed nickel and cobalt acquisition systems of *H. mustelae*. Nickel and cobalt are transported over the outer membrane by NikH is energized by TonB2 (probably in combination with ExbB2 and ExbD2, given in grey) and is subsequently transported to the cytoplasm via the NixA inner membrane transporter, but may also use the FecDE/CeuE system. Whether the FecDE/CeuE system requires an outer membrane transporter or porin is unknown (indicated by the question mark), but is independent of TonB2, and is probably also involved in acquisition of cobalt.

Nickel and cobalt acquisition pathways often overlap this is likely due to the similarities between these two transition metals (26, 48). In *H. pylori* it was demonstrated that a *nikR* mutant was affected in cobalt resistance, and nickel was able to rescue cobalt toxicity (5, 7), which is suggestive for competition in transport. The increased cobalt resistance observed in *fecD* mutants (Fig. 3B) suggests that FecD is also involved in cobalt acquisition, and this was confirmed by the decreased cellular cobalt levels in this mutant. The nickel regulatory protein

NikR seemed not able to fully discriminate between cobalt and nickel, since the addition of 1 µM cobalt in the absence of nickel resulted in the repression of UreB2 expression (Fig. 3B), similar to what happens in nickel-sufficient conditions (33, 34). These results are in agreement with the finding that cobalt-cofactored NikR from *H. pylori* and *E. coli* is able to bind target DNA *in vitro*, although with less affinity than nickel- cofactored NikR (42, 47). Similar, the specificity of the *Mycobacterium tuberculosis* NmtR repressor is not only determined by the protein itself but also depends on the metals imported (6).

In conclusion, the available genome sequences of gastric *Helicobacter* species contain multiple genes annotated as putatively involved in iron acquisition, of which the *H. mustelae ceuE* and *fecDE* genes are likely to encode a novel nickel and cobalt acquisition system. This redresses the apparent imbalance between iron- and nickel-acquisition genes in the *H. mustelae* genome sequence (4, 21, 48), since several genes previously annotated as involved in iron-acquisition can now be reclassified.

ACKNOWLEDGMENTS

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SUPPLEMENTARY INFORMATION

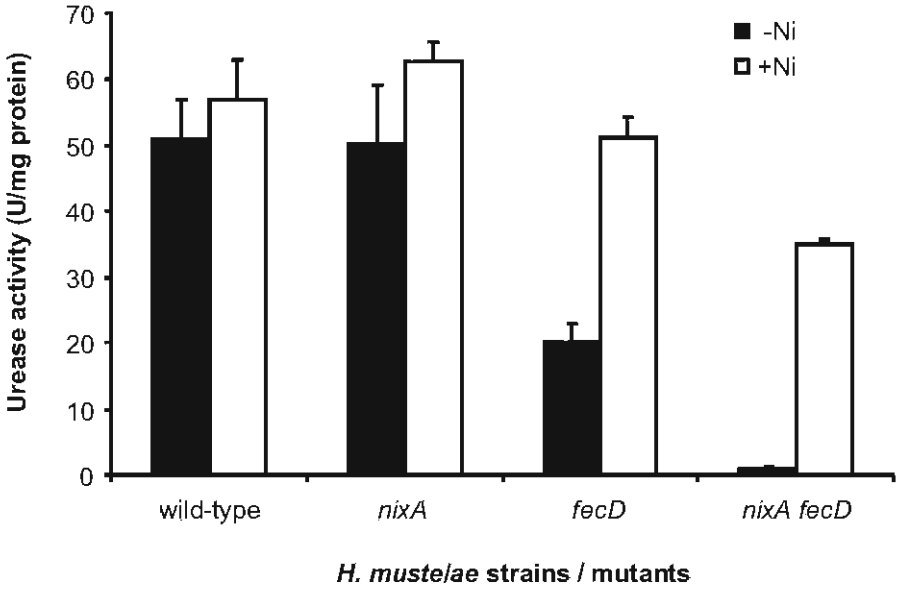

Figure S1. Supplementation of brucella broth with nickel restores urease activity in the *H. mustelae fecD* mutant to levels comparable to the wild-type strain, similar to the *H. mustelae nikH* mutant (4), but only partially restores urease activity in the *nixA fecD* double mutant. Black bars represent unsupplemented brucella broth, white bars represent brucella broth supplemented with $NiCl_2$ to a final concentration of 100 µM. Results shown are the average of three independent experiments. Error bars represent standard deviation.

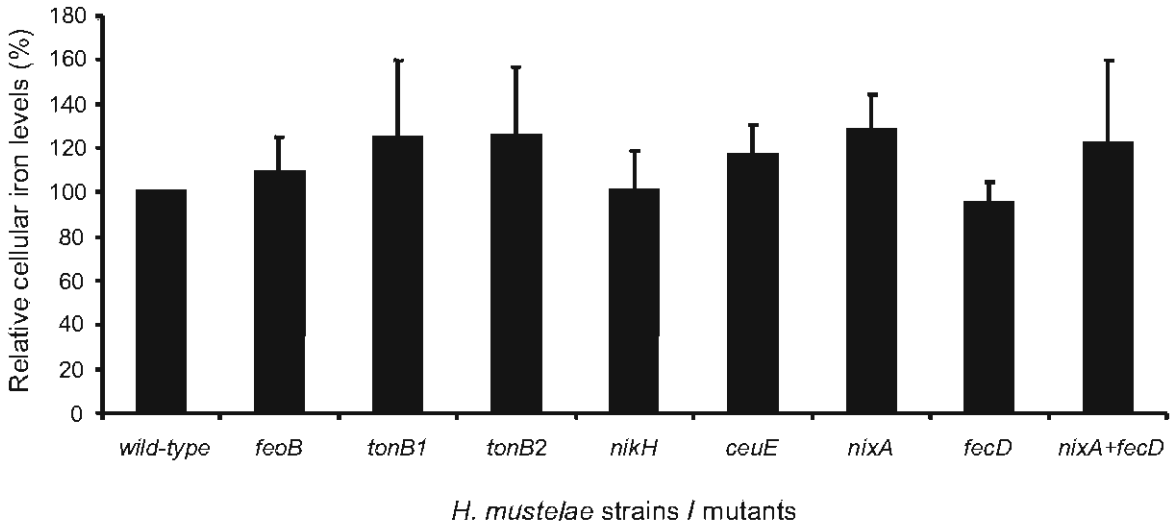

Figure S2. Cellular levels of iron as determined by using HR-ICPMS, expressed as percentage of the levels observed in *H. mustelae* wild-type NCTC 12198. The 100% value for the wild-type strain for iron is 4.31×10^6 atoms/cell.

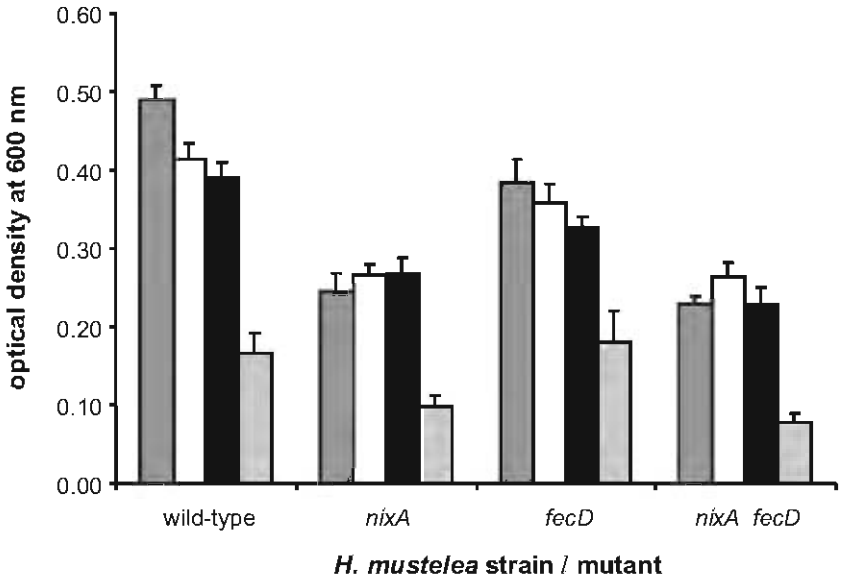

Figure S3. Inactivation of the *fecD* gene does not affect nickel sensitivity of *H. mustelae*. Nickel-sensitivity of wild-type *H. mustelae* NCTC 12198 and its isogenic *nixA*, *fecD* and *nixA fecD* double mutant, grown in Ham-F12 medium, as assessed by the optical density at 600 nm after 24 h of growth. Results shown are the average of three independent experiments. Error bars represent standard deviation. Mutants are shown on the X-axis; dark grey bars represent no addition of nickel, whereas white, black and light grey bars represent a final concentration of 10, 100 and 1000 µM nickel in the medium, respectively.

Table S1. Annotated nickel- and iron-homeostasis genes in the *H. mustelae* genome

Gene name [a]	predicted / annotated function [b]	Hm gene [a]	Ortholog of: [c]
tonB1	Energy transduction for OM transport	HMU04830	*hp0582*
tonB2	Energy transduction for OM transport	HMU02910	*hp1341*
exbD2	ExbD family accessory protein	HMU02920	*hp1340*
exbB2	ExbB family accessory protein	HMU02930	*hp1339*
cfrA	TonB-dependent OM receptor	HMU11450	*cj0755*
fecA	TonB-dependent OM receptor	HMU05510	*hp0686*
frpB1	TonB-dependent OM receptor	HMU00720	*hp0876*
frpB2	TonB-dependent OM receptor	HMU00730	*hp0876*
feoB	IM ferrous iron transporter	HMU00740	*hp0687*
ceuE	periplasmic binding protein	HMU03030	*hp1561 / hp1562*
fecD	IM permease	HMU01890	*hp0889*
fecE	IM ATPase	HMU01900	*hp0888*
nixA	IM nickel/cobalt transporter	HMU02270	*hp1077*
nikH	TonB-dependent OM receptor	HMU03020	*hh0418*
hm0418-2	TonB-dependent OM receptor	HMU03010	*hh0418*
hm0418-3	TonB-dependent OM receptor	HMU02990	*hh0418*
nikR	Nickel-responsive regulatory protein	HMU11920	*hp1338*
fur	Iron-responsive regulatory protein	HMU11690	*hp1027*
pfr	Ferritin	HMU14370	*hp0653*

a) Gene designation and gene number based on homology studies and annotation of the *H. mustelae* genome (1), or on experimental studies on *H. mustelae* (3, 4).

b) Predicted function of the protein encoded by the *H. mustelae* gene (1). OM = outer membrane, IM = inner membrane.

c) Gene number of ortholog in the genome sequences of *H. pylori* 26695 (*hp*), *H. hepaticus* ATCC 51449 *hh*) or *C. jejuni* NCTC 11168 (*cj*) (2, 5, 6).

Table S2. Oligonucleotide primers used in this study

Primer	Sequence (5' → 3') [a]
Hmus tonb1 KO F	CCTGCTGCATGCCCTAATC
Hmus tonb1 KO R	CCCAGTACCCAAAGCACTTAG
Hmus tonb1 ORS F	AGCATTGGCATGAAGGATCG
Hmus tonb1 ORS R	CATGCCAAAGTCCACCAACTC
Hmus tonb2 KO F	CACAGTGGACGCAAACAATG
Hmus tonb2 KO R	ATGAGCTCGCGGTTGCAAAG
Hmus tonb2 ORS F	AGGCAAACTCAGCACAACAG
Hmus tonb2 ORS R	CAGCGCATATCCATGAGTG
Hmus frpb1 KO F	GCTAGGCTCACCCTCAATG*
Hmus frpb1 KO R	ATAGCCTGGCTTATCCACTTG
Hmus frpb1 ORS F	TGAAAGAATCTGCGGCCATC
Hmus frpb1 ORS R	TATGGCGCGGAGGTATGTAG
Hmus frpb2 probe F	GCTAGGCTCACCCTCAATG*
Hmus frpb2 probe R T7 [b]	ctaatacgactcactatagggagaTGTTTGACCTCAAAGTCCATTC
Hmus frpb2 KO F	GCTAGGCTCACCCTCAATG*
Hmus frpb2 KO R	ATAGGTATTGCCCACGGATGC
Hmus frpb2 ORS F	ATCCCGCGCCACAAGACAAG
Hmus frpb2 ORS R	AAGCGCTGCACAAATCTACC
Hmus fecA KO F	TCAAGGAATCCGGCTACAAC
Hmus fecA KO R	TGTATAGCGCATTCCAAAGACG
Hmus fecA ORS F	AGCCCACATAACCCACTAAGC
Hmus fecA ORS R	GGTCTGCCTTGCCATTTCTG
Hmus cfrA KO f	TGACCTCAGCCTCTGGATAC
Hmus cfrA KO R	AGGGCTGCTTCACCACAAC
Hmus crfA ORS F	AGCTCCTAGCATGTCCTAAATG
Hmus cfrA ORS R	CTTGGCGAGACATGAAAGCC
Hmus feoB KO F	CCACGCCCAACCATTTCATC
Hmus feoB KO R	TGGCATCTCCATGACAAAGG
Hmus feoB ORS F	CAATCTCACGCTAAGCACAC
Hmus feoB ORS R	GGATACTGGGAGGCAAACC
Hmus fecD probe F	GCGCTACTTGTTGGTGGAG
Hmus fecD probe R T7 [b]	ctaatacgactcactatagggagaTAACGGAGCGCCAATAATCG
Hmus fecD KO F	TTTGTTGGCCCTTGGAGTAG
Hmus fecD KO R	AAACACCCTGCCTCTCATAC
Hmus fecD ORS F	CGCGGAAGTTTGTCGTATCTG
Hmus fecD ORS R	ATTAGGCGCAAGCACAGC
Hmus nixA KO F	TCATGCGCGAATTTCTACCC
Hmus nixA KO R	AAAGCGCCAGATGCCATAAG
Hmus nixA ORS F	GTTTGAATCCCGCTACTCCG
Hmus nixA ORS R	CGCGAGGCTTTGATCTTTAG
Hmus ceuE probe F	CCATTGATGTGGAGCGTCTG
Hmus ceuE probe R T7 [b]	ctaatacgactcactatagggagaTCGCCTCTTGGTTGGCTTAC
Hmus ceuE KO F	TAGTGGCGCTTTTGGCATAG
Hmus ceuE KO R	GTGCAGGGCAGCAATTAGAG
Hmus ceuE ORS F	CAATATCACATCGCGCTTCC
Hmus ceuE ORS R	TGACCCACTTTGCATCAATCC

a) Primer sequences were based on the *H. mustelae* NCTC 12198 genome sequence (1).

b) Primer contains a 5'-extension with T7 promoter sequence (in lowercase letters) for the creation of an antisense RNA probe (3, 7).

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Chapter 8

Summary & Conclusions

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Summary & Conclusions

Gastric *Helicobacter* species are colonizers of one of the harshest niches of the mammalian body. This colonization can lead to gastritis, peptic ulcer disease and eventually to gastric cancer (2). One of the major acid resistance factors of gastric *Helicobacter* species is the nickel co-factored enzyme urease. Nickel is also required as cofactor by hydrogenase enzyme, that provides energy for the bacterium using hydrogen (4). Inactivation of either urease (12, 13) or hydrogenase (9), prevents *Helicobacter* species from colonising the gastric niche in diverse animal models. Thus potentially, deprivation of nickel may also lead to an inability to colonize the stomach. This hypothesis is supported by experiments that demonstrated that inactivation of NixA, partially responsible for nickel transport across the inner membrane, resulted in reduced colonization (8).

In order to be able to inhibit nickel acquisition, more knowledge was needed about the mechanisms of nickel acquisition by *Helicobacter species*. The only information available at the start of the research presented in this thesis, was that NixA partially contributed to the levels of nickel transport across the inner membrane in *H. pylori* (11). This suggested that an additional inner membrane transport protein should be present. Furthermore, nickel was presumed to pass the outer membrane without the need for dedicated transport proteins. In this thesis we have used the availability of genome sequences to identify putative nickel regulated genes in different *Helicobacter* species and have assessed their function in nickel uptake and utilization.

Comparison of the putative metal acquisition systems present in the *H. hepaticus*, *H. pylori* and *H. mustelae* genomes revealed major differences in the prevalence of these genes. Most strikingly were the differences in TonB-dependent outer membrane proteins and the absence of a dedicated ABC transporter for nickel in the gastric *Helicobacter* species (chapter 2). To identify which of these putative metal transporters are involved in nickel transport, we hypothesized that genes involved in nickel transport would be nickel-repressed to prevent nickel transport in nickel-sufficient conditions, as was demonstrated for NixA (3). Therefore we investigated nickel-responsive patterns of transcriptional regulation of *H. mustelae* (chapter 4 and 5) and *H. pylori* genes (chapter 6), and assessed the role of the nickel responsive regulator NikR in such transcriptional regulation.

The first genes in this thesis shown to be directly repressed by nickel and NikR were the genes encoding the *H. pylori* TonB dependent transporters FrpB3 and FecA3 (chapter 6). These genes were annotated as putative outer membrane receptors for iron, but their expression was not iron responsive (10). Although no actual nickel transport was demonstrated, the nickel dependent regulation of these TonB dependent transporters, was suggestive of involvement in nickel transport. Hitherto, nickel was thought to pass freely across the outer membrane. Involvement of these putative TonB gated channels, suggests the presence of ligand mediated, and active nickel transport across the outer membrane. Actual

nickel transport by this TonB dependent system was later demonstrated (1) although overall nickel transport by the cell was only partially affected, similar to inactivation of *H. pylori nixA*.

The *H. pylori* TonB-dependent transporters for nickel and *nixA* were not the only genes repressed by nickel in *Helicobacter* species. The second urease (UreA2B2) present in *Helicobacter felis* (chapter3), *H. mustelae* and *H. acinonychis*, was also demonstrated to be repressed by nickel while the UreAB urease was nickel-induced (chapter 4). *H. mustelae* was chosen for further characterisation of the UreA2B2 urease since the complete genome sequence of this *Helicobacter* species was available, and this species is readily amenable to genetic manipulation. Due to the iron-induced and nickel repressed regulatory pattern, the independency of the standard accessory proteins and the oxygen sensitivity of UreA2B2, it was hypothesized that the UreA2B2 urease may use an alternative cofactor (chapter 4). Analysis of the metal content of UreA2B2 indeed revealed the presence of iron and the absence of nickel (unpublished results), although it is still unclear whether iron functions as a co-factor. The three *Helicobacter* species encoding UreA2B2 have in common that they colonise the stomach of strict carnivores. However the major dietary sources of nickel are vegetarian, and include roots, nuts and vegetables (14). This poses a potential problem for *Helicobacter* species colonizing the gastric environment of obligate carnivores, since meat is unlikely to contain sufficient amounts of nickel to cater for the highly expressed urease enzyme, and sufficient nickel will probably only be available when the intestinal contents of the prey is consumed (7). An iron co-factored urease may therefore ensure urea mediated acid resistance under nickel limited conditions. Although UreA2B2 is present in *H. acinonychis*, the closest relative of *H. pylori*, it is not present in *H. pylori*, which therefore may be more acid sensitive under nickel-limited conditions.

Since *H. pylori* mutants lacking the nickel-transporters NixA, TonB2 or FrpB4 show only a partial reduction in nickel acquisition (1, 11), the presence of additional nickel acquisition proteins for both inner and outer membrane transport can be predicted. Identification of the binding site of the nickel responsive regulator NikR in the promoters regions of *Hp-frpB3*, *Hp-fecA3* (chapter 6), *Hm-ureA2* and *Hm-ureA* (chapter 5) and comparison with the previous identified NikR binding site of *Hp-ureA* and *Hp-nixA (3)*, allowed the definition of an improved consensus sequence (chapter 5). Screening the genomes of *H. hepaticus*, *H. acinonychis, H. mustelae* and *H. pylori*, pointed to several genes preceded by such a binding site. As proof of principle, the expression and function of a putative TonB dependent receptor in *H. mustelae* (Hmu03020) was studied. This gene had no significant homology with either *H. pylori* FecA3 or FrpB4 but contained a putative NikR binding site in its promoter region. Expression of this gene was shown to be nickel responsive, while inactivation of this gene resulted in a partial reduced urease activity suggestive for involvement in nickel transport. We named this protein NikH, and hypothesize that NikH is an *H. mustelae* analog of Hp-FrpB4/ Hp-FecA3 (chapter5).

Surprisingly, upstream of the putative inner membrane transporter for ferric citrate, *Hp-fecDE,* a putative NikR box was identified (chapter 5). As for *Hp-frpB3* and *Hp-fecA3*, expression of the *Hp-fecDE* genes was reported to be iron-independent both in *H. pylori* (10) and *H. mustelae* (chapter 7). Inactivation of *Hm-fecD*, but also inactivation of the periplasmic component of an ABC transporter, *Hm-ceuE,* resulted in a decreased urease activity and cellular nickel content in *H. mustelae* (chapter 7). In order to get a better understanding of nickel acquisition in *H. mustelae*, double mutants of all known nickel transporters were created in *H. mustelae*. Screening of these mutants for urease activity and intracellular nickel content, led to the generation of our hypothesis of the presence of two separate nickel acquisition systems in *H. mustelae*, while a TonB independent outer membrane nickel transporter remained unidentified (chapter 7).

In the genome era, many microbial genomes are sequenced while relatively limited experimental data is available to support the published annotation. In most cases gene annotation is inferred from homology with genomes of only distantly related species, with experimental evidence of those annotations often lacking too. In this thesis we demonstrate that several of these genes actually may fulfil an alternative function. The identification of operators in these genomes, like NikR boxes (Chapter 5, 6 and 7) are a useful tool to support and predict gene function, although experimental evidence for confirmation of function remains essential.

For gastric *Helicobacter* spp. nickel is toxic at high concentrations. However, to skew nickel homeostasis in *Helicobacter* species towards toxicity is difficult due to allergy and nickel toxicity to the host at high concentrations (7), moreover *Helicobacter* species are able to protect themselves to certain levels against overload by repression of the nickel uptake systems and storage of nickel in urease and other nickel sinks (6).
A better alternative to disturb nickel homeostasis of *Helicobacter* spp. could be nickel depletion, since nickel is essential for urease- and hydrogenase dependent gastric survival *of H. pylori.* Gastric *Helicobacter* species naturally encountering decreased nickel concentrations due to nickel limited feeding patterns like *H. felis*, *H. mustelae* and *H. acinonychis*, have complemented their nickel-dependent urease enzyme with a nickel-independent one, minimizing the need for nickel as co-factor. Nickel in food is likely to be the main source of nickel available for the bacterium, therefore a nickel limited diet may be sufficient to induce nickel starvation. In *Helicobacter* species that do not encode a second urease like *H. pylori*, nickel starvation may lead to decreased urease activity resulting in increased acid sensitivity.
Competition in acquisition between nickel and another metal might be an option to reduce nickel acquisition. In *H. pylori* cobalt toxicity was decreased by nickel (5) and increased by inactivation of *nikR (6)* both suggestive for competition between transport of nickel and cobalt. Similar an *H. mustelae fecD* mutant was demonstrated to have reduced cellular cobalt and nickel concentrations (chapter 7) while nickel reduced cobalt toxicity (unpublished

results). However, cobalt is even more toxic to the host than nickel and therefore not suitable for therapeutics.

Alternatively, molecules that resemble the nickel ligands required by *H. pylori* for nickel transport across the outer membrane may specifically inhibit nickel acquisition by this pathogen. The identification of these nickel ligands could be a task for future projects, as is the identification of the TonB independent transport mechanism of nickel across the outer membrane.

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Chapter 8

Samenvatting & Conclusies

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Samenvatting & Conclusies

Gastrische *Helicobacter* species koloniseren het meest zure orgaan van hun gastheer. Kolonisatie door *Helicobacter pylori* kan bij de mens leiden tot maagontsteking, maagzweren en maagkanker (2). Eén van de eiwitten die *Helicobacter* species in staat stelt het zure milieu van de maag te koloniseren is het enzym urease. Dit enzym zet ureum om in ammonia en CO_2 en zorgt voor neutralisatie van de directe omgeving van de bacterie. De activiteit van urease is afhankelijk van de aanwezigheid van nikkel, dat als cofactor dient. Nikkel is ook nodig als cofactor voor hydrogenase, een enzym dat er voor zorgt dat de bacterie waterstof kan gebruiken als energie bron (4). Uitschakeling van urease of hydrogenase, leidt er toe dat *Helicobacter* species de maag niet meer kunnen koloniseren, zoals gebleken is uit verschillende diermodellen (12, 13, 9). Beperking van de nikkel beschikbaarheid leidt tot lagere activiteit van beide enzymen en zou dus er toe kunnen leiden dat *Helicobacter* species de maag niet meer kunnen koloniseren. Deze hypothese wordt ondersteund door experimenten waaruit blijkt dat een *Helicobacter pylori* mutant die minder nikkel kan opnemen, minder goed de gastheer koloniseert dan het wildtype (8).

In dit proefschrift is onderzocht welke mechanismen door *Helicobacter* species worden gebruikt om nikkel op te nemen. De enige informatie die beschikbaar was bij de aanvang van het onderzoek, was dat NixA maar gedeeltelijk bijdraagt aan het transport van nikkel over het binnenmembraan (11). Dit impliceert dat er een alternatief system voor transport over het binnenmembraan aanwezig moet zijn. Verder werd verondersteld dat nikkel het buitenmembraan van de *Helicobacter* species kon passeren zonder dat daarvoor specifieke transport eiwitten nodig zijn. In dit proefschrift hebben we de beschikbaarheid van bacteriële genoom sequenties gebruikt om genen te identificeren die mogelijk betrokken zijn bij nikkel opname in verschillende *Helicobacter* species.

Een vergelijking van potentieel metaal opname systemen in *H. hepaticus*, *H. pylori* en *H. mustelae,* liet grote verschillen zien in de aanwezigheid van zulke genen tussen de verschillende species. Het duidelijkst waren de verschillen tussen de TonB afhankelijke buitenmembraan transporters en de afwezigheid van een ABC transporter voor nikkel in gastrische *Helicobacter* species (hoofdstuk 2). Om te identificeren welke van deze genen betrokken zijn in nikkel transport hebben we aangenomen dat genen, die betrokken zijn bij nikkel transport, uitgeschakeld moeten worden onder hoge nikkel concentraties, om nikkel toxiciteit te voorkomen. Dit was eerder aangetoond voor NixA (3). Om die reden hebben we nikkel afhankelijke transcriptionele regulatie van *H. mustelae* (hoofdstuk 4 and 5) en *H. pylori* (hoofdstuk 6) onderzocht en hebben we de rol van de nikkel afhankelijke regulator NikR in deze regulatie bepaald.

De eerste genen waarvan we konden aantonen dat de transcriptie direct gereguleerd werd door nikkel en NikR, waren de genen die codeerden voor de TonB afhankelijke transporters FrpB3 en FecA3 in *H. pylori* (hoofdstuk 6). Deze genen waren oorspronkelijk geannoteerd als

potentiële buitenmembraan transporters voor ijzer, maar de transcriptie werd niet geremd door ijzer (10). Hoewel we niet in staat waren om directe betrokkenheid in nikkel transport aan te tonen, was de nikkel afhankelijke regulatie suggestief voor een rol in nikkel transport. Het definitieve bewijs van nikkel transport door dit TonB afhankelijke systeem werd kort daarna door een andere groep aangetoond (1). Echter mutatie van dit transport systeem, bleek net als de uitschakeling van NixA, niet voor een complete inhibitie te zorgen van nikkel transport, wat de aanwezigheid van een alternatief buitenmembraan transport systeem voor nikkel suggereert.

De TonB afhankelijke transporters en *nixA* bleken niet de enige nikkel gerepresseerde genen te zijn in *Helicobacter* species. Ook het tweede urease (UreA2B2) zoals aanwezig in *H. felis* (hoofdstuk 3), *H. acinonychis* en *H. mustelae* werd gerepresseerd door nikkel (hoofdstuk 4). *H. mustelae* werd uitgekozen om UreA2B2 verder te karakteriseren omdat de complete genoom sequentie van deze bacterie bekend was en deze bovendien relatief eenvoudig was te muteren. Omdat expressie van het tweede urease geïnduceerd werd door ijzer maar gerepresseerd werd door nikkel en het enzym bovendien zuurstof gevoelig was veronderstelden we dat dit urease een ander cofactor gebruikte (hoofdstuk 4). Analyse van het metaal gebonden door UreA2B2 liet inderdaad de afwezigheid van nikkel, maar aanwezigheid van ijzer zien (niet gepubliceerde resultaten). Nikkel komt voornamelijk in hogere concentraties voor in vegetarische producten zoals wortels, noten en sommige groenten (14), vleesproducten bevatten echter maar zeer lage concentraties nikkel. Dit veroorzaakt een potentieel probleem voor *Helicobacter* species die de maag van strikt carnivoren koloniseren (7). Het tweede urease zou een aanpassing kunnen zijn van deze *Helicobacter* species om toch te kunnen overleven onder nikkel beperkte condities. Dit zou kunnen verklaren waarom UreA2B2 alleen kon worden aangetoond in *Helicobacter* species die de maag van strikt carnivoren koloniseren. *Helicobacter pylori* die de mens koloniseert heeft deze aanpassing echter niet en zou dus daarvoor gevoeliger moeten zijn voor zuur onder nikkel beperkende condities.

Omdat *H. pylori* stammen waarvan het *nixA*, *tonB2* of *FrpB4* gemuteerd is, alleen een verminderde nikkel opname laten zien maar geen complete afwezigheid van opname (1, 11), kon de aanwezigheid van een additioneel transport systeem worden voorspeld. Identificatie van de bindingsplaats van de nikkel afhankelijke regulator NikR in de promoter regio's van Hp-frpB3, Hp-fecA3 (hoofdstuk 6), Hm-ureA2 en Hm-ureA (hoofdstuk 5) en vergelijking met eerder gekarakteriseerde NikR bindingsplaatsen van Hp-ureA en Hp-nixA (3), maakte het mogelijk een verbeterde consensus sequentie te definiëren (hoofdstuk 5). Screening van de DNA sequenties van *H. hepaticus*, *H. acinonychis*, *H. mustelae* and *H. pylori* voor de aanwezigheid van deze bindingsplaats voorspelde de regulatie van een aantal nog niet eerder gekarakteriseerde genen. Als proef op de som, werden de expressie en functie van een TonB afhankelijke receptor in *H. mustelae* (Hmu03020) bestudeerd. Dit gen had geen significante homologie met *H. pylori FecA3* of *FrpB4,* maar bevatte wel een potentiële NikR binding plaats in de promoter regio. Expressie van dit gen bleek inderdaad gereguleerd te zijn door

nikkel, terwijl inactivatie van dit gen resulteerde in een verminderde urease activiteit, wat suggestief was voor verminderd nikkel transport. Dit gen werd geannoteerd als NikH en is waarschijnlijk een analoog van Hp-FrpB4/ Hp-FecA3 (hoofdstuk5).

Verrassend genoeg werd ook een NikR bindingsplaats gevonden in de promoter regio van een gen dat geannoteerd was als een binnenmembraan transporter voor ijzer citraat (*Hp-fecDE*) (hoofdstuk 5). Net zoals gerapporteerd was voor *Hp-frpB3* and *Hp-fecA3*, was de transcriptie van *Hp-fecDE (10)* en *Hm-fecDE* (hoofdstuk 7) niet ijzer afhankelijk. Mutatie van *Hm-fecD*, maar ook mutatie van het periplasmatische component van de ABC transporter, *Hm-ceuE*, resulteerde in een verminderde urease activiteit en een verminderde hoeveelheid nikkel in de bacteriële cellen (hoofdstuk 7). Om een goed overzicht te krijgen welke eiwitten met elkaar samenwerkten in nikkel opname, werden dubbelmutanten gemaakt van alle eiwitten die betrokken zijn in nikkel transport in *H. mustelae*. Screening van deze mutanten op urease activiteit en intracellulaire nikkel hoeveelheid, leidde tot de hypothese dat er twee verschillende nikkel opname systemen zijn in *H. mustelae* die verschillen in specificiteit. (hoofdstuk 7).

In dit tijdperk waarin vele bacteriële genoom sequenties bepaald worden moet er aan veel genen een naam worden gegeven. In de meeste gevallen is deze naamgeving niet gebaseerd op experimenteel bewijs, maar op basis van zwakke homologie met DNA sequenties van verwante of minder verwante species. In dit proefschrift hebben we aangetoond dat verschillende genen in *H. mustelae* en *H. pylori* een andere functie hebben dan kon worden voorspeld op basis van homologie. We lieten zien dat de identificatie van potentiële operators zoals NikR boxen (hoofdstuk 5, 6 and 7) in de promoter regio van genen waardevol kunnen zijn bij het voorspellen van een genfunctie, hoewel experimenteel bewijs altijd noodzakelijk zal zijn.

In hoge concentraties is nikkel toxisch voor gastrische *Helicobacter* spp. Echter dit gegeven is niet te gebruiken in de behandeling van deze bacteriën omdat nikkel ook toxisch is voor de gastheer en al in lagere concentraties kan lijden tot allergie (7). Bovendien zijn deze bacteriën tot op zekere hoogte in staat zichzelf te beschermen tegen hoog nikkel concentraties door repressie van de nikkel opname systemen en het teveel aan nikkel op te slaan in urease en andere nikkel opslag eiwitten (6).
Een beter alternatief om nikkel homeostase in *Helicobacter* species te verstoren is het beperken van de nikkel beschikbaarheid. *Helicobacter* species die van nature in lage nikkel concentraties moeten koloniseren door een nikkel beperkt dieet van hun gastheer (zoals *H. felis*, *H. mustelae* and *H. acinonychis*), hebben zich aangepast aan deze condities door het ontwikkelen of opnemen van een nikkel onafhankelijk urease. In *Helicobacter* species die geen tweede urease coderen, zoals *H. pylori,* kan nikkel beperking leiden tot verminderde urease activiteit en zuurgevoeligheid. Nikkel opname in *Helicobacter* spp. kan theoretisch geremd worden door andere metalen die op de zelfde manier de cel binnen komen. In *H. pylori* werd kobalt toxiciteit verminderd door nikkel (5) terwijl een *nikR* mutant meer

gevoelig was voor kobalt (6), beide bevindingen zijn suggestief voor competitie tussen nikkel en kobalt transport. Een *H. mustelae fecD* mutant liet niet alleen een verminderde nikkel opname zien maar ook de kobalt opname was zeer sterk afgenomen (hoofdstuk 7), terwijl nikkel de kobalt toxiciteit remde (niet gepubliceerde resultaten). Echter kobalt is meer toxisch voor de gastheer dan nikkel en daarom niet toepasbaar als antibioticum.
Een andere mogelijkheid om nikkel opname te remmen zou kunnen voortkomen uit de identificatie van de moleculen die *Helicobacter* species gebruiken voor nikkel transport over het buitenmembraan. Moleculen die hier op lijken kunnen de eigenschap bezitten dat ze de nikkel opname inhiberen. De identificatie van deze nikkel bindende moleculen, net als de identificatie van de voorspelde TonB onafhankelijke buiten membraan transporter voor nikkel zou een goed onderwerp zijn voor vervolg studies.

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S **C** **I** **N** **D** **Y** **A** **L** **E** **G** **N** **A** N D R E
L R D M E **N** R **N** H A N N **E** K **E** S
X E L A L **O** **O** T U G Y **U** D **D** **T** S
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G K E A **I** **A** A **N** **A** **I** **R** **O** **L** **F** **E** R
H **A** **N** **S** R **R** J T **N** **S** **N** D L **M** **L** E
G **C** D E A T R **O** N **R** **O** O L **A** **O** I
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E **A** E M C N W T O **A** H O **H** **T** **I** O
M **R** O K R E P S **A** J P S R **I** **N** **R**
G **A** G E R A R **D** L L P A A **J** **E** **A**
E S A N O U K **D** **U** **U** **R** T P **N** N **Y**
R T **E** **R** **N** **S** **T** M A R J A R D K **M**
S R C S I E W D P O E E R E L **O**
P I U F I M E L L I W E K O H **N**
D D L V H **L** **I** **N** **D** **A** A K R A M **D**

Curriculum vitae

Jeroen Stoof was born on the 28th of November 1974 in Nieuwer Ter Aa, the Netherlands. He attended "De Driestar college" in Gouda and after graduation in 1992, he started his study at the Department of Laboratory Science and Chemical Engineering, the Utrecht University of Professional Education (HLO), Utrecht, The Netherlands, with as specialisation Medical Microbiology. After an internship studying molecular typing of hospital-acquired infections at the Department of Medical Microbiology and Infection Prevention at the Vrije Universiteit Amsterdam, he started as technician at the same department in 1997, working in the group of Dr. Savelkoul. In 2001, he moved to the Department of Gastroenterology and Hepatology of the Erasmus MC - University Medical Center Rotterdam to start a job as research technician on the metal-responsive gene regulation in *Helicobacter pylori* under supervision of Dr. Arnoud van Vliet and Prof. Ernst Kuipers. From 2005 onwards he has combined a position of PhD student and research technician at the same department, with a research project on acid resistance and metal-responsive gene regulation in pathogenic *Helicobacter* species. In 2010 he has started a position as postdoctoral research associate at the University of Nottingham, United Kingdom, focusing on host pathogen interaction of *Campylobacter jejuni*.

List of publications

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1. **Stoof, J., E. J. Kuipers, G. Klaver, and A. H. M. van Vliet.** 2010. TonB2 and an ABC transporter contribute to *Helicobacter mustelae* nickel and cobalt acquisition. Infect. Immun **78:** 4261-7
2. **Stoof, J., E. J. Kuipers, and A. H. M. van Vliet.** 2010. Characterization of NikR-responsive promoters of urease and metal transport genes of Helicobacter mustelae. Biometals **23:**145-59.
3. **Belzer, C., J. Stoof, S. Breijer, J. G. Kusters, E. J. Kuipers, and A. H. M. van Vliet.** 2009. The Helicobacter hepaticus hefA gene is involved in resistance to amoxicillin. Helicobacter **14:**72-9.
4. **Moons, L. M., J. G. Kusters, J. H. van Delft, E. J. Kuipers, R. Gottschalk, H. Geldof, W. A. Bode, J. Stoof, A. H. M. van Vliet, H. B. Ketelslegers, J. C. Kleinjans, and P. D. Siersema.** 2008. A pro-inflammatory genotype predisposes to Barrett's esophagus. Carcinogenesis **29:**926-31.
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10. **Gerrits, M. M., A. P. Godoy, E. J. Kuipers, M. L. Ribeiro, J. Stoof, S. Mendonca, A. H. M. van Vliet, J. Pedrazzoli, Jr., and J. G. Kusters.** 2006. Multiple mutations in or adjacent to the conserved penicillin-binding protein motifs of the penicillin-binding protein 1A confer amoxicillin resistance to Helicobacter pylori. Helicobacter **11:**181-7.
11. **Belzer, C., J. Stoof, C. S. Beckwith, E. J. Kuipers, J. G. Kusters, and A. H. M. van Vliet.** 2005. Differential regulation of urease activity in Helicobacter hepaticus and Helicobacter pylori. Microbiology **151:**3989-95.

12. **Ernst, F. D., S. Bereswill, B. Waidner, J. Stoof, U. Mader, J. G. Kusters, E. J. Kuipers, M. Kist, A. H. M. van Vliet, and G. Homuth.** 2005. Transcriptional profiling of Helicobacter pylori Fur- and iron-regulated gene expression. Microbiology **151:**533-46.
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23. **Spaargaren, J., J. Stoof, H. Fennema, R. Coutinho, and P. Savelkoul.** 2001. Amplified fragment length polymorphism fingerprinting for identification of a core group of Neisseria gonorrhoeae transmitters in the population attending a clinic for treatment of sexually transmitted diseases in Amsterdam, The Netherlands. J Clin Microbiol **39:**2335-7.

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Presentations

Oral presentations (English):
NVMM2006, HPIG2006, NVGE2006, IFR2007 invited speaker, NVGE 2008, NVMM2009, SGM 2010

Poster presentations:
NVMM2005, NVMM2007, CHRO2007, SGM2008, Biometals 2008,.
NVGE 2009

NVMM: Dutch society for Medical Microbiology
NVGE: Dutch society for Gastroenterology
HPIG: International Workshop on Pathogenesis and Host Response in Helicobacter Infections
CHRO: Campylobacter, Helicobacter and Related Organisms
SGM: U.K. Society General Microbiology
IFR: Institute of Food Research