Surviving the Enterohepatic Tract:

Molecular Mechanisms of Stress Adaptation in *Helicobacter hepaticus*

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Molecular Mechanisms of Stress Adaptation in *Helicobacter hepaticus*

Overleven in het enterohepatische milieu: *Helicobacter hepaticus* gebruikt moleculaire mechanismen om zich aan te passen aan stress factoren

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CONTENT

Chapter 1

Aims & Outline

AIMS & OUTLINE

Members of the genus *Helicobacter* are pathogens of mammals and are associated with a wide range of diseases of the digestive tract. The genus is best known for the human gastric pathogen *Helicobacter pylori*, but also contains a large number of other *Helicobacter* species, all colonizing the gastrointestinal tract of man and animals. One of these species is the murine pathogen *Helicobacter hepaticus*, which causes enteritis, hepatitis, hepatocellular carcinoma, typhlocolitis and has recently been implicated to contribute to the formation of cholesterol gallstones. Although to date *H. hepaticus* has only been isolated from rodents, it may be representative for other enterohepatic *Helicobacter* species suggested to exist in humans. A better understanding of *H. hepaticus* biology may therefore contribute to a better understanding of these diseases.

One of the hallmarks of bacterial infections is the capacity of the pathogenic microorganism to adapt to the multitude of stresses encountered during infection of the host. Many features of this adaptation are controlled through the process of regulation of gene transcription in response to such stresses. This thesis is focused on stress adaptation and gene regulation of *H. hepaticus*. In particular, the objective of this study was to study metal-responsive transcriptional and posttranscriptional regulation, of urease, and the mechanisms that lead to *H. hepaticus* resistance to antimicrobials. This should give us a better insight in the mechanisms allowing chronic colonization by this organism, and its association with diseases of the digestive tract.

In **CHAPTER 2,** aspects of metal-responsive gene regulation and metal transport in the genus *Helicobacter* is reviewed. This chapter contains comparative genome analyses of the human gastric pathogen *H. pylori*, the murine enterohepatic pathogen *H. hepaticus* and the ferret gastric pathogen *Helicobacter mustelae*. The complete genome sequences of these species were used to identify potential metal transporters, metal storage proteins and metal-responsive regulators. Subsequently, the possible contributions of the differences in metal metabolism in adaptation to the gastric or enterohepatic niches occupied by *Helicobacter* species are discussed.

Chapter 3 describes a comparative experimental analysis of the urease systems of *H. hepaticus* and *H. pylori*. Urease is a major virulence factor and is required for gastric colonization by *H. pylori*, but its role in enterohepatic *Helicobacter* species was not yet established. The experiments described in this chapter indicate that unlike with gastric *Helicobacters* like *H. pylori* and *H. mustelae* urease activity does not contribute to acid-resistance of *H. hepaticus*. A second major difference with the Urease of *H. pylori* is that the *H. hepaticus* urease activity is posttranslationally but not transcriptionally regulated by nickel. **CHAPTER 4** further analyzes the regulation of urease expression in *H. hepaticus*, which is demonstrated to be iron-responsive through the transcriptional regulator Fur.

The number of regulatory proteins in the genome of *H. hepaticus* is relatively low, suggesting a limited capacity for gene regulation. Three potential metal-responsive regulatory systems were identified in **CHAPTER 2**: the nickel-responsive regulator NikR, the iron-responsive regulator Fur and the peroxide stress regulator PerR. In **CHAPTER 5** it is shown that the PerR mediates iron- and peroxide-responsive regulation of the peroxide stress resistance determinant catalase (KatA) of *H. hepaticus*. Furthermore we

demonstrate that KatA modulates peroxide resistance and aerotolerance of *H. hepaticus*, and thus can be predicted to contribute to survival of the bacterium during transmission as well as during interaction with the mammalian immune system.

In addition to being exposed stress due to Ph and peroxide pathogenic bacteria are often confronted with antimicrobials. The importance of efflux pumps in the resistance of *H. hepaticus* to antimicrobial agents such as bile acids and antibiotics is analyzed in **Chapter 6.** Here it is demonstrated that *H. hepaticus* has a natural resistance to bile acids and to the antibiotic amoxicillin, and that resistance requires the HefA efflux pump. Bile acid resistance of *H. hepaticus* is essential, as it has been hypothesized that in order to reach the liver, *H. hepaticus* migrates from the intestines through the bile ducts. It has also been suggested that the bacteria contribute to the formation of cholesterol gallstones. **Chapter 7** describes a mechanism can lead to the development of gallstones as a result of *H. hepaticus* colonization. It is proposed in this chapter that the urease enzyme is involved in this process and concluded that urease activity together with the ability to survive the harmful environment of the bile duct, can lead to the development of gallstones in infected host.

Finally the research described in this thesis is summarized and discussed in **Chapter 8**. The overall conclusion states that *H. hepaticus* is an organism able to adapt to the specific stresses encountered in its natural niche, through a variety of mechanisms. This allows survival and growth in different niches and contributes to the longevity of *H. hepaticus* infection and to the development of diseases. The activities of iron-regulated enzymes like urease and catalase is essential for the survival of *H. hepaticus* when stressed. Finally, resistance to antimicrobials stress factors such as amoxicillin and bile can be overcome by *H. hepaticus* through the carriage of efflux pump gene *hefA*.

Chapter 2

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

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ABSTRACT

Helicobacter species are among the most successful colonizers of the mammalian gastrointestinal and hepatobiliary tract. Colonization is usually lifelong, indicating that _Helicobacter_ species have evolved intricate mechanisms of dealing with stresses encountered during colonization of host tissues, like restriction of essential metal ions. The recent availability of genome sequences of the human gastric pathogen _Helicobacter pylori_, the murine enterohepatic pathogen _Helicobacter hepaticus_ and the unannotated genome sequence of the ferret gastric pathogen _Helicobacter mustelae_ has allowed for comparitive genome analyses. In this review we present such analyses for metal transporters, metal storage and metal-responsive regulators in these three _Helicobacter_ species, and discuss possible contributions of the differences in metal metabolism in adaptation to the gastric or enterohepatic niches occupied by _Helicobacter_ species.

INTRODUCTION

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 (70). 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).

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 (70).

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 $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^-$).

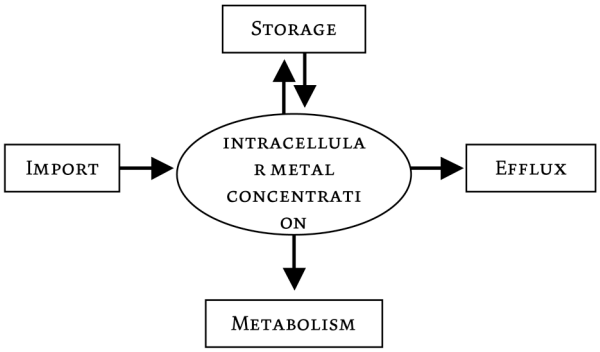

FIGURE 1. Schematic representation of the mechanisms involved in maintaining homeostasic control of the intracellular metal concentration via concerted action of import, efflux, storage and metabolism.

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, 54, 60, 81). Many of the components of oxidative stress defense are essential for gastric colonization by *H. pylori* in animal models (4, 37, 55), and are likely to be of similar importance to colonization of other *Helicobacter* species.

Helicobacter species can be subdivided into two major lineages (32, 61). 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 *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, 61). 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, 46, 61).

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, 66) and *H. hepaticus* (64) 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* (58) this has allowed for comparative genomics, and here we will review the current knowledge on metal metabolism and metal-responsive gene regulation of *H. pylori* and *H. hepaticus*, focussing on the role of the

metals iron, nickel and copper. We will also present hypotheses on metal-transport and metal-responsive regulation in *H. mustelae*, based on comparisons with *H. pylori* and *H. hepaticus*.

METAL-TRANSPORT AND STORAGE OF *HELICOBACTER* SPECIES

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 comparitive 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, ferric iron transport requires an outer membrane receptor to transport the iron over the outer membrane, as well as a ABC transporter which transports the iron from the periplasm to the cytoplasm. In contrast, ferrous iron is soluble and only requires a 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 display 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, 77), 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

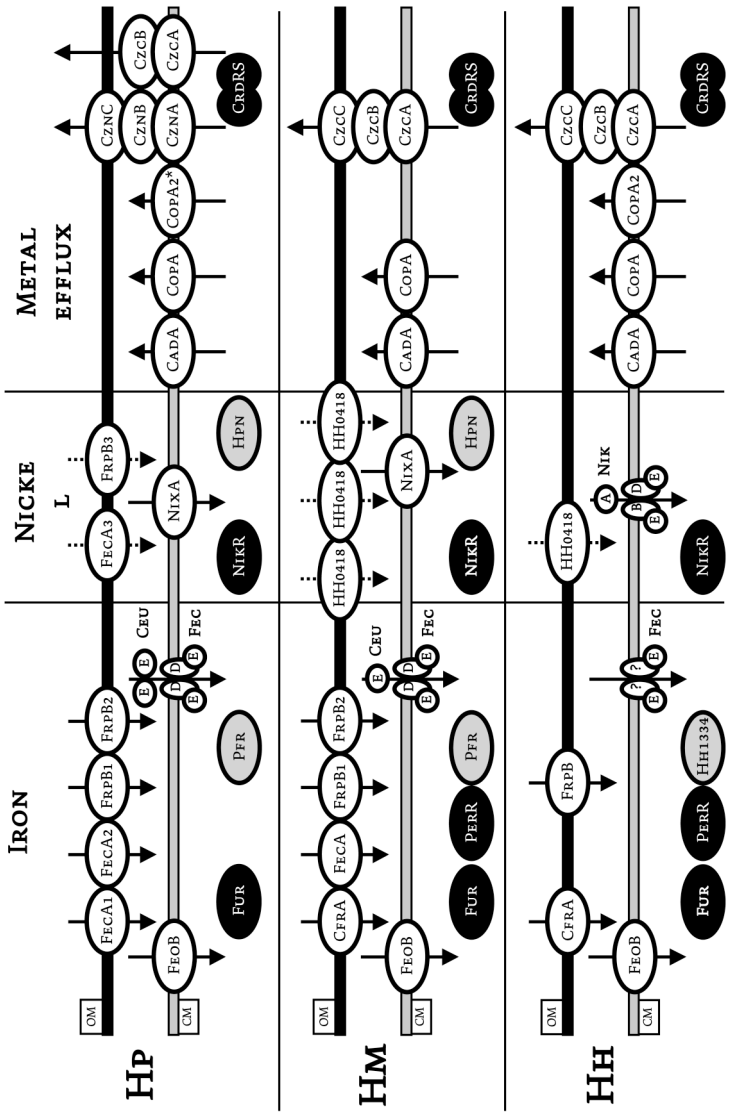

FIGURE 2. Comparison of iron-transporters, nickel-transporters, metal efflux systems, metal storage and metal-responsive regulatory systems of *Helicobacter pylori* (Hp, top), *Helicobacter mustelae* (Hm, middle) and *Helicobacter hepaticus* (Hh, bottom). The ion transported and the direction of transport 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.

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 (77). Rather surprisingly, this did not affect iron transport. Thus the contribution of ferric iron uptake 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 (77). FeoB-mediated iron acquisition is of major importance to *H. pylori*, as isogenic *feoB* mutants were unable to colonize the gastric mucosa of mice (77).

Two iron-storage proteins have been characterized in *H. pylori*, the Pfr ferritin and HP-NAP (Dps) bacterioferritin (Fig. 2) (14, 67). The Pfr ferritin serves as an intracellular iron deposit and protects *H. pylori* against intracellular iron toxicity and free iron-mediated oxidative stress (78). Iron stored in Pfr can be released and reused to support growth under iron-limited conditions (78). The HP-NAP protein is both homologous to bacterioferritins as well as to the DNA-binding proteins of the Dps family (67). 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 (63). 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, and mutation of the *fecA* gene significantly reduces growth when ferric citrate is used as sole iron source (Stoof & van Vliet, unpublished results). 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 (Fig. 2). The functions of the CfrA, FrpB and HH0418 orthologs are 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 (56). 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 (76). 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 (65). 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 (50). 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 (53). 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). Finally, two TonB-dependent outer membrane proteins (FecA3 [HP1400] and FrpB3 [HP1512, also called FrpB4]) have recently been shown to be regulated by nickel and the NikR-regulator (29), and may thus represent a novel type of nickel-transporters.

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 (42). 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 (51), 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 HH0418, which may fulfill a similar role as FecA3/FrpB3 in *H. pylori*. However, this awaits experimental validation. Finally, *H. mustelae* also expresses the histidine-rich Hpn protein (34), which is thought to function in nickel storage.

(iii) *Helicobacter hepaticus.* *H. hepaticus* expresses two nickel-cofactored enzymes, urease and the Ni/Fe hydrogenase (44, 47). 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 (52). 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 HH0418 protein may function in nickel uptake, although this hypothesis may be farfetched and awaits rigorous experimental validation.

<u>Copper</u>

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 (51). 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 (48, 79). 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 (79, 80), but a role in copper metabolism is not yet established.

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
hho418	-	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	-	-	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 (66).
b. Gene numbers from the genome sequence of *H. hepaticus* strain ATCC51449 (64).
c. Alternative designation *frpB4*.
d. The *H. pylori* Dps protein is also known as Neutrophil Activating Protein (HP-NAP).

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, 59), 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 (49). 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 an 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) (62). The latter system is also implicated in acid-resistance (possibly via nickel efflux) (62) and efflux of metronidazole (68).

(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, 64), the nickel-responsive regulator NikR (73) and the copper-responsive two-component regulatory system CrdRS (80). 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, 69), 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.

(i) Fur. The Fur protein is primarily known for its role as repressor of iron acquisition systems (35). It fulfills this classical function in all three *Helicobacter* species as well, as

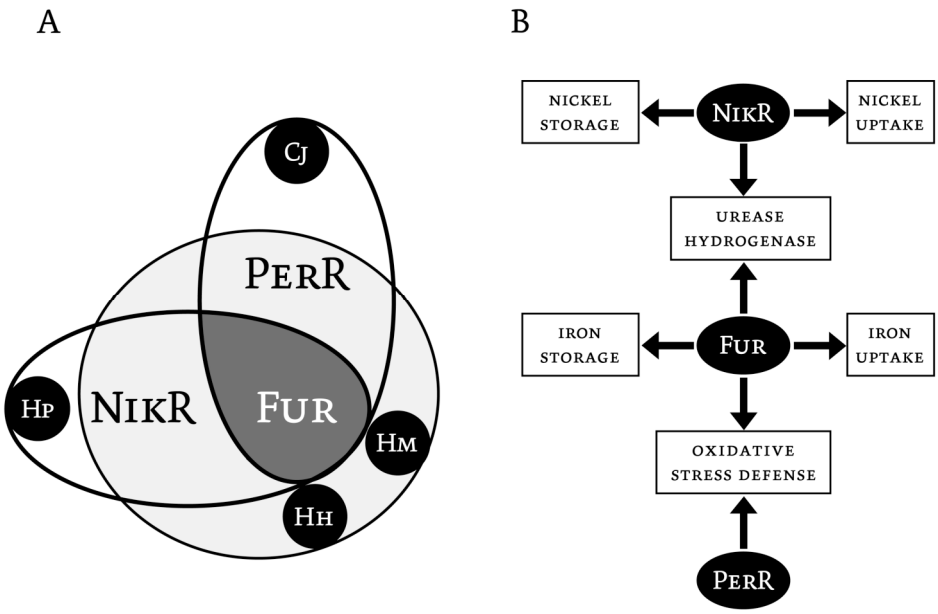

FIGURE 3. Distribution and overlapping functions of the Fur, NikR and PerR regulatory systems in *Helicobacter* and *Campylobacter* species. (A) Distribution of the regulators in *Helicobacter pylori* (Hp), *Helicobacter mustelae* (Hm), *Helicobacter hepaticus* (Hh) and *Campylobacter jejuni* (Cj). The 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*.

the *H. pylori* Fur mediates regulation of iron acquisition outer membrane proteins (24, 75). However, the Fur protein also regulates part of the nitrogen metabolism of *H. pylori*, via iron-responsive repression of the AmiE aliphatic amidase (74). 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 (45), 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, 69). 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 (69). 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* (71). 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, 72), 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, 73). A similar regulation of urease expression has been observed in *H. mustelae* (Stoof & van Vliet, unpublished results). 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 (79, 80). It is not yet known how the CrdRS system senses copper, but absence of either CrdR or CrdS disturbs regulation of Crd (80). The CrdRS system plays an important role in gastric colonization in a mouse infection model for *H. pylori* (57) and is also implicated in acid resistance of *H. pylori* (43). Whether *H. mustelae* and *H. hepaticus* contain 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.

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Chapter 3

Differential regulation of urease activity in *Helicobacter hepaticus* and *Helicobacter pylori*

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ABSTRACT

Helicobacter hepaticus **is a pathogen of rodents, which causes diverse enteric and hepatic inflammatory diseases and malignancies. The urease enzyme is an important colonization factor of gastric *Helicobacter* species like *Helicobacter pylori*, but little is known about the role and regulation of urease in enterohepatic *Helicobacter* species. Here it is reported that *H. hepaticus* urease activity does not contribute to acid-resistance, and that *H. hepaticus* urease activity is nickel-responsive at the post-translational level. *H. hepaticus* strain ATCC51449 did not grow or survive at pH 3.0, and supplementation with urea or $NiCl_2$ did not complement this acid-sensitivity. Furthermore urease enzyme activity of *H. hepaticus* was acid-independent, which contrasts with the acid-induced urease system of *H. pylori*. Nickel-supplementation of Brucella media resulted in a tenfold increase in urease activity in both *H. hepaticus* and *H. pylori*, but the maximum level of urease activity in *H. hepaticus* was still three- to fivefold lower when compared to *H. pylori* in the same conditions. The increase in urease activity of *H. hepaticus* was not associated with elevation of urease mRNA or protein levels. Inhibition of protein synthesis by chloramphenicol did not affect nickel-responsive induction of urease activity in *H. hepaticus*, and confirmed that nickel-induction occurs at the post-translational level, likely by activation of preformed apo-enzyme. In conclusion, both the role of the urease enzyme as well as the regulation of urease activity differ between the enterohepatic pathogen *H. hepaticus* and the gastric pathogen *H. pylori*.**

INTRODUCTION

Helicobacter hepaticus is a pathogen of rodents, and colonization with *H. hepaticus* may result enteric and hepatic inflammatory diseases and malignancies (11, 36). *H. hepaticus* has recently also been implicated to participate in the formation of cholesterol gallstones (18). Members of the genus *Helicobacter* are gram-negative, microaerophilic bacteria. While the genus is best known for the human gastric pathogen *Helicobacter pylori*, a large number of other species are classified (26). Based on their target organ, *Helicobacter* species are subdivided into gastric and enterohepatic lineages (26). Both enterohepatic and gastric *Helicobacter* species can colonize humans and a wide variety of animals, resulting in chronic infections and chronic inflammation. With gastric *Helicobacter* species, this can progress to gastric ulceration, atrophy, metaplasia and malignancies (5). Enterohepatic *Helicobacter* species like *H. hepaticus* have been less thoroughly studied, but depending upon the species are associated with inflammatory and proliferative bowel lesions, hepatitis, and in some cases, hepatic cancer (12, 13, 36). There is increasing evidence linking infection with *Helicobacter* species to liver cancer in humans (20, 21, 35), and *H. hepaticus* infection of mice may prove to be an excellent model for the study of hepatic carcinogenesis (27).

H. hepaticus expresses a nickel co-factored urease enzyme (4). Urease is a

multimeric, nickel-containing enzyme produced by many pathogenic and non-pathogenic bacteria (6). In *Helicobacter* species, the urease enzyme consists of UreA and UreB subunits (4). These are encoded by the *ureA* and *ureB* genes and are located in an operon, also containing the *ureI* gene (encoding a urea channel), and the *ureEFGH* genes encoding accessory proteins involved in enzyme activation (4, 6, 25). Active urease converts urea into ammonia and bicarbonate, and the ammonia produced is thought to mediate protection against acidic microenvironments, but may also serve as a nitrogen source (38).

In gastric *Helicobacter* species like *H. pylori* and *Helicobacter mustelae*, urease is an important virulence factor (2, 29). The ammonia produced is thought to allow protection against acidic environments, and mutants lacking urease are unable to colonize the gastric environments in different animal models (2, 29). In *H. pylori*, urease expression and enzyme activity is controlled by an intricate regulatory network, centered around the environmental pH and the availability of the cofactor nickel (24, 31). For *H. hepaticus* and other urease-positive enterohepatic *Helicobacter* species, the role of urease in metabolism or pathogenesis has not yet been established.

Recently differences between the urease and nickel transport genes of *H. hepaticus* and *H. pylori* were reported (4, 27), which suggested that there may be differences in regulation of urease expression and activity between these two *Helicobacter* species. Here it is demonstrated that urease activity in *H. hepaticus* is nickel-responsive, but solely at the post-translational level. In contrast to *H. pylori*, *H. hepaticus* urease activity is not induced at acidic pH, and *H. hepaticus* does not grow or survive at a pH 3.0. This suggests that the urease system is not a universal acid-resistance factor of ureolytic *Helicobacter* species, and that its functions may differ between gastric and enterohepatic *Helicobacter* species.

RESULTS

H. hepaticus is acid-sensitive and does not grow at acidic pH

In gastric *Helicobacter* species, urease activity is required for growth in acidic conditions and survival of acid shock. To determine whether *H. hepaticus* is able to grow at acidic conditions, *H. hepaticus* was resuspended in BBC of pH 7.0, 5.5 and 3.0 in the presence or absence of 100 μM $NiCl_2$ (Fig. 1a). There was no increase in OD_{600} at pH 3.0, indicating absence of growth. *H. hepaticus* was able to grow at pH 5.5, although the final OD_{600} values were slightly lower than at pH 7.0. Supplementation of media with $NiCl_2$ did not affect growth at pH 7.0 or 5.5, nor did it allow growth at pH 3.0 (Fig. 1).

To determine whether *H. hepaticus* is able to survive acid shock, *H. hepaticus* was resuspended in PBS of pH 7.0, 5.5 and 3.0 in the presence or absence of either 100 μM $NiCl_2$ or 0.5 mM urea, and incubated for 30 min at microaerobic conditions. Viability was not affected by incubation at pH 5.5 (Fig. S1). *H. hepaticus* was not able to survive incubation at pH 3.0, even in the presence of either $NiCl_2$ or urea (Fig. S1), suggesting that *H. hepaticus* is acid-sensitive, and that urease is not involved in acid-resistance of *H. hepaticus*.

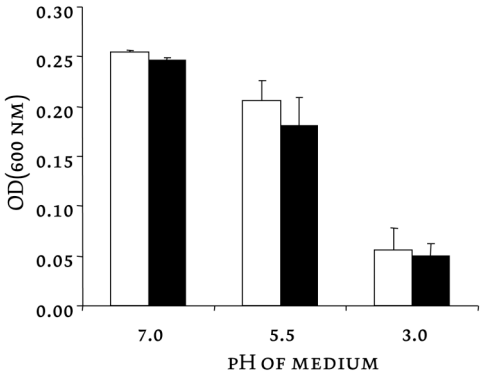

FIGURE 1. ***H. hepaticus*** **is acid-sensitive and does neither grow nor survive at pH 3.0.** ***H. hepaticus*** **ATCC51449 was grown for 24 h in BBC with a pH of 7.0, 5.5 or 3.0 in the absence (white bars) or presence (black bars) of 100 μM $NiCl_2$. Data points shown are the average of three independent experiments, error bars denote standard deviation.**

H. hepaticus urease activity is acid-independent, but nickel-induced

To determine the effect of medium pH on growth and urease activity, *H. hepaticus* was grown for 24 h in BBC media adjusted to pH 5.5, 6.0 and 7.0. These pH values were selected on the basis of their use in previous studies with *H. pylori,* as pH 5.5 and 6.0 are thought to resemble the pH of the mucus layer (31). pH 5.5 was the lowest pH value allowing growth of *H. hepaticus* (Fig. 1). The pH of the medium did not change significantly during 24 h growth. Urease activity in *H. hepaticus* was not significantly affected by the pH of the medium (Fig. 2A), while urease activity in the positive control *H. pylori* increased at pH 6.0 and pH 5.5 (Fig. 2B), consistent with earlier data (24, 31).

To determine the effect of nickel-supplementation of BBC medium on *H. hepaticus* urease activity, *H. hepaticus* was grown for 24 h in BBC media of pH 7.0 supplemented with $NiCl_2$ to a final concentration of 100 μM. Urease activity of *H. hepaticus* increased tenfold in nickel-supplemented BBC medium when compared to unsupplemented BBC medium (Fig. S2A). Likewise, urease activity of *H. pylori* grown with nickel-supplementation was also tenfold increased (Fig. S2B), as was reported previously (32). Overall, *H. hepaticus* urease activity was three- to fivefold lower than that of *H. pylori* in comparable conditions.

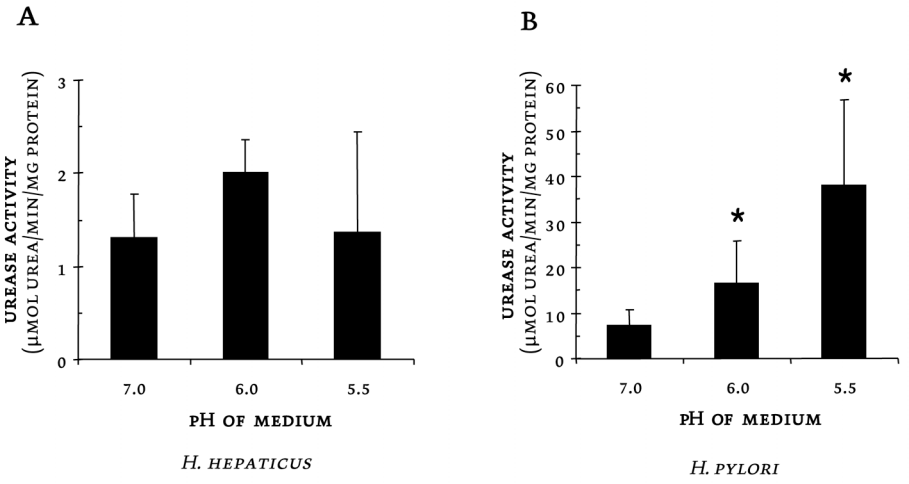

FIGURE 2. Urease activity is acid-independent but nickel-induced in *H. hepaticus*. Urease activity of *H. hepaticus* strain ATCC 51449 (A) and *H. pylori* strain 26695 (B) was assessed after 24 h growth in BBC medium with a pH of 7.0, pH 6.0 or pH 5.5. Note the difference in the scale of the y-axis. Each bar represents data from three to five independent experiments for each strain; error bars denote SD. Asterisks indicate a significant increase of urease activity in the test condition (pH 6.0 or 5.5) when compared to the standard growth conditions (pH 7.0) ($P \leq 0.05$, Mann–Whitney U test).

Nickel-responsive induction of urease activity in *H. hepaticus* is mediated at the post-translational level

To identify at which level nickel-responsive induction of urease activity in *H. hepaticus* was mediated, the effect of nickel on transcription of *H. hepaticus* urease genes and expression of urease protein was determined (Fig. 3). Nickel-supplementation of BBC medium did not affect transcription of either the *ureA* or *ureB* genes (Fig. 3A). Likewise, expression of the UreA and UreB proteins was not altered by nickel supplementation, as was shown for both UreA and UreB by Western immunoblot using antibodies to *H. felis* UreA and UreB protein (Fig. 3B). The Western blot data were independently confirmed using isogenic *ureB* mutant of *H. hepaticus* ATCC51449 (Fig. 3C). Next to an absence in UreB expression, this *ureB* mutant did not show significant changes in growth under the tested conditions, and did not have any detectable urease activity (data not shown).

The bacteriostatic antibiotic chloramphenicol blocks protein synthesis (14), and was used to confirm that nickel-responsive induction of urease activity in *H. hepaticus* is indeed mediated solely at the post-translational (enzyme activity) level. Supplementation of BBC medium with chloramphenicol to a final concentration of 20 µg ml^{-1} resulted in cessation of growth of *H. hepaticus*, but did not significantly affect cell viability as determined by colony counts (not shown). Addition of chloramphenicol

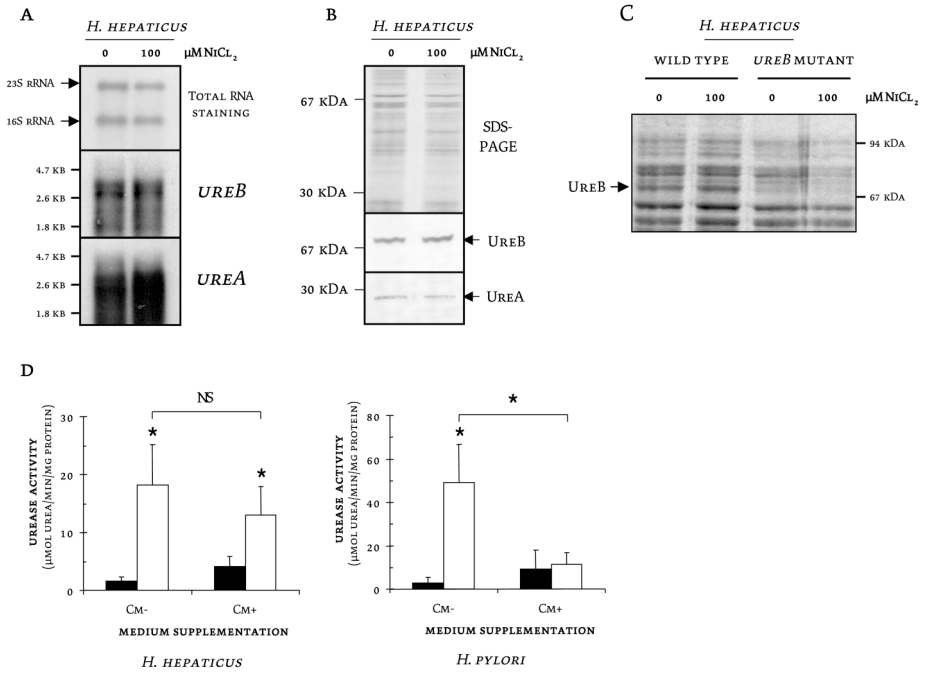

FIGURE 3. Nickel-responsive induction of urease activity in *H. hepaticus* is mediated at the post-translational level. (A) Analysis of *ureA* and *ureB* transcription using Northern hybridisation. Top panel, staining of total RNA with methylene blue; middle panel, visualisation of *ureB* mRNA; lower panel, visualisation of *ureA* mRNA. Relevant marker sizes are indicated on the left. (B) Comparison of UreB (middle panel) and UreA (lower panel) protein expression levels using Western immunoblot with antibodies raised against *H. felis* UreA and UreB protein. The top panel displays the total protein profile of the lysates used for immunoblot, stained using Coomassie Brilliant Blue. Relevant marker sizes are indicated on the left. (C) Comparison of UreB protein expression levels using SDS-PAGE followed by Coomassie Brillant Blue staining. Total protein of *H. hepaticus* ATCC51449 wild-type strain and isogenic *ureB* mutant, respectively, grown in unsupplemented BBC medium (Lanes 1 and 3) and BBC medium supplemented with 100 μM $NiCl_2$ (Lanes 2 and 4). Lanes 3 and 4 are included to facilitate identification of the UreB protein (indicated by an arrow). Relevant marker sizes are indicated on the right. (D) Effect of the protein synthesis inhibitor chloramphenicol on nickel-responsive induction of urease activity in *H. hepaticus* (left panel) and *H. pylori* (right panel). Activity of urease was assessed after 24 h of growth in unsupplemented BBC medium (black bars) and BBC medium supplemented with $NiCl_2$ to a final concentration of 100 μM (white bars). Cells were either grown in the absence of chloramphenicol (-Cm) or in the presence of 20 μg ml^{-1} chloramphenicol (+Cm). Data are from three to five independent experiments; error bars denote standard deviation. Asterisks indicate statistically significant increase in urease activity in nickel-supplemented versus unsupplemented medium ($P \leq 0.05$, Mann-Whitney U test).

did also not affect nickel-responsive induction of urease activity of *H. hepaticus* when compared to cultures grown in BBC medium without chloramphenicol (Fig. 3D), demonstrating that this induction does not require protein synthesis. In contrast, chloramphenicol supplementation almost completely abolished nickel-responsive induction of urease activity in *H. pylori* (Fig. 3D), consistent with the requirement for additional urease protein synthesis in nickel-responsive induction of urease activity (32).

Nickel-responsive induction of urease activity in *H. hepaticus* is growth phase-independent

The effect of growth phase on nickel-responsive induction of urease was determined for *H. hepaticus* and *H. pylori* (Fig. 4). Nickel supplementation did not significantly affect the growth of *H. hepaticus* or *H. pylori*, and both *Helicobacter* species reached late-log phase after 24 h (Fig. 4A). *H. hepaticus* already reached maximum urease activity at approximately 12 h of growth in nickel-supplemented media (Fig. 4B). In contrast, urease activity in *H. pylori* increased steadily in nickel-supplemented cultures, and reached its maximum at approximately 18 h of growth, consistent with the requirement for transcription and translation of extra urease protein for increased urease activity during this period of time (Fig. 4B). This indicates that in *H. hepaticus* urease activity peaks during early log-phase, further supporting the hypothesis that nickel-induction of urease activity is mediated by enzyme activation rather than increased expression of urease enzyme.

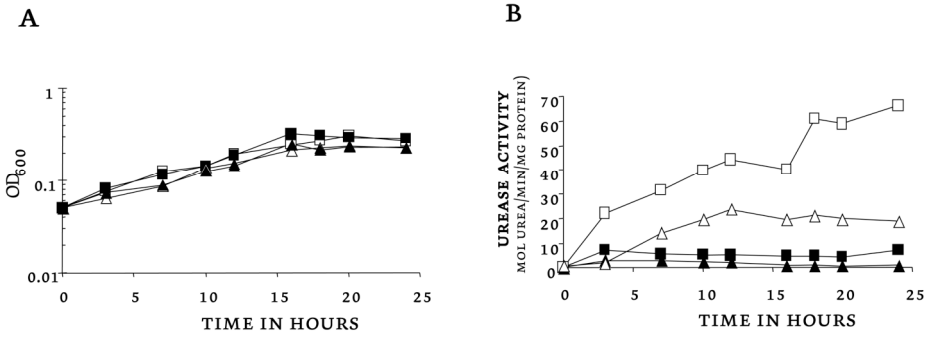

FIGURE 4. Effect of growth phase on nickel-responsive induction of urease activity in *H. hepaticus* and *H. pylori*. (A) Growth curves of *H. hepaticus* and *H. pylori* in unsupplemented and nickel-supplemented BBC medium monitored over a time period of 24 hours. *H. hepaticus* (triangles) and *H. pylori* (squares) were grown in unsupplemented BBC medium (solid symbols) and in BBC medium with 100 μM $NiCl_2$ (open symbols). (B) Urease activity of *H. hepaticus* and *H. pylori* grown in unsupplemented and nickel-supplemented BBC medium monitored over a period of 24 hours. *H. hepaticus* (triangles) and *H. pylori* (squares) were grown in unsupplemented BBC medium (solid symbols) and in BBC medium with 100 μM $NiCl_2$ (open symbols). Data points shown are the average of two to eight independent experiments per timepoint.

DISCUSSION

The nickel containing enzyme urease is an important virulence factor of many bacterial pathogens (6). All gastric *Helicobacter* species known to date are urease-positive, and this urease activity is thought to be essential for their ability to colonize the gastric environment. In contrast, only a third of enterohepatic *Helicobacter* species are urease-positive, but are still unable to colonize the gastric environment (26), with the exception of *Helicobacter muridarum* (16). Thus it is likely that not just the presence of urease activity, but also the absolute level of urease activity or its regulation may be required to allow gastric colonization. While the operon encoding the *H. hepaticus* urease enzyme was previously described (4), to our knowledge the role of the urease enzyme in *H. hepaticus* and other enterohepatic *Helicobacter* species has not yet been reported.

In this study it has been demonstrated that *H. hepaticus* is acid-sensitive, and that addition of urea does not increase survival of *H. hepaticus* at pH 3.0 (Fig. 1). In contrast, addition of urea does allow gastric *Helicobacter* species like *H. pylori* to survive such acidic conditions. Taken together, this suggests that the urease enzyme of *H. hepaticus* is not involved in acid-resistance. Consistent with this was the lack of acid-responsive induction of urease activity in *H. hepaticus* (Fig. 2A). However, urease activity in *H. hepaticus* is nickel-responsive, but this regulation is mediated solely at the post-translational level, probably through the activation of preformed apo-enzyme (Fig. 3, 4). During revision of this manuscript, nickel-responsive induction of *H. hepaticus* urease activity was confirmed in an independent study (19), indicating that the observed nickel-induction of *H. hepaticus* urease activity is not due to the conditions employed in our study.

Despite overall similarities of *H. hepaticus* and *H. pylori* urease gene clusters, there are notable differences. Both urease gene clusters consist of two structural genes (*ureAB*) and five accessory genes (*ureIEFGH*) (4), but the *ureB-ureI* intergenic distance of *H. hepaticus* is much shorter (9 bp) than that of *H. pylori* (200 bp). This indicates that in *H.hepaticus* there is probably no promoter directly upstream of the *ureI* gene, thus limiting the possibilities of transcriptional and post-transcriptional regulation (1, 32). Furthermore, the overall amino acid sequence of UreI in both species is well conserved, but several amino acid residues which were shown to be required for acid-activation of the *H. pylori* UreI urea channel are absent in the *H. hepaticus* UreI protein (37). This is consistent with the extragastric lifestyle of *H. hepaticus* and its inability to grow or survive at a pH of 3.0 (Fig. 1). Whether this phenotype is due to the lack of acid-responsive regulation of urease activity, absence of a *ureI* promoter or absence of the acid-activation of the UreI urea channel remains to be established.

Enzymatic degradation of urea by urease results in the production of ammonia and bicarbonate, and both reaction products are thought to play an important part in bacterial pathogenesis. Ammonia serves as nitrogen source and may function in acid resistance (6, 24, 38), whereas bicarbonate may also function in acid resistance as well as in modulating the immune system of the host (15, 17). Expression of urease in bacteria is controlled by different stimuli, such as urea availability, environmental pH, nitrogen status of the cell, or growth phase (6).

In this study it has been demonstrated that urease activity in *H. hepaticus* is nickel-responsive. This form of urease regulation has to date only been identified for two other urease systems, of *H. pylori* (32) and *Streptococcus salivarius* (9). The enzymatic activity of *H. hepaticus* urease is induced by nickel-supplementation of the growth medium, but this induction is mediated solely at the post-translational level (Fig. 3), probably by activation of urease apo-enzyme, as was previously described for *S. salivarius* (9). In contrast, the *H. pylori* urease system is nickel-induced at the enzyme activity and transcriptional level (32). The nickel- and acid-responsive transcriptional regulation of urease observed in *H. pylori* may be a specific adaptation to the gastric lifestyle. This suggests that the proposed link in *H. pylori* between intracellular nickel availability and environmental pH (30) may not be universal in the genus *Helicobacter*.

In *H. pylori*, regulation of urease activity is dependent on nickel and the NikR regulatory protein (33). Mutants lacking the NikR regulators are unable to colonise the murine gastric mucosa (8). A NikR homolog is also present in *H. hepaticus* (27), and it can be envisaged that NikR can indirectly affect urease activity via regulation of nickel uptake. In *H. pylori*, NikR regulates NixA-mediated nickel-uptake (10), and while the *H. hepaticus* genome sequence does not contain a *nixA* ortholog (27), it does contain a putative nickel-uptake ABC transporter located adjacent to the urease operon (4). However, up to now the *H. hepaticus nikR* gene has proven refractory to insertional mutagenesis (C.B. and A.H.M.v.V., unpublished results), and thus a role of NikR in regulation of urease expression of *H. hepaticus* cannot be established yet.

In conclusion, *H. hepaticus* is acid-sensitive and lacks regulatory mechanisms mediating acid- and nickel-responsive regulation of urease expression. These may contribute to its extragastric niche, and may be directly linked with the animal host colonised and/or the respective target organ. We hypothesise that enterohepatic *Helicobacter* species do not require high levels of urease activity in the rodent gut. The high levels of urease activity observed in gastric *Helicobacter* species (7, 23) are likely to be an adaptation that allows them to thrive in the gastric environment, albeit at a high metabolic cost. Regulation of urease expression and activity will allow them to adapt to changes in acidity observed during fasting or feeding. In contrast, urease-positive enterohepatic *Helicobacter* species might use their urease system for nitrogen metabolism, securing a constant supply of ammonia. The diversity in urease function and regulatory responses in *Helicobacter* species are a prime example of adaptation required for chronic colonisation of host tissues.

MATERIAL & METHODS

Bacterial strains, plasmids and growth conditions

H. hepaticus strain ATCC51449 (11) and its isogenic *ureB* mutant (this study), and *H. pylori* strain 26695 (28) were routinely cultured at 37 ℃ in microaerobic conditions (10% CO_2, 5% O_2 and 85% N_2) on Dent agar (Biotrading, Mijdrecht, The Netherlands) (32). Liquid growth was performed in Brucella broth (Difco, Sparks, MD, USA) supplemented with 0.2% (w/v) -cyclodextrins (Fluka, Buchs, Switserland) (BBC). Bacteria were inoculated at a starting OD_{600} of 0.05. The pH of liquid media was adjusted from pH 7.0

to 6.0, 5.5 or 3.0 using HCl and did not change more than 0.5 pH unit during the experiments. $NiCl_2$ was purchased from Sigma, filter sterilised, and used at a final concentration of 100 μM. *Escherichia coli* ER1793 was grown aerobically in Luria-Bertani medium (22) at 37 °C. When indicated, growth media were supplemented with chloramphenicol to a final concentration of 20 μg ml^{-1}.

Acid shock survival

H. hepaticus was grown for 24 hours in BBC at pH 7.0 at a starting OD_{600} of 0.05. Subsequently, bacteria were harvested by centrifugation for 10 minutes at 4000 *g* and resuspended to a final OD_{600} of 0.2 in PBS adjusted to pH 7.0, 5.5 or 3.0. When indicated, PBS was supplemented with $NiCl_2$ or urea to a final concentration of 100 μM and 0.5 mM, respectively. Cells were incubated for 30 minutes at 37 °C in microaerobic conditions. Subsequently 5 μl of tenfold dilutions were spotted on Dent agar. Plates were incubated for two days at 37 °C under microaerobic conditions and the presence or absence of growth at the spots was assessed by visual inspection (3). Differences in the highest dilution still containing growth between different conditions indicated difference in survival rates.

Urease enzyme assay

Urease enzyme activity was determined in freshly sonicated lysates by measuring ammonia production from hydrolysis of urea, as described previously (32). The concentration of ammonia in the samples was inferred from a standard NH_4Cl concentration curve. Enzyme activity was expressed as micromoles of urea substrate hydrolysed per minute, per milligram protein.

RNA analysis

RNA was isolated from *H. hepaticus* using Trizol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Gel electrophoresis of RNA, transfer to positively charged nylon membranes (Roche), crosslinking, hybridisation to DIG-labelled specific RNA probes and detection of bound probe was performed as described previously (32). Probes specific for *H. hepaticus ureA and ureB* were synthesised by *in vitro* transcription using T7 RNA polymerase (Roche) and PCR products obtained with primers HhUreA-F1 (5'-TGCATTATGCTGGGGCACTA) and HhUreA-R1-T7 (5'-ctaatacgactcactatagggaga ATAGGTCTATCGCCCTTATG), HhUreB-F1 (5'-TGGTAAAGCGGGAAATCCAG), and HhUreB-R1 T7 (5'-ctaatacgactcactatagggagaGTGTGCAGGTAGTAGCGTTTG). Lowercase letters indicate the T7 promoter used for *in vitro* transcription.

Construction of an *H. hepaticus ureB* mutant

The *ureB* gene of *H. hepaticus* strain 51449 was amplified using primers HhUreB-mutF1 (5'-TCCTGTGCCTCCACCAAT) and HhUreB-mutR1 (5'-GCATTATGCTGGGGCACT), and cloned in pGEM-T_{easy} (Promega), resulting in plasmid pJS10. The *ureB* gene was subsequently interrupted by insertion of the chloramphenicol resistance gene from pAV35 (34) in the unique *Bsm*I site, resulting in plasmid pJS11. This plasmid was introduced into *E. coli* ER1793 and subsequently used for natural

transformation of *H. hepaticus* 51449. Chloramphenicol-resistant colonies isolated were designated 51449*ureB*. Two colonies derived from independent transformations were tested, and both colonies gave identical results in all experiments. Correct allelic replacement of the wild type *ureB* gene with the interrupted version was confirmed by PCR.

Protein analysis

H. hepaticus wild-type and *ureB* mutant cells were grown for 24 h in unsupplemented BBC medium or BBC medium supplemented with 100 μM $NiCl_2$, centrifuged at 4000 *g* for 10 min at room temperature and resuspended in PBS pH 7.4 to a final OD_{600} of 10. *H. hepaticus* cells were subsequently lysed by sonication for 15 sec on ice, using a MSE Soniprep 150 (MSE, Crawley, UK) at amplitude 6. The protein concentration of lysates was determined using the bicinchoninic acid method (Pierce) using bovine serum albumin as standard. Proteins were separated by SDS-PAGE on a 10% (w/v) polyacrylamide gel and stained with Coomassie Brilliant Blue. Westernblots were performed by electrotransfer of proteins onto nitrocellulose membrane (Roche). The blot was probed with antibodies raised in rabbits to *Helicobacter felis* UreA or UreB (Intervet International BV; Boxmeer, Netherlands). Bound antibodies were visualised with swine anti-rabbit antibodies labelled with alkaline phosphatase (Promega), using BCIP and NBT (Promega) as substrate.

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SUPPLEMENTARY DATA

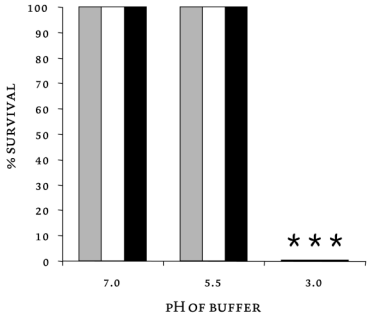

FIGURE S1. ***H. hepaticus*** **does not survive acid shock.** ***H. hepaticus*** **ATCC 51449 was incubated for 30 min in PBS of pH 7.0, pH 5.5 and pH 3.0 in either unsupplemented PBS (grey bars),PBS with 0.5 mM urea (white bars), or PBS with 100 µM Ni Cl2 (black bars). Asterisks at pH3.0 indicate that there was no survival of** ***H. hepaticus*** **at this condition. Data points shown are the mean of three independent experiments.**

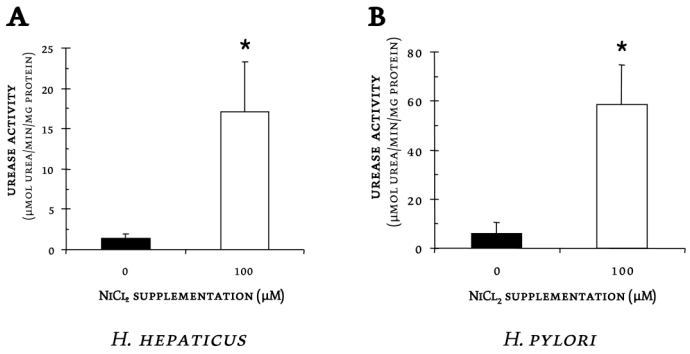

FIGURE S2. Urease activity is nickel-induced in ***H. hepaticus.*** **Urease activity of** ***H. hepaticus*** **strain ATCC 51449 (A) and** ***H. pylori*** **strain 26695 (B) was assessed after 24 h of growth at pH 7.0 in unsupplemented BBC medium (black bars) and BBC medium supplemented with NiCl2 to a final concentration of 100 µM (white bars). Note the difference in the scale of the** ***y*****-axis. Each bar represents data from three to five independent experiments for each strain; error bars denote standard deviation. Asterisks indicate a significant increase of urease activity in the test condition (100 µM Ni Cl2) when compared to the standard growth conditions (unsupplemented medium) (*P*≤0.05, Mann-Whitney U test).**

Chapter 4

Iron-responsive repression of urease expression in *Helicobacter hepaticus* is mediated by the transcriptional regulator Fur

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ABSTRACT

Persistent colonization of mucosal surfaces by bacteria in the mammalian host requires concerted expression of colonization factors, depending on the environmental conditions. *Helicobacter hepaticus* is a urease-positive pathogen which colonizes the intestinal and hepatobiliary tract of rodents. Here it is reported that urease expression of *H. hepaticus* is iron-repressed by the transcriptional regulator Fur. Iron-restriction of growth medium resulted in a doubling of urease activity in wild-type *H. hepaticus* strain ATCC51449 and was accompanied by increased levels of urease subunit proteins and *ureA* mRNA. Insertional inactivation of the *fur* gene abolished iron-responsive repression of urease activity, whereas inactivation of the *perR* gene did not affect iron-responsive regulation of urease activity. The iron-responsive promoter element was identified directly upstream of the *H. hepaticus ureA* gene. Recombinant *H. hepaticus* Fur protein bound to this *ureA* promoter region in a metal-dependent matter, and binding resulted in the protection of a 41 bp, Furbox-containing operator sequence located at positions -35 to -75 upstream of the transcription start site. In conclusion, *H. hepaticus* Fur controls urease expression at the transcriptional level in response to iron-availability. This represents a novel type of urease regulation in ureolytic bacteria, and extends the already diverse regulatory repertoire of the Fur protein.

INTRODUCTION

Urease is considered to be an important colonization factor for many pathogenic bacteria (8). The urease enzyme catalyzes the hydrolysis of urea into bicarbonate and ammonia. Both ammonia and bicarbonate are thought to contribute to acid-resistance (27), whereas ammonia may also aid in injury of the surrounding epithelial cells (40) and serves as nitrogen source (52). In most bacterial species expression of urease is strictly regulated in response to environmental or intracellular stimuli, like substrate availability, growth phase, nitrogen status or pH (1, 8, 11, 33).

Helicobacter species colonize the mammalian gastrointestinal and hepatobiliary tract resulting in chronic inflammation, which may progress to ulceration and carcinogenesis (21, 25). The murine pathogen *Helicobacter hepaticus* colonizes the intestine, bile ducts and liver, and this can result in hepatitis, hepatic malignancies and possibly cholesterol gallstones (22, 29, 50, 51). The urease enzyme is an important virulence factor in gastric *Helicobacter* species like *Helicobacter pylori* and *Helicobacter mustelae* (2, 15, 34), but a contribution of urease in the development of *H. hepaticus*-associated disorders is yet to be demonstrated. We have previously demonstrated that urease expression in *H. hepaticus* is neither nickel- nor pH-responsive, nor growth phase-regulated (5). This contrasts with the situation in *H. pylori*, where urease expression is controlled by nickel and pH at the transcriptional, post-transcriptional and translational level (1, 39, 43, 44). Although urease expression in *H. hepaticus* is lower than in *H. pylori* (5, 30), it is still relatively high when compared to many other urease-positive pathogens (10). In view of the metabolic cost of urease

expression and the importance of the enzyme in other bacteria, we hypothesized that urease expression in *H. hepaticus* is also regulated, but may be responsive to a different environmental signal.

Iron is an important micronutrient and required by almost all organisms (37). Iron is used as a co-factor of enzymes and participates in redox reactions. In the mammalian host, iron is mostly complexed into host proteins like hemoglobin and ferritin, whereas at mucosal surfaces, iron availability is restricted due to chelation of iron by lactoferrin (37). Conversely, pathogenic bacteria often use iron-restriction as environmental cue for the expression of virulence factors like hemolysins, toxins and iron-acquisition systems (38). Many of these iron-responsive virulence factors are under the control of the Ferric Uptake Regulator (Fur) protein. Fur acts as an transcriptional repressor of iron-regulated promoters via iron-dependent DNA-binding activity (20). In iron-replete conditions, Fur complexes with Fe^{2+} can recognize and bind to operator sequences (Fur boxes) in promoters. When iron is scarce Fur will dissociate from the DNA, allowing access of RNA polymerase to the promoter and transcription of the iron-regulated genes. The functions of Fur even extends outside iron metabolism, as the protein has also been reported to regulate oxidative stress resistance, acid-resistance and virulence factors, suggesting a key role for this protein in chronic colonization by pathogenic bacteria (7, 14, 16, 17, 23, 28).

In this study it is demonstrated that *H. hepaticus* uses iron as a signal for the regulation of urease expression, and that this iron-responsive regulation of urease is mediated by the binding of the Fur protein to an operator sequence in the *H. hepaticus ureA* promoter. To our knowledge, this is the first demonstration of a direct role of Fur and iron in regulation of urease expression, and thus this represents a novel function for Fur in regulation of metabolic pathways and colonization factors.

RESULTS

Induction of *H. hepaticus* urease expression and activity by iron-restriction

The effect of iron on *H. hepaticus* growth was determined by growing bacteria in BBC medium (standard conditions) or BBC medium supplemented with either deferoxamine mesylate (iron-restricted conditions) or deferoxamine mesylate and 100 μM $FeCl_3$ (iron-replete conditions). Iron-restriction resulted in reduced growth of *H. hepaticus* and cells entered late log-phase between 16 and 24 hours of growth (Fig. 1A). In contrast, growth in standard conditions and in iron-replete conditions saw cells in late-log phase after approximately 24 hours. At 24 h cells were still viable and examination of cells by gram-staining indicated that after 24 h in iron-restricted and iron-replete conditions the cultures still contained >95% of bacillary forms of *H. hepaticus*. Changes in gene expression after 24 hours are therefore likely to be a result of changes in iron-availability rather than growth phase-dependent (16, 31), and thus we decided to use the 24 h time point for phenotypic analyses.

Growth of *H. hepaticus* in iron-restricted conditions resulted in a twofold increase in urease activity when compared to cells grown in iron-replete conditions (Fig. 1B).

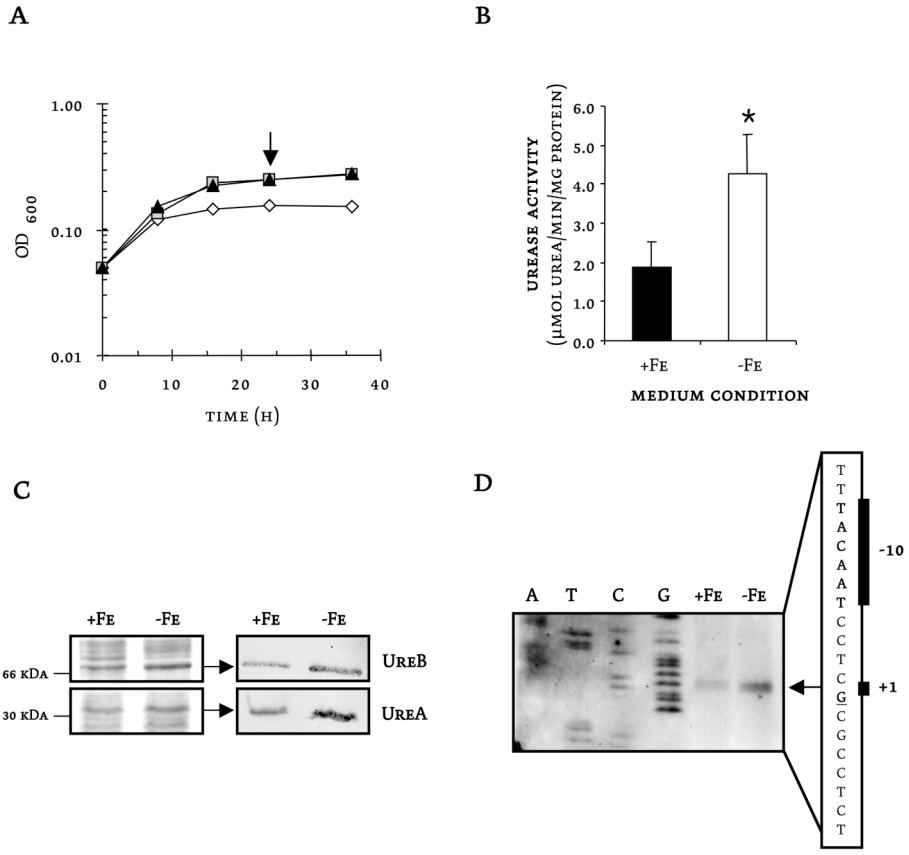

FIGURE 1. Induction of *H. hepaticus* urease transcription, expression and activity induced at iron-restricted conditions. (A) *H. hepaticus* ATCC51449 was grown in standard BBC medium (grey squares), iron-restricted medium (white diamonds) and iron-replete medium (black triangles) and the OD_{600} was monitored over a time period of 36 hours. The arrow indicates the 24 h time point representing late log phase, which was chosen for comparison of iron-restricted and iron-replete conditions. (B) Urease activity in *H. hepaticus* is iron-repressed, as measured after 24 h growth in iron-restricted medium (white bars) or iron-replete medium (black bars). Each bar represents data from seven independent experiments, error bars denote standard deviation. An asterisk indicate a significant increase of urease activity after growth at iron-resticted conditions when compared to growth iron replete conditions ($P < 0.05$, Mann-Whitney U test). (C) The increase in *H. hepaticus* urease activity in iron-restricted conditions is associated with increased expression of the UreA and UreB enzyme subunits, as shown by SDS-PAGE (left panel) and immunoblotting (right panel) with antibodies to *H. felis* urease. The left panel displays the relevant section of the protein profile of the lysates used for immunoblots, stained using Coomassie Brilliant Blue. Relevant marker sizes are indicated on the left. (D) Identification of the *H. hepaticus ureA* transcription start site by primer extension analysis, using RNA purified from *H. hepaticus* ATCC51449 grown in iron-restricted (-Fe) and iron-replete (+Fe) conditions. The sequence of the respective region is displayed on the left, with the +1 residue and the -10 promoter sequence indicated. Please note that the primer extension product displays iron-responsive repression of transcription.

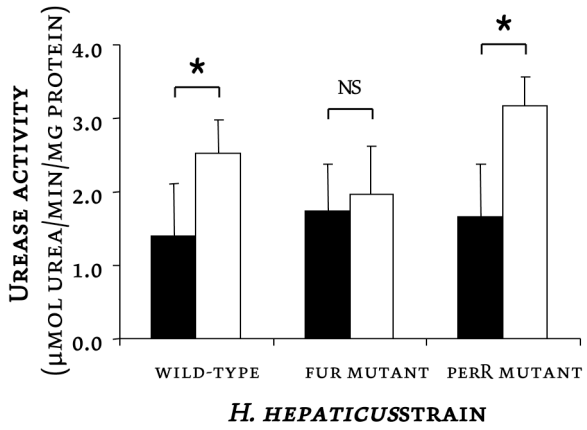

Figure 2. Fur but not PerR mediates iron-responsive regulation of urease expression in *H. hepaticus*. Urease activity of *H. hepaticus* strain ATCC51449 and *fur* and *perR* mutants was assessed after 24 h of growth in iron-restricted medium (white bars) or iron-replete medium (black bars). Each bar represents data from three independent experiment for each strain, error bars denote standard deviation. Asterisks indicate a significant increase of urease activity after growth at iron-restricted conditions when compared to growth iron replete conditions ($P \leq 0.05$, Mann-Whitney U test, NS = not significant).

This iron-responsive urease activity resulted from increased expression of urease subunit proteins UreA and UreB, as was demonstrated by SDS-PAGE and immunoblot assay using both UreA and UreB antibodies (Fig. 1C). These findings contrast with nickel-induction of urease expression in *H. hepaticus*, as this is mediated solely by increased enzyme activity without additional UreA or UreB protein expression (5). Induction of urease activity and expression by iron-restriction was mediated at the transcriptional level and mediated from the *ureA* promoter, as was shown by primer extension analysis (Fig. 1D). The transcription start site of the *ureA* gene was identified to be the G residue 47 bp upstream of the *ureA* ATG start codon (Fig. 1D). There was only a single transcription start site, which displayed iron-responsive repression (Fig. 1D) similar to the regulation of expression and activity of *H. hepaticus* urease (Fig. 1B, C). Upstream of the transcription start site there is a TACAAT hexamer sequence identical to the -10 sequence of the *H. pylori ureA* promoter (12), which suggests that the *H. hepaticus* urease cluster is transcribed via the σ^{80} sigma factor. Further comparison of the *H. hepaticus ureA* promoter sequence with that of *H. pylori* indicated the absence of the recently identified NikR binding sequence (13, 18, 19), consistent with the absence of nickel-responsive transcription of the urease genes in *H. hepaticus* (5).

Fur but not PerR mediates iron-responsive regulation of urease expression in *H. hepaticus*

Since iron-responsive regulation of urease expression is mediated at the transcriptional level, this suggested the involvement of an iron-responsive regulatory protein. The *H. hepaticus* genome encodes two orthologs of such regulators: the peroxide stress regulator PerR (HH0942) and the iron-responsive regulator Fur (HH0893) (41). Both genes are predicted to be transcribed as a monocistronic mRNA (41), and thus mutation of these genes is unlikely to result in polar effects on surrounding genes. To determine whether Fur or PerR were involved in the iron-responsive regulation of urease expression, isogenic *H. hepaticus fur* and *perR* mutants were created by insertion of a chloramphenicol resistance gene.

Mutation of either the *perR* or *fur* gene did not significantly affect growth of *H. hepaticus*, since growth of the mutants was similar to that of the wild-type strain in both iron-restricted and iron-replete conditions (data not shown). Mutation of the *perR* gene did not affect iron-responsive regulation of urease activity and the resulting urease activities were similar to that of the wild-type strain (Fig. 2), while mutation of *fur* abolished regulation of urease activity at the tested conditions (Fig. 2). It should be noted that urease activity in the *fur* mutant does not show complete derepression when compared to the wild-type strain in iron-restricted conditions, and thus it is possible that other regulatory mechanisms play a role in urease regulation in *H. hepaticus*.

Fur binds to the *H. hepaticus ureA* promoter in a metal dependent matter

To determine whether the Fur-mediated regulation of urease transcription in *H. hepaticus* is direct or indirect, the *H. hepaticus* Fur protein was expressed as recombinant protein in *E. coli*. Recombinant *H. hepaticus* Fur protein was mixed with DIG-labeled *ureA* promoter, and tested by electrophoretic mobility shift assay in the presence and absence of metal. Fur displayed concentration- and metal-dependent binding to the *ureA* promoter region of *H. hepaticus*, since the *ureA* promoter fragment shifted in the presence of Fur and $MnCl_2$ (Fig. 3, top panel), whereas this shift was not detected in the presence of Fur and EDTA (Fig. 3, middle panel). As a negative control, Fur protein was also incubated with the promoter region of the *ksgA* gene of *H. hepaticus* in the presence of metal, and this did not result in a shift of the DNA fragment (Fig. 3, lower panel), indicating that binding of Fur to the *ureA* promoter is sequence-specific.

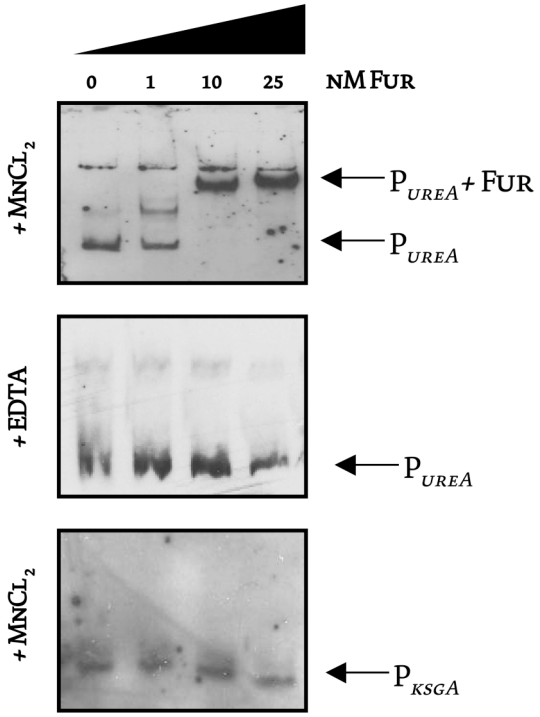

FIGURE 3. ***H. hepaticus*** **Fur displays metal-dependent binding to the** ***ureA*** **promoter. Electrophoretic mobility shift assay using recombinant** ***H. hepaticus*** **Fur protein and DIG-labeled** ***ureA*** **promoter DNA (P_{ureA}). In the absence of the iron-substitution manganese (+EDTA; middle panel), Fur is unable to complex with the** ***ureA*** **promoter region, and a shift is not observed. Only in the presence of manganese (+$MnCl_2$, top panel), Fur is able to bind to the** ***ureA*** **promoter region and cause a mobility shift (indicated as Fur-P_{ureA} complex). No shift is observed when the Fur protein is incubated with the promoter region of the** ***ksgA*** **gene (P_{ksgA}) in the presence of manganese (+$MnCl_2$, bottom panel). The concentration of Fur in nM is indicated above the lanes, the concentration of DNA was 0.5 nM** ***ureA*** **or** ***ksgA*** **promoter DNA.**

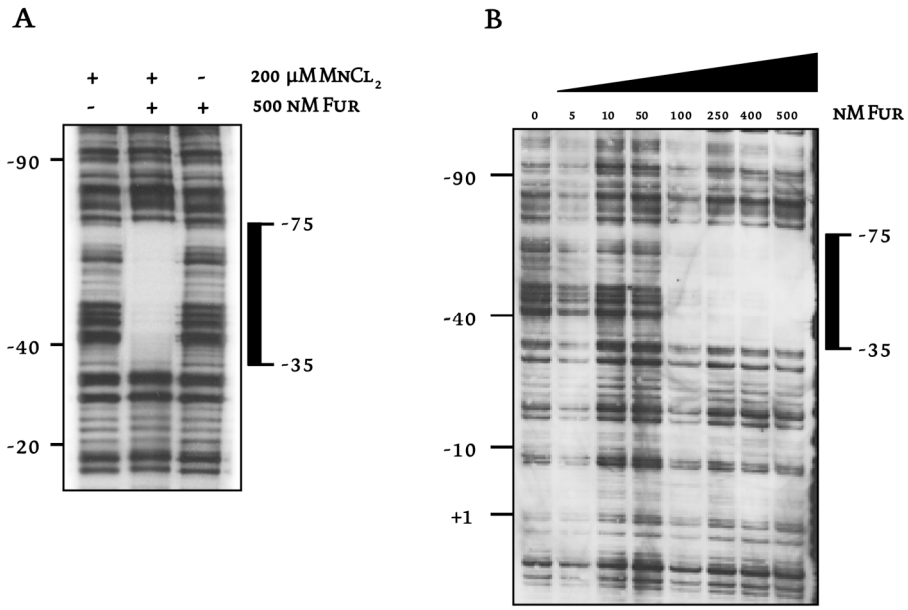

FIGURE 4. Identification of the Fur operator sequence in the *H. hepaticus* ATCC51449 *ureA* promoter. (A) DNase I footprinting assay performed in the presence and absence of the iron substitute $MnCl_2$, using 50 nM DIG-labeled *H. hepaticus* ATCC51449 *ureA* promoter DNA and 500 nM Fur. On the left side are indicated the positions relative to the *ureA* transcription start site. On the right hand side is indicated the position of the protected region (located from -35 to -75 relative to the transcription start site). Note that a protected region is only observed in the presence of both Fur and $MnCl_2$. (B) Binding of the *ureA* promoter DNA is dependent on the Fur concentration. Increasing concentrations of Fur were mixed with 20 nM DIG-labeled *H. pylori* ATCC51449 *ureA* promoter in the presence of 200 µM $MnCl_2$. The concentration of Fur protein used is indicated above the lanes. Positions relative to the *ureA* transcription start site are indicated on the left, the protected region is indicated on the right.

Fur binds to an operator sequence in the *H. hepaticus ureA* promoter

The Fur-binding site in the *ureA* promoter was subsequently identified by using DNase I footprinting. Addition of recombinant Fur resulted in the protection of a single region in the *ureA* promoter only in the presence of metal. Absence of either Fur or metal did not result in protection of the *ureA* promoter DNA (Fig. 4A), consistent with the data obtained using the electrophoretic mobility shift assays (Fig. 3). Fur-mediated protection of the region in the *ureA* promoter was concentration-dependent (Fig. 4B). The protected sequence was located between positions -35 and -75 upstream of the *ureA* transcription start site (Figs. 4, 5A), and the operator sequence contained a single sequence resembling a Fur box, consisting of three NAT(A/T)AT hexamers in a F-F-x-R formation (26).

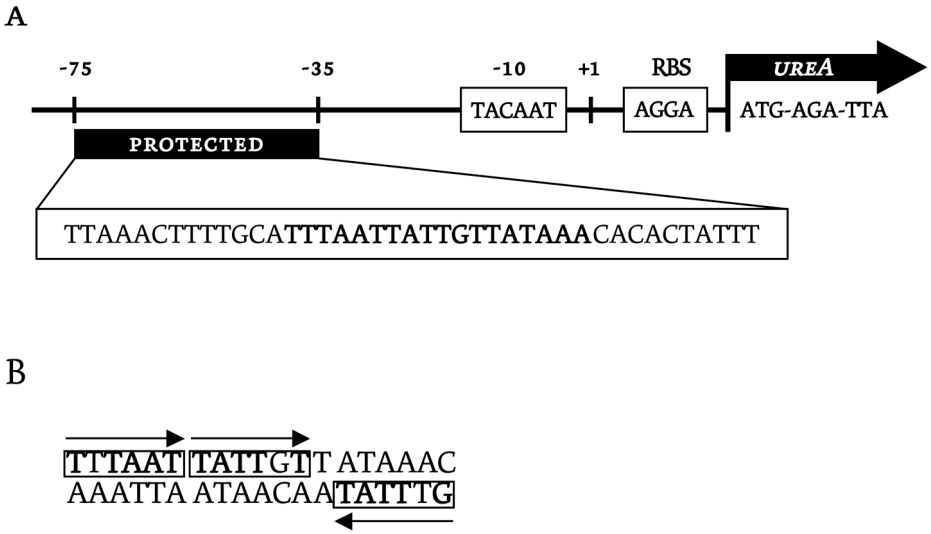

FIGURE 5. (A) Schematic representation of the *H. hepaticus ureA* promoter region with the location and sequence of the Fur-binding site indicated. The proposed ribosome binding site (RBS), -10 promoter sequence and transcription start site (+1) are indicated. (B) Annotation of the proposed Furbox present in the Fur-bound sequence in the *H. hepaticus ureA* promoter region, according to the F-F-x-R hexamer consensus sequence. Residues indicated in bold typeface match the 5'-NAT(A/T)AT consensus hexamer sequence

DISCUSSION

Urease is an important colonization and virulence factor for many pathogenic bacteria (8). In view of both the possible deleterious effects of uncontrolled ammonia production and the metabolic cost of urease production, expression and activity of urease is often controlled by specific environmental signals. These include substrate availability, nitrogen status of the cell, growth phase and pH (1, 8, 11, 33). *Helicobacter* species are known to have some of the highest urease activities among ureolytic

bacteria studied. This can be accompanied by urease protein expression reaching up to 10% of the total cell protein fraction in *H. pylori* (3). With the exception of pH, the aforementioned regulatory mechanisms of urease expression are absent in the few tested *Helicobacter* species (5, 9, 39). To date, only the molecular mechanisms governing urease activity in *H. pylori* have been reported, where urease expression is regulated at the transcriptional and post-transcriptional level by nickel and pH (1, 44), with transcriptional regulation being controlled via the NikR and HP0166 (ArsR) regulatory proteins (13, 18, 35, 43, 45).

Urease activity of *H. hepaticus* is lower than that of *H. pylori,* but still reaches high

levels when compared to many ureolytic bacterial species (5, 8, 10, 30). We previously demonstrated that the urease system *in H. hepaticus* is nickel-responsive at the enzyme activity level (5), but not at the transcriptional level. The *H. hepaticus* genome encodes for three orthologs of metal-responsive regulatory proteins that are all at different locations in the genome, the nickel-responsive regulator NikR (HH 0352), the peroxide stress regulator PerR (HH 0942), and the iron-responsive regulator Fur (HH0893) (41). The absence of nickel-responsive transcriptional regulation of urease in *H. hepaticus* (5) already suggested that NikR is not involved in urease regulation. Since the regulatory repertoire of *H. hepaticus* is relatively limited (41), we hypothesized that one of the other metal-regulatory proteins could be responsible for urease regulation in *H. hepaticus.* Here it is reported that *H. hepaticus* uses iron-restriction as a signal for induction of urease transcription, expression and activity (Fig. 1), that PerR is not involved in urease regulation (Fig. 2), and that the iron-responsive transcriptional regulator Fur directly mediates this process (Figs. 2, 3, 4). Both *fur* and *perR* are predicted to be transcribed as a monocistronic mRNA, and thus the observed phenotype of the *fur* mutant is unlikely to result from polar effects of the insertion of the chloramphenicol resistance cassette. In addition, both the *fur* and *perR* genes are surrounded by genes annotated as hypothetical proteins, without any predicted function in urease activity or metal metabolism (41).

Fur was first identified in *E. coli* as the repressor of iron acquisition systems, and was subsequently shown to be a DNA-binding protein that requires iron as co-factor (20). In recent years it has been shown that the regulatory role of Fur extends beyond iron-uptake systems, since Fur is involved in regulation of many cellular processes (20). In several bacterial species, Fur may regulate oxidative stress defense genes, acid-resistance, and metabolic routes (often via small RNA molecules) (28, 42). To date, Fur has not been shown to directly mediate urease regulation. There is a single report of a Fur-box in front of a (silent) urease gene cluster in enterohaemorrhagic *E. coli* (EHEC) strain EDL933, and mutation of *fur* resulted in decreased urease activity in a different EHEC isolate (24). However, direct involvement of Fur in urease regulation in *E. coli* was so far not conclusively demonstrated, and thus it remains possible that the observed phenotypes in *E. coli* are not mediated by Fur, but are indirectly regulated via a cascade or through polar effects. Therefore our study remains to our knowledge the first report of a direct role of Fur in regulation of a urease gene cluster.

The Fur protein has also been described to be involved in acid resistance for several bacterial species, including *Salmonella enterica* serovar Typhimurium and *H. pylori* (7, 23). Urease expression of *H. pylori* is also acid-responsive, and urease activity is required for colonization of the stomach (15, 34). Although it is tempting to suggest a role for Fur in acid-resistance of *H. hepaticus*, it was previously shown that the urease system of *H. hepaticus* is not acid-responsive and that urease activity does not allow survival at low pH (5).

H. hepaticus urease activity is not completely repressed by iron, since even in iron-replete conditions there is urease activity (Fig. 1A), and the *fur*-mutant does not show a complete derepression of urease activity (Fig. 2). This could be due to a role of other regulatory systems in urease expression, like in *H. pylori* (35). Alternatively, binding of Fur may only result in incomplete or intermediate repression. The region protected

from DNase I digestion in the footprint assay is located from -35 to -75, and thus is located just on the edge of the canonical σ^{80} promoter sequence of the *ureA* promoter. This may be an indication for the presence of a binding site for an additional regulatory system, upstream of the *ureA* promoter, as has been described for NikR and ArsR in the *H. pylori ureA* promoter (13, 18, 35). Alternatively, binding of Fur may only reduce but not completely block access of RNA polymerase. However, it should be noted that urease activity is only an indirect readout, and is subject to regulation at the post-transcriptional or post-translational level (5). The demonstration of sequence-specific binding of Fur to the *ureA* promoter (Fig. 3, 4) confirms the direct role of Fur in urease regulation in *H. hepaticus.* The *ureA* operator sequence bound by Fur contains a putative Furbox (Fig. 5B) which matches well with the proposed consensus sequence (20, 26, 42).

The known environmental signals used for regulation urease expression or activity are all somehow involved in the urease reaction or downstream processes, be it enzyme co-factor, substrate availability, nitrogen status or pH. The link between iron and urease is not directly apparent, since iron is not involved in any of these pathways, with the exception of iron-responsive regulation of ammonia-producing enzymes in *H. pylori* (46). However, iron-restriction is often used by pathogenic bacteria as a signal for entering the host and is a well known signal for regulation of virulence gene expression (32). The sites of colonization of *H. hepaticus* are the murine intestine and liver, and both environments are thought to be iron-limited (22). In the intestine, *H. hepaticus* has to compete for iron with many other bacteria, including the resident flora. In contrast, the liver is thought to be a rather sterile environment containing high concentrations of bile and urea (4). Bile components can chelate iron, and this may make the hepatobiliary tract also an iron-restricted site of colonization for *H. hepaticus* (4). Increased urease expression may help *H. hepaticus* to overcome such detrimental conditions through yet unknown mechanisms. Similarly, iron-restriction functions as signal for increased expression of the ammonia-producing enzyme amidase of *H. pylori* (46), but the physiological role of this regulatory response is also still unknown. It is therefore conceivable that low iron can serve as an indirect signal for encountering unfavourable conditions requiring additional urease activity. Such increases in urease activity may help the bacteria to colonize their niche, for instance through competition for nitrogen sources with other bacteria.

In conclusion, *H. hepaticus* uses iron and Fur for regulation of urease expression, a type of regulation not previously described for a urease system in ureolytic bacteria. These findings are another example of the versatility of the members of the genus *Helicobacter*, which extend the possibilities of their relatively limited regulatory repertoire by using well known regulatory proteins like Fur to mediate expression of novel target systems.

MATERIALS & METHODS

Bacterial strains, plasmids and growth conditions

H. hepaticus strain ATCC51449 (22, 41) and its isogenic *fur* and *perR* mutants (this study), were routinely cultured at 37 °C in microaerobic conditions (10% CO_2, 5% O_2 and 85% N_2) on Dent agar (Oxoid) (44). Liquid growth was performed in Brucella broth (Difco) supplemented with 0.2% (w/v) -cyclodextrins (Fluka) (BBC). Cultures were inoculated with a starting OD_{600} of 0.05, and were shaken at 40 rpm. For growth under iron-restricted conditions 20 μM deferoxamine mesylate (desferal, Sigma) was added to Brucella broth before adding the cyclodextrins. Iron-replete media were prepared by the addition of 100 μM $FeCl_3$ (Sigma) to the iron-restricted medium. In case of transcriptional analyses (primer extension analysis) iron-restriction for was achieved by addition of 2,2'-dipyridyl (Sigma) or 2,2'-dipyridyl and $FeCl_3$ to final concentrations of 100 μM to an overnight culture of *H. hepaticus*, followed by incubation for 30 min at 37°C (14). The reason for the use of different chelators was that *H. hepaticus* RNA isolated after overnight growth in the presence of desferal was of lower quality than RNA isolated from cells incubated with dipyridyl (data not shown) and could not be used for primer extension analysis, whereas incubation with 2,2'-dipyridyl was too short to result in phenotypic changes (protein profile and urease activity). Both chelators have been used previously for iron-restriction in *H. pylori* and gave similar results for iron-responsive regulation of iron-uptake and iron-storage genes (6, 14, 16). Incubation of *H. hepaticus* with 2,2'-dipyridyl resulted in absence of growth, probably due to toxicity of the chelator (data not shown). *Escherichia coli* strains ER1793 and DH5α were grown aerobically in Luria-Bertani medium (36) at 37 °C. When indicated, growth media were supplemented with chloramphenicol and ampicillin to final concentrations of 20 μg/ml and 100 μg/ml, respectively.

TABLE 1. Oligonucleotide primers used in this study.

Primer name	Sequence (5' → 3')[a]
HhureA-F1	TGCATTATGCTGGGGCACTA
HhureA-DIG	GTGCCCCAGCATAATGCAAC.
Hhfur-mutF1	AGAATCGCAATCCTAAAAGC
Hhfur-mutR1	TATTGCCACAAAGCGGTGTC
Hhfur-outF1	GTGCTTTGCTGGAACCAAAC
Hhfur-outR1	CCAAGAATGGCGACTACTAC
HhperR-mutF1	TTGGAGAATCTTTAGAG
HhperR-mutR1	GGAGCATTTCACATATTCTG
HhperR-outF1	ATCTTGGAGCGCATTTATGA
HhperR-outR1	CTCACGCGAGATGATTGTAG
Hhfur-overF1	GGATCCATGAATATGAATGCAAATCATCGCGTGGAA
Hhfur-overR1	CTGCAGCCTTTAGAGTCCTTGACATTT
HhksgA-F1	GCGTGATAGATTCTGTCGTG
HhksgA-DIG	TCGCTTTACCGAAACTTTCAAC

a. Primer sequences were derived from the *H. hepaticus* ATCC51449 genome sequence (41).

Urease enzyme assay

Urease enzyme activity was determined in freshly sonicated lysates by measuring ammonia production from hydrolysis of urea as described previously (44). The concentration of ammonia in the samples was inferred from a standard NH_4Cl concentration curve. Enzyme activity was expressed as micromoles of urea substrate hydrolysed per minute, per milligram protein.

Primer extension analysis

RNA was isolated from *H. hepaticus* using Trizol reagent (Invitrogen), according to the manufacturers instructions. Primer extension analyses were performed using the reverse primer HhureA-DIG (Table 1) essentially as described previously (17). The digoxygenin-labeled primer was annealed to 10 μg of total RNA from *H. hepaticus* strain ATCC51449, and cDNA was synthesized after addition of 5 units of AMV-reverse transcriptase (Promega) and incubation for 1 h at 42°C. The cDNA product was separated on a 7% acrylamide-8 M urea sequencing gel, and then blotted onto a nylon membrane (Roche), followed by chemiluminescent DIG-detection (16). A sequencing reaction made with the same primer was used to determine the transcriptional start site.

Construction of *H. hepaticus fur* and *perR* mutants

The *fur* gene of *H. hepaticus* strain 51449 was amplified using primers Hhfur-mutF1 and Hhfur-mutR1 (Table 1), and cloned in pGEM-T_{easy} (Promega), resulting in plasmid pCB1. The *fur* gene was subsequently interrupted by insertion of the chloramphenicol resistance gene from pAV35 (47) in the unique *Bsm*I site, resulting in plasmid pCB2. The *perR* gene of *H. hepaticus* strain 51449 was amplified using primers HhperR-mutF1 and HhperR-mutR1 (Table 1), and cloned in pGEM-T_{easy} (Promega), resulting in plasmid pCB3. The *perR* gene was subsequently interrupted by insertion of the chloramphenicol resistance gene from pAV35 (47) in the unique HindIII site, resulting in plasmid pCB4. Both plasmids pCB2 and pCB4 were first introduced into *E. coli* ER1793 and plasmids isolated from *E. coli* ER1793 were subsequently used for natural transformation of *H. hepaticus* 51449 (5). Chloramphenicol-resistant colonies isolated were designated 51449*fur* and 51449*perR*, respectively. Two colonies derived from independent transformations were tested, and both colonies gave identical results in all experiments. Correct allelic replacement of the wild type *fur* and *perR* genes with the interrupted version was confirmed by PCR using combinations of the primers Hhfur-outF1/Hhfur-outR1 and HhperR-outF1/HhperR-outR1, respectively (Table 1).

Protein analysis

H. hepaticus ATCC51449 was grown for 24 hours in iron-restricted and iron-replete BBC media, centrifuged at 4000 × *g* for 10 min at room temperature and resuspended in PBS pH 7.4 to a final OD_{600} of 10. *H. hepaticus* cells were subsequently lysed by sonication for 15 sec on ice, using a MSE Soniprep 150 at amplitude 6. Proteins were separated by SDS-PAGE on a 10% (w/v) polyacrylamide gel and stained with Coomassie Brilliant Blue. Immunoblotting was performed after electrotransfer of proteins from a 10% SDS-PAGE gel onto nitrocellulose membrane (Roche). The blot was subsequently probed with antibodies raised in rabbits to *Helicobacter felis* UreA or UreB (Intervet

International BV; Boxmeer, Netherlands) (5). Bound antibodies were visualized with swine anti-rabbit antibodies labeled with alkaline phosphatase (Promega), using BCIP and NBT (Promega) as substrate (5).

Recombinant expression of *H. hepaticus* Fur protein.

The *fur* gene was amplified from *H. hepaticus* ATCC51449 using the primers Hhfur-overF1 and Hhfur-overR1 (Table 1). The resulting fragment was digested with *Bam*HI and *Pst*I, and ligated into pASK-IBA7 (IBA, Gottingen, Germany) to create pCB10. The wild-type sequence of the *fur* gene was confirmed by DNA sequencing. *H. hepaticus* Fur was expressed with an N-terminal Streptag, which has been used with the *H. pylori* Fur, NikR and CrdR proteins, and which does not influence DNA-binding activity (17, 18, 48, 49) and therefore the Streptag was not removed prior to use. The recombinant protein was purified as described in the manufacturer's instructions (48). The recombinant protein was over 90% pure as determined by staining with Coomassie Brilliant Blue following electrophoresis on 12% SDS-polyacrylamide gels. Purified protein was dialyzed and stored in 50 mM Tris pH 8.0, 100 mM NaCl, 1 mM $MgCl_2$, 2 mM DTT and 50% glycerol and was used without further purification for electrophoretic mobility shift and DNase I footprinting assays.

Electrophoretic mobility shift assays

DIG-labeled *ureA* promoter DNA was amplified using primers HhureAF1 and HhureA-DIG (Table 1). As a negative control, the promoter of the *ksgA* gene (HH1174) (41) was amplified using primers HhksgA-F1 and HhksgA-DIG (Table 1). The binding buffer used for the gel-shift assays contained 24% glycerol, 40 mM Tris pH 8.0, 150 mM KCl, 2 mM DTT, 600 μg/ml bovine serum albumin and 1 _g denatured salmon sperm DNA. Gel-shift assays were performed with 0.5 nM DIG-labeled *ureA* or *ksgA* promoter DNA, which was mixed with increasing concentrations of recombinant Fur protein, and was subsequently incubated for 30 minutes in binding buffer. Reactions were separated for 35 minutes at 200 V on a 8% polyacrylamide gel. The assay was performed in the presence of either 200 μM $MnCl_2$ or 200 μM EDTA in the binding buffer, polyacrylamide-gel and electrophoresis buffer (2.5 M Tris and 0.19 M Glycine). Gels were blotted onto a positively charged nylon membrane (Roche), followed by chemiluminescent DIG detection (44).

DNase I footprinting assays

DNase footprint assays were performed using 500 nM recombinant Fur protein and 50 nM DIG-labeled *ureA* promoter DNA or different concentration of recombinant Fur in combination with 20nM DIG-labeled *ureA* promoter DNA. Binding buffer consisted of 50 mM Tris pH 8.0, 250 mM NaCl, 50 mM KCl, 5 mM DTT, 0.5% NP-40, 50% glycerol, 100 μg/ml denatured salmon DNA, in the presence or absence of 200 μM $MnCl_2$. DNase I digestion was carried out by addition of 0.25 units of DNase I (Promega) for one minute at room temperature, and the reaction was stopped by the addition of 2.5 M sodium acetate, 20 mM EDTA and 10 μg denatured salmon DNA. Subsequently the DNA was ethanol precipitated and resuspended in 6 μl of loading buffer (0.05% bromophenol blue (Sigma) and 0.05% xylene cyanol (Merck) in dionized formamide (Sigma)),

denatured and seperated on a 7% polyacrylamide gel containing 8M urea. Gels were blotted onto a positively charged nylon membrane (Roche), followed by chemiluminescent DIG detection (44).

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Chapter 5

The PerR-regulated catalase *(katA)* of *Helicobacter hepaticus* contributes to peroxide stress resistance and aerotolerance

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ABSTRACT

Chronic intestinal and hepatic colonization with the microaerophilic murine pathogen *Helicobacter hepaticus* can lead to a range of inflammatory diseases of the digestive tract. Infection with *H. hepaticus* is associated with an active cellular immune response with production of oxygen radicals, and thus the bacterium needs to cope with such oxidative stresses. In this study we have investigated the expression and regulation of oxidative stress resistance genes of *H. hepaticus*. The *H. hepaticus* genome sequence contains a gene encoding catalase (*katA*), as well as an ortholog of the peroxide stress regulator PerR. Expression of catalase was induced by hydrogen peroxide, and was also increased upon iron-restriction. The iron- and hydrogen peroxide-responsive regulation of *katA* was mediated at the transcriptional level, from a promoter directly upstream of the *katA* gene. Transcription of the *perR* gene was also iron-and hydrogen peroxide-responsive and mutation of the *perR* gene resulted in constitutive high-level expression of the *katA* gene and protein. Finally, absence of catalase resulted in increased sensitivity of *H. hepaticus* to hydrogen peroxide and reduced aerotolerance. In *H. hepaticus*, iron metabolism and oxidative stress defense are intimately connected via the PerR regulatory protein. This regulatory pattern resembles that seen in the enteric pathogen *Campylobacter jejuni*, but contrasts with the regulatory patterns observed in the human gastric pathogen *Helicobacter pylori*. Therefore, iron-dependent regulation of peroxide stress defense may be an adaptation beneficial for enteric colonization.

INTRODUCTION

Members of the genus *Helicobacter* are microaerophilic bacteria that colonize the different organs of the mammalian digestive tract and due to their immunostimulative properties they are associated with the development of immunoproliferative diseases. *Helicobacter hepaticus* is a murine pathogen, which colonizes the intestines, but can also be found in the liver and bile ducts. Infection with *H. hepaticus* is associated with typhlocolitis, hepatitis, hepatocellular carcinoma and may also induce the formation of cholesterol gallstones (3, 9, 16, 24). *H. hepaticus* infection is widespread in mouse colonies used for experimental infections (20), and its presence may well influence the outcome of animal experiments in infection and immunology.

Colonization of host tissues by bacterial pathogens usually results in an active cellular immune response associated with the production of reactive oxygen species (ROS) by inflammatory cells. ROS can damage lipids, proteins and DNA by oxidation, and thus cells will attempt to remove ROS before they cause significant damage. Pathogens have evolved protective mechanisms aimed at defusing the activity of ROS. Next to external sources, ROS are also produced by cellular metabolism, and this is intimately coupled to cellular iron metabolism (11, 23). In the presence of oxygen, iron can generate reactive oxygen species like superoxide anions (O_2^-), peroxides (RO_2) and hydroxyl radicals (OH^-) through the Haber–Weiss and Fenton reactions ($Fe^{2+}+O_2\_Fe^{3+}+O_2^-$ and $Fe^{2+}+H_2O_2\_Fe^{3+}+OH^-+OH^-$). To combat and prevent oxidative stress,

cells have evolved well-regulated systems that can sense and respond to changes in intracellular concentrations of iron and ROS, and both stimuli are major environmental signals for pathogenic bacteria to trigger expression of virulence determinants.

Peroxide stress is a subset of oxidative stress, and resistance to peroxides is mediated by peroxidases (23). Analysis of the *H. hepaticus* genome sequence (5, 19) has indicated that the components of the *H. hepaticus* peroxide stress resistance system resembles that of the related pathogen *C. jejuni*, and contains several peroxidases including a single catalase (12) and an alkyl hydroperoxide reductase (2), and also a putative PerR regulatory protein (14). Expression of oxidative stress defense protein is usually coupled to the presence of the stressor, and different forms of oxidative stress regulatory proteins have been described. The PerR regulatory protein is similar to the Fur class of metal-responsive repressor proteins (14), which require a metal-cofactor for their activity. PerR is thought to use either manganese or iron to detect oxidative stress, and changes to the cofactor affect its DNA-binding ability (14, 15).

In this study we have investigated the function and regulation of expression of different members of the peroxide stress defense components of *H. hepaticus*. We demonstrate that catalase is required for survival of peroxide stress and aerotolerance. The expression of catalase is demonstrated to be iron-repressed and peroxide-induced, and this regulation is mediated by PerR at the transcriptional level.

RESULTS

Expression of *H. hepaticus* catalase is iron-repressed at the transcriptional level

Comparison of *H. hepaticus* protein profiles in iron-restricted and iron-replete conditions indicated that two proteins of approximately 25 and 55 kDa displayed iron-responsive repression of their expression (Fig. 1A). The 25 and 55 kDa proteins were identified using MALDI-TOF as alkyl hydroperoxide reductase (AhpC, also known as TsaA) and catalase (KatA), respectively. Both proteins are involved in the degradation of peroxide compounds, and are known to contribute to bacterial oxidative stress defense.

The iron regulation of the *katA* gene was selected for further investigation. Primer extension, qRT-PCR and Northern hybridization were used to study the mRNA levels and identify the transcriptional start site of *katA*. Transcription of *katA* appeared to be induced after cells were incubated at iron limited conditions (Fig.1 B,C). The transcription start site of the *katA* gene was identified to be the T residue 13 bp upstream of the *katA* ATG start codon . Upstream of the transcription start site there is a ACAAAT hexamer sequence , which could potentially function as -10 promoter motif. Catalase mediates the breakdown of peroxides, and thus we investigated whether hydrogen peroxide had a regulatory effect on *katA* transcription. Incubation of *H. hepaticus* cells with a sublethal concentration of H_2O_2 led to the induction of the *katA* gene transcription, as was demonstrated using quantitative qRT-PCR (Fig.1C).

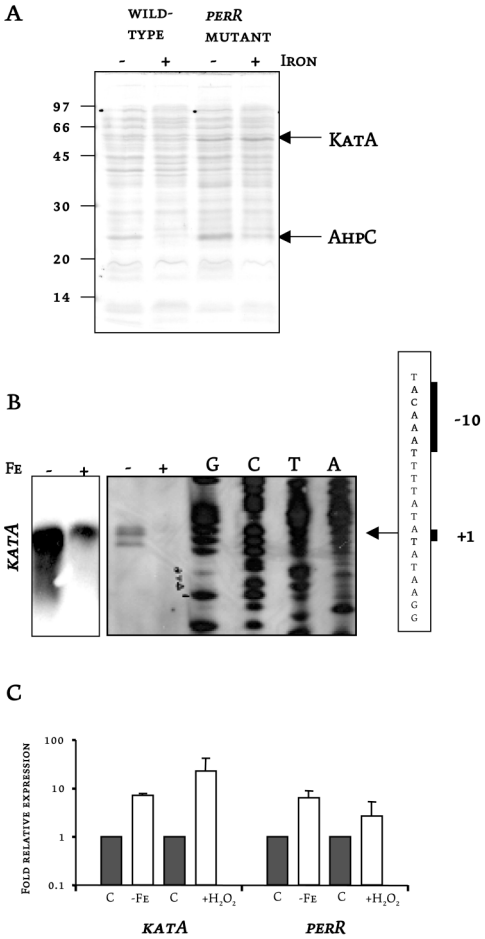

FIGURE 1. (A) Catalase and AhpC expression of *H. hepaticus* is iron-repressed via PerR. Total protein of *H. hepaticus* ATCC51449 and *perR* mutant, grown in iron-restricted medium and iron-replete medium protein was separated by SDS-PAGE and stained using Coomassie Brilliant Blue. Relevant marker sizes are indicated on the left. Identification of the *H. hepaticus* KatA and AhpC bands as a result of MALDI-TOF analysis are indicated on the right. (B) Iron-repression of *katA* is mediated at the transcriptional level. Northern hybridazation of *H. hepaticus katA* (left) and identification of the *katA* transcription start site by primer extension analysis (right), using RNA purified from *H. hepaticus* ATCC51449 grown in iron-restricted (-Fe) and iron-replete (+Fe) conditions. The sequence of the respective region is displayed on the left, with the +1 residue and the -10 promoter sequence indicated. Please note that the primer extension product displays iron-responsive repression of transcription. (C) *katA* and *perR* transcription in *H. hepaticus* is iron-repressed and peroxide induced, as measured by mRNA levels after over night growth in iron-restricted medium compared to iron-replete medium (set as one) or after peroxide shock compared to the reference condition (set as 1). Each bar represents relative expression data from three independent experiments, error bars denote standard deviation. Al the genes are corrected for the levels of 16S rRNA.

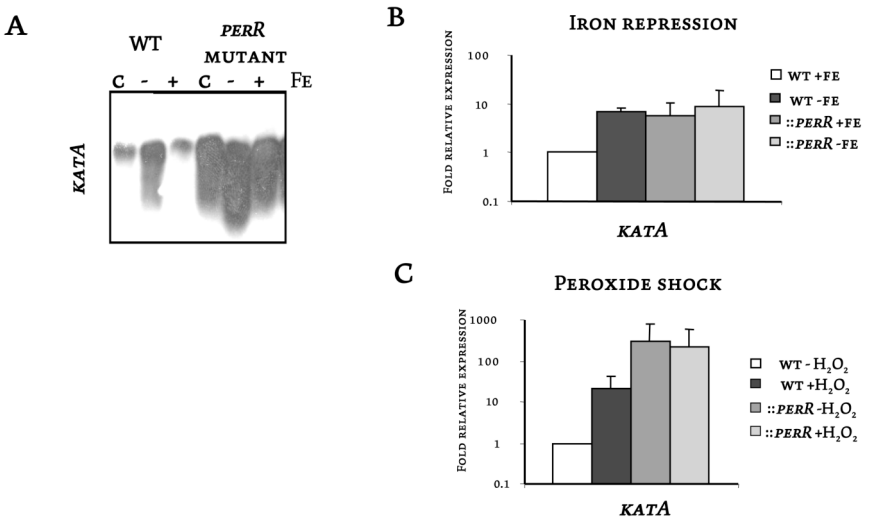

FIGURE 2. *H. hepaticus katA* is regulated by PerR. (A) Northern hybridization of *H. hepaticus* ATCC51449 and *perR* mutant with probe specific for *katA,* using RNA isolated from cells iron-restricted medium and iron-replete medium. (B) *katA* transcription in *H. hepaticus* wild type and *perR* mutant as measured after 24 h growth in iron-restricted medium compared to iron-replete medium (set as 1). Each bar represents relative expression data from three independent experiments, error bars denote standard deviation. Expression levels are normalized for levels of 16S rRNA. (C) *katA* transcription in *H. hepaticus* wild type and *perR* mutant after peroxide shock compared to the reference condition (set as one). Each bar represents relative expression data from three independent experiments, error bars denote standard deviation. Expression levels are normalized for the levels of 16S rRNA.

Transcription of *H. hepaticus katA* is regulated by PerR.

Analysis of the *perR* gene transcription in *H. hepaticus* demonstrated that *perR* transcription is peroxide-induced and iron-repressed similar to *katA* (Fig.1C). To investigate if PerR is involved in the regulation of *katA* transcription, an isogenic *perR* mutant was created. Inactivation of the *H. hepaticus perR* gene resulted in high-level, iron- and peroxide independent, expression of KatA protein (fig. 1A) and *katA* mRNA (fig.2).

KatA is required for peroxide stress resistance and aerotolerance of *H. hepaticus*.

The role of KatA in peroxide stress resistance and aerotolerance of *H. hepaticus* was investigated using a *H. hepaticus katA* mutant. *H. hepaticus* wild-type ATCC 51449 and its isogenic *katA* mutant were exposed to different concentrations of hydrogen peroxide for 30 minutes, after which the viability of the cells was analyzed. The wild-type strain was able to survive incubation with H_2O_2 to a concentration of at least 2 mM. In contrast the *H. hepaticus katA* mutant strain was highly sensitive to hydrogen peroxide, displaying 90 percent loss in viability at 1 μM H_2O_2 (Fig. 3A).

To examine the aerotolerance of *H. hepaticus*, wild-type and *katA* mutant cells were exposed to atmospheric oxygen levels and viability was compared with cells kept at microaerophilic conditions. The *katA* mutant displayed a reduced tolerance to atmospheric oxygen conditions when compared to the wild-type strain (Fig. 3B), and viability of the *katA* mutant decreased below detection levels ~ 2 hours earlier than the wild-type strain.(Fig. 3B)

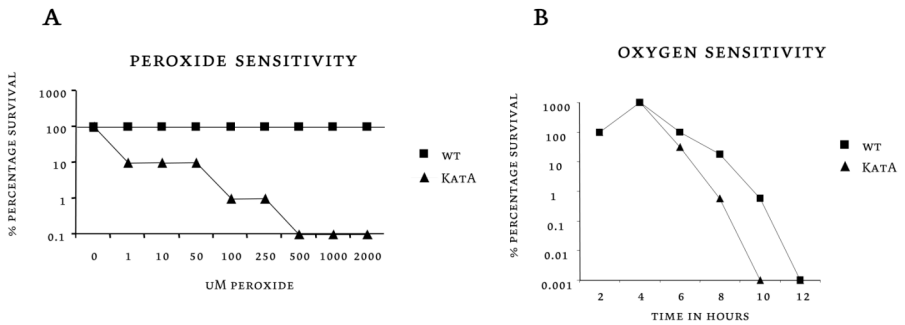

FIGURE 3. KatA mediates hydrogen peroxide resistance and aerotolerance in *H. hepaticus*. (A) *H. hepaticus* peroxide sensitivity was measured by incubating strain ATCC51449 (squares) and *katA* mutant (triangles) in PBS and incubated with different H_2O_2concentrations. Survival of cells was monitored by spotting tenfold dilutions on Dent plates, and visual assessment of growth after 2 days. Data points shown are the average of three independent experiments. (B) *H. hepaticus* oxygen sensitivity was determined by incubating ATCC51449 (squares) and *katA* mutant (triangles) atmospheric oxygen conditions. Survival of cells was monitored using viability counts on Dent plates. Data points shown are the average of three independent experiments.

DISCUSSION

Almost all cells produce the enzyme catalase, which mediates the breakdown of hydrogen peroxide to water and oxygen. This protects the cells against the toxic features of peroxides which range from production of hydroxyl radicals, DNA strand breaks, lipid peroxidation and oxidative damage of proteins (23)

H. hepaticus produces high levels of catalase, of almost 1% of total protein content (12). The high energy costs that the organism put in to this suggests the importance of the enzyme for the cell. This study s demonstrates the role of catalase in *H. hepaticus* peroxide resistance and aerotolerance, and is in agreement with other studies on oxidative stress resistance in *H. hepaticus* (12, 17). Consistent with findings in other organisms, disruption of the catalase encoding gene *katA*, results in bacteria hypersensitive to hydrogen peroxide and oxygen relative to the wild type strain (23) PBS OD_{600} of 0.5 (12). Mice infected with *H. hepaticus* demonstrated immune responses to murine and *H. hepaticus* catalase, suggesting that apart from oxidative stress defence *Helicobacter* catalase contains may also contribute to autoimmune responses (1).

H. hepaticus katA is transcriptionally regulated by iron and peroxide. The presence of peroxide and the low iron concentrations lead to an induction of *katA* transcription. Furthermore, the iron and peroxide regulation of *katA* are dependent on PerR. *H. hepaticus perR* mutants show high-level, iron- and peroxide independent, expression of KatA protein and mRNA. This ability of the preoxide stress regulator has also been reported for the organisms *C. jejuni, Streptococcus pyogenes* and *B. subtilis* (7, 13).

Low iron and peroxide might be linked as it has been reported for that PerR uses metal catalyzed oxidation reactions to sense peroxide-stress. This means that the iron bound by histidine residues inside PerR are oxidized when hydrogen peroxide is present. Iron is required as a ligand for the binding abbility of the PerR. The regulatory capability of PerR can therefore be similar in low iron and peroxide conditions as in both cases the protein is inactivated (14, 15)

The regulatory pattern described for *H. hepaticus* resembles that seen in the enteric pathogen *C. jejuni*, but contrasts with the regulatory patterns observed in the human gastric pathogen *H. pylori*. *H. pylori* has no gene coding for the *perR* protein (5). and Fur regulation has been reported for *katA* regulation (10). In *C. jejuni*, apart from PerR regulation of the *katA* (Cj1385) Fur also seems be involved in regulation of these genes (22). Competition of Fur and PerR to *katA* promoter in *H. hepaticus* is a possibility, and remains to be studied in the near future. Initial attempts to produce recombinant *H. hepaticus* PerR protein for in vitro studies has so far failed, possibly due to toxicity of the protein to *E. coli* (data not shown). A similar problem has been reported for *C. jejuni* PerR (B.M. Pearson, personal communication).

In conclusion, we have demonstrated the involvement of KatA in *H. hepaticus* oxidative stress resistance, and its regulation by iron and peroxide via the PerR regulatory protein. Iron-restriction and increased peroxide levels may be linked, since peroxides can oxidize iron, thus preventing the co-factor to bind to the PerR protein. Alternatively, iron-restriction can be a signal for entering the host and a signal indicating the necessity to protect the cell against peroxides that will be produced by the immune cells. Therefore, iron-dependent regulation of peroxide stress defense may

be an advantage in host colonization.

MATERIALS & METHODS

Bacterial strains, plasmids and growth conditions

H. hepaticus strain ATCC51449 (9) and its isogenic *perR* (6) and *katA* mutants (this study), were routinely cultured at 37 °C in microaerobic conditions (5% O_2, 7.5% CO_2, 7.5% H_2 and 80% N_2) on Dent agar (Oxoid, Basingstoke, UK). Liquid growth was performed in Brucella broth (Difco, Sparks, MD, USA) supplemented with 0.2% (w/v) _-cyclodextrins (Fluka, Buchs, Switserland) (BBC) (6). For growth under iron-restricted conditions 20 μM of the iron chelator desferoxamine mesylate (desferal, Sigma) was added the medium. Iron-replete media were obtained by the addition of 100 μM of $FeCl_3$ (Sigma) to desferal-treated medium (6). Bacteria were inoculated with a starting OD_{600} of 0.05. For primer extension analysis, cells were grown over night in BBC and were then incubated for 30 minutes in the presence of 100 μM 2,2'-dipyridyl (Sigma) or 100 μM 2,2'-dipyridyl and 100 μM $FeCl_3$ (6).

Escherichia coli DH5α and ER1793 were grown aerobically in Luria-Bertani medium (18) at 37 °C. When indicated, growth media were supplemented with or ampicilin to a final concentration of 100 μg ml^{-1}, chloramphenicol to a final concentration of 20 μg ml^{-1} or erythromycin to a final concentration of 10 μg ml^{-1}.

TABLE 1: Oligonucleotide primers used in this study.

primer name	sequence
HhperR-F1	5'-TCCTCAACGCATTGCTACAC-3
HhperR-R1-T7	5'-ctaatacgactcactatagggagaGCTTTGCACAGCGCATTGAC-3
HperR FQ_	5'-ACTCCTCAACGCATTGCTAC-3'
HHperRRQ_	5'-GCTTTGCACAGCGCATTGAC-3'
HhpeR-mutF1	5'-TTGGAGAATCTTTAGAG-3
HhperR-mutR1	5'-GGAGCATTTCACATATTCTG-3
HhperR-outF1	5'-ATCTTGGAGCGCATTTATGA-3
HhperR-outR1	5'-CTCACGCGAGATGATTGTAG-3
HhkataF1	5'-AGGAGTTTCTCCAAGTGTAT-3
HhkatAR1	5'-TGCGTCTATCGCCAAACTGA-3
HH katA FQ_	5'-GGTTTTGCACTCAAGCTTTA-3'
HHkatA RQ_	5'-AAATCCCACATTGCCGTAGG-3'
HhkatA-F2	5'-GTGAGCGAGGAGCAGCAGAT-3
HhkatA-R1-T7	5'-ctaatacgactcactatagggagaGGCGATAACTGCCTCTGA-3
Hh katA-DIG	5'-GCCTGCTGTAATTGAGTTTT-3
HH16SFQ_	5'-GCGACCTGCTGGAACATTAC-3'
HH16SRQ_	5'-CCCAGGCGGGATGCTTAATG-3'

Primer sequences were derived from the *H. hepaticus* ATCC51449 genome sequence .Lowercase letters indicate the T7 promoter used for *in vitro* transcription.

Sensitivity studies

Strains were grown overnight (of: for 20-24h) in BBC at a starting OD_{600} of 0.05. In case of peroxide shock experiments, cells were resuspended in PBS to an OD_{600} of 0.5 and incubated with 100 μM H_2O_2 for 15 minutes. Subsequently RNA was be isolated for further analysis, and 5 μl of tenfold dilutions of the cells were spotted on Dent agar for viability testing. Plates were incubated for two days at 37 °C under microaerobic conditions (2). For the aero-tolerance assay, cells were put under atmospheric conditions for 2, 4, 6, 8, 10 or 12 hours. Hereafter, 5 μl of tenfold dilutions were spotted on Dent agar. Plates were incubated for two days at 37 °C under microaerobic conditions (2).

RNA analysis

RNA was isolated from *H. hepaticus* using Trizol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Gel electrophoresis of RNA, transfer to positively charged nylon membranes (Roche), crosslinking, hybridization to DIG-labeled specific RNA probes and detection of bound probe was performed as described previously (4). Probes specific for *H. hepaticus perR* and *katA* were synthesized by *in vitro* transcription using T7 RNA polymerase (Roche) and PCR products obtained with primers HhperR-F1, HhperR-R1-T7, HhkatA-F1, HhkatA-R1-T7. Primer extension analyses were performed using the reverse primers, Hh katA-DIG. cDNA was created using AMV reverse transcriptase (Promega)(6). All primers used are listed in Table 1.

Quantitative PCR analyses

RNA was isolated from *H. hepaticus* using Trizol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. All reverse transcription reactions were performed using 100 ng of RNA, and gene specific primers, cDNA was used directly for Quantitative RT-PCR (qRT-PCR). qRT-PCR reactions contained 5 μl of cDNA template, 25 pM gene specific primers, 10 nM dNTP 1 unit Taq polymerase and dH_2o to a final volume of 25 μl. SYBR green was added in a 10.000 fold dilution as instructed by the manual (Bio-Rad IQ5 manual) qRT-PCR assays were performed using RNA isolated in three independent growth experiments. Transcript levels were normalized to the levels of 16S rRNA in each sample (DD Ct analysis, as described in the Bio-Rad IQ5 manual). All primer sequences are listed in table1.

Construction of *H. hepaticus* mutants

The *katA* gene of *H. hepaticus* strain ATCC51449 were amplified using primers HhkataF1 and HhkatAR1, and the resulting PCR product was cloned in pGEM-T_{easy} (Promega). The *katA* gene was subsequently interrupted by insertion of the chloramphenicol (Cm^R) from pAV35 (21) resistance gene resulting in plasmid pCB41(*katA*::Cm^R). This plasmid was introduced into *E. coli* ER1793 and subsequently used for natural transformation of *H. hepaticus* ATCC51449 (6). Chloramphenicol-resistant colonies were isolated, and two colonies derived from independent transformations were tested, and both colonies gave identical results in all experiments. Correct allelic replacement of the *katA* gene with their interrupted versions was confirmed by PCR using the primers. In addition, the *katA* mutant was also confirmed

by absence of the KatA protein on SDS-PAGE and absence of KatA activity in a peroxide assay (data not shown).

Protein analysis

H. hepaticus ATCC51449 wild-type and isogenic mutant cells were grown for 16h in BBC supplemented with 20 _M desferal or 20 _M desferal and 100 _M $FeCl_3$, centrifuged at 4000 _ *g* for 10 min at room temperature and resuspended in PBS pH 7.4 to a final OD_{600} of 10. *H. hepaticus* cells were subsequently lysed by sonication for 15 sec on ice, using a MSE Soniprep 150 (MSE, Crawley, UK) at amplitude 6. Proteins were separated by SDS-PAGE on a 10% (w/v) polyacrylamide gel and stained with Coomassie Brilliant Blue. Proteins were identified with MALDI-TOF as described before (8).

ACKNOWLEDGEMENTS

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Chapter 6

Amoxicillin resistance of enterohepatic *Helicobacter* species: role of the *hefA* gene in antimicrobial resistance of *H. hepaticus*

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ABSTRACT

***Helicobacter* species colonize the gastrointestinal and hepatobiliary tract of mammals, and often cause inflammation-associated diseases. A major stress factor for pathogenic bacteria during colonization host is due to antimicrobial agents, either produced by the host, the resident flora, or by antibiotic treatment of the infection. Bacteria often use efflux mechanisms to transport toxic components out of the cells. In this study we have compared the antimicrobial resistance profiles of a collection of *Helicobacter* species, and show that several enterohepatic *Helicobacter* species are resistant to amoxicillin, and report on the resistance mechanism in *Helicobacter hepaticus*. Three enterohepatic and three gastric *Helicobacter* species were tested for amoxicillin resistance, and all three tested enterohepatic *Helicobacter* species were amoxicillin-resistant, while all gastric colonizers were amoxicillin-sensitive. Amoxicillin resistance of *H. hepaticus* was further investigated, and was shown to be mediated by the HefA component of the HefABC efflux system. Mutation of *hefA* resulted in amoxicillin and bile acid-sensitivity, whereas the wild-type strain was resistant to these compounds. Transcription of the *hefA* gene was bile acid-induced, but amoxicillin-independent. In conclusion, three enterohepatic *Helicobacter* species are resistant to amoxicillin. This has implications for the treatment of infection with enterohepatic *Helicobacter* species colonizing mouse strains used in animal experiments.**

INTRODUCTION

One of the problems that all living cells have to deal with is the handling of toxic substances accumulating in the cytoplasm. To detoxify such compounds cells can transport them out of the cell. This process is usually mediated by efflux pumps, involved in the extrusion of toxic substrates (including bile acids and all classes of clinically relevant antibiotics). Efflux pumps may be specific for one substrate or may transport a range of structurally dissimilar compounds; such pumps can be associated with multiple drug resistance (MDR). Bacterial efflux systems are classified into several families based on sequence similarities (20). The resistance-nodulation-division (RND) family of efflux proteins is of particular interest because of their unusually broad substrate specificity (29), and are ubiquitous among gram-negative bacteria (26). RND proteins are involved in the resistance to multiple antibiotics and antimicrobial compounds (19), their activity is energized via the proton motive force, and they consists of three components that include an integral membrane pump protein (IEP), a periplasmatic membrane fusion protein (PEP) and an outer membrane pore protein (OEP). These three proteins together function as a continuous channel for extrusion of substrates from within the cell envelope back to the external environment (26).

Members of the genus *Helicobacter* chronically colonize the different organs of the digestive tract in mammals. *Helicobacter* species are subdivided in the gastric *Helicobacter* species, which colonize the gastric mucosa (e.g. *H. pylori*) and the

enterohepatic *Helicobacter* species, which colonize the intestines and hepatobiliary tract. Colonization with enterohepatic *Helicobacter* species results in chronic inflammation and is associated with disease including typhlocolitis, hepatitis and development of hepatocellular carcinoma (22, 31). *H. hepaticus* is the best studied enterohepatic *Helicobacter* species, and colonizes the intestines of rodents (11, 31). However, in certain mouse strains the bacterium also colonizes the liver and bile duct and this can result in development of hepatocellular carcinoma or can contribute to the formation of cholesterol gallstones (3, 11, 31). In the intestinal and hepatobiliary tract *H. hepaticus* will be exposed to the antimicrobial activity of bile, and the concentration of bile acids the organism is exposed to in the bile duct will probably be even higher. Therefore it can be predicted that *H. hepaticus* harbors protein systems that help the organism to resist this bile stress. Furthermore, infection of laboratory mice with *H. hepaticus* is a serious problem for many animal facilities worldwide. Not much is known about *H. hepaticus* antimicrobial resistance, although different multiple antibiotic therapy has been used for treatment of *H. hepaticus* in laboratory mice (9, 33).
In this study we have compared the antimicrobial resistance profiles of a collection of enterohepatic and gastric *Helicobacter* species, and show that several enterohepatic *Helicobacter* species are resistant to amoxicillin. Subsequently we have used *H. hepaticus* as model organism for this group and show that the HefA RND efflux protein is involved in amoxicillin and bile acid-resistance, and that transcription of the *hefA* gene is bile acid-induced. Finally, we discuss the implications of amoxicillin resistance in enterohepatic *Helicobacter* species on treatment of this infection, especially with respect to mouse strains used in animal experiments.

RESULTS

Amoxicillin sensitivity of *Helicobacter spp.*

Several *Helicobacter* species were tested for amoxicillin resistance (Table 1). Surprisingly, many tested enterohepatic *Helicobacter* species displayed a high level of amoxicillin resistance. *H. hepaticus*, *H. bilis* and *H. rappini* were resistant to amoxicillin with MIC values of 64, 16 and 8 mg/L respectively. The gastric *Helicobacter* species *H. pylori*, *H. acinonychis*, and *H. mustelae* were all sensitive to amoxicillin in our assay with MIC values of 0.047, <0.016 and 0.25 mg/L respectively. Consistent with previous reports, the *H. pylori* Hardenberg strain was amoxicillin resistant with an MIC of 4 mg/L (12).The break-point of amoxicillin for *H. pylori* was determined as 0.5 mg/L.

TABLE 1. Amoxicillin sensitivity of *Helicobacter* spp.

species	natural host	site of colonization	MIC (mg/L)
H. hepaticus ATCC 51449	mouse	enterohepatic	64
H. hepaticus 1701	mouse	enterohepatic	8
H. hepaticus MU94 (1)	mouse	enterohepatic	6
H. rappini (field isolate)	mouse	enterohepatic	8
H. bilis (field isolate)	mouse, rat, human	enterohepatic	16
H. mustelae ATCC 2647	ferret	gastric	0.25
H. acinonychis ATCC 3003	cheetah	gastric	<0.016
H. pylori ATCC 26695	human	gastric	0.047
H. pylori BH9802-108	human	gastric	4

Helicobacter strains, site of colonization and host. Analysis of amoxicillin sensitivity of different gastric and enterohepatic *Helicobacter* species. All values are means derived from of three independent experiments.

Identification of putative multi-drug efflux pumps in *H. hepaticus*

H. hepaticus was selected as model organism for the further analysis of the mechanism of amoxicillin resistance in enterohepatic *Helicobacter* species. The genome of *H. hepaticus* (24) encodes three putative RND efflux systems homologous to several gram negative bacteria (fig.1). The HH0175-HH0174 genes encode homologues of the *C. jejuni* CmeAB periplasmic and inner membrane fusion proteins, respectively, which are part of the CmeABC cluster in *C. jejuni*, where this system mediates bile resistance (16).

The *hefABC* (HH0222-HH0223-HH0224) is annotated as an efflux system with OMP, PMP and IMP homologous to *C. jejuni cmeDEF* operon and the *H. pylori* efflux system HP0605-0606-0607. Both clusters are involved in multiple antibiotic resistance. For *H. pylori* the efflux system is also involved in cholic acid resistance (27). Furthermore the OMP shows similarities to *tolC*, the PMP to *acrA* and the IMP to *acrB* (fig.1). Finally, the *hefDEF* (HH 0623-0624-0625) operon is annotated as an efflux sysytem with OMP, PMP and IMP homologues to *H. pylori* HP0971-0970-0969. The OMP is similar to *tolC* and involved in *H. pylori* antimicrobial and metal resistance (23, 27). The PMP and IMP are *acrA* and *acrB* similar to to *cznB* and *cznA* from *H. pylori* respectively where this system modulates resistance to cobalt, zinc and cadmium (27).

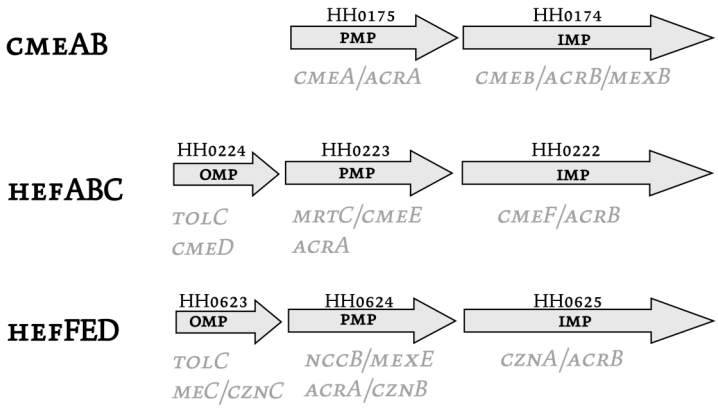

FIGURE 1. Schematic overview of the RND efflux pumps of *H. hepaticus*. Th e *H. hepaticus* ATCC51449 genome sequence (24) was used to study potential RND efflux pumps in *H. hepaticus*. Each of the three operons found contains a inner membrane protein (imp) and a periplasmatic protein (pmp) the cmeAB operon lacks a outer membrane protein (omp). All efflux pumps have high homology to membrane transporters of other bacteria.

HefA is involved in antimicrobial resistance

To investigate if efflux pumps of *H. hepaticus* are involved in antimicrobial resistance we created an isogenic *hefA* mutant by insertional mutagenesis. Several attempts to mutate the other efflux components *hefC, cmeA* and *cmeB* were unsuccessful to date, and this suggests these genes may be essential to *H. hepaticus*, although this requires further investigation (data not shown). The *H. hepaticus* wild-type strain and *hefA* mutant cells were screened for antimicrobial susceptibility. The most apparent difference between the wild-type strain and the *hefA* mutant was that the *hefA* mutant is sensitive to amoxicillin on E-test, and this was confirmed by agar dilution. The agar dilution assay for amoxicillin confirmed the differences observed with E-test as wild-type *H. hepaticus* and its isogenic *hefA* mutant had an MIC of 64 mg/L, and 0.25 mg/L respectively. The *hefA* mutant was also more sensitive to the bile acids cholic acid and deoxycholic acid, when compared to the wild-type strain, suggesting that absence of HefA results in a more generalized defect in efflux properties in *H. hepaticus*.

To investigate if *hefA* expression was responsive to the presence of antimicrobials in the growth medium, the expression of *hefA* mRNA was monitored. mRNA levels of the *hefA* gene were twofold increased in the presence of the bile acids cholic acid and deoxycholic acid, whereas the presence of amoxicillin had no significant effect on *H. hepaticus hefA* transcription (Fig. 2).

TABLE 3. Antimicrobial resistance of *H.hepaticus* ATCC51449 and its isogenic *hefA* mutant.

Compound	MIC (mg/L)	
	wild-type strain	*hefA*-mutant
Amoxicillin	64 (R)	0.25 (S)
Cefotaxim	32	32
Vancomycin	256	256
Trimetroprim	32	32
Erythromycin	0.016	0.016
Metranidozole	0.047	0.078
Tetracyclin	0.38	0.48
Gentamycin	0.357	0.106
Ciprofloxacin	0.172	0.0.14
Ofloxacin	0.121	0.033
Rifamycin	0.232	0.025
SDS	>0.5	>0.5
Ethidium bromide	>2	0.5
Cholic acid	2150	860
Deoxycholic acid	216	21.6
Nickel chloride	119	119

Table shows MIC values of *H. hepaticus* 51449 reference strain and its isogenic *hefA* mutant. All values are means derived from of three independent experiments.

DISCUSSION

A major stress factor for pathogenic bacteria during colonization of the mammalian host is due to antimicrobial agents, either produced by the host, the resident flora, or by antibiotic treatment of the infection. Multidrug efflux pumps have been shown to contribute to antimicrobial resistance in gram-negative bacteria by removing the toxic components that get through the outer membrane (2, 8). The carriage of efflux pump genes on the chromosome may give these pathogens an intrinsic mechanism that allows survival in a hostile environment.

Bile is an antimicrobial agent that bacteria encounter when colonizing the gut and bile ducts. Resistance to bile acids is often dependent upon enzymes that modify and transform bile salts, permeability properties of the outer membrane, or the production of certain cell envelope proteins including energy-dependent efflux systems. This study describes the involvement of *hefA,* a *tolC*-like outer membrane protein, in *H. hepaticus* resistance to amoxicillin and bile acids.

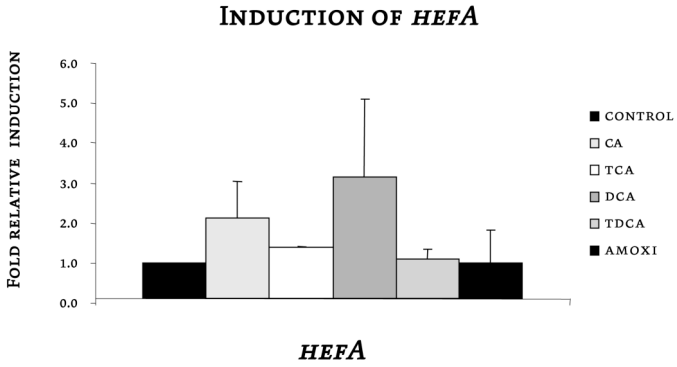

FIGURE 2. Transcription of the *H. hepaticus hefA* gene is induced by bile acids. mRNA levels of *H. hepaticus hefA* after cells were grown in the presence of antimicrobials compared to the reference condition (normalized to 1). Antimicrobials used were amoxicillin (amoxi), cholic acid (ca), taurocholic acid (tca), deoxycholic acid (dca) and taurodeoxycholic acid (tdca). Each bar represents relative expression data from three independent experiments, error bars denote standard deviation. Samples are corrected for the levels of 16S rRNA

The HefA protein is also involved in amoxicillin resistance of *H. hepaticus*. Efflux systems that contribute to antibiotic resistance have been described from a number of clinically important bacteria, including *H. pylori* (27), *C. jejuni* (7, 15), *E. coli* (17), *Pseudomonas aeruginosa* (8) and *Salmonella typhimurium* (6). Especially the RND type pumps can transport several structurally unrelated drugs (18, 32). However, the penicillin type antibiotics (to which amoxicillin belongs) are usually not transported by RND pumps (28). To our knowledge the role of RND outer membrane efflux mechanism in amoxicillin resistance has not been reported before.

Although HefA plays a key role in *H. hepaticus* antimicrobial resistance, a role of the inner membrane components in the *hefABC* operon cannot be excluded and is likely. Mutation of the *hefA* gene may also lead to polar effects on the other genes in the operon. Furthermore, two other RND efflux systems were identified as well in the *H. hepaticus* genome sequence, and the contribution of these systems to *H. hepaticus* antimicrobial resistance is yet to be determined. The result that several of the other *H. hepaticus* RND transporter genes could not be mutated in this study implies that these proteins are important for cell survival. The potential bile specific efflux system *cmeAB* is not in a three gene operon with a outer membrane component, as it is in *C. jejuni*. It has been described before for *acrAB* of *E. coli* for example (17) that the outer membrane component can be encoded separately, also because the OM pumps usually transport several antimicrobial agents and can thus be functional with more than one efflux system (17). The bile resistance of *H. hepaticus,* as shown to modulated by *hefA* may well be the result of a *cmeAB* transfusion with the *hefA* OMP. Evaluation of the uncharacterized systems in *H. hepaticus* will improve our understanding of its antimicrobial resistance.

Nowadays, the triple therapy (proton pump inhibitor, amoxicillin and clarithromycin) is often described for the eradication of *H. pylori* in humans, and is also used for treatment of *H. hepaticus* in laboratory mice (33). Antibiotic resistance to components of the multiple antibiotic therapy is known to negatively affect efficacy of the treatment (13). However, not much is known about amoxicillin susceptibility of *H. hepaticus* or other enterohepatic *Helicobacter* species. The data presented here show that the tested *H. hepaticus*, *H. bilis* and *H. rappini* isolates are resistant to amoxicillin. The eradication therapy used so far may possibly not meet up to the expectations for complete eradication of the bacteria, and should be reviewed. In agreement with this result it was reported that a two week triple treatment (metranidozole, amoxicillin and bismuth) of A/JCr mice does eradicate *H. hepaticus*, however similar therapy administered in drinking water was less effective than oral gavage; this study group isolated *H. hepaticus* from livers a month after treatment (9, 10). The amoxicillin triple treatment is not effective for eradicating *H. bilis* and *H. rodentium* or for treatment of rats, in the latter case the treatment is only sufficient when three cycles of a two week treatment is used and even than the animals will test negative on pcr for a period of only 8 weeks (33). Treatment with combination therapy consisting of four antibiotic including amoxicillin, metranidozole, claritomicine and omeprazole does eradicate *H. hepaticus* (14).

Mice are the natural hosts of *H. hepaticus.* Since 1994 investigators have recognized endemic *Helicobacter* infection in research colonies and their impact on research programs (25, 33). Molecular techniques based on PCR are the used for the detection. Managing a rodent facility with a *Helicobacter*-free health status requires the same effort as required for excluding a broad range of murine pathogens. The knowledge that enterohepatic *Helicobacter* species can be amoxicillin resistant could be beneficial when developing eradication programs in animal facilities worldwide.

MATERIALS & METHODS

Bacterial strains, plasmids and growth conditions

All *Helicobacter* strains (listed in table 1), were routinely cultured at 37 ℃ in microaerobic conditions (5% O_2, 7.5% CO_2, 7.5% H_2 and 80% N_2) on Dent agar (Oxoid, Basingstoke, UK) (5). Liquid growth was performed in Brucella broth (Difco, Sparks, MD, USA) supplemented with 0.2% (w/v) β-cyclodextrins (Fluka, Buchs, Switserland) (BBC). *Escherichia coli* strains DH5α and ER1793 were grown aerobically in Luria-Bertani medium (21) at 37 ℃. When indicated, growth media were supplemented with chloramphenicol to a final concentration of 20 μg ml^{-1} or ampicillin to a final concentration of 100 μg ml^{-1}.

TABLE 2. Oligonucleotide primers used in this study.

primer name	sequence
hefAmutF	TGAACGCAATGCTGCCTCTG
hefAmutR1	AGCGTAATAGTCGCACTTCG
hefA outF	CCAAGCGCAAGATAATGCAC
hefAoutR	TTGTTGCAAATCTTGTCCGC
hefA FQ1	TGCTACCGAGCACGATATTG
hefA RQ1	TGAACGCAATGCTGCCTCTG
16S FQ1	GCGACCTGCTGGAACATTAC
16S RQ1	CCCAGGCGGGATGCTTAATG

Primer sequences were derived from the *H. hepaticus* ATCC51449 genome sequence (24).

Assessment of susceptibilities to antimicrobials.

Helicobacter species were grown on plates for 24 h at 37 °C. Thereafter cells were resuspended in PBS and adjusted to an concentration of 2 x 10^9 cells/ml. This suspension was spread on dent plates with a swab stick. The MICs value's were determined by E-test (AB Biodisc, Solna Sweden). Agar dilution assays were performed as described before (12). Susceptibility to ethidium bromide (Promega), sodium dodecyl sulfate (Sigma), $NiCl_2$ (Sigma), cholic acid (Sigma) and deoxy cholic acid (Sigma) were determined by broth dilution assay (16).

Construction of *H. hepaticus* mutants.

The *hefA* gene of *H. hepaticus* strain 51449 was amplified using specific primers (Table 2) and cloned in pGEM-T_{easy} (Promega), resulting in plasmid pCB20. The gene was subsequently interrupted by insertion of the chloramphenicol resistance gene from pAV35 (30) in a unique *Eco*RV restriction site, resulting in plasmid pCB21. This plasmid was introduced into *E. coli* ER1793 and used for natural transformation of *H. hepaticus* 51449 as described before (4). Colonies derived from two independent transformations were tested. Correct allelic replacement of the wild-type *g*ene with the interrupted version was confirmed by PCR.

Quantitative PCR analyses

RNA was isolated from *H. hepaticus* using Trizol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. All reverse transcription reactions were performed using 100 ng of RNA, and gene specific primers, cDNA was used directly for Quantitative RT-PCR (qRT-PCR). qRT-PCR reactions contained 5 µl of cDNA template, 25 pM gene specific primers, 10 nM dNTP 1 unit Taq polymerase and dH_2o to a final volume of 25 µl. SYBR green was added in a 10.000 fold dilution as instructed by the manual (IQ5 Cycler System Bio-Rad manual) qRT-PCR assays were performed using RNA isolated in three independent growth experiments. Transcript levels were

normalized to the levels of 16S rRNA in each sample (DD Ct analysis, as described in the Bio-Rad IQ5 Cycler System manual). All primer sequences are listed in table1.

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Chapter 7

Urease-induced calcium precipitation by *Helicobacter* species may initiate gallstone formation

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Urease-induced calcium precipitation by *Helicobacter* species may initiate gallstone formation

Helicobacter species can colonize the mammalian gastrointestinal and hepatobiliary tract, which usually results in a chronic infection coupled to an inflammatory host response. It is therefore not surprising that colonization with *Helicobacter* species is linked with a range of inflammation-associated gastrointestinal and hepatobiliary diseases (3). Recently this range has been expanded with an association of infection with enterohepatic *Helicobacter* species and the formation of cholesterol gallstones (8).

In their study, Maurer and colleagues (8) demonstrated that murine infection with the enterohepatic *Helicobacter* species *H. bilis* and *H. hepaticus* accelerated the formation of cholesterol gallstones in mice fed with a lithogenic diet. Although the gallbladder mucosa in mice with gallstones displayed signs of inflammation, *Helicobacter* species were not cultured from the inflamed gallbladder or bile. Therefore Maurer and colleagues hypothesized that the chronic immune stimulation caused by *Helicobacter* species, rather than a direct bacterial factor, lead to the production of nucleating agents, thus indirectly linked *Helicobacter* species and cholesterol gallstone formation (8). Although a role for inflammation cannot be excluded, we believe that the identification of *Helicobacter* DNA in gallstones (1, 11) is an indication that *Helicobacter* species may also play a direct role in gallstone formation.

Gallstones are crystalline bodies formed by accretion or concretion of bile components. About 80% of gallstones are cholesterol gallstones, and 20% are pigment stones consisting of bilirubin and calcium, two components both present in bile. There is a connection between the two types of gallstones, since calcium-bilirubin salts form surfaces that are highly attractive for cholesterols to adhere to (10). In addition, almost all types of gallstones contain a nidus of calcium (2, 6). Bacteria have been shown to be able to initiate calcium precipitation and subsequent stone formation (4, 5), like with *Proteus mirabilis* and kidney stones (7). An important factor in bacteria-induced stone formation is the enzyme urease, which hydrolyses urea into ammonia and bicarbonate. Ammonia increases the pH and thereby favors generation of an insoluble form of calcium and subsequent precipitation, and this may be a mechanism shared between different types of stones.

We noticed in the study of Maurer and colleagues (8) that cholesterol gallstone formation was only detected after infection with the urease-positive species *H. hepaticus* and *H. bilis*, but not with the urease-negative species *H. rodentium* or *H. cinaedi* (8). This prompted us to investigate whether *Helicobacter* urease activity is involved in precipitation of calcium. For this purpose we developed a precipitation agar that allows for simultaneous growth of *Helicobacter* species and testing of their ability to precipitate calcium. We have tested four different calcium concentrations (30, 10, 5 and 1 mM $CaCl_2$); best results were seen at 30 mM of calcium (fig. 1), however calcium precipitation also occured at more physiologically relevant calcium concentrations (10, 5 and 1 mM) (12), although the crystals were smaller. All four urease-positive *Helicobacter* species tested (*H. hepaticus, H. bilis, H. pylori* and *H. mustelae*) were capable of precipitating calcium in our assay (Fig. 1), whereas isogenic urease-negative mutants of three species as well as urease-negative *Helicobacter* species (*H. pullorum*

and *H. cinaedi*) were unable to do so (Fig. 1). Purified urease enzyme alone was also capable of precipitating calcium.

This suggests that urease-positive *Helicobacter* species that are able to survive in or colonize the bile ducts (which excludes *H. pylori* (9)), may induce the formation of gallstones both directly via their urease activity, and indirectly via the immune response. Our observations extend those previously reported (1, 8, 9, 11), and combined these provide a possible mechanism explaining the association between hepatobiliary colonization with urease-positive *Helicobacter* species and gallstone formatio

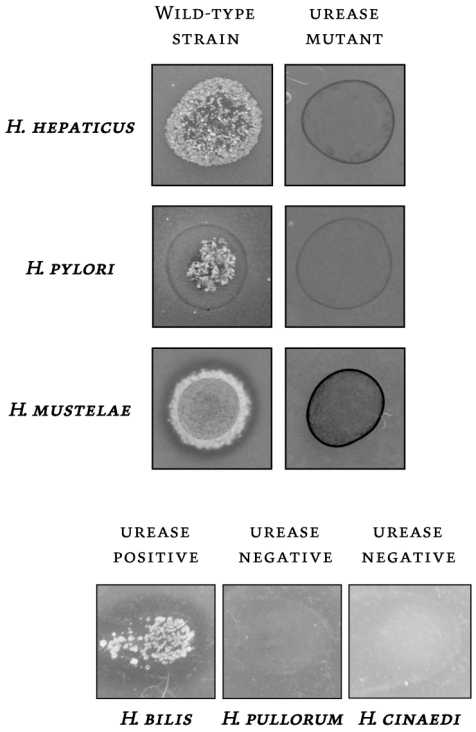

Figure 1. Urease activity is required for calcium precipitation by *Helicobacter* species. Top panel: comparison of *H. hepaticus* strain ATCC51449, *H. pylori* strain 26695, and *H. mustelae* strain NCTC12198 (left side) and their isogenic urease-negative mutants (right side). Bottom panel: comparison of wild-type *H. bilis, H. pullorum* and *H. cinaedi* isolates. All *Helicobacter* species were grown on precipitation agar (Columbia agar supplemented with Dent selective supplement (Oxoid), 0.2% _-cyclodextrins, 333 mM Urea, 100 μM $NiCl_2$, 20 mM $NaHCO_3$, 187 mM NH_4Cl, 30 mM $CaCl_2$, 0.04% triphenyl tetrazolium chloride; all concentrations given are final concentrations). After overnight growth in microaerobic conditions (5% O_2, 10% CO_2, 85% N_2), calcium crystals are visible with urease positive *Helicobacter* species, but not with urease-negative *Helicobacter* species or with the isogenic urease-negative mutants. Magnification 3×, photos taken with 3 megapixel digital camera. The figure represents a representative example of the three independent experiments performed.

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Chapter 8

SUMMARY & DISCUSSION

SAMENVATTING & CONCLUSIES

CURRICULUM VITAE

LIST OF PUBLICATIONS

AKNOWLEDGMENTS

SUMMARY & DISCUSSION

Chronic inflammation is a precursor for metaplastic and dysplastic changes in diverse organs of the mammalian digestive tract, and can lead to carcinogenesis. Chronic hepatitis is a precursor for hepatocellular carcinoma, and can be caused by an infection with hepatitis B and C virus, alcohol and medicine abuse (3). Approximately ten percent of the patients with chronic hepatitis in a non-cirrhotic liver do not have any of the known risk factors, and it has been suggested that chronic colonization by an unknown *Helicobacter* species may contribute to disease development in these cases (7). The role of *Helicobacter* spp. in the development of hepatitis and hepatocellular carcinoma could be similar to that of *Helicobacter pylori* and the development of gastric ulceration and gastric carcinoma. *Helicobacter hepaticus* colonizes the intestinal and hepatobiliary tract of rodents, and is thought to represent an excellent model for the research on yet unknown human enterohepatic *Helicobacter* species.

H. hepaticus colonizes different niches: the intestine, liver and bile ducts. This suggests that the organism is well adapted to the variable and hostile conditions thought to occur there. The ability to survive and grow in these different environments is thought to be established through the capability to cope with toxic environmental factors derived from both medication and the host immune system. The research presented in this thesis was aimed to investigate the molecular mechanisms underlying stress adaptation mechanisms of *H. hepaticus*. This should give us a better insight in the development of the diseases related to *H. hepaticus*, and the role that such mechanisms play in the evolutionary differentiation of *Helicobacter* species as shown by their ability to colonize different niches.

CHAPTER 2 describes the similarities and differences among three *Helicobacter* species (*H. hepaticus, H. pylori* and *H. mustelae*) based on comparative genomic analyses of systems proposed to be involved in metal transport, metal storage and metal-responsive regulation. The three analyzed species share many similarities with regard to the genes involved in metal metabolism, but also some striking differences are apparent. These differences may represent adaptations of the different species to their specific host and target organs, thus contributing to the host- and organ-specificity associated with *Helicobacter* infections. Based on 16S rRNA phylogeny (11) it could be assumed that the adaptation of the three *Helicobacter* species appeared early in *Helicobacter* evolution, emphasizing the coevolution of *Helicobacters* with their host.

CHAPTER 3 describes the difference in urease regulation between *H. hepaticus* and *H. pylori*. While urease activity is essential for acid resistance of *H. pylori* (1), urease activity does not contribute to acid resistance in *H. hepaticus*. This may be one of the reasons why *H. hepaticus* is not found in the acidic gastric mucosa. Furthermore it was demonstrated that urease regulation is different in *H. hepaticus* when compared to *H. pylori*. In *H. hepaticus* urease activity is nickel-induced only at the post-translational level (enzyme activity), while in *H. pylori* this regulation occur on both transcriptionally and post-translational level. The latter response may be required for gastric colonization, and hints again at the presence of organ-specific responses in *Helicobacter* species. In **CHAPTER 4** the urease regulation of *H. hepaticus* is further

investigated. Expression of *H. hepaticus* urease is iron-responsive through the transcriptional regulator Fur. Although, iron is not involved in urease enzyme activity, the iron-responsive regulation is in agreement with the well-known hypothesis that low iron availability in the host is used by bacteria as a signal for expression of colonization factors (10). Whether urease activity contributes to the colonization of the intestinal and hepatobiliary tract is not yet known. The enzyme is predicted to be involved in nitrogen metabolism, as the ammonia produced by urea hydrolysis can be used by the cell. In the competitive niche of the intestine this may give *H. hepaticus* an advantage over other bacterial colonizers. However, urease enzyme activity may also be important for the pathogenicity of *H. hepaticus* infection as it might contribute to gall stone formation as described in **Chapter 7**. Precipitation of calcium and stone formation is a feature harbored by other bacteria as well, like with *Proteus mirabilis* and kidney stones (8). The results of this chapter could have a future clinical application; instead of removing the gallbladder in patients with recurring gallstone formation, antibiotic treatment of the underlying infection may be used for prevention of further gallstone development. Further understanding of how bacteria can play a role in gallstone development is therefore required.

The presence of *H. hepaticus* in the gallbladder and bile ducts poses another question. Bile acids are toxic to many microbes and apart from the bile duct the intestine and liver also contain bile acids. What mechanisms are involved in *H. hepaticus* bile survival? In **Chapter 6** putative bile efflux systems are identified using the complete genome sequence of *H. hepaticus* (12), which revealed the presence of an operon orthologous to the *C. jejuni* bile-efflux system *cmeABC*. Although the operon is lacking the gene coding for an outer membrane protein (OMP), its presence suggests that this operon might be involved in bile acid-resistance of *H. hepaticus*. The bile resistance of *H. hepaticus,* as shown to modulated by *hefA* may well be the result of a *cmeAB* transfusion with the *hefA* OMP. Evaluation of this uncharacterized systems in *H. hepaticus* will improve our understanding of its antimicrobial resistance.

This chapter also describes the resistance of *H. hepaticus* and *H. pylori* to specific bile acids. Both species have the ability to grow and survive in the presence of bile acids but *H. hepatic*us can resist higher concentrations of cholic acid and taurocholic acid and taurodeoxycholic acid compared to *H. pylori*. This is inherent to the place of colonization and the possibility of being confronted with bile acids. The resistance of *H. pylori* to cholic acid has been ascribed to the HefABC system (14). However this system is a multi drug transporter system, which is also effective in the transport of other antimicrobial agents, whereas the Cme-efflux system of *C. jejuni* is described to be highly specific for bile acid transportation and is regulated by the bile acid-responsive regulator CmeR (9). The *cme* operon is absent in *H. pylori*, supporting the hypothesis that *H. pylori* lacks systems required for biliary/intestinal colonization, while containing systems required for gastric colonization. Comparison of additional gastric and enterohepatic *Helicobacter* species should allow more detailed searches for gastric and enterohepatic colonization-specific factors.

The HefA outer membrane transporter also appeared to be involved in amoxicillin resistance. Although the gastric *H. pylori* has a *hefABC* operon the organism is

naturally sensitive to amoxicillin, resistance can be gained in *H. pylori* but this is by a completly different mechanism of penicillin binding protein (4). Antibiotic resistance of *Helicobacter* species has a clinical importance in view of animal experiments. Mice can be naturally colonized with *H. hepaticus*, differences in infection rates and strains may influence the outcome of experimental infections of mice or studies on intestinal and hepatic systems. Mice are often treated with a mix of antibiotics including amoxicillin (6, 13), and antibiotic resistance to one of the components is well known to reduce efficacy of the treatment, and thus may be insufficient to eradicate the infection with enterohepatic *Helicobacter* species. With **CHAPTER 6** we hope to create awareness of the shortcomings of amoxicillin-containing regimens, and it may be advisable to verify the absence of enterohepatic *Helicobacter* species (13).

One of the main defenses of the human host to bacterial infections is via the production of reactive oxygen species. In addition, microaerophilic organisms like *H. hepaticus* also need to be able to handle oxidative stress caused by atmospheric oxygen. The main defense against oxidative stress is via enzymes which remove the reactive oxygen species, like superoxide dismutase and catalase. The ability of bacteria to cope with such oxidative stresses is thought to comprise an important virulence factor. **CHAPTER 5** describes the iron-responsive regulation of expression of the *H. hepaticus katA* gene, which encodes the catalase enzyme. This regulation is mediated via the PerR regulatory protein, in *H. pylori*, who doesn't have a *perR* gene, this is done by Fur (5). Although, both organisms have an iron-responsive oxidative stress response (2) *H. hepaticus* is more like the intestinal colonizer *C. jejuni* with the iron responsive PerR regulating oxidative stress response (15) It would be interesting to know how *perR* is distributed among enterohepatic and gastric *Helicobacter* species.

Overall, *H. hepaticus* is well adapted to environmental stress factors. Low iron seems to be an important signal that leads to activation of *H. hepaticus* genes required for survival under these stressful conditions. The right antibiotic treatment of *H. hepaticus* infections may prevent gallstone formation, hepatitis and liver cancer in rodents, and may also be helpful in preventing inflammatory differences in animal experiments using rodents, thus ensuring better reproducibility between laboratories. If there is an enterohepatic *Helicobacter* species in humans, this could explain the development of hepatitis and gallstones in patients without any of the known risk factors, and these patients may benefit from targeted antibiotic treatment

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SAMENVATTING & CONCLUSIES

Een chronische ontsteking is een voorbode voor metaplasie en dysplasie van het betreffende weefsel en kan leiden tot kanker. Lever kanker kan het gevolg zijn van chronische hepatitis veroorzaakt door een infectie met het hepatitis B of C virus, alcohol en medicijn misbruik. Echter, in een aanzienlijk percentage van de patiënten is de oorzaak van hepatitis niet te herleiden naar een van deze factoren. Een infectie met de bacterie *Helicobacter* is mogelijk de oorzaak bij deze patiënten. Het mechanisme dat ten grondslag ligt aan de door *Helicobacter pylori* geïnduceerde ontsteking van de maag en de ontwikkeling van maag kanker zou tevens kunnen gelden voor de door een *Helicobacter* geïnduceerde hepatitis en de ontwikkeling van lever kanker. *Helicobacter hepaticus* is een bacterie die muizen infecteert. Een infectie met deze bacterie kan leiden tot hepatitis, leverkanker, chronische darm ontsteking en zelfs galstenen. Deze bacterie is daarom te gebruiken als model organisme voor een mogelijke associatie van *Helicobacter* in de humane hepatitis.

H. hepaticus koloniseert zowel de darmen de lever als de galwegen van zijn gastheer. Dit duidt er op dat de bacterie in staat is zich aan te passen aan de verschillende omstandigheden die zich voordoen in deze organen. In de gastheer wordt de bacterie geconfronteerd met veel schadelijke componenten als het gevolg van zowel de afweer van de gastheer en eventuele medicatie die bedoeld is de bacterie te bestrijden.

Dit proefschrift beschrijft moleculaire mechanismen die *H. hepaticus* gebruikt om zich te wapenen tegen en aan te passen aan verschillende stress volle situaties die zich kunnen voordoen in de gastheer. De verkregen resultaten geven ons mogelijk een beter inzicht in de rol van *H. hepaticus* in ziekte ontwikkeling. Alsmede, de mogelijke rol van zulke moleculaire mechanismen in de evolutie van *Helicobacter* soorten.

HOOFDSTUK 2 beschrijft de overeenkomsten en verschillen tussen drie *Helicobacter* soorten (*H. pylori, H. hepaticus* en *H. mustelae*) op basis van vergelijking van DNA sequenties die betrokken zijn bij het metaal metabolisme van deze bacteriën. De drie beschreven *Helicobacter* soorten vertonen veel gelijkenissen, er zijn echter ook een aantal opvallende verschillen. Deze verschillen duiden erop dat elke afzonderlijke soort zich heeft aangepast aan specifieke organen binnen een specifieke gastheer. De fylogenie van het 16S rRNA van bekende *Helicobacters* doet vermoeden dat de afsplitsing van de verschillende soorten vroeg heeft plaats gevonden in de evolutie en dat elke *Helicobacter* soort mee geëvalueerd is met zijn specifieke gastheer.

HOOFDSTUK 3 bestudeerd het verschil in urease regulatie tussen *H. hepaticus* en *H. pylori*. Urease enzym activiteit is essentieel voor *H. pylori* om het zure milieu van de maag te kunnen overleven. *H. hepaticus* bezit ook een actief urease enzym maar dit stel de bacterie niet in staat te overleven in een zure omgeving. Dit is in overeenstemming met het feit dat *H. hepaticus* nog nooit is aangetroffen in de maag of het maag epitheel. **HOOFDSTUK 3** demonstreert tevens dat de regulatie van het urease gen verschilt tussen *H. hepaticus* en *H. pylori*. Activatie van het urease eiwit is in beide soorten afhankelijk van nikkel, echter in *H. hepaticus* is geen sprake van inductie van het urease gen door nikkel zoals dat het geval is bij *H. pylori*. Dit doet wederom vermoeden dat *H. pylori* zich heeft aangepast aan de maag en het zure milieu aldaar, terwijl *H. hepaticus*

waarschijnlijk een ander mechanisme hanteert dat deze bacterie in staat stelt in zijn eigen specifieke milieu te overleven.

In **hoofdstuk 4** is de urease regulatie van *H. hepaticus* verder onderzocht. De expressie van het *H. hepaticus* urease gen blijkt afhankelijk te zijn van ijzer en de transcriptionele regulator Fur. De ijzer afhankelijke regulatie is in overeenstemming met de hypothese dat een lage concentratie ijzer in de gastheer door bacteriën gebruikt wordt als signaal voor de expressie van kolonisatie factoren. Of urease werkelijk een bijdrage levert aan de mogelijkheid van *H. hepaticus* om te koloniseren is echter niet bekend. Men verwacht dat de urease enzym activiteit het stikstof metabolisme van de cel ten goede komt, omdat er ammonia vrij komt na de hydrolyse van ureum. Zo zou *H. hepaticus* in het voordeel zijn wanneer het zich in een competitieve omgeving bevind zoals de darmen. Echter, de urease enzym activiteit van *H. hepaticus* zou ook een rol kunne spelen bij de pathogeniteit aangezien het een mogelijke rol speelt bij het ontstaan van galstenen zoals beschreven in **hoofdstuk 7**. Het precipiteren van calcium en het ontstaan van steentjes is een eigenschap die reeds is beschreven voor andere bacteriën. De associatie van *Proteus mirabilis* met nierstenen is hier een goed voorbeeld van. De resultaten gepresenteerd in dit hoofdstuk zouden in de toekomst misschien van betekenis kunnen zijn voor patiënten die veel last ondervinden van galstenen. Mogelijkerwijs is het bij deze patiënten niet meer nodig de galblaas te verwijderen maar kan een antibiotica behandeling volstaan om de infectie en de galsteen formatie tegen te gaan. Dit vereist echter verder onderzoek naar de betrokkenheid van bacteriën bij het ontstaan van galstenen.

Galzuren komen voor in zowel de galwegen, de lever als de darmen en zijn schadelijk voor microörganismen. Welke mechanismen hanteert *H. hepaticus* om de aanwezigheid van gal te overleven in de betreffende organen? In **hoofdstuk 6** worden genen van *H. hepaticus* geïdentificeerd die mogelijkerwijs coderen voor eiwitten die galzuren uit de cel kunnen transporteren. In *H. hepaticus* blijkt een operon aanwezig te zijn dat ortoloog is aan een galzuur transport systeem *cmeABC* van *Campylobacter jejuni*. Ondanks het feit dat er geen gen aanwezig is in het operon dat codeert voor het buiten membraan eiwit zouden deze genen toch betrokken kunnen zijn bij de galzuur resistentie. Dit hoofdstuk beschrijft dat de galzuur resistentie van *H. hepaticus* afhankelijk is van het HefA buitenmembraan eiwit. Mogelijkerwijs, treed het HefA eiwit op als buitenmembraan component van CmeAB. Verder onderzoek naar dit nog ongekarakteriseerde systeem zal onze kennis over de galzuur resistentie van *H. hepaticus* kunnen vergroten.

Hoofdstuk 6 beschrijft tevens dat *H. hepaticus* minder gevoelig is voor galzuren dan *H. pylori*. Dit is te relateren aan de verschillende organen die deze bacteriën infecteren. In de maag zijn geen galzuren aanwezig terwijl die in de lever, galwegen en darmen wel aanwezig zijn. Dit ondersteunt de hypothese dat deze bacterie niet is aangepast aan een omgeving waar gal aanwezig is. Door meer *Helicobacter* soorten uit verschillende organen met elkaar te vergelijken zouden factoren betrokken bij de specialisatie van de verschillende soorten geïdentificeerd kunnen worden.

De HefA transporter van *H. hepaticus* blijkt ook te zijn betrokken bij de resistentie tegen het antibiotica amoxicilline. Ondanks de aanwezigheid van een HefABC transport

systeem in *H. pylori* is deze bacterie gevoelig voor amoxicilline. *H. pylori* kan resistentie verkrijgen tegen amoxicilline maar het mechanisme dat hieraan ten grondslag ligt betreft een penicilline bindend eiwit. Inzicht in antibiotica resistentie van *Helicobacter* bacteriën is klinisch relevant wanneer men het in het licht stelt van dier experimenten. Muizen kunnen van nature gekoloniseerd zijn met *H. hepaticus*, deze infectie kan interfereren met de beoogde studie en de uitkomst ervan. Om muizen vrij te krijgen van een *Helicobacter* infectie worden deze doorgaans behandeld met een mix van antibiotica waaronder amoxicilline. Resistentie tegen een van de componenten uit zo'n behandeling kan de efficiëntie van de behandeling verminderen of zelf te niet doen. De gepresenteerde resultaten leiden mogelijk tot bewustwording van de tekortkomingen van een amoxicilline kuur wanneer het de eradicatie van enterohepatische *Helicobacters* betreft.

Een van de belangrijkste afweermechanismen van de gastheer om bacteriële infecties tegen te gaan is de productie van reactief zuurstof. Bovendien moeten microearofiele bacteriën in staat zijn om te gaan met atmosferische zuurstof concentraties. Deze oxidatieve stress kan worden overkomen door enzymen te hanteren zoals superoxide dismutase en katalase die het reactief zuurstof omzetten. De mogelijkheid van een bacterie om met zulke oxidatieve stress te kunnen omgaan wordt dan ook gezien als een belangrijke virulentie factor. In **HOOFDSTUK 5** word de ijzer en peroxide afhankelijke regulatie van het *H. hepaticus* katalase (*katA*) gen beschreven. Deze regulatie is afhankelijk van de transcriptie factor PerR. In *H. pylori*, die geen *perR* bezit, wordt deze regulatie toegeschreven aan Fur. Ondanks het feit dat beide organismen een ijzer afhankelijke respons hebben om zich te beschermen tegen oxidatieve stress vertoont *H. hepaticus* op dit punt meer gelijkenis met de intestinale *C. jejuni* die ook een ijzer afhankelijke PerR eiwit hanteert. Het zou interessant zijn om te weten hoe het PerR eiwit gedistribueerd is in *Helicobacter* soorten en of dit verschilt tussen enterohepatische en gastrische *Helicobacter* soorten.

Tot slot, kunnen we stellen dat *H. hepaticus* goed is aangepast aan stress factoren uit zijn omgeving. Een lage ijzer concentratie is een belangrijk signaal voor de bacterie en leidt tot activatie van genen die betrokken zijn bij het overleven van deze stress situaties. Een juiste antibiotica kuur gericht tegen *H. hepaticus* zou het ontstaan van zowel gastenen, hepatitis en lever kanker in knaagdieren kunnen voorkomen, alsmede de reproduceerbaarheid van dier experimenten kunnen verbeteren. Mocht er een enterohepatische *Helicobacter* in mensen gevonden worden dan zou dit mogelijk het ontstaan van hepatitis en galstenen kunnen verklaren in patiënten waar geen van de bekende oorzaken te achterhalen zijn. Deze patiënten zouden dan gebaat zijn bij een antibiotica kuur gericht tegen deze enterohepatische *Helicobacter* soort.

CURRICULUM VITAE

Clara Belzer was born on the 20th of March 1978 in Rotterdam, the Netherlands. She attended 'het Emmauscollege' in Rotterdam and after her graduation started her biology study at the University of Utrecht. For her masters degree she did an internship at the Phytopathology group of prof. dr. van Loon (University Utrecht) studying the involvement of pathogenic microorganisms in plant disease. This work was followed by a literature study about the plant microbe interactions under supervision of prof. dr. Wösten (Utrecht University, Molecular Microbiology).

In 2003 she moved to Spain for a research internship at the Centro National the Biotechnologia (CNB) in Madrid under supervision of dr. Perez. The study aimed to characterize the pathogenic mechanisms of *Ustilago maydis*.

From August 2003 until November 2007 she worked as a PhD student at the department of Gastroenterology and Hepatology of the ErasmusMC in Rotterdam. Under supervision of prof. dr. Kuipers, dr. van Vliet and dr. Kusters she performed the research that is presented in this thesis. Hereafter she will continue her scientific carrier in Boston, USA.

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NOTES