Transcriptional Regulation of the Nickel and Iron Metabolism in *Helicobacter pylori*

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Transcriptionele regulatie van nickel en ijzer metabolisme in *Helicobacter pylori*

Thesis

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CONTENTS

CHAPTER 1

The role of nickel in environmental adaptation of the gastric pathogen *Helicobacter pylori*

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1 INTRODUCTION

Gastric problems represent a common problem that nearly everybody encounters during life. Before the discovery of the gastric pathogen *Helicobacter pylori*, such gastric problems were often debilitating and incurable. The first report that bacteria might cause gastric diseases by colonization of the stomach dates back over 100 years (18). However, proof that bacterial infections are indeed the causative agent of gastric disorders came not until 1982, when two Australian scientists, Robin Warren and Barry Marshall, were able to culture *H. pylori* (originally named *Campylobacter pyloridis*) from gastric biopsy samples (100). Subsequently, Marshall fulfilled Koch's postulates for *H. pylori* in a self-infection experiment, which resulted in *H. pylori*-induced gastritis. *H. pylori* was than re-isolated from gastric biopsies, and the inflammatory disease was cured by subsequent treatment with antibiotics (99). The discovery of *H. pylori* and the associated disorders was a major breakthrough in gastroenterology. It offered not only a novel way for the understanding of mechanisms underlying ulcerations, lymphomas and gastric cancer, but also for the treatment of gastric diseases by antibiotic treatment.

1.1 Microbiology and Molecular Biology of *Helicobacter pylori*

H. pylori is a non-spore forming, Gram-negative, curved bacterium with an average size of 0.2 x 0.5 μm. It is highly motile via its 5 to 6 unipolar flagella, which are sheathed and end in a terminal bulb ((128), Fig. 1). *H. pylori* requires microaerobic conditions for growth, and is usually cultivated in an atmosphere of 2-5% O_2 and 5-10% CO_2 (7). The standard identification method for *H. pylori* is Gram-staining followed by microscopic identification. Other identification methods are tests for urease, catalase and oxidase, as *H. pylori* is positive for all three enzymes (7).

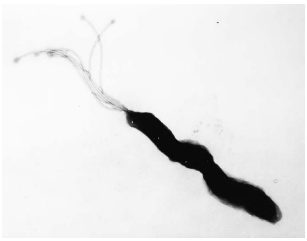

Fig. 1. EM picture of *H. pylori* (source: www.oka.urban.ne.jp/ home/yoshiro/hp1.jpg).

H. pylori belongs to the ε-subdivision of the proteobacteria, and is classified in the order of *Campylobacterales*, family of *Helicobacteraceae* (51). In 1997 the genome sequence of *H. pylori* strain 26695 became available (156), which was followed in 1999 by the sequence of another strain (J99; (6)). The *H. pylori* genome has an average size of 1.6 Mbp with an overall G/C-content of 35%. Approximately 1600 open reading frames (ORF) were identified which cover 90% of the *H. pylori* genome. One-third of the identified ORFs are considered to be *Helicobacter*-specific (6, 97, 156). Furthermore, about 50% of all *H. pylori* strains carry plasmids, however these plasmids cannot be used for cloning experiments (131). *H. pylori* strains are highly diverse and adapt to their host due to constant changes of its genome by point mutations, substitutions, insertions and deletions (36, 55, 153). In addition, *H. pylori* is able to switch transcription and translation

of genes on and off through phase variation (35).

1.2 H. pylori Colonization and Infection

Infection with *H. pylori* occurs worldwide. However, the prevalence of infection varies between different parts of the world. Infection with *H. pylori* mostly occurs in families in early childhood via the oral-oral transmission route (110). The prevalence of *H. pylori* infection is associated with the socio-economic status of the host; in developing countries around 90% of the population is infected, and this is potentiated by low income, high numbers of children per household, and lower hygienic standards (110). In contrast, only 20 to 50% of the people are infected in industrialized countries. As a result of higher hygienic standards, as well as active treatment of infection with antibiotics, the prevalence of infection with *H. pylori* has decreased significantly in the Western world over the last two decades (153).

H. pylori colonizes the mucus layer overlaying the epithelial cells of the human stomach, and this leads to lifelong chronic infection unless treated (77, 119, 153). With the help of sequence comparison of gene fragments from *H. pylori* found in the stomach of humans from all over the world, it was demonstrated that *H. pylori* is colonizing the human stomach for thousands of years (32), and that *H. pylori* populations arose in Africa and Asia and were spread worldwide through human migratory fluxes (56).

Course of H. pylori Infection. It is generally accepted that infection with *H. pylori* causes acute gastric inflammation initiated by autoimmune reactions. Gastritis develops predominantly in the antrum of the stomach, as there is a higher local acid concentration (44), where it might further develop into duodenal ulcer. In contrast, patients with gastritis in the corpus are predominantly at risk of developing gastric ulcers, which can develop into gastric atrophy, intestinal metaplasia and gastric carcinoma ((153); Fig. 2). As *H. pylori* infection is the main cause of atrophic gastritis in human, *H. pylori* was classified a class I carcinogen by the WHO in 1994 (9). It was furthermore demonstrated that *H. pylori* infection increases the risk of developing MALT-lymphoma ((153); Fig. 2).

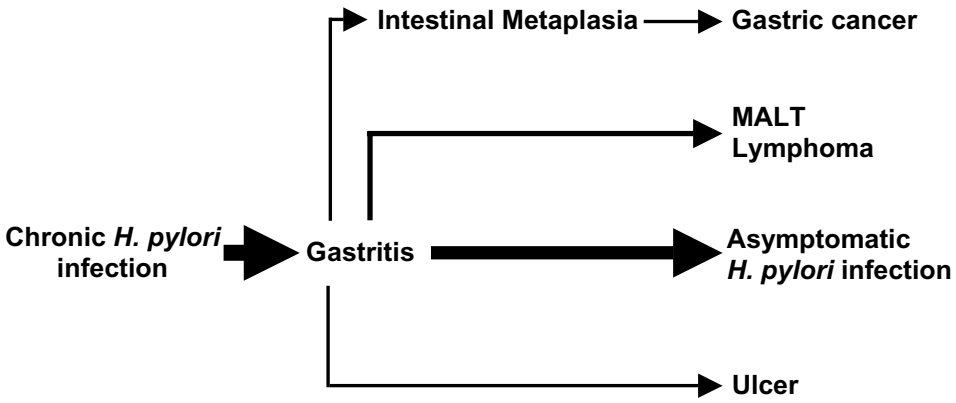

Fig. 2. Chronic infection with *H. pylori* always leads to gastritis. The further outcome of infection can differ, as indicated by the arrows. The thickness of the arrows indicates the approximate quantities of disease outcome.

An interaction of *H. pylori* and gastroesophageal reflux disease (GERD) was suggested as it was shown that GERD patients had a lower prevalence for *H. pylori* infections. It is thought that *H. pylori* infection might prevent GERD due to suppression of the acid-producing parietal cells, which may lead to an increase of the pH in the stomach. However, no increased risk for the subsequent development of GERD was found so far in patients after eradication of *H. pylori* (87).

Diagnostics. Several methods are used to diagnose *H. pylori* infection. They can be divided into invasive and non-invasive techniques. Invasive techniques were the first diagnostic tools and they always include obtaining a gastric biopsy. The most standardized technique is subsequent culture of *H. pylori* from these obtained biopsies, or a quick urease test (CLO-test). Other tests involving gastric biopsies are PCR on 16S rRNA or the urease genes. Non-invasive techniques are serology against *H. pylori* antigens, as well as stool antigen tests or urea breath-tests using radioactively labeled urea (95, 153).

Treatment. Eradication of *H. pylori* infection usually results in cure of the underlying *H. pylori*-associated disorder (153). *H. pylori* is susceptible to most antibiotics in in-vitro experiments (102), however many antibiotics are not very effective for treatment of *H. pylori*-infected patients (69). This failure may be due to the slow growth rate of *H. pylori*, an inability of antibiotics to attain effective concentrations in the gastric mucus layer (103), or inactivation of antibiotics by the low pH at the site of infection (70). Therefore, therapy with one antibiotic alone is mostly ineffective. The standard treatment of *H. pylori* eradication consists of a combination therapy of antibiotics and acid-suppressive drugs. Usually a triple therapy is given for 7 to 14 days, consisting of two antibiotics (either amoxicillin, clarithromycin, metronidazole, or tetracycline (105)) together with a proton pump inhibitor to raise the pH of the stomach and/or ranithidine bismuth citrate (153). However, as the prevalence of antibiotic resistance is rising, other strategies like vaccination or treatment with inhibitors against essential colonization factors like the urease will become necessary (see section 6).

1.3 Gastric Adaptation

The acidity in the mucus layer of the human stomach is thought to vary around pH 5, with occasional acid shocks occurring when the mucus layer is damaged (141). This is still under debate, as the proton protective ability of the mucus layer has been questioned (98). However, despite its acidic habitat, *H. pylori* is a neutralophilic bacterium which maintains its cytoplasmic pH around 7 (140), and under *in vitro* conditions in the absence of urea, the lowest pH allowing growth of *H. pylori* in vitro is about pH 5 (25, 161).

H. pylori is able to respond actively to changes in the environmental proton concentrations. At low pH, acidity induces changes in the composition of lipopolysaccharides (118), elevates the expression of chaperone-like proteins (78) and affects transcription of many genes (25, 108, 161). One of the most important factors to survive the acidic pH in the stomach is the *H. pylori* urease, which is a nickel-containing enzyme. *H. pylori* also possesses alternative systems for ammonia production in times of urea shortage, the amidase (HP0294) and formamidase (HP1238) enzymes ((25, 161); Fig. 3). In general, amidases catalyze the conversion of amide substrates to the corresponding carboxylic acid and ammonia, however both amidases use different substrates. AmiE hydrolyses propionamide, acetamide and acrylamide, but not formamide, whereas AmiF exclusively hydrolysed formamide (147, 148). It was previously demonstrated that

regulation of transcription of the urease structural genes, as well as induction of urease activity, is regulated by the nickel responsive regulator NikR and dependent on nickel cofactor as ((25, 161-163), Fig. 4). It is thought that nickel bioavailability is an indicator for sensing the environmental pH (161). Therefore, it has been demonstrated that under mildly acidic conditions control of both the amidase and the formamidase genes is mediated in a regulatory cascade by NikR and a second metal regulatory protein, the iron uptake regulator Fur ((25, 160); Fig. 3).

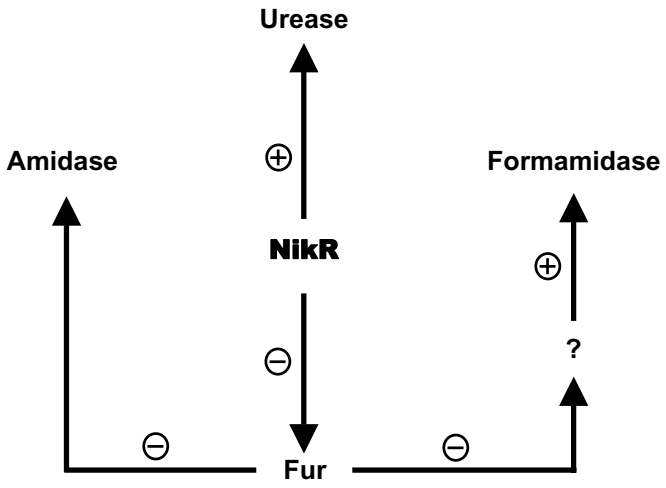

Fig. 3. Transcription of ammonia-producing genes is mediated via a cascade involving Fur and NikR. Transcription of urease is induced, whereas transcription of the iron-uptake regulator Fur is repressed by NikR. The Fur regulator in turn represses transcription of the amidase gene, while regulation of the formamidase gene by Fur involves a currently unknown mediator.

The importance of urease for *H. pylori* is demonstrated by the inability of urease mutants to colonize the gastric mucosa of nude mice or cynomolgus monkeys (46, 47, 116, 149, 159). And even when the pH of the stomach of gnotobiotic piglets was raised to neutral pH with proton pump inhibitors, only very low numbers of urease mutants were recovered (33).

1.4 Mechanisms of Pathogenesis

The mucus layer overlaying the gastric epithelial cells is the primary niche of *H. pylori*, which is characterized by acidic pH, rapid changes in nutrient availability. As also a possibly aggressive host immune response can occur, *H. pylori* requires several systems for colonization and survival in this hostile environment.

Motility and Chemotaxis. After primary infection, *H. pylori* moves rapidly to the mucus layer which is thought to be more pH-neutral, to escape the acidity of the gastric lumen. *H. pylori* uses its flagella to reach the mucus layer and orients itself with the help of a potent chemotaxis system (122), which, amongst others, responds to the urea gradient

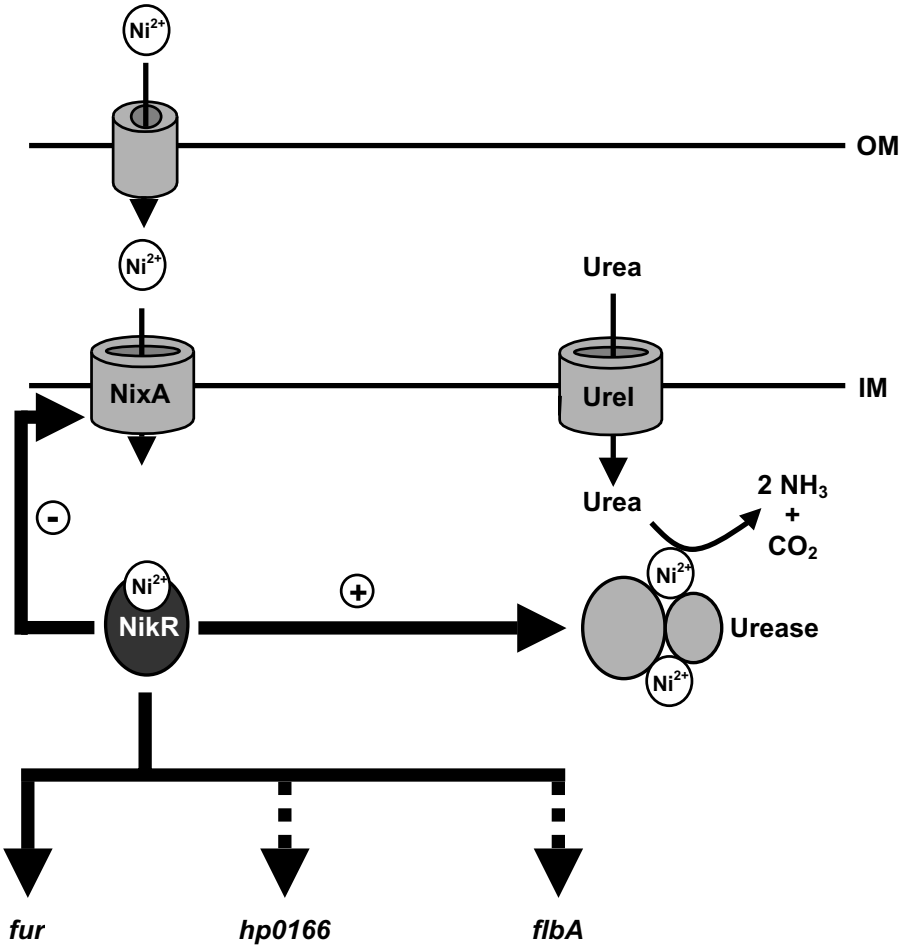

Fig. 4. The nickel-uptake by NixA is nickel-dependent repressed, whereas the urease activity is nickel-dependent induced by the nickel responsive regulator NikR. Further genes regulated by NikR are the *fur* regulatory gene and possibility *hp0166* and *flbA*. Increased nickel-availability is thought to be a signal for a low pH of the environment. Transcription of urease is induced to convert urea which is entering through UreI under low pH into NH_3 and CO_2; OM: outer membrane; IM: inner membrane.

present in the gastric mucus layer (142). As the amount of substrates for bacterial growth and metabolism is varying in the stomach and as the mucus layer itself is thought to be rather poor of nutrients (126), both the flagella and the chemotaxis enables *H. pylori* to adequately react on resource changes.

CagA/VacA. *H. pylori* produces several factors that allow the bacteria to actively modulate immune responses. The exact role of these alterations is still unknown, but there are two major factors that interact directly with host cells; the vacuolating cytotoxin VacA and the cytotoxin-associated gene pathogenicity island (*cag* PAI).

VacA is a cytotoxin that causes the formation and accumulation of endosomal vacuoles in epithelial cells. Furthermore, it forms pores in the cytoplasmic membrane that

lead to leakage of anions and urea out of cells, which can serve as nutrients for *H. pylori*. The structure of the *vacA* gene is highly variable, and certain variants of the VacA protein are associated with increased risk of ulcer disease and cancer (10, 19, 77, 104, 119).

The genes of the *cag* PAI code for a type IV secretion system, which injects the CagA protein into host epithelial cells (151). Inside the cells, CagA is phosphorylated and influences proliferation, apoptosis, cytokine release and cytoskeleton rearrangements, which is leading to a "hummingbird" morphology in AGS cells (19, 77, 104, 119). Although the biological consequences of the cellular CagA effects are not understood so far, one can speculate that most of them are of advantage for the bacterium and may lead to an increased release of nutrients to *H. pylori*. In addition, *H. pylori* induces inflammatory responses, like induction of IL-8 (23), and the infection results in a strong accumulation of neutrophils and macrophages in the inflamed gastric mucosa. These cells of the innate immune system play an important role in the defense against bacterial infections, as they are specialized on the eradication of pathogens by phagocytosis. *H. pylori* is able to interact directly with these immune cells, as it downregulates phagosome formation and is able to persist within macrophages for extended time periods (3-5, 138). The *H. pylori* urease, which produces carbonate and ammonia from urea, is suspected to play a key role in the interaction with immune cells. Macrophages produce both nitric oxide (created by inducible nitrite oxide synthase (iNOS)) and superoxides, which in turn react to peroxynitrite (81). Peroxynitrite can be scavenged and detoxified by carbonate, which is an aqueous product of the *H. pylori* urease reaction (88). It has been demonstrated that urease induces iNOS (68), which is associated with epithelial cell damage (89) and apoptosis (96). Furthermore, it is speculated that urease may directly induce cytokine-induced apoptosis of epithelial cells as the apoptosis levels observed were correlated with *H. pylori* urease activity. Finally, it was demonstrated that ammonia released by the urease reaction is able to accelerate apoptosis via tumor necrosis factor α (TNFα; (79, 84)). Taken together, these mechanisms indicate that *H. pylori* is well-adapted to interactions with host cells and gains access to nutrients by active induction of apoptosis and cell death.

Adhesins. Histological investigations revealed that *H. pylori* is often located in close proximity to epithelial cells, probably because of the less acidic pH and the availability of nutrients there. To protect itself from peristaltic movements of the mucus and to gain easier access to nutrients, as well as to allow interaction with the host cells, *H. pylori* expresses adhesins on its surface. Several adhesins, like BabA or SabA, which attach to $Lewis^b$ and $Lewis^x$ antigens of epithelial cells, have been described (20, 80, 92). $Lewis^x$ antigen is an epitope of glycoproteins that is only present in low concentrations in healthy epithelial cells. Upon *H. pylori* infection, expression of $Lewis^x$ antigen is stimulated, leading to increase of these glycoproteins that can be exploited for further adherence by *H. pylori* (92). Adhesion furthermore is thought to be involved in triggering the immune response leading to increased risk of gastric inflammation, ulceration and gastric cancer (33, 65) and therefore damage of gastric epithelial cells. *H. pylori* can take advantage of the nutritional exudates leaking from the damaged cells (22).

Defence against Reactive Oxygen Species (ROS). Neutrophils and macrophages not only interact directly with bacteria, they are also are able to create reactive oxygen species (ROS) like superoxides and hydrogen peroxide. Furthermore, ROS are also a byproduct of respiration and electron transporters, as well as from the metal-catalyzed oxidation of metabolites (59). The danger of ROS is their reactivity with macromolecules like DNA,

proteins or components of the cell wall, leading to subsequent damage or destruction (152, 158). ROS can be produced via the Haber-Weiss-Fenton-reaction:

Eq. 1: $O_2^- + Fe^{3+} \rightarrow Fe^{2+} + O_2$

Eq. 2: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO^{\bullet}$

Eq. 3: Net: $O_2^- + H_2O_2 \rightarrow HO^{\bullet} + OH^- + O_2$

To survive increased ROS production at the site of inflammation, *H. pylori* expresses several defence mechanisms against oxidative stress. These include its iron-cofactored superoxide dismutase SodB (Hp0389), which detoxifies superoxide radicals into hydrogen peroxide molecules (Eq. 4), which are in turn catalyzed to water and molecular oxygen by the catalase enzyme (KatA (Hp0875); Eq. 5; (152, 153)).

Eq. 4: $2\ O_2^- + 2\ H^+ \rightarrow H_2O_2$

Eq. 5: $H_2O_2 + O_2 \rightarrow H_2O + O_2$

In view of its microaerophilic requirements and low oxygen tolerance, it is not surprising that enzymes involved in the detoxification of ROS are important *H. pylori* colonization factors. In *H. pylori*, this is demonstrated by the inability of mutants in genes encoding components or regulators of detoxification enzymes to colonize the gastric environment in animal models (2, 31, 74, 75, 125, 168, 169).

1.5 Mechanisms of Metal Homoestasis

Metal ions like iron and nickel favour the creation of ROS, but are also essential for metabolism. Therefore, detoxification of ROS needs to be coupled tightly to the control of intracellular metal concentrations, which secures metal ion homeostasis by regulation of metal uptake, export and/or storage. Most metal ions are small enough to enter the periplasm via unspecific pores (123), whereas transport into the cytoplasm is mediated by specific uptake systems, because the cytoplasmic membrane is not permeable for ions. Metal homeostasis is usually mediated by transcriptional regulation via ion-specific regulatory proteins, which combine detector and effector functions in one molecule by sensing the intracellular metal concentration and modulation of transcription by DNA binding. When activated, the regulator protein can induce or repress transcription of the corresponding ion uptake, ion efflux and/or ion storage genes. In *H. pylori* only two metal regulatory proteins have been identified to date, the nickel responsive regulator NikR (36) and the iron-uptake regulator Fur (16).

The metal regulatory protein Fur functions as a repressor. It is a homodimer composed of 17 kDa subunits. It possesses a Helix-Turn-Helix N-terminal DNA binding domain and a C-terminal oligomerization and metal binding domain (136). To repress transcription of its target genes, Fur binds ferrous ions as cofactor (8). The ferric uptake regulator Fur has been found so far in all Gram-negative and many Gram-positive bacteria, as well as in cyanobacteria (54). It was first identified as *fur* mutants of *E. coli* were overexpressing siderophores (73). Therefore, the classical regulation of Fur is regulation of the iron uptake. Upon binding of Fe^{2+} Fur changes its conformation, which has an increased affinity for its DNA binding site in the promoter regions of target genes, called Fur box (41). Fur boxes are mostly located around the -10 and -35 boxes of Fur repressed genes. When the concentration of ferrous ions in the cell decreases, Fur loses its cofactor and subsequently leaves the Fur box (8). The Fur box was first identified as a 19 bp palindromic region in *E. coli* (GATAATGAT(a/t)ATCATTATC), but has been reinterpreted as a region of multiple repeats of NAT(a/t)AT (8).

Apart from the classical repressor function of the Fur protein, Fur had also been

described in *E. coli* as an activator of gene transcription. Several genes like the ferritins *bfr* and *ftn*, the aconitase *acnA*, or the superoxide dismutase *sodB* are Fur- and iron-dependent upregulated. Interestingly, these genes do not possess an apparent Fur-box (8, 73). Recently, the Fur regulation of positive gene transcription had been discovered (101). It is an indirect regulation, where Fur represses the transcription of the small RNA *rhyB*, which acts as a Fe^{2+}-Fur repressed negative regulator of gene regulation for these genes (101). In the absence of Fe^{2+}, Fur is inactive and the small RNA *rhyB* is transcribed, thus repressing transcription of its target genes. Upon increasing ferrous ion concentrations in the cell, a Fe^{2+}-Fur-complex is formed, repressing transcription of *rhyB* and thereby inducing transcription of *rhyB* target genes (101).

H. pylori also possesses a Fur regulatory protein (6, 156), which was demonstrated to partially complement a *fur*-mutant of *E. coli* (16, 57), suggesting that Hp-Fur and Ec-Fur both behave similarly. Interestingly, comparison between the sequences of Hp-Fur and Fur of *Pseudomonas aeroginosa* (136) revealed a great homology in the oligomerization domain and only a low homology in the DNA-binding domain (41), suggesting that the consensus Fur box of *H. pylori* differs significantly from that of other bacteria.

The Fur protein of *H. pylori* directly regulates sets of target genes in two different manners. The classic iron-dependent regulation was confirmed in several studies where Fur binds Fe^{2+} and represses the iron uptake by binding to Fur-boxes on its iron uptake genes (39, 42, 57, 165). The second manner of direct regulation by Fur was first demonstrated with the *H. pylori* ferritin gene (*pfr*, *hp0653*; (15)). With the help of DNAse footprinting experiments (42) it was demonstrated that *pfr* is directly repressed by the iron-free form of Fur.

Apart from regulating metal uptake and storage, Fur of *H. pylori* had also been implicated to be involved in the regulation of acid resistance (17, 25, 161), nitrogen metabolism (164) and oxidative stress resistance (31, 74). Fur is an autoregulator in *H. pylori* that is regulated by the nickel responsive regulator NikR (25, 40, 41, 160, 161). Additionally, Fur of *H. pylori* is necessary for colonization as demonstrated in a mouse model (25).

2 NICKEL ENZYMES AND ENVIRONMENTAL ADAPTATION

2.1 Urease

The nickel-containing metalloenzyme urease (urea amidohydrolase, EC 3.5.1.5) is present in bacteria, plants and animals (112, 113, 115). Urease catalyzes the hydrolysis of urea into ammonia and carbamate (Eq. 6), which decomposes spontaneously into another molecule of ammonia and carbon dioxide (Eq. 7).

Eq. 6: $H_2N\text{-}CO\text{-}NH_2 + H_2O \rightarrow NH_3 + H_2N\text{-}HCO_2$

Eq. 7: $H_2N\text{-}HCO_2 \rightarrow NH_3 + CO_2$

Carbon dioxide is subsequently converted into carbonic acid mediated by the periplasmic carbonic anhydrase (Cah, Hp1186; (98)) and the cytosolic carbonic anhydrase (IcfA, Hp0004). Both carbonic anhydrases were demonstrated to be required for urease activity (98, 150).

Eq. 8: $CO_2 + H_2O \rightarrow H_2CO_3$

Protonation of ammonia and carbonic acid in equilibrium in aqueous solutions is occurring:

Eq. 9: $2\ NH_3 + 2\ H^+ \leftrightarrow 2\ NH_4^+$

Eq. 10: $H_2CO_3 \leftrightarrow H^+ + HCO_3^-$

The protonation of ammonia leads to an increase in pH and the NH_3 /NH_4^+ couple reaches its equilibrium at pH 9.2. The H_2CO_3/HCO_3^- couple is in equilibrium at pH 6.1 (98). In most organisms, including the neutralophilic bacterium *H. pylori* (172), urease activity functions to produce ammonia for nitrogen metabolism. However, as the protonation of ammonia leads to an increase in pH, in *H. pylori* the urease activity is primarily used to neutralize the acidic surrounding of the human stomach.

The activity of the urease enzyme in *H. pylori* does not essentially differ much from ureases of other bacteria (113, 115). However, as up to 10% of the whole cell protein can consist of urease protein, the enzyme activity in *H. pylori* is 10-100 fold higher than observed in most other urease-positive bacteria (114, 161). The K_m of *H. pylori* urease is somewhere between 0.17 to 0.48 mM [urea/min/mg protein]. The physiological concentration of urea in the mucus layer of the human stomach is thought to be around 1.7 to 3.4 mM, therefore, the *H. pylori* urease is saturated under physiological conditions (115). In comparison, the K_m of ureases of microorganisms, which are exposed to high concentrations of urea, is usually higher (113). For example, K_m values between 13 and 130 mM have been observed in *P. mirabilis*, *Sporosarcina ureae* and *B. pasteurii* (113). Urease of *H. pylori* is composed of two subunits UreA (27 kDa) and UreB (62 kDa, (71, 115, 135)). In the quaternary structure, the subunits form a multimeric enzyme complex with spherical assembly. The native urease holoenzyme is a dodecameric protein structure consisting of four UreAB trimers $((AB)_3)_4$ (71, 135). Each UreAB subunit needs two nickel ions for activity. Therefore 24 nickel ions are necessary for a native $((UreAB)_3)_4$ holoenzyme.

The urease genes are evolutionary related and share a common ancestor. In general, ureases consist of the structural subunits UreABC (113). In *H. pylori* the ancestral *ureA* and *ureB* genes are fused and create *ureA*, whereas the ancestral *ureC* gene is unfortunately labeled *ureB* in this bacterium (Fig. 5). The *H. pylori ureA* and *ureB* (*hp0073-hp0072*) structural genes for the urease subunits are located in a large gene cluster together with the *ureI* (*hp0071*) gene, encoding a pH regulated urea channel, and with the *ureEFGH* (*hp0070-hp0069-hp0068-hp0067*) genes for urease accessory proteins, which mediate proper formation of the complex quaternary structure and transport nickel ions in the active center. The *ureABIEFGH* operon of *H. pylori* is coordinately expressed from two different promoters situated in front of *ureA* and *ureI* genes (1). The *ureI* gene codes for a pH-regulated urea channel situated in the cytoplasmic membrane.

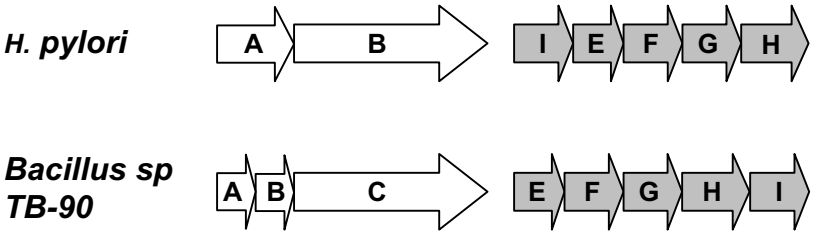

Fig. 5. Genetic organisation of the urease operon of *H. pylori* and *Bacillus* sp TB90. The *ureAB* genes of the ancestral urease operon are fused and labeled *ureA*, the ancestral *ureC* gene is labeled *ureB* in *H. pylori*.

This gene was thought to be unique for *Helicobacter* species (115), however, over the last few years at least two publications described UreI urea transporters (91, 117). When the periplasmic pH drops below pH 6.1, protons bind to protonable amino acid residues in the periplasmic part of the UreI transporter, presumably leading to conformational changes in the transmembrane domain (171). The UreI channel opens and environmental urea molecules, which are present at concentrations of around 10 mM (115), enter the cell (170). Preliminary experiments indicated that UreI and UreA might interact, suggesting that urea hydrolysis may take place in close proximity of the cell wall to allow fast diffusion of ammonia and CO_2 into the periplasm to effectively protect *H. pylori* against the acidic environment (167). The accessory proteins UreEFGH are necessary for incorporation of the nickel cofactor into the apo-urease, as it was shown that urease activity was lost or diminished in accessory gene mutants (113). The UreH protein of *H. pylori*, which is homologous to UreD in other bacteria (113), was proposed to be a chaperone to facilitate proper assembly of the metallo center, as interaction between UreD and the apo-urease in the presence of CO_2 was elucidated in *Klebsiella aerogenes* (129, 130). In *H. pylori*, interaction was described between UreH and UreF (167). The function of UreF is still unknown. UreE of *H. pylori* is a metallo chaperone, which does sequester nickel, but interestingly, in contrast to other UreE proteins, *H. pylori* UreE lacks the histidine rich C-terminal domain (13).

The accessory proteins HypAB, normally involved in incorporation of nickel into the *H. pylori* hydrogenase, were also shown to be necessary for the activation of urease (106, 107, 127). The HypB protein is a homolog of UreG, an enzyme that possesses GTP-hydrolyzing activity (106, 107, 174). Furthermore, interaction between UreG with UreE was demonstrated (137, 167).

The GroES homologue of *H. pylori*, the HspA protein, was also hypothesized to be involved in the regulation of the urease, as it contains a histidine- and glutamine-rich C-terminus (83, 154). An *E. coli* strain harbouring plasmids with the urease gene cluster and the *hspA* gene displayed significantly higher urease activity under low nickel concentrations added compared to absence of nickel (83, 154), and therefore it was concluded that HspA is a carrier for nickel incorporation into urease (83, 154).

Urease is now thought to be a cytoplasmic protein. However, in previous studies the urease enzyme was shown to be present on the surface of viable *H. pylori* cells in aging cultures (134, 166). The importance of this finding is being debated and may represent an artefact of lysed cells, as the urease enzyme was shown to be inactive at pH < 5 (144). Additionally, urease needs the accessory proteins to be activated, and these are not available on the outside of *H. pylori* cells.

2.2 The Role of Urease in Acid Resistance

Urease converts urea into ammonia and carbon dioxide (Eq. 6,7), which both can be protonated (Eq. 8,9). However, the equilibrium of the NH_3 /NH_4^+ couple is at pH 9.2, and therefore its buffering capacity is too small to maintain the neutral pH of the cytoplasm (98). The equilibrium of H_2CO_3/HCO_3^- couple is at pH 6.1, close to the pH of the cytoplasm of the neutralophilic *H. pylori*. The following model for the role of the urease and carbonic anhydrase was suggested (Fig. 6): Urea is hydrolyzed in the cytoplasm into two molecules of ammonia and one molecule of carbon dioxide. All products diffuse into the periplasm, where the carbonic anhydrase converts CO_2 into H_2CO_3, which dissolves into HCO_3^- and H^+. This proton is detoxified by one molecule of ammonia, whereas the

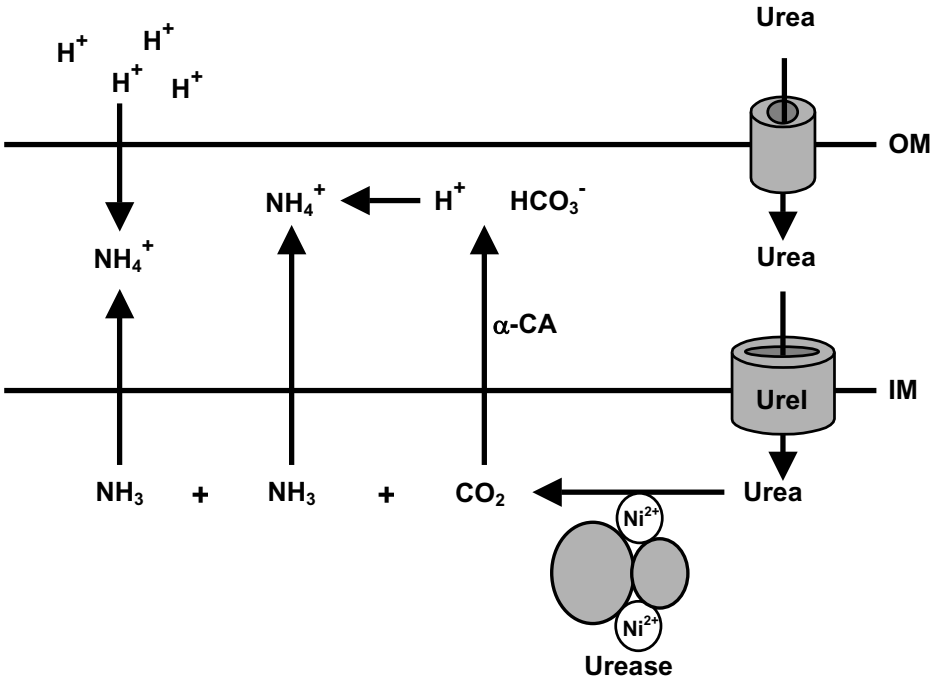

Fig. 6. Model of acid resistance in *H. pylori* (modified from (98)): Urea enters the periplasm via pores in the outer membrane (OM). The inner membrane (IM) transporter UreI transports urea under acidic conditions into the cytoplasm. Here, urea is converted into NH_3 and CO_2 by the urease, and the gases diffuse back into the periplasm. There, the α-carbonic anhydrase hydrates CO_2 to H_2CO_3, which dissociates into H^+ and HCO_3. The created proton is accepted by one NH_3 to create NH_4^+, leaving the second NH_3 to neutralize a proton from acid leaking into the periplasm.

second molecule of ammonia can be used to neutralize protons entering the periplasm from the surrounding medium (98).

Transcriptional regulation of bacterial ureases is usually mediated by nitrogen level, urea level, growth phase or pH (24, 115). Transcription of urease genes in *H. pylori* is not altered upon changes in nitrogen and urea level, and therefore it was thought for a long time that urease expression was constitutive and thus not regulated at all. However, only recently it was demonstrated that transcription of the *H. pylori* urease is acid-regulated (1, 25, 161). Northern hybridization analysis revealed elevated levels of mRNA fragments that contain the *ureAB* and *ureIE'* genes, respectively, in cells incubated for 30 minutes in medium at pH 6 (1, 25, 161). Urease transcription was furthermore shown to be nickel-regulated via the nickel regulator NikR (Table 1; see section 4; (161-163)). It is believed that the nickel concentration may reflect the level of acidity (see section 4, (160, 161)).

In *H. pylori*, urease enzyme activity is regulated both by posttranscriptional and posttranslational mechanisms (Table 1). These include the acid-dependent access to the urea substrate due to the opening and closing of the UreI channel, a posttranscriptional stabilization of the urease mRNA under acidic pH and a nickel-induced activation of apo-urease. If urease activity is measured at neutral pH, it is relatively low, however, when the pH was decreased to 5.5 or lower, there is a 10-20-fold induction of urease activity. This induction of the urease activity was demonstrated to be due to increased influx of urea, as

mediated by the pH-dependent opening of the UreI channel, which has increased urea permeability at low pH. The UreI channel opens at a pH of 6.1 and activity was maintained down to a pH as low as 2.5 (144, 145). At neutral pH UreI is closed to circumvent the raise of the cytoplasmic pH above neutral (170).

Table 1. Transcriptional and posttranscriptional mechanisms of regulation of the urease activity

	Mechanism	Reference
Transcriptional	Acid-dependent induction of transcription	(25, 161-163)
	Nickel-dependent induction of transcription	
	Modification of urease transcripts	(1)
Posttranscriptional	Stabilization of urease mRNA	(1)
Posttranslational	Nickel-dependent induction of urease activity	(162)
	Acid- and UreI-dependent induction of urease activity	(170)

A second posttranscriptional induction mechanism of urease was reported to be pH-dependent mRNA stability at low pH. Urease mRNA is stabilized at acidic pH. Additionally, under acidic conditions higher levels of mRNA transcripts were found as well as different transcripts, especially of the mRNA of the urease accessory genes (1). It was concluded that the posttranscriptional stabilization of the accessory genes increased the efficiency of the urease assembly (1).

The third mechanism is post-transcriptional induction of urease activity at the protein level. It was found that the increase in urease activity is much higher than the increase in protein levels after nickel-supplementation of growth medium (162). Furthermore, a nickel-dependent increase of urease activity is still observed when nickel-dependent transcription is blocked (163). It was concluded that nickel may be an indicator for low pH for *H. pylori*, as it has been suggested that the bioavailability of nickel ions is positively affected under low pH (86, 161-163).

The importance of urease for *H. pylori* is demonstrated by the inability of urease mutants to colonize the gastric mucosa of nude mice, cynomolgus monkeys and gnotobiotic piglets, even when the pH was neutral (46, 47, 116, 149, 159).

2.3 Ni/Fe-Hydrogenase

The membrane-bound hydrogenase enzyme (cytochrome c_3 oxidoreductase, EC 1.18.99.1) catalyzes the "splitting" of electrons from hydrogen (Eq. 11), which are in turn used for energy generating purposes (61).

Eq. 11: $H_2 \leftrightarrow 2H^+ + 2e^-$

H. pylori expresses a Ni/Fe-hydrogenase which displays many features common to

bacterial Ni/Fe-hydrogenases, which use O_2 as terminal end electron acceptor (94). The hydrogenase operon consists of five genes: *hydA* (*hp0631*) is coding for the small 26 kD subunit and *hydB* (*hp0632*) for the large 65 kD subunit, *hydC* (*hp0633*) encodes a cytochrome c subunit, and *hydD* (*hp0634*) seems to be similar to a protease involved in the maturation of the hydrogenase large subunit of *Wollinella succinogenes*. The function of gene product *hydE* (*hp0635*) is currently unknown (14).

Similar to the urease enzyme activation, the hydrogenase enzyme requires, besides nickel and iron incorporation, a set of accessory proteins encoded by the *hypABCDEF* genes (6, 156), which are splitted in three gene clusters over the genome: *hypA* (*hp0869*), *hypBCD* (*hp0900-hp0899-hp0898*) and *hypEF* (*hp0047-hp0048*) located at different places in the chromosome. HypA was shown to have nickel-binding activity, whereas HypB displayed GTPase activity (106, 127). Activity of the *H. pylori* hydrogenase enzyme is 2 to 4-fold induced by addition of 10% H_2 (126).

Inactivation of all the hydrogenase accessory genes leads to reduced activity or complete absence of hydrogenase activity (127), and it was demonstrated that the HypA and HypB proteins are also necessary for urease activity (127). Nickel supplementation of a *hypAB* mutant restored urease activity, but not the hydrogenase activity (127). Addition of nickel ions may induce the activity of the urease accessory proteins leading to urease activity (127), as the urease system itself possesses UreE and UreG similar to HypAB.

2.4 Hydrogenase and Gastric Adaptation

The hydrogenase system with O_2 as end-acceptor is proposed to help *H. pylori* to adapt to the gastric mucosa. This assumption is based on the observation that *H. pylori* seems to be limited in its use of oxidizable organic compounds, and that the mucus layer of the human stomach is thought to be relatively poor in nutrients (126). Furthermore, the nature of primary carbon and nutrient sources of *H. pylori* in the gastric mucosa of the human stomach is still unknown (45, 93, 126).

Hydrogen is produced by colonic bacteria and from there transported via the bloodstream to the gastric mucus layer. *H. pylori* can efficiently use its hydrogenase system, as the hydrogen concentration of the mucus layer of the stomach of living mice was shown to be 43 µM. Therefore, the hydrogen concentration is high enough to saturate the *H. pylori* hydrogenase, which has a K_m of 1.8 µM (126). As human cells cannot use hydrogen as energy source, there is no competition between *H. pylori* and its host for hydrogen. Evidence for the use of hydrogenase to adapt to the human stomach is the fact that hydrogenase mutants are deficient in colonizing a mouse model (126).

3 NICKEL UPTAKE SYSTEMS

Two classes of bacterial nickel transporters have been described. In *Escherichia coli* nickel is imported into the cytoplasm via an ABC-transporter, consisting of the NikA periplasmic binding protein, the NikBC proteins, which encode inner membrane permease proteins and finally the NikDE ATP-binding proteins (121). The *E. coli* NikABCDE system is transcriptionally regulated by NikR, which is encoded downstream of the *nik* gene cluster (34). Orthologs of the Nik ABC-transporter are found in *Brucella* (82), and *Yersinia* species (146), and in *Actinobacillus pleuropneumoniae* (21). A second class of nickel transporters is represented by the *Alcaligenes eutrophus* HoxN protein, which is a single protein, high-affinity nickel permease (48, 173) that has eight transmembrane

segments (49, 50). Other homologs of HoxN were identified in *Bradyrhizobium japonicum* (62), *Rhodococcus rhodochrous* (85) and in thermophilic *Bacillus* species (91).

3.1 Nickel Import by the NixA Protein

H. pylori possesses a nickel-specific uptake system designated NixA, which was discovered by the finding that additional nickel was needed to activate recombinant *H. pylori* urease in an *E. coli* strain (111). NixA belongs to the family of HoxN-type single protein, high-affinity nickel permeases (111). The *nixA* (*hp1077*) gene encodes a monomeric, 37 kDa cytoplasmic protein (111), which possesses eight transmembrane domains (64). Both the carboxy- and amino-terminal ends of the protein are situated in the cytoplasm (64), and motives in transmembrane domain II ($GX_2HAXDADADH$) and III ($GX_2FX_2GHSSVV$) are essential for nickel transport (63, 64). The nickel concentration in the gastric mucosa is presumably similar to the concentration of nickel in the serum, which is 2 to 11 nM (63, 155). However, the nickel concentration can change due to the nutritional status of the host as nutrients like tea, chocolate, cornflakes or nuts are rich in nickel (155). The affinity of NixA for nickel is 11.3 nM, and thus in the gastric mucus layer nickel uptake via NixA will be efficient (63). High affinity nickel uptake is an energy-dependent process, as experiments with the NixA homologue NhlF of *Rhodococcus rhodochrous* demonstrated. However, the energy source in *H. pylori* is currently unknown (37, 120).

Nickel uptake via NixA is thought to be the major nickel uptake route in *H. pylori*, as inactivation of *nixA* resulted in a 75% reduction of nickel uptake (12). Competition experiments indicated that other divalent ions like Co^{2+}, Cu^{2+} and Zn^{2+} ions may be cotransported by NixA (63). The importance of NixA for the colonization of the stomach was demonstrated *in vivo.* In mice, the gastric colonization capacity of a *H. pylori nixA* mutant was decreased compared with the wild-type strain, and *nixA* mutants where overgrown by the wild-type strain (124).

3.2 Possible Alternative Nickel Transporters

Two additional putative alternative nickel uptake transporters have been identified in *H. pylori*. The first one is the magnesium transporter CorA (Hp1344), a cytoplasmic membrane protein. With the help of growth experiments it was suggested that CorA co-transports Ni^{2+} ions under very high concentrations of Ni^{2+} in the medium (132). An *E. coli corA* mutant displayed nickel resistance in the presence of 3 mM $NiCl_2$ (72). In contrast, an *E. coli corA* mutant harbouring a plasmid with an intact *corA* gene of *H. pylori* displayed nickel sensitivity. Subsequent supplementation of the medium with Mg^{2+} abolished the sensitivity of the *E. coli corA* mutant, indicating that Mg^{2+} is the dominant substrate for CorA. Mutation of *corA* had no effect in *H. pylori* 26695 on nickel metabolism, as a *corA* mutant displayed no difference of urease activity or expression compared to the *H. pylori* wild-type (132).

The *abcABCD* operon may encode for a second nickel transporter. The AbcC protein displays some homology to the ATP-binding protein NikD from *E. coli*, however the other three proteins displayed no significant homology to any known proteins (76). The AbcABCD proteins may be involved in nickel uptake, as it was demonstrated that an *abcD-nixA* double mutant nearly abolished urease activity without altering synthesis of the urease, whereas a *nixA* single mutant only lowered the nickel uptake to 1/3 (12, 76). However, nickel transport experiments with *abcD* and *abcC-nixA* mutants revealed no

significant difference in nickel uptake (76).

4 MECHANISMS OF NICKEL REGULATION

4.1 The NikR Regulator

So far, the only nickel regulator described and biochemically characterized is NikR of *E. coli* (26, 28, 34), however, NikR orthologs are also described in other Gram-negative bacteria and Archaea (26, 29, 30, 163). NikR is a member of the Arc/CopG/MetJ/Mnt family of ribbon-helix-helix (RHH) family of DNA binding proteins, which function as transcriptional regulators. *E. coli* NikR possesses an N-terminal DNA-binding domain, which recognizes a 28 bp palindromic region (GTATGA-N_{16}-TCATAC) in the *nikA* promoter. The C-terminal domain of NikR contains a nickel-binding motif. Binding of nickel ions induces changes in the secondary structure of NikR, and thereby enhances the DNA binding capacity (26-28, 143). Interestingly, *E. coli* NikR was only shown to regulate the nickel uptake via *nikA*, but other targets of NikR have not been identified.

Analysis of the genome sequence of *H. pylori* (6, 156) allowed the identification of a NikR ortholog (Hp1338), which displays 30% identity and 68% similarity with *E. coli* NikR (163). It is predicted to be a 17 kDa transcriptional repressor protein that is supposed to be a direct cytoplasmic nickel sensor, changing its conformation upon binding intracellular free nickel. Subsequently, the NikR-nickel complex binds to the promoter region of target genes and regulate transcription (160). In contrast to *E. coli*, where NikR only seems to regulate the nickel uptake, the NikR regulator of *H. pylori* has also been shown to be involved in the regulation of several metabolic pathways like regulation of the nickel and iron homeostasis, as well as regulation of motility and chaperones (25, 30, 161-163). Above all, our work indicates that *H. pylori* NikR functions as master regulator of acid adaptation (160). The importance of NikR in colonization and adaptation of the stomach was demonstrated as NikR mutants were shown to be attenuated in colonizing the mouse stomach (25).

4.2 Genes Regulated by NikR

In *H. pylori,* several genes and metabolic pathways have been demonstrated to be regulated by NikR and/or by nickel (Fig. 4).

Regulation of Nickel Uptake by NixA. As its counterpart in *E. coli*, the NikR protein of *H. pylori* is controlling nickel uptake via direct nickel-dependent binding to the promoter region of the gene for the high-affinity nickel uptake protein NixA (30, 52). However, there is no consensus on the exact mechanism of how NikR exerts its regulatory function: using array and spot blot analysis, it was suggested that *nixA* transcription is nickel- and NikR- induced (30). However, nickel-dependent transcription of *nixA* would result in continuous influx of nickel in wild-type cells, and this is contradicted by the high-level nickel-resistance of the wild-type strain and nickel-sensitivity of a *nikR* mutant (30, 163). Furthermore, mutation of the *nixA* gene was shown to complement nickel-sensitivity of an *H. pylori nikR* mutant (53). In-vitro binding assays using recombinant NikR demonstrated nickel- and NikR-dependent repression of the *nixA* gene by binding to an operator sequence overlapping the –10 and +1 sequence (53). The *nixA* binding sequence recognized by *H. pylori* differs significantly from the well-characterized binding sequence of *E. coli* NikR.

ureAB. Another promoter directly regulated by *H. pylori* NikR is the *ureA* promoter.

During growth at mildly acidic pH and under high nickel concentrations urease expression is induced by nickel at the transcriptional level. Mutational and Northern hybridisation analysis revealed that NikR is involved in the transcription of the urease genes and expression of the urease activity (25, 161-163). Gelshift and DNAseI footprint experiments allowed the identification of the NikR binding site at nucleotides -56 to -91 upstream of the canonical sigma^{80} promoter (53). The binding sequence displayed a partial overlap with an imperfect palindromic region at position –49 to –69 upstream of the transcriptional start site of the *ureA* gene (53). It was hypothesized that binding of NikR to this region would give easier access for the RNA polymerase to bind to the promoter (163), however the exact role of this palindrome is still not clear. Analysis of the NikR binding sites in the *ureA* and *nixA* genes revealed no similarities of these sites (53). Therefore, further analysis is necessary to define a NikR-box in *H. pylori*.

***fur* and amidase genes.** The transcription of the iron-uptake regulator Fur is acid- and nickel-dependent regulated by NikR ((25, 30, 160); Fig. 3) and also the ammonia-producing enzymes amidase and formamidase are significantly upregulated during growth under mildly acidic conditions (pH 5-5.5; (25, 30, 160-163)). The latter are indirect effects as it was shown that the regulation of the amidase and formamidase is indirectly regulated by NikR involving the iron-uptake regulator Fur (25, 30, 161).

***nikR*.** The NikR protein displays nickel-dependent autoregulation by repressing the promoter of its own gene, which was shown by gelshift and *lacZ*-fusion experiments (30). Additionally, transcription of NikR is induced by acid (160). The autoregulation of *Hp* NikR is in contrast to NikR of *E. coli*, which so far is only involved in the nickel-dependent regulation of the *nikABCDE* operon, but not involved in nickel-dependent autoregulation (34).

Other genes. NikR may regulate the hydrogenase as transcription of the hydrogenase operon was repressed in a *nikR* mutant, but induced in a *H. pylori fur* mutant, suggesting that transcription of hydrogenase is similar to the transcription of the amidases (25, 30, 161). Other genes indicating NikR-dependent regulation are members of the copper metabolism, respiration, stress response as well as motility and chemotaxis and regulators like *hspR* as was demonstrated by transcriptional array analysis (30).

4.3 Metal Metabolism and Acid Regulation

The *H. pylori* genome contains a relatively low number of regulatory proteins, consistent with its restricted host range and target organs. However, three of them are involved in acid regulation: the nickel responsive regulator NikR, the ferric-uptake regulator Fur and the two-component regulatory system HP0165/0166 (25, 160). Presumably the acid regulation is mediated via the cascade NikR>Fur>Hp0166 (Fig. 4), as expression of Fur and Hp0166 proteins is acid-repressed (160), and expression of Hp0166 is absent in a *fur* mutant background (90). It was suggested that *H. pylori* is detecting changes in the external pH through sensing the availability of nickel ions in the cytoplasm, as the solubility of metal ions, like nickel and iron, is enhanced under acidic conditions (86, 160). The enhanced solubility of metal ions might be a) due to increased bioavailability of nickel under acidic conditions, b) increased expressions of nickel transporters, or c) increased efficiency of the nickel transporter NixA (160).

Under low nickel concentrations the imported free nickel ions will activate the pool of preformed urease apo-enzyme. When under higher nickel ion concentration all urease apo-enzyme is activated, further imported free nickel-ions are available in the cytoplasm.

This leads to formation of NikR-nickel complexes. These NikR-nickel complexes subsequently bind to target promoters with different affinities. Repression of the *nixA* promoter will occur even at low nickel concentrations, and therefore lead to decrease and subsequently cessation of NixA-mediated nickel uptake. The upstream binding site for NikR in the *ureAB* operon is supposed to have a lower binding affinity compared to the *nixA* promoter. As a consequence, it will be induced when the intracellular nickel concentration further increases, leading to an induction of transcription of the *ureAB* genes. Moreover, NikR will bind to the promoter sites of other target genes with low binding affinity sites regulators such as the *fur* gene and probably also the *hspR*, *Hp0166* and *flbA* genes ((160); Fig. 4).

5 NICKEL METABOLISM AND STORAGE

5.1 Nickel Binding by the Hpn Protein

As nickel ions are a necessity for *H. pylori* to survive the acidic conditions in the stomach, and as nickel availability may change, expression of a nickel storage system would be beneficial for *H. pylori*. The hydrophilic protein Hpn (*H. pylori* nickel-binding protein, Hp1427) was identified as being a possible storage protein. It is a 180 bp gene that codes for a 7 kD protein. Nearly half of its 60 aa are histidines (28 aa), which have a high affinity for nickel. Indeed, an *hpn* mutant was more susceptible for Ni_2^+ added to the medium compared to the wild-type (66). Transcriptome analysis demonstrated a nickel- and NikR-dependent regulation of *hpn* (30), as well as an acid induced induction (25). Furthermore, transcription of *hpn* is affected by iron in a Fur-dependent fashion (52).

Another protein that might be involved in nickel binding is Hp1432, the histidine- and glutamine-rich metal-binding protein. Like its homolog Hpn, Hp1432 is also rich in histidines, which have nickel-binding properties (16 out of 72 aa are histidines, clustered in 3 domains). The regulation of Hp1432 is similar to *hpn*, as it is nickel- and NikR-dependent as well iron- and Fur-dependent regulated (25, 30, 52). In addition, Hp1432 is acid-dependent induced by the HP0165-HP0166 two-component system (133).

6 METAL METABOLISM AS DRUG TARGET / THERAPEUTICAL CONSIDERATIONS

The most effective treatment for *H. pylori* infection so far is an antibiotic triple therapy. However, the prevalence of resistance against the most frequently used antibiotics is rising, especially in developing countries, where most antibiotics can be obtained without special prescription (60). It is estimated that in Europe approximately 10% of *H. pylori* are resistant against clarithromycin and even 30% against metronidazole (these numbers increase in developing countries, where the resistance varies between 25 to 50% for clarithromycin and to near complete resistance for metronidazole; (67, 109, 157)). Therefore, new strategies to treat *H. pylori* infections are necessary and desirable. As *H. pylori* uses a metal-cofactored enzyme as a first line defence to colonize the human stomach, the metal metabolism is a potential drug target. Three possible new lines of treatment of *H. pylori* infection will be discussed: i) vaccines against antigens of the metal metabolism like UreAB, HspAB or NapA (58), ii) use of inhibitors against enzymes essential for *H. pylori*, and iii) a metal diet.

Vaccination. One of the best ways to battle pathogens is the use of vaccination. Two

possible vaccination strategies can be envisioned for treatment of *H. pylori* infection: A prophylactic vaccine, where antigens are provided prior to infection in order to try and prevent new infections, and a therapeutic vaccine, which is used to treat existing infections. The most suitable antigens for both types of vaccines are thought to be abundantly expressed and well conserved surface-exposed proteins. The UreAB and HspAB subunits are cytoplasmic proteins, however, both are described to be surface-bound in stationary *H. pylori* cultures (166). In addition, urease is an important virulence factor and therefore, these proteins have already been used in several vaccination experiments. Prophylactic vaccination with these recombinant proteins induced good protection against *H. pylori* and *H. felis* in mouse models, however, they failed or at best gave modest protection in monkeys or humans (58). Also vaccination with a triple vaccine consisting of recombinant NapA (an iron-binding protein that shows homology to the Dps family of iron-binding proteins (31)), CagA and VacA only showed moderate protection of beagle dogs (139).

So far, it is unknown why vaccines are quite effective in a rodent model but not in humans. An explanation might be that *H. pylori* is able to alter the human immune response against itself in humans, but these mechanisms might not be as effective in its non-natural host (38).

Inhibition of Metallo-Enzymes. The availability of whole genome sequences, transcriptomics and proteomics made it possible to identify genes unique for *H. pylori*. Subsequent colonizing or growth experiment mutants indicate essential genes. With further analysis of the regulation and mechanism of action, it is feasible to identify possible targets for inhibitors, which can in turn alter expression of essential genes or block enzyme activity. Possible targets for inhibitors are the hydrogenase and the urease system of *H. pylori*. Both nickel-cofactored systems are lacking in the human metabolism, but are present in the periplasm (hydrogenase and UreI) or cytoplasm (urease) of *H. pylori*. Mutations in both systems were shown to be at least growth deficient (115, 127). Both enzymes have unique active centers and a complicated mechanism of activation by assembly. Therefore, the design and testing of therapeutical inhibitors of the hydrogenase and urease active enzymes or its assembly function may be of interest, especially in regard of the increasing resistance of *H. pylori* against antibiotics (93, 94, 113).

Metal Diet. *H. pylori* has a strong need in nickel ions as it is used as a cofactor for the virulence factor urease. On the other hand, nickel ions are not used by the human metabolism (43). However, several human food sources like coffee, tea, nuts and chocolate are rich in nickel ions (155). As nickel depletion or probably even a nickel shortage is detrimental for *H. pylori* growth (162, 163), a nickel-deficient diet might help to treat *H. pylori* infection. Alternatively, a nickel-rich diet might be successful as well, however, this might be too dangerous, as nickel is also harmful for human cells due to carcinogenesis (43).

7 CONCLUSIONS

Although *H. pylori* colonizes a very harsh environment, this bacterium is well adapted to the mucus layer of the human stomach. *H. pylori* can overcome shortage of nutrients by turning the defence mechanisms of its host against the host itself, leading to damage of epithelial cells and outpour of nutrients. The exact way of *H. pylori* tricking the human host is still poorly understood therefore more research is necessary. It may help to

establish vaccination against *H. pylori*, which is so far only effective in rodents.

A second strategy to overcome nutrient shortage used by *H. pylori* is to use resources, which are not in competition with its host. For example, *H. pylori* uses hydrogen to gain energy, a resource that humans can not use. Furthermore, it uses urea, a waste-product of the nitrogen metabolism of humans, to survive the acidity of its niche. For both processes, the energy metabolism and the acid adaptation nickel-cofactored enzymes are necessary, again something that is not used by humans. However, due to the fact that nutrients used by humans are rich in nickel, there will be no shortage of nickel.

Due to its small genome *H. pylori* features a paucity of regulators. From the few regulators identified and characterized at present, the nickel responsive regulator NikR is one of the most important regulators, as many metabolic pathways, essential for gastric adaptation, are regulated by NikR. Furthermore, NikR is master regulator for the acid metabolism, which it mediates, often in cascade form. The nickel ions itself are of importance for *H. pylori*, as the concentration of nickel ions is an indicator of the acidity of the environment.

Finally, as nickel ions are not used in the human metabolism, the nickel metabolism of *H. pylori* is a drug target for inhibitors, especially as urease and hydrogenase are activated in a complicated way but are essential for survival in the stomach. However, further research is necessary to unravel the complete regulatory network of adaptation in *H. pylori*, especially with NikR as master regulator.

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CHAPTER **2**

Aims and outline of this thesis

The aim of this thesis was to gain more insight into the regulation of the iron- and nickel metabolism of *H. pylori* by its metal-dependent regulators Fur and NikR.

The molecular mechanism by which the ferric uptake regulator Fur regulates its target genes is by now quite well understood in *H. pylori*. Fur was known to not only be involved in the regulation of iron metabolism, but also in several other metabolic pathways. In Chapter 3, an iron- and Fur-dependent regulon is characterized, of which some target genes had been already known, like the iron uptake gene *fecA* or iron storage gene *pfr*. Furthermore, several genes encoding proteins involved in nitrogen metabolism, motility, cell wall and cofactor synthesis, respiration, chemotaxis or energy metabolism have been detected as Fur-dependent regulated. The superoxide dismutase SodB, whose gene was also identified as a new member of the Fur regulon in Chapter 3, is characterized in Chapter 4 as being directly iron- and Fur-dependent regulated. This is the first direct regulation of a *sodB* gene by Fur and this regulation contrasts with the indirect regulation of *sodB* through Fur and small RNAs in *E. coli*.

Regulation by the nickel-responsive regulator NikR was so far only well characterized in *E. coli* for the Nik nickel uptake system. For *H. pylori*, it was known that a nickel-dependent ortholog of NikR is involved in regulation of urease expression. In Chapter 5, it is demonstrated that the urease gene is directly nickel-dependent induced by NikR by binding to a sequence upstream of the -35 region, and that the nickel uptake gene *nixA* is directly nickel-dependently repressed by NikR by binding to a region overlapping with the -10 and +1 region of the promoter of *nixA*. Whether NikR of *H. pylori* is repressing or inducing its target genes is therefore dependent on the binding region in the promoter of the target gene. NikR not only nickel-dependently regulates genes of the nickel metabolism, but is also involved in the regulation of the iron metabolism in *H. pylori*. In Chapter 6, it is demonstrated that NikR is directly nickel-dependently repressing the putative iron uptake genes *frpB3* and *fecA3*, as well as the *tonB2*-operon. Finally, the knowledge on nickel- and iron-metabolism is summarized in Chapter 7, and a model of a regulatory network of NikR and Fur is suggested, where NikR functions as master regulator.

CHAPTER **3**

Transcriptional profiling of *Helicobacter pylori* Fur- and iron-regulated gene expression

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Intracellular iron homeostasis is a necessity for almost all living organisms, since both iron restriction and iron overload can result in cell death. The ferric uptake regulator protein, Fur, controls iron homeostasis in most Gram-negative bacteria. In the human gastric pathogen *Helicobacter pylori*, Fur is thought to have acquired extra functions to compensate for the relative paucity of regulatory genes. To identify *H. pylori* genes regulated by iron and Fur, we used DNA array-based transcriptional profiling with RNA isolated from *H. pylori* 26695 wild-type and *fur* mutant cells grown in iron-restricted and iron-replete conditions. Sixteen genes encoding proteins involved in metal metabolism, nitrogen metabolism, motility, cell wall synthesis and cofactor synthesis, displayed iron-dependent Fur-repressed expression. Conversely, sixteen genes encoding proteins involved in iron storage, respiration, energy metabolism, chemotaxis, and oxygen scavenging, displayed iron-induced Fur-dependent expression. Several Fur-regulated genes have been previously shown to be essential for acid resistance or gastric colonisation in animal models, such as those encoding the hydrogenase and superoxide dismutase enzymes. Overall, there was a partial overlap between the sets of genes regulated by Fur and those previously identified as growth phase, iron or acid regulated. Regulatory patterns were confirmed for five selected genes using Northern hybridisation. In conclusion, *H. pylori* Fur is a versatile regulator involved in many pathways essential for gastric colonisation. These findings further delineate the central role of Fur in regulating the unique capacity of *H. pylori* to colonize the human stomach.

Infection with the human pathogen *Helicobacter pylori* results in persistent gastritis which can develop into peptic ulcer disease and adenocarcinoma of the distal stomach (9). Approximately half of the world's human population is colonized by *H. pylori*, leading to significant morbidity and mortality. For these reasons, the infection is considered an important public health problem with serious economic consequences. The only known habitat of *H. pylori* is the mucus layer overlaying the epithelial cells in the human stomach. Colonisation of this acidic and variable environmental niche has necessitated the development of adaptive stress responses by *H. pylori*.

In the gastric environment, changes in iron availability represent one of the important environmental stimuli for *H. pylori*. Iron is an essential element for almost all living organisms, as it is a cofactor of many enzymes, and acts as a catalyst in electron transport processes. However, in the presence of oxygen, iron potentiates the formation of toxic oxygen radicals. Therefore regulation of intracellular iron homeostasis, as mediated by the Ferric Uptake Regulator (Fur) protein, is of critical importance (4). Regulation of gene expression via Fur has been extensively investigated in several Gram-negative and Gram-positive bacteria, where it is involved in regulation of many cellular processes, including iron-metabolism, oxidative stress defense and central intermediary metabolism (4, 21). However, while the absence of Fur affects many cellular processes, several of the regulatory phenomena described mostly in *E. coli* are only indirectly affected by Fur (28).

Fur is a transcriptional repressor protein, which displays iron-dependent binding to conserved DNA sequences (Fur boxes) located in the promoters of iron-regulated genes (21). In most bacteria, including *H. pylori*, the iron-complexed form of Fur binds to promoters of iron uptake genes, thus repressing iron uptake in iron-replete conditions (15, 49). However, *H. pylori* Fur has acquired the thus far unique possibility also to bind the *pfr* promoter in its iron-free form, thus repressing expression of iron storage proteins in iron-restricted conditions (7, 16, 50).

The relative paucity of transcriptional regulators in *H. pylori*, combined with the necessity to respond to environmental stresses, may have resulted in *H. pylori* Fur being involved in the regulation of other adaptive responses. Other than regulation of iron metabolism, *H. pylori* Fur has also been implicated in regulation of acid resistance (8, 11, 46), nitrogen metabolism (47, 48) and oxidative stress resistance (6, 14, 22). Fur-mediated regulation is also required for gastric colonisation by *H. pylori*, as demonstrated in a mouse model of infection (11).

DNA array technology has proved to be a powerful technique for the study of global gene regulation in many organisms (13), and has also been successfully applied to study alterations in *H. pylori* gene expression (11, 19, 27, 29, 30, 43, 51) and genetic variation between isolates (24, 38). In this study we have applied DNA array technology to growth experiments with the well-characterised *H. pylori* reference strain 26695, both to define the *H. pylori* responses to variation in iron-availability, and to identify new members of the *H. pylori* Fur regulon.

MATERIALS AND METHODS

Bacterial strains, plasmids, media and growth conditions. The *H. pylori* strains used in this study were reference strain 26695 (44) and its isogenic *fur* mutant (8, 49). *H. pylori* strains were routinely cultured on Dent agar at 37°C under microaerophilic conditions (10% CO_2, 5% O_2, and 85% N_2). Broth cultures were grown in Brucella Broth

(Difco) supplemented with 3% Newborn Calf Serum (Gibco, Life technologies, Breda, The Netherlands) (BBN). Broth cultures were continuously shaken at 40 rpm under microaerophilic conditions. Iron restriction was achieved by supplementing BBN with desferal (deferoxamine mesylate, Sigma) to a final concentration of 20 μM. Iron-replete conditions were achieved by supplementing desferal-treated BBN with ferric chloride (Sigma) to a final concentration of 100 μM (49).

Purification and analysis of RNA. Total RNA was isolated using Trizol (Gibco), according to the manufacturer's instructions. The amount of RNA was determined spectrophotometrically. RNA electrophoresis, blotting, hybridisation with DIG-labelled RNA probes, and detection of bound probe were carried out as described previously (23). Directly after transfer, the membranes were stained with methylene blue to confirm the integrity of the RNA samples, and to confirm loading of equal amounts of RNA based on the relative intensities of the 16S and 23S rRNA (47). Chemiluminescence was detected using a Lumi-Imager (Roche Diagnostics) and chemiluminographs were quantified using the Lumi-Analyse-software package (Roche Diagnostics). The DIG-labelled specific RNA probes were synthesized by *in vitro* transcription using T7 RNA polymerase (Roche Diagnostics) and PCR products amplified using primers listed in Table 1.

Synthesis of labelled cDNA for transcriptome analysis. *H. pylori* strain 26695 and its isogenic *fur* mutant were grown in iron-restricted and iron-replete conditions (49), and total RNA was isolated from cells grown for 20 h and checked by Northern hybridisation using an *amiE*-specific probe (Fig. 1). For annealing of the specific oligonucleotide primers complementary to the mRNAs specified by all *H. pylori* genes, 1 μg of total RNA (concentration determined photometrically) was hybridised to 4 μl cDNA primer mix (0.05 pmol μl^{-1}) (Eurogentec, Seraing, Belgium) in hybridisation buffer (10 mM Tris-HCl, pH 7.9; mM dTTP, 4.5 μl of [α-^{33}P]dCTP [10 μCi μl^{-1}, Amersham Pharmacia], 1.5 μl of reverse transcriptase [Superscript II; Gibco-BRL]) was added, and reverse transcription

Table 1. Oligonucleotide primers used in this study

Primer name	Sequence (5' → 3')*
AmiE-F1	AGTAGCAGCCCAGATACTGT
AmiE-R-T7	ctaatacgactcactatagggagaTCGCTACCGCTACATAACAT
HP1432-F1	GGCACACCATGAACAACAAC
HP1432-R-T7	ctaatacgactcactatagggagaTGTTGGTTTTGTTGTTGCGC
Hp0388-F1	TGATGACATGCTGGAGCGAT
HP0388-R-T7	ctaatacgactcactatagggagaTCCACATGCTTAAACCCCAC
SerB-F1	TGACTCCACGCTAGTCAATG
SerB-R-T7	ctaatacgactcactatagggagaGGGCTAAATCAGGCTCATTG
Pfr-F1	AGACATCATTAAGTTGC
Pfr-R-T7	ctaatacgactcactatagggagaAGATTTCCTGCTTTTAG

* Primer sequences were derived from the *H. pylori* 26695 genome sequence (44). Lowercase letters indicate a 5′-extension with T7 promoter sequence for creation of an antisense RNA probe.

was carried out for 1.5 h at 42°C. Next, 2 µl of 0.5 M EDTA were added to stop all reactions. Alkaline hydrolysis of the RNA was performed by addition of 6 µl of 3.0 M NaOH and incubation of the solution for 30 min at 65°C, followed by 15 min at RT. The solution was neutralised with 20 µl of 1 M Tris-HCl (pH 8.0) and 6 µl of 2N HCl. Finally, the cDNA was precipitated overnight at -20°C after addition of 10 µl of 3 M sodium acetate (pH 5.2) and 400 µl ethanol. The cDNA was pelleted by centrifugation at 17.600× g for 15 min at 4°C, washed with 70% (vol/vol) ethanol, dried, and resuspended in 100 µl of sterile water. Labelling efficiency was determined by liquid scintillation measurement.

Hybridisation of labeled cDNA to DNA macroarrays. *H. pylori* arrays (Eurogentec, Seraing, Belgium): Nylon membranes carrying PCR products which represent 97% of all *H. pylori* 26695 and J99 protein-encoding genes (n = 1578), were incubated for 10 min in 50 ml of SSPE buffer (0.18 M NaCl; 10 mM sodium phosphate, pH 7.7; 1 mM EDTA). Prehybridisation was carried out in 10 ml of hybridisation solution (5× Denhardt solution; 5× SSC, where 1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate; 0.5% SDS; 100 µg of denatured salmon sperm DNA (Sigma) ml^{-1}) for 2 h at 65°C. Subsequently, hybridisation was performed for 20 h at 65°C in 5 ml of hybridisation solution containing the labeled cDNA probe which had been boiled for 5 min and rapidly cooled on ice before hybridisation. Arrays were washed twice with 200 ml of 2× SSC and 0.1% (wt/vol) SDS (5 min at RT and 20 min at 65°C) and once with 200 ml of 0.2× SSC and 0.1% (wt/vol) SDS. Finally, arrays were air dried for 2 min, sealed in plastic bags, and exposed to PhosphorImager screens. The transcriptome analysis was carried out twice, using two independently isolated sets of RNA preparations and two different array batches. Exposed PhosphorImager screens were scanned with a Storm 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA) at a resolution of 50 µm and a color depth of 16 bit. To remove the labeled cDNA from the arrays prior to subsequent hybridisations, the membranes were incubated three times (2, 5, and 60 min) in 300 ml of boiling stripping buffer (10 mM Tris/HCl, pH 8.0; 1mM EDTA; 1% SDS). Exposure of the arrays after stripping revealed that the complete activity was successfully removed from the membrane. Using this method, it was possible to use the macroarrays five times without significant loss in quality.

Analysis of transcriptome data. For quantification of the hybridisation signals and background values, the ArrayVision software (Imaging Research, St. Catherines, Ont., Canada) was used (17). Subsequently, a quality score was calculated for each spot reflecting the ratio between the signal intensity and the background intensity. This quality score was utilised to identify hybridisation signals close to the detection limit. Data normalisation and data anlysis were done with the GeneSpring software (Silicon Genetics, Redwood City, CA, USA). After background subtraction, normalised intensity values of the individual spots were calculated (median normalisation). Only genes specifying signals which significantly exceeded the background signal level (determined by the quality scores) under at least one condition were included in further data analysis. The average of the normalised intensity values of the duplicate spots of each gene was used to calculate the expression level ratios. Induction or repression ratios ≥ 2 in both experiments were considered as significant and used in subsequent analysis (17).

Final evaluation of the macroarray data included the consideration of putative operon structures derived from the genome sequence as well as previously known operons. Genes exhibiting significant expression ratios were analysed for their transcriptional organisation using the PyloriGene database (http://genolist.pasteur.fr/PyloriGene/; (10)). The complete

dataset is shown in Supplementary Table S1, which is available online as supplementary data with the online version of this paper at http://mic.sgmjournals.org.

Furbox Analyses. Sequences of Fur-regulated genes (Table 2) were obtained from the *H. pylori* 26695 genome sequence using the PyloriGene database (http://genolist.pasteur.fr/PyloriGene/). Sequences included the intergenic regions upstream of the regulated gene when applicable. Genes were designated as being located downstream of co-transcribed genes (*fliP*, *murB*, *ispE*, *pdxJ* and *hp0241*) when there was less than 10 bp between the stopcodon of the preceding gene and the startcodon of the following gene. All genes included in this analyses had putative ribosome-binding sites (RBS) located upstream of the translational start codon.

To find putative binding sequences for iron-cofactored Fur, promoter sequences were analysed for the presence of consecutive NAT triplets (45) using the GeneRunner program (http://www.generunner.com). Putative binding sequences for apo-Fur were identified by aligning the promoter sequences with the Pfr-boxes I and II (16) using the Clone Manager 7 Suite (Scientific and Educational Software, Cary, NC, USA).

RESULTS

Identification of iron- and Fur-regulated *H. pylori* genes by transcriptional profiling. For characterising Fur- and iron-regulated gene expression, *H. pylori* strain 26695 was selected, as this allowed direct comparison with the available genome sequence (44). In addition, *H. pylori* 26695 has been extensively characterised for the role of Fur and iron in regulation of genes putatively involved in iron-transport (49). The *H. pylori* 26695 isogenic *fur* mutant used in this study contains the *Campylobacter coli* chloramphenicol resistance cassette in the unique *Bcl*I restriction inside the *fur* coding region, and was characterised previously with regard to acid resistance (8), iron uptake (49) and iron- and acid-responsive regulation (11, 46).

We selected a single time-point (20 h) to compare gene expression, as this is when *H. pylori* 26695 reaches the late log-phase (Fig. 1A). To confirm that the 20 h time-point was representative for identification of iron- and Fur-regulated genes, RNA samples isolated at 8, 20 and 30 h post-inoculation were hybridised with a probe specific for the *amiE* gene (Fig. 1B). The *amiE* gene was previously demonstrated to be iron- and Fur-repressed (48), and this regulation is apparent at each of the three time-points (Fig. 1B). The *amiE* mRNA, with a size of ~1 kb, was detected in the wild-type strain only in iron-restricted conditions, but was constitutively expressed in the *fur* mutant (Fig. 1B). Although the *amiE* gene has also been reported to be growth-phase regulated (30, 43), this was not apparent in the conditions used in this study.

RNA for array testing was isolated from two independent cultures of *H. pylori* 26695 and its isogenic *fur* mutant, grown in iron-restricted and iron-replete conditions. Subsequently, the RNA samples were used for transcriptional profiling using the Eurogentec *H. pylori* DNA array, which contains 97% of all ORFs of *H. pylori* strain 26695. To exclude potential artefacts, only genes with a signal to noise ratio > 3 were included in the subsequent data analysis. In total, 1248 out of 1551 genes (80.5%) fullfilled these criteria for at least one of the conditions in both array experiments, and this percentage of genes exhibiting significant expression signals is relatively high compared

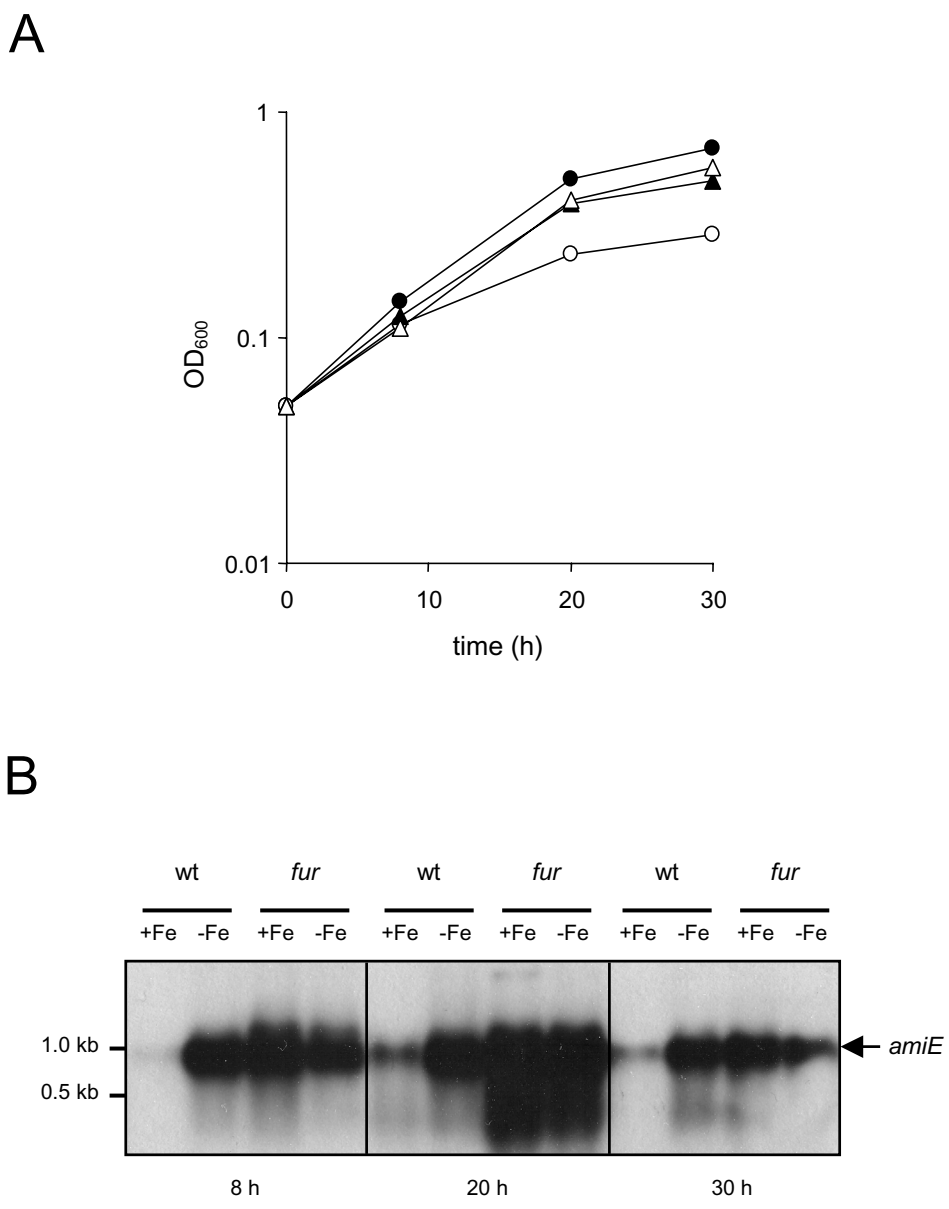

Fig. 1. Selection of the growth-phase of cultures of *H. pylori* 26695 wild-type and isogenic *fur* mutant for isolation of RNA and subsequent transcriptome analysis. (A) Growth curves of *H. pylori* 26695 wild-type (circles) and *fur* mutant (triangles) grown in iron-restricted (open symbols) and iron-replete conditions (closed symbols). Growth is expressed as OD_{600}. (B) Verification of iron- and Fur-responsive regulation at 8, 20 and 30 h of growth in iron-restricted (-Fe) and iron-replete (+Fe) conditions using the iron- and Fur-repressed *amiE* gene. Northern hybridisation of RNA isolated from *H. pylori* 26695 (wt) and its isogenic *fur* mutant (*fur*) with a probe specific for the *amiE* gene. The position of the two relevant RNA marker sizes (in kb) is indicated on the left.

to that reported in previous studies (~50%) (5, 29, 43). In total, data for iron regulation were available for 1241 genes in the wild-type strain, and for 964 genes in the *fur* mutant. Data for Fur-regulation were available for 994 genes in iron-restricted conditions, and 909 genes in iron-replete conditions.

For each of the 1248 genes, iron regulation was assessed by calculating the ratio between expression levels in iron-restricted conditions (-Fe) and the expression levels in iron-replete conditions (+Fe). To assess Fur-regulation, the ratio between expression levels in the wild-type strain (wt) was compared with the expression levels in the *fur* mutant strain (*fur*/wt ratio). Since *H. pylori* Fur affects transcription both in iron-replete and iron-restricted conditions, the *fur*/wt ratio was calculated for both -Fe and +Fe conditions. Genes were considered to be regulated by either iron or Fur when the repression or induction ratio was > 2 in both independent RNA preparations. Genes regulated by Fur, together with the different ratios, are presented in Table 2.

In total, 61 genes (4.9%) displayed iron-repressed expression in the wild-type strain, whereas 36 genes (2.9%) displayed iron-induced expression. Of these 97 iron-regulated genes, only 10 still displayed iron-dependent regulation in the *fur* mutant, with data for 22 genes not being available in the *fur* mutant. This underlines the central role of Fur in iron-regulated gene expression in *H. pylori*. Sixteen genes displayed derepressed expression in the *fur* mutant in iron-replete conditions, and thus these genes probably are regulated by the iron-complexed form of Fur. Conversely, sixteen genes displayed derepressed expression in iron-restricted conditions, possibly representing repression by the iron-free form of Fur.

Fur-repressed genes. In most Gram-negative bacteria, Fur binds its target promoters in an iron-dependent fashion, in other words. only in iron-replete conditions. Uniquely, the iron-free form of *H. pylori* Fur is also capable of repressing transcription, thus allowing differential expression of genes depending on iron-availability in the cytoplasm (16). Surprisingly, not all Fur-regulated genes identified in this study displayed iron-responsive expression (Table 2).

(i) Genes repressed by the iron-complexed form of Fur (Fe-Fur): Of the 16 genes demonstrating derepressed expression under iron-replete conditions in the *fur* mutant, 10 also demonstrated iron-repressed expression in the wild-type strain (Table 2), with four displaying iron-independent expression; for two genes, data were not available. As predicted in previous studies (15, 16, 18, 49), several of these genes (*fecA1*, *fecA2*, *frpB1*, *exbB2*) encode homologues of iron transport and binding proteins, and probably play a role in the uptake and transport of iron to the cytoplasm. In addition, the *hp1432* gene, encoding a nickel-binding histidine- and glutamine-rich protein (20) is also regulated by Fur, and this regulation was confirmed by Northern hybridisation (Fig. 2) and RNA slotblot hybridisation (data not shown). This gene has also been classified as nickel- and NikR-activated (12), and acid-induced (29).

The *fur* mutation also influenced several other classes of genes (Table 2). These included genes putatively involved in: (i) biosynthesis of cofactors and prosthetic groups: biotin (*bioB*), isoprenoid (*ispE*) and pyridoxal phosphate (*pdxA*); (ii) production of cell envelope and surface structures such as the *murB* peptidoglycan synthesis gene and the *flaB* and *fliP* flagellar biosynthesis genes (26); (iii) energy metabolism, with both the paralogous amidases *amiE* and *amiF* (41, 48); (iv) protein synthesis, in which the the 16S rRNA dimethyltransferase gene *ksgA* is putatively involved. Finally, expression of the

Table 2. Fur-regulated genes of *Helicobacter pylori* strain 26695

Gene number*		Predicted function†	Ratio§			
26695	J99		wt-Fe / wt+Fe	*fur*-Fe / *fur*+Fe	*fur*-Fe / wt-Fe	*fur*+Fe / wt+Fe
Repressed by iron-bound Fur						
Biosynthesis of cofactors, prosthetic groups, and carriers						
HP1406	JHP1298	biotin synthetase (*bioB*)	2.6	0.6	1.4	**6.7**
HP1443	JHP1336	4-disphosphocytidyl-2-C-methyl-D-erythritol kinase (*ispE*)	**3.7**	0.6	1.5	**9.2**
HP1583	JHP1490	pyridoxal phosphate biosynthetic protein A (*pdxA*)	**9.5**	2.3	0.9	**4.0**
Cell envelope and surface structures						
HP0685	JHP0625	flagellar biosynthetic protein (*fliP*)	**2.4**	0.8	1.3	**4.2**
HP1418	JHP1313	UDP-N-acetylenolpyruvoylglucosamine reductase (*murB*)	**2.8**	0.6	1.6	**7.8**
Cellular processes						
HP0115	JHP0107	flagellin B (*flaB*)	ND‡	*0.4*	ND	**3.0**
HP0870	JHP0804	flagellar hook (*flgE*)	ND	*0.4*	ND	**4.2**
Energy metabolism						
HP0294	JHP0279	aliphatic amidase (*amiE*)	**2.2**	0.9	0.8	**2.1**
HP1238	JHP1159	formamidase (*amiF*)	0.9	0.5	1.7	**3.5**
Hypothetical proteins						
HP0906	JHP0842	*H. pylori* predicted coding region HP0906	0.8	*0.3*	1.4	**3.0**
Protein synthesis						
HP1431	JHP1322	16S rRNA (adenosine-N6,N6-)-dimethyltransferase (*ksgA*)	**3.5**	1.4	1.4	**3.7**
Transport and binding proteins						
HP0686	JHP0626	iron(III) dicitrate transport protein (*fecA1*)	**8.5**	1.2	0.6	**4.1**
HP0807	JHP0743	iron(III) dicitrate transport protein (*fecA2*)	**30.2**	1.7	0.5	**8.3**
HP0876	JHP0810	iron-regulated outer membrane protein (*frpB1*)	**5.5**	0.7	0.9	**6.8**
HP1339	JHP1258	biopolymer transport protein (*exbB2*)	1.6	1.4	2.1	**2.2**
HP1432	JHP1321	histidine and glutamine-rich protein	**4.0**	0.7	1.8	**9.5**
Repressed by iron-free Fur						
Amino acid biosynthesis						
HP0652	JHP0597	phosphoserine phosphatase (*serB*)	0.7	2.3	**8.6**	2.9
Cellular processes						
HP0389	JHP0992	superoxide dismutase (*sodB*)	*0.2*	0.6	**8.7**	1.9
HP0616	JHP0559	chemotaxis protein (*cheV2*)	*0.2*	1.8	**14.1**	1.9
HP0922	JHP0856	toxin-like outer membrane protein / VacA paralogue	1.1	2.3	**2.4**	1.3
Energy metabolism						
HP0631	JHP0574	quinone-reactive Ni/Fe hydrogenase, small subunit (*hydA*)	*0.4*	1.2	**3.4**	1.2
HP0632	JHP0575	quinone-reactive Ni/Fe hydrogenase, large subunit (*hydB*)	*0.3*	0.9	**3.2**	1.2
HP0633	JHP0576	quinone-reactive Ni/Fe hydrogenase, cytochr. b subunit (*hydC*)	*0.3*	1.0	**4.2**	1.2
HP1227	JHP1148	cytochrome c553	0.4	1.0	**4.3**	1.5

Hypothetical proteins / unknown function						
HP0241	JHP0226	predicted coding region HP0241	*0.3*	1.1	**2.6**	0.7
HP0388	JHP0993	conserved hypothetical protein	*0.3*	0.9	**4.9**	1.4
HP0629	JHP0572	predicted coding region HP0629	0.8	1.3	**2.6**	1.7
HP1094	none#	predicted coding region HP1094	ND	**2.2**	**3.2**	ND
HP1502	JHP1395	predicted coding region HP1502	0.9	1.7	**3.6**	1.8
HP1524	JHP1413	predicted coding region HP1524	*0.4*	0.9	**2.3**	0.8
Protein synthesis						
HP1253	JHP1174	tryptophanyl-tRNA synthetase (*trpS*)	ND	1.2	**3.0**	1.0
Transport and binding protein						
HP0653	JHP0598	nonheme iron-containing ferritin (*pfr*)	*0.2*	**2.5**	**53.1**	**2.3**
Fur-induced						
Cell envelope and surface structures						
HP0638	JHP0581	outer membrane protein (*omp13*; *oipA*)	1.0	ND	*0.2*	*0.1*
HP0855	none#	alginate O-acetylation protein (*algI*)	**5.8**	ND	*0.3*	ND
HP1494	JHP1387	UDP-MurNac-tripeptide synthetase (*murE*)	**4.3**	2.1	*0.5*	0.9
DNA metabolism, restriction and modification						
HP0260	JHP0244	adenine specific DNA methyltransferase (*mod*)	1.8	1.0	*0.4*	0.9
HP1323	JHP1243	ribonuclease HII (*rnhB*)	**4.4**	1.6	*0.4*	0.9
Hypothetical proteins / unknown function						
HP0207	JHP0193	ATP-binding protein (*mpr*)	**4.0**	**2.6**	*0.4*	0.5
HP0236	JHP0221	predicted coding region HP0236	1.4	0.8	*0.4*	0.8
HP0248	JHP0233	conserved hypothetical protein	2.6	0.9	*0.4*	1.1
HP0322	JHP0305	poly E-rich protein	*0.3*	0.8	0.9	*0.4*
HP0424	none#	predicted coding region HP0424	2.0	ND	*0.4*	ND
HP0773	JHP0710	predicted coding region HP0773	2.6	1.8	*0.5*	0.7
HP1117	JHP1045	cysteine-rich protein X (*hcpX*)	1.8	1.5	0.9	*0.4*
HP1142	JHP1070	predicted coding region HP1142	*0.5*	ND	ND	*0.4*
Protein synthesis						
HP1141	JHP1069	methionyl-tRNA formyltransferase (*fmt*)	**2.3**	1.3	*0.4*	0.7
HP1160	JHP1087	tRNA(5-methylaminomethyl-2-thiouridylate)-methyltransferase	**2.6**	1.2	*0.4*	0.8
Transport and binding proteins						
HP1172	JHP1099	glutamine ABC transp., peripl. glutamine-binding protein (*glnH*)	**3.2**	1.5	*0.4*	0.7

* Gene number in the complete genome sequences of *H. pylori* strain 26695 (44) and strain J99 (3).
† Function and functional category as defined on the PyloriGene database (10).
‡ ND = not detectable: the signal on the array was below the detection threshold in both array experiments.
§ Ratio of expression levels in *H. pylori* wild-type (wt) or *fur* mutant (fur) strain, in iron-restricted (-Fe) or iron-replete (+Fe) conditions. Value shown is the average ratio of the two independent array experiments. Values in italic type indicate significant downregulation of expression, values in bold type indicate significant upregulation of expression. Significant upregulation was defined as at least twofold changes in the mRNA levels in both independent array experiments.
# This gene is absent in the *H. pylori* J99 complete genome sequence (3).

hypothetical protein HP906 was repressed by Fe-Fur. For two of the iron-repressed, Fur-regulated genes (*amiE* and *hp1432*), the transcriptional pattern was confirmed using Northern hybridisation (Fig. 2).

(ii) Genes repressed by the iron-free form of Fur: Sixteen genes demonstrated increased expression in the *fur* mutant, when compared to the wild-type strain grown in iron-restricted conditions (Table 2). This unique form of regulation has so far only been reported for the *pfr* gene (7, 16, 50), but is probably more widespread in *H. pylori*. Of the 16 genes demonstrating derepressed expression under iron-restricted conditions in the *fur* mutant, nine genes also demonstrated iron-induced expression in the wild-type strain (Table 3), with five genes displaying iron-independent expression; for two genes data were not available.

Several genes associated with energy and oxygen metabolism displayed Fur-mediated repression of transcription, including the nickel/iron-cofactored hydrogenase subunit genes (*hydABC*) (33), a putative cytochrome c553, and the *sodB* gene encoding the iron-cofactored superoxide dismutase (37, 40, 42). Further genes regulated by the iron-free form of Fur included the chemotaxis gene *cheV2* , the *hp0922* gene encoding a toxin-like outer membrane protein, the *serB* gene which is cotranscribed with *pfr*, the tryptophanyl-tRNA synthetase gene *trpS*, and five genes encoding hypothetical proteins (Table 2). For three of the iron-induced, Fur-regulated genes (*pfr*, *serB* and *hp0388*) the transcriptional pattern was confirmed using Northern hybridisation (Fig. 2).

(iii) Fur-induced genes: Sixteen genes displayed decreased expression in the *fur* mutant when compared to the wild-type strain (Table 2). This inverse regulation is atypical for a repressor like Fur, and is likely to represent indirect regulation. This cluster of genes included the *oipA* gene encoding an outer membrane protein, the *murE* gene involved in peptidoglycan synthesis, a DNA methyltransferase (*mod*), the ribonuclease HII-encoding *rnhB* gene, the periplasmic binding protein of the glutamine ABC transporter, two genes involved in protein synthesis, and eight genes encoding hypothetical proteins.

Identification of putative binding sequences for Fe-Fur and apo-Fur. All Fur-repressed genes listed in Table 2 were further investigated for the presence of putative Fur boxes in their respective promoters (Fig. 3). Firstly, the putative promoter region was identified for each gene using the PyloriGene database (see Materials and Methods for details). Putative binding sites for Fe-Fur were identified as up to six consecutive nAT triplets, with n representing any nucleotide (15, 45). Each of the promoters included in this analysis contained a putative Fur box, with identities to the $(nAT)_6$ sequence ranging from 6 to 11 per 12 residues (Fig. 3A).

The identification of binding sites of apo-Fur is currently hampered by the absence of a consensus sequence, since only the *pfr* promoter has been analysed to date (16). In this promoter there are two high-affinity sites for apo-Fur, designated Pfr-box I and Pfr-box II (16). Therefore these boxes were aligned with the promoters of the genes putatively regulated by apo-Fur (Fig. 3B). All promoters contained sequences similar to each of the Pfr-boxes, although this identity ranged from 17 to 24 per 41 residues (Pfr-box I) and 15 to 22 per 37 residues (Pfr-box II), respectively (Fig. 3B).

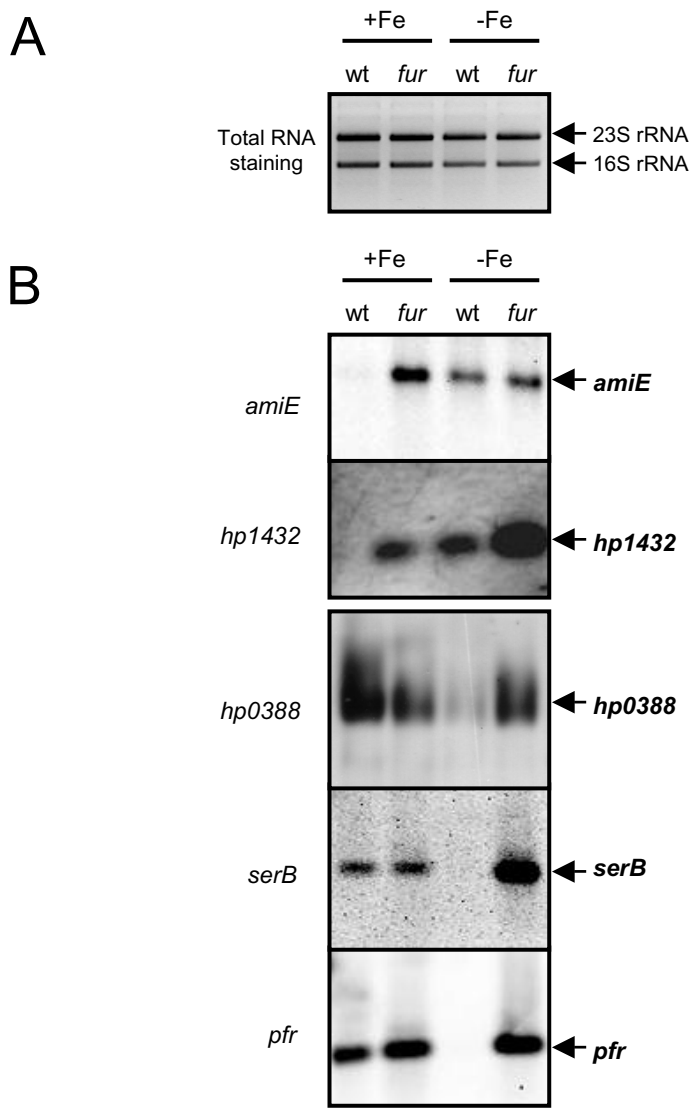

Fig. 2. Confirmation of Fur- and iron-responsive regulation of a subset of genes selected from Table 2. (A) Staining of transferred RNA by methylene blue to allow for comparison of RNA amounts. (B) Northern hybridisation with probes specific for five genes using RNA purified from *H. pylori* wild-type (wt) and *fur* mutant (*fur*) cells grown in iron-restricted (-Fe) and iron-replete (+Fe) conditions. Probes used are indicated on the left; the specific mRNA is indicated on the right.

Iron-responsive regulation independent of Fur. While only approximately half of the Fur-repressed genes displayed iron-responsive expression, several other genes displayed iron-responsive expression which was not significantly altered in the *fur* mutant (Table 3). Forty-five genes displayed iron-repressed expression in the wild-type strain, i.e. higher mRNA levels in iron-restricted conditions, whereas twenty-five genes displayed

iron-induced expression. As with the Fur-repressed genes, genes belonging to several functional classes were affected by iron restriction when compared to iron-replete conditions.

(i) Iron-repressed genes: This group of iron-repressed genes includes five motility-associated genes, the *fliD*, *fliI*, *fliM, fliY* and *flgI* genes, encoding components of the flagellum of *H. pylori* (34). Their regulation by iron may explain the effect of iron restriction and acid-exposure on motility of *H. pylori* (30). In addition to motility-associated genes, iron repressed the expression of genes involved with the cell envelope and surface structures (*lpp20*), cell division (*ftsH*), peptidoglycan synthesis (*murZ*), LPS (*kpsF*, *rfaC*) and phospholipid synthesis (*plsX*). Other membrane-associated structures repressed by iron included a putative phosphate permease and one of the ferric citrate outer membrane receptors (*fecA*3). In addition to these genes, genes involved in protein synthesis, stress response, nucleotide metabolism and modification, and cofactor biosynthesis were also induced by iron restriction, as were several genes encoding hypothetical proteins (Table 3).

(ii) Iron-induced genes: Many genes subject to Fur-independent, iron-induced transcriptional regulation encode major virulence factors of *H. pylori*. These include the VacA vacuolating cytotoxin, the CagA cytotoxin and the HopA and HP0492 outer membrane proteins. Also included in this category are genes encoding proteins functioning in stress-response, such as the KatA catalase and the chaperones DnaK and ClpB (Table 3). Chemotaxis may also be iron-responsive via the *tlpB* gene, which encodes a methyl-accepting chemotaxis protein. Finally, two genes involved in nucleotide metabolism/modification and eight genes encoding hypothetical proteins displayed Fur-independent, iron-induced expression.

(iii) Abberantly regulated genes: Seven genes displayed iron-regulated expression in the *fur* mutant only, but were transcribed in an iron-independent manner in the wild-type strain. This cluster includes the HP0004 gene encoding carbonic anhydrase, HP1458 gene encoding a putative thioredoxin, which is transcribed at higher levels in iron-replete conditions in the *fur* mutant; the HP0220 *nifS* gene, which is involved in formation of Fe-S clusters (32), and three copies of the IS605 transposase (*tnpB*), whose expression is decreased in iron-replete conditions (Table 3).

Table 3. Iron-regulated (Fur-independent) genes of *Helicobacter pylori* strain 26695

Gene number*		Predicted function†	Ratio§			
26695	J99		wt -Fe / wt +Fe	*fur* -Fe / *fur* +Fe	*fur* -Fe / wt -Fe	*fur* +Fe / wt +Fe
Iron-repressed						
Biosynthesis of cofactors, prosthetic groups, and carriers						
HP0625	JHP0569	1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthetase (*ispG*)	**2.7**	2.6	0.7	0.8
HP0798	JHP0734	molybdenum cofactor biosynthesis protein C (*moaC*)	**4.2**	ND‡	ND	ND
HP0804	JHP0740	GTP cyclohydrolase II/3,4-diOH-2-butanone 4-phosphate synthase (*ribBA*)	**2.5**	ND	0.5	ND
HP0841	JHP0779	pantothenate metabolism flavoprotein (*dfp*)	**2.9**	**2.5**	0.9	ND

Cell envelope and surface structures						
HP0279	JHP0264	lipopolysaccharide heptosyltransferase-1 (*rfaC*)	**2.6**	ND	ND	ND
HP0648	HP0593	UDP-N-acetylglucosamine enolpyruvyl transferase (*murZ*)	**2.7**	1.0	0.5	1.1
HP1429	JHP1324	polysialic acid capsule expression protein (*kpsF*)	**9.0**	**4.7**	1.0	ND
HP1456	JHP1349	membrane-associated lipoprotein (*lpp20*)	**2.7**	1.8	0.7	1.0
Cellular processes						
HP0246	JHP0231	flagellar basal-body P-ring protein (*flgI*)	**2.3**	1.4	0.7	1.1
HP0752	JHP0689	flagellar hook-associated protein 2 (*fliD*)	**4.2**	2.2	0.6	1.2
HP0792	JHP0728	Predicted DNA transformation competence protein (*comM*)	**2.7**	2.0	0.7	0.8
HP1030	JHP0394	fliY protein (*fliY*)	**2.6**	ND	0.5	ND
HP1031	JHP0393	flagellar motor switch protein (*fliM*)	**4.5**	2.1	0.4	ND
HP1069	JHP0356	cell division protein (*ftsH2*)	**2.6**	2.1	0.7	1.0
HP1420	JHP1315	flagellar export protein ATP synthase (*fliI*)	**4.1**	**2.1**	0.4	0.7
DNA metabolism, restriction and modification						
HP1114	JHP1041	excinuclease ABC subunit B (*uvrB*)	**3.2**	2.0	0.6	1.0
HP1478	JHP1371	DNA helicase II (*uvrD*)	**3.2**	2.5	0.6	ND
Energy metabolism						
HP1103	JHP1029	glucokinase (*glk*)	**2.2**	ND	ND	ND
Fatty acid and phospholipid metabolism and biosynthesis						
HP0201	JHP0187	fatty acid/phospholipid synthesis protein (*plsX*)	**2.8**	3.3	0.6	0.6
Hypothetical proteins / unknown function						
HP0066	JHP0061	conserved hypothetical ATP-binding protein	**2.7**	ND	0.8	ND
HP0258	JHP0242	conserved hypothetical integral membrane protein	**2.7**	ND	ND	ND
HP0346	none[#]	predicted coding region HP0346	**5.6**	ND	ND	ND
HP0347	JHP0321	conserved hypothetical protein	**3.1**	ND	ND	ND
HP0356	JHP0330	predicted coding region HP0356	**2.1**	ND	ND	ND
HP0726	JHP0663	predicted coding region HP0726	**3.3**	**3.5**	0.7	ND
HP0806	JHP0742	predicted coding region HP0806	**4.0**	1.5	0.5	ND
HP1335	JHP1254	conserved hypothetical protein	**4.2**	**2.3**	0.4	ND
HP1336	JHP1255	predicted coding region HP1336	**2.7**	2.3	0.7	ND
HP1343	JHP1262	conserved hypothetical integral membrane protein	**2.2**	ND	ND	0.7
HP1424	JHP1319	predicted coding region HP1424	**3.5**	**3.3**	0.7	ND
HP1428	JHP1325	conserved hypothetical protein	**4.8**	**3.9**	1.0	ND
HP1430	JHP1323	conserved hypothetical ATP-binding protein	**4.6**	7.6	1.0	1.0
HP1454	JHP1347	predicted coding region HP1454	**3.4**	2.3	0.5	0.8
HP1467	JHP1360	predicted coding region HP1467	**2.4**	1.6	0.7	1.1
HP1567	JHP1475	conserved hypothetical GTP-binding protein	**2.3**	ND	ND	ND
Protein synthesis						
HP1201	JHP1124	ribosomal protein L1 (*rplI*)	**3.5**	2.6	0.7	0.9

HP1480	JHP1373	seryl-tRNA synthetase (*serS*)	**3.0**	2.2	0.6	0.7
HP1547	JHP1452	leucyl-tRNA synthetase (*leuS*)	**2.6**	1.9	0.6	0.8
Purines, pyrimidines, nucleosides, and nucleotides						
HP0043	JHP0037	mannose-6-phosphate isomerase (*pmi*) or (*algA*)	**2.7**	ND	0.8	ND
HP0854	JHP0790	GMP reductase (*guaC*)	**4.4**	**4.4**	0.6	ND
Regulatory functions						
HP0278	JHP0263	guanosine pentaphosphate phosphohydrolase (*gppA*)	**3.3**	2.2	0.5	0.7
Transport and binding proteins						
HP0724	JHP0660	anaerobic C4-dicarboxylate transport protein (*dcuA*)	**2.3**	3.4	2.0	ND
HP0818	JHP0754/7¶	osmoprotection protein (*proWX*)	**3.2**	ND	0.5	ND
HP1400	JHP1426	iron (III) dicitrate transport protein (*fecA3*)	**2.5**	3.2	0.9	0.7
HP1491	JHP1384	phosphate permease	**2.7**	2.0	0.7	0.9
Iron-induced						
Cell envelope and surface structures						
HP0229	JHP0214	outer membrane protein (*omp6*; *hopA*)	*0.1*	0.4	2.2	0.7
HP0492	JHP0444	neuraminyllactose-binding hemagglutinin homolog/paralog of HpaA	*0.5*	0.6	1.5	1.1
Cellular processes						
HP0103	JHP0095	methyl-accepting chemotaxis protein (*tlpB*)	*0.4*	0.6	1.1	0.6
HP0522	JHP0471	*cag* pathogenicity island protein (*cag3*)	*0.5*	0.9	1.2	0.6
HP0523	JHP0472	*cag* pathogenicity island protein (*cag4*)	*0.3*	ND	ND	0.6
HP0547	JHP0495	*cag* pathogenicity island protein (*cag26*; *cagA*)	*0.2*	0.5	1.0	0.5
HP0875	JHP0809	catalase (*katA*)	*0.4*	0.5	1.7	1.4
HP0887	JHP0819	vacuolating cytotoxin (*vacA*)	*0.2*	*0.3*	3.7	2.0
DNA metabolism, restriction and modification						
HP0091	JHP0084	type II restriction enzyme R protein (*hsdR*)	*0.4*	ND	ND	ND
HP0481	JHP0433	type II adenine specific DNA methyltransferase (MFOKI)	*0.4*	ND	ND	0.7
Hypothetical proteins / unknown function						
HP0097	JHP0089	predicted coding region HP0097	*0.4*	0.7	1.4	0.8
HP0102	JHP0094	predicted coding region HP0102	*0.4*	ND	ND	ND
HP0119	none#	predicted coding region HP0119	*0.5*	0.9	1.3	0.7
HP0120	none#	predicted coding region HP0120	*0.5*	1.0	1.4	0.7
HP0130	JHP0119	predicted coding region HP0130	*0.4*	0.7	1.0	0.6
HP0377	JHP1004	thiol:disulfide interchange protein (dsbC), putative	*0.4*	ND	ND	0.5
HP0762	JHP0699	predicted coding region HP0762	*0.4*	0.9	1.3	0.6
HP0938	JHP0873	predicted coding region HP0938	*0.4*	ND	ND	1.0
HP1143	JHP1071	predicted coding region HP1143	*0.5*	ND	ND	0.6
HP1175	JHP1102	conserved hypothetical integral membrane protein	*0.5*	0.5	1.1	1.0
Protein fate						
HP0109	JHP0101	chaperone and heat shock protein 70 (*dnaK*)	*0.4*	0.7	1.1	0.6
HP0264	JHP0249	ATP-dependent protease binding subunit (*clpB*)	*0.4*	0.6	0.9	0.6

HP0470	JHP0422	oligoendopeptidase F (*pepF*)	*0.4*	0.8	1.2	0.7
Purines, pyrimidines, nucleosides, and nucleotides						
HP0404	JHP0977	Predicted ADP hydrolase of the HIT protein family (HINT)	*0.4*	0.5	ND	0.8
Transport and binding proteins						
HP1082	JHP0343	multidrug resistance protein (*msbA*)	*0.5*	1.0	1.3	0.7
Other						
HP0472	JHP0424	outer membrane protein (*omp11*)	ND	*0.3*	1.1	ND
HP0708	JHP0647	predicted coding region HP0708	0.5	*0.3*	0.8	1.3
HP0909	JHP0845	predicted coding region HP0909 (pseudogene)	0.6	*0.5*	1.6	1.2
HP1458	JHP1351	thioredoxin	0.6	*0.3*	1.0	1.3
HP0004	JHP0004	carbonic anhydrase (*icfA*)	1.9	**3.6**	1.2	ND
HP0220	JHP0101	synthesis of [Fe-S] cluster (*nifS*)	2.3	**3.6**	1.1	0.7
HP0438	JHP0249	IS605 transposase (*tnpB*)	1.4	**2.1**	1.3	0.9
HP1095	JHP0422	IS605 transposase (*tnpB*)	1.6	**2.5**	1.6	1.0
HP1387	JHP1438	DNA polymerase III epsilon subunit (*dnaQ*)	2.7	**3.4**	1.2	ND
HP1534	JHP0977	IS605 transposase (*tnpB*)	2.0	**2.7**	1.7	1.1

* Gene number in the complete genome sequences of *H. pylori* strain 26695 (44) and strain J99 (3).
† Function and functional category as defined on the PyloriGene database (10).
‡ ND = not detectable: the signal on the array was below the detection threshold in both array experiments.
§ Ratio of expression levels in *H. pylori* wild-type (wt) or *fur* mutant (fur) strain, in iron-restricted (-Fe) or iron-replete (+Fe) conditions. Value shown is the average ratio of the two independent array experiments. Values in italic type indicate significant downregulation of expression, values in bold type indicate significant upregulation of expression. Significant regulation was defined as at least twofold changes in the mRNA levels in both independent array experiments.
# This gene is absent in the *H. pylori* J99 complete genome sequence (3).
¶ The HP0818 (*proWX*) gene is a single in *H. pylori* strain 26695, but consists of two genes (JHP0754 and JHP0757) in *H. pylori* strain J99.

DISCUSSION

In many bacteria, the Fur repressor is the central regulator of iron homeostasis (4, 21). Fur mediates its iron homeostasis function via careful regulation of iron acquisition and iron storage systems: in iron-restricted conditions iron-uptake systems are expressed and iron-storage is repressed, but conversely in iron-replete conditions iron-storage systems are expressed and iron-uptake is abolished (7, 16, 49). The switch between repression and induction of iron-uptake is coupled to the iron availability in the cytoplasm: when iron is available, a Fur dimer forms a complex with ferrous iron and binds to Fur binding sequences (Fur boxes) in the promoters of iron-uptake genes (21). This situation is, however, not as clear for the switch in the repression and induction of ferritin-mediated iron-storage: while iron-induction of ferritin expression is found in several bacteria, the role of Fur in this process is not universal.

In this study, transcriptional profiling was used to identify *H. pylori* genes that are regulated by Fur and iron at the transcriptional level. Recent studies focussing on the effects of iron restriction, growth phase and acidic pH on gene expression in *H. pylori* indicated that many genes classified in different functional categories are affected by these conditions (2, 5, 27, 29, 30, 43, 51). For 1248 genes, data were obtained on their

regulation by iron or by Fur. In our study using the wild-type *H. pylori* strain 26695, 97 genes displayed iron-responsive regulation and 43 genes displayed Fur-dependent regulation.

Genes regulated by Fe-Fur and apo-Fur are classified in several functional categories (Table 2), indicative of the role of Fur as global regulator in *H. pylori*. This is consistent with the phenotypes reported for the *fur* mutant thus far, which displays increased iron uptake (49), decreased acid-resistance (8) and attenuation in a mouse model of *H. pylori* infection (11). Rather surprisingly, while mutation of *fur* affects many cellular processes, the *fur* mutant is not significantly affected in growth under *in vitro* conditions (Fig. 1A).

Other than the genes functioning in metal metabolism, many of the genes regulated by Fe-Fur and apo-Fur have not been investigated previously and require experimental confirmation of their predicted function. However, based on homology, several of the proteins encoded by Fur-regulated genes are predicted to be iron-cofactored, like the biotin synthetase BioB (39). The *E. coli* BioB protein also requires pyridoxal phosphate (31), as synthesized by the PdxA protein, and this gene displays in *H. pylori* similar regulation to the *bioB* gene (Table 2). Furthermore, in *E. coli*, the *ksgA* gene is cotranscribed with the *pdxA* gene and both are growth-phase regulated (36), while in *H. pylori* both genes are subjected to regulation by Fe-Fur (Table 2). Other Fe-Fur regulated genes include the *flaB* and *flgE* genes, and taken together with the iron-responsive regulation of several *fli* genes (Table 2), this may explain the effect of iron on motility of *H. pylori* (30). Finally, genes regulated by apo-Fur encode iron-cofactored enzymes like hydrogenase and superoxide dismutase (Table 2), and this form of regulation may ensure that these enzymes are only expressed when iron is available. Comparison with Fur- and iron-regulons in other bacteria is hampered by the lack of operon structure in the *H. pylori* genome sequence. The most closely related bacterium is *Campylobacter jejuni*, and recently its Fur- and iron-regulons were determined (35). Interestingly, both in *H. pylori* and *C. jejuni*, motility-associated genes were affected by iron and the mutation of *fur*, suggesting a common mechanism behind iron-responsive regulation of motility.

Iron-responsive genes were also recently identified in the mouse-adapted *H. pylori* strain SS1 (30, 43), and show partial overlap with the iron-responsive genes in our study. Unfortunately a direct comparison with the two related studies is hampered by the use of different strains of *H. pylori* and differences in the experimental setup. An important difference may be that in the previously published studies (30, 43), iron restriction was achieved via the use of 2,2-dipyridyl, which has a high affinity for ferrous iron and is membrane permeable, whereas in our study we used desferal, which is a siderophore-based iron chelator that removes ferric iron from the medium and makes it unavailable for *H. pylori* (49). Comparison of the data sets is further complicated by the difference in *H. pylori* strains used. The complete genome sequence of *H. pylori* 26695, the strain used in this study, is available (44), whereas the other two studies were based on *H. pylori* strain SS1 (30, 43), whose genome sequence is not yet known. Thus the gene order, promoter sequences and regulatory responses of *H. pylori* SS1 are unknown and may differ significantly from those in *H. pylori* 26695 (3, 24, 25, 38, 44). However, the majority of Fur-regulated genes identified in our study display iron-responsive regulation (Table 2) and cluster mostly in the group of stationary-phase induced genes (30).

A

Fur-Box

Gene	Prom	nATnATnATnATnATnATn		RBS	Start
flaB	HP0115	AATAAAATGTTTATACCTATTAATGAATG	<113 bp >	AAGGATGCAAACATG	
amiE	HP0294	TCGCCCACAATAATCATAATGATTAAAGT	< 81 bp >	AAGGAACATAATATG	
fliP	HP0684*	TCATTGATTTTAGTGATGACGAGTTTCAC	< 27 bp >	AAGGACCGCTTTG	
fecA1	HP0686	AAACTAATAATGGTTATTATAAATTTCAC	< 28 bp >	AAAGGTGGTAAATG	
		ATTAATATTATCTAAATAATATATTTTCA	< 70 bp >	AAAGGTGGTAAATG	
fecA2	HP0807	AAGTTAATTTTAATAATTATTTTATAGT	< 24 bp >	AAAGGTTATCACAATG	
flgE	HP0870	CAACCAATCATTCTAAAAAGCTATTTAGG	< 45 bp >	AAGGATAACCATG	
frpB1	HP0876	CTGGTTTTAATAATAATTATTATACTATT	< 72 bp >	GAGAGTTGTTGGATG	
hp0906	HP0906	CCCACCATGTAAAATATAAAGAAATAAAA	< 13 bp >	AAGGATAACCATG	
amiF	HP1238	CTCGCAATAATTGTTATTGTTATTGCGAC	< 11 bp >	AAGGAGTTATTATG	
exbB2	HP1339	GTAATTATTAGCTTAATCATTATTGACTT	<152 bp >	AAGGAGTTTTTATG	
		GATTTGATTGTAATTATTAGCTTAATCAT	<161 bp >	AAGGAGTTTTTATG	
bioB	HP1406	TCAAAAATCAAAATAATCAAATTTTTTA	< 80 bp >	AAAGGAATGATTATG	
murB	HP1423*	GCTAAAACTAAAACCCTTATAAGTTATGG	< 17 bp >	AGGGTAAAATAAACGCATG	
ksgA	HP1431	CTTTGAATGAAAAAGCTATAATAAAGAA	< 56 bp >	AGAAGCGTTTCTTATG	
hpn2	HP1432	GTATTAAAAATTAGTATTAAAATCTTTTT	< 74 bp >	AAGGAGTCATCATG	
ispE	HP1441*	TAATAGAGCATTTGAATGACAAAAGCTTA----------TCGCAAAGGTTTATG			
pdxAJ	HP1582*	ATTCCAAAAATCATTATTATAAA----------TAAAGGATAGTCATG			

B

Pfr-box I

Gene	Prom	TTACTTTTTCATTATCATTTATGCTATAATTATGGGACAAC		RBS	Start
hp0241	HP0243*	AAAATTTTTTATTAACTCTTTTGGTGTAGGATAGCGATCAA	< 40 bp>	AGGACTTTTGATG	
sodB	HP0389	TCCATTTTTAAGAAAAGATTTACCAAAAAGTATTAAAAAAT	< 26 bp>	AGGAGAAAACATG	
cheV	HP0616	AAAACCTTTTAAACTAATTAGGGTAGTATTATAGCATTTA	< 19 bp>	GGGAAAATAGGTG	
hp0629	HP0629	TAAGCTCTTTTTTTTTTTTTGATTTAATACTCGGTGTGA	< 17 bp>	AGGAAAAGCTATG	
hydABC	HP0631	TGTTCCTTTTCTAATAAAATAGGGTGGCATTCTATCCAAAT	<106 bp>	AGGAGTGGTCATG	
pfr	HP0653	TTACTTTTTCATTATCATTTATGCTATAATTATGGGACAAC	< 15 bp>	AGGAGATACTATG	
hp0922	HP0922	TTGAGTTTGTTTAACATCAAATTGTATAATGTGGGATTTAA	< 11 bp>	GGAGTTTTAAATG	
hp1094	HP1094	CCACTTGATTATGAGCGTCCATTTTAAGATAGAGCCATTCC	< 73 bp>	GTATAGGCGGTG	
hp1227	HP1227	TCTCATTTTCACTTCATTTTAAGCAAAACTTAAACTTGTAA	< 34 bp>	GAGACAAACGATG	
trpS	HP1253	ACTCTTTTCTGGGATCAAGCGTTCTTAAAAACTAAGATAAA	< 47 bp>	GAGAAACAACATG	
hp1502	HP1502	TAATTATTTTATTATAGCTAATTGCAATCTTAAAAGTCAAA	< 95 bp>	AGGATTTTAAATG	
hp1524	HP1524	GCCAATCCTTAATCATTTTATGATAAGATAGTCAATTTAT	< 11 bp>	AGGAAATTTAATTG	

Pfr-box II

Gene	Prom	GGTGTTCTTTCTCATTTTTGTAAATTTTTAAAAATTT		RBS	Start
hp0241	HP0243*	AATGAGTTTTACTATAAAACAAAATTTTAAAAAGATT	<135 bp>	AGGACTTTTGATG	
sodB	HP0389	AAAAGTCGTTTCATTTTAAAAACCCCTTAAAATCC	< 85 bp>	AGGAGAAAACATG	
cheV	HP0616	CATGTTTGTAAAAACCTTTTAAACTAAATTAGGGTAG	< 33 bp>	GGGAAAATAGGTG	
hp0629	HP0629	GGTGTGATTTTGATTTTATTTAAA----------AGGAAAAGCTATG			
hydABC	HP0631	TGTATTATTTTTATTATGTTAAGATAATGAAAATTTC	< 6 bp>	AGGAGTGGTCATG	
pfr	HP0653	GGTGTTCTTTCTCATTTTTGTAAATTTTTAAAAATTT	< 68 bp>	AGGAGATACTATG	
hp0922	HP0922	AGTCATTTTTGCGAATCTTTTTGAGTTTGTTTAACATC	< 34 bp>	GGAGTTTTAAATG	
hp1094	HP1094	TTTAAGATAGAGCCATTCCAAAAGCTTGTAAAAATTT	< 55 bp>	GTATAGGCGGTG	
hp1227	HP1227	TTGTAATTGTATCATTTTAAGATCATTTTAAAAATTT	<135 bp>	GAGACAAACGATG	
trpS	HP1253	TCTGGGATCAAGCGTTCTTAAAAACTAAGATAAATTA	< 44 bp>	GAGAAACAACATG	
hp1502	HP1502	CCGCTTAAGTGTTACTTTTTAATTATTTTATTATAG	<119 bp>	AGGATTTTAAATG	
hp1524	HP1524	GCAATTTGTTCTTACCCTAGCCAATCCTTAATCATTT	< 34 bp>	AGGAAATTTAATTG	

Fig. 3. Identification of putative binding sequences for Fe-Fur and for apo-Fur. (A) Putative Fe-Fur-repressed promoter sequences were searched for the presence of consecutive nAT-triples, indicative of binding sequences for Fur (45). Residues with black background are identical to the $(NAT)_6$ Fur box, whereas residues with gray background represent A/T and T/A substitutions. (B) Putative apo-Fur-regulated promoters were aligned with the two high-affinity apo-Fur binding sequences identified in the *H. pylori pfr* promoter (Pfr-box I and Pfr-box II) (16). Residues with black background are identical to the respective Pfr-box, whereas residues with gray background represent A/T and T/A substitutions. For all aligned promoters, the position respective to the underlined ribosome-binding site (RBS) and underlined translational start codon (Start) are given. Designations above the alignments: Gene, gene designation given in Table 2; Prom, putative promoter of regulated gene. An asterisk indicates that the regulated gene is likely to be transcribed as a member of an operon, and the putative promoter of the gene at the beginning of the operon was analysed for the presence of a binding sequence for Fur-Fe or apo-Fur.

This is consistent with our experimental setup, since we used late-exponential phase cells to isolate RNA for the transcriptome studies (Fig. 1A).

Rather surprising was the relative lack of operon structure in the transcriptome data. While several of the genes identified in this study are predicted to be transcribed as part of a multicistronic mRNA, this was not apparent from the array data. An example of this is the *pdxA* gene, which is predicted to be an operon with the upstream *pdxJ* gene. However, expression of the *pdxJ* gene seems not to be affected by the *fur* mutation or iron restriction (see supplementary data). This may be partially due to differential mRNA degradation, as was described for the urease operon (1), and it is interesting to see that a ribonuclease gene (*rnhB*) is included in the list of iron-regulated genes (Table 3).

Despite its small genome, *H. pylori* is a highly successful colonizer of the human gastric mucosa, and is present for life unless eradicated by antibiotic treatment (9). Its potential to adapt to hostile environmental niches with changing conditions is apparent, despite the relative paucity of transcriptional regulators. One of the possibilities explaining such adaptive capacity with relatively few regulators is that these regulators have broadened their regulatory potential, and this seems to be the case for *H. pylori* Fur. This protein, well known for its central role in iron homeostasis in bacteria, controls the expression of different pathways involved in normal metabolism, stress resistance, motility and virulence. This central role in these important pathways makes it a prime candidate for further study on the role of bacterial adaptation in long-term colonisation of hostile environmental niches.

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CHAPTER **4**

Iron-responsive regulation of the *Helicobacter pylori* iron-cofactored superoxide dismutase SodB is mediated by Fur

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Maintaining iron homeostasis is a necessity for all living organisms, as free iron augments the generation of reactive oxygen species (ROS) like superoxide anions, at the risk of subsequent lethal cellular damage. The iron-responsive regulator Fur controls iron metabolism in many bacteria, including the important human pathogen *Helicobacter pylori*, and thus is directly or indirectly involved in regulation of oxidative stress defense. Here we demonstrate that Fur is a direct regulator of the *H. pylori* iron-cofactored superoxide dismutase SodB, which is essential for the defense against toxic superoxide radicals. Transcription of the *sodB* gene was iron-induced in *H. pylori* wild-type strain 26695, resulting in expression of the SodB protein in iron-replete conditions, but an absence of expression in iron-restricted conditions. Mutation of the *fur* gene resulted in constitutive, iron-independent expression of SodB. Recombinant *H. pylori* Fur protein bound with low affinity to the *sodB* promoter region, but addition of the iron-substitute Mn^{2+} abolished binding. The operator sequence of the iron-free form of Fur, as identified by DNAse I footprinting, was located directly upstream of the *sodB* gene at positions -5 to -47 from the transcription start site. The direct role of Fur in regulation of the *H. pylori sodB* gene contrasts with the small RNA-mediated *sodB* regulation observed in *Escherichia coli*. In conclusion, *H. pylori* Fur is a versatile regulator involved in many pathways essential for gastric colonization, including superoxide stress defense.

The human gastric pathogen *Helicobacter pylori* is the causative agent of gastritis and peptic ulcer disease, and infection with *H. pylori* is associated with the development of adenocarcinoma of the distal stomach (20, 34). About one half of the worlds' population is infected with *H. pylori*, making it a very successful pathogen. The bacterium colonizes the mucus layer overlaying the gastric epithelial cells, and in this niche it is exposed to hostile environmental conditions, caused by the acidic pH and by an active immune response (34). The extraordinary success of *H. pylori* in its hostile niche is indicative of a very effective adaptation to these conditions.

The strong immune response of the host induces oxidative stress in *H. pylori* (34). In addition, reactive oxygen species (ROS) like superoxides are also generated during bacterial respiration and metabolism (33). In view of the microaerophilic requirements of this organism and its low oxygen tolerance, it is not surprising that enzymes involved in the detoxicification of ROS are important *H. pylori* colonization factors. In *H. pylori* this is demonstrated by the inability of mutants in genes encoding components or regulators of detoxification enzymes to colonize the gastric environment in animal models (1, 3, 8, 10, 16, 17, 26, 27, 30, 41, 42).

Iron and oxidative stress defense are intimately linked, as iron potentiates the formation of toxic oxygen radicals through the Fenton and Haber-Weiss reactions (24). Therefore, the modulation of intracellular iron levels is of critical importance in oxidative stress defense (15). In many bacteria, intracellular iron levels are controlled by the ferric uptake regulator Fur, which acts as a transcriptional repressor protein that displays iron-dependent binding to conserved DNA sequences (Fur boxes) located in the promoters of iron-regulated genes (15). In *H. pylori*, Fur displays differential binding to promoters depending on the presence or absence of the iron cofactor. As in other bacteria (15), the iron-complexed form of Fur in *H. pylori* binds to promoters of iron-uptake genes, thus repressing iron-uptake in iron-replete conditions (12, 39). Uniquely, the iron-free form of *H. pylori* Fur (apo-Fur) is also able to bind promoters, as was exclusively shown for the *pfr* gene, which encodes the *H. pylori* iron-storage protein Pfr (4, 13, 40). Effectively, the binding of apo-Fur to the *pfr* promoter results in repression of ferritin expression in iron-restricted conditions (4, 40). In addition to regulation of iron metabolism, *H. pylori* Fur has been implicated in regulation of acid resistance (6) and oxidative stress resistance (3, 10, 16).

H. pylori expresses only a single superoxide dismutase (SOD), the iron-cofactored SodB protein (29, 32). Expression of SodB is essential for gastric colonization by *H. pylori* and is also required for growth under microaerophilic conditions (30). Recently, it was shown that expression of the *sodB* gene is subjected to regulation in response to varying environmental conditions, including iron (14, 19). In this study, we demonstrated that Fur mediates iron-responsive regulation of *sodB* expression in *H. pylori* by direct binding of apo-Fur to the *sodB* promoter region. To our knowledge, this is the first description of regulation of oxidative stress defense by apo-Fur.

MATERIALS AND METHODS

Bacterial strains, plasmids, media and growth conditions. *H. pylori* strain 26695 (35) and an isogenic *fur* mutant (6, 39) were routinely cultured on Columbia agar plates supplemented with 7% saponin-lysed horse blood and Dent Selective Supplement (Oxoid) at 37°C under microaerophilic conditions (10% CO_2, 5% O_2, and 85% N_2). Broth cultures were grown in Brucella Broth (Difco) supplemented with 3% Newborn or Fetal Calf

Table 1. Oligonucleotide primers used in this study

Primer name	Sequence (5' → 3') [a]
SodB-F1	GCTAAAGACAGCATGGGAGA
SodB-R-T7	ctaatacgactcactatagggagaTCCACTGATCCTAAGCCTTC
TagD-R1	CTTTATCGCCCACCTTTAAGGCT
SodB-R1	ATGGTGGAAATCAAACGCTACAG

[a)] Primer sequences were derived from the *H. pylori* 26695 genome sequence (35). Lowercase letters indicate a 5´-extension with a T7 promoter sequence for creation of an antisense RNA probe.

Serum (Life Technologies, Breda, The Netherlands) (BBN), and shaken continuously at 40 rpm. Iron-restriction was achieved by supplementing BBN with desferal (deferoxamine mesylate, Sigma) at a final concentration of 20 μM (39). Iron-replete conditions were achieved by supplementing desferal-treated BBN with ferric chloride (Sigma) at a final concentration of 100 μM (39).

Two-dimensional (2D) polyacrylamide gel electrophoresis. Cells were harvested by centrifugation. After removal of the supernatant, the pellets were washed with PBS pH 7.3, resuspended in 10 μl lysis buffer A (8 M urea, 4% [wt/vol] CHAPS, 40 mM Tris) and incubated for 45 min on a Eppendorf shaker. Then, 450 μl of reswelling solution (8 M urea, 2 M thiourea, 20 mM dithiothreitol [DTT], 1% [wt/vol] CHAPS, 0.52% Pharmalyte (pH 3-10)) was added. After vortexing, cell debris was removed by centrifugation at 18186 × g for 10 min at room temperature. The supernatant was used for the rehydration of the of IPG strips in the pH range 3-10 (Amersham Biosciences, Uppsala, Sweden). Proteins were subsequently size separated using the Investigator 2-D electrophoresis system (Genomic Solutions, Chelmsford, MA, USA) as described previously (9), fixed with 40% [vol/vol] methanol / 7% [vol/vol] acetic acid, and stained with SYPRO Ruby protein gel stain (Molecular Probes, Eugene, OR, USA). Fluorescence was detected using a Storm 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA) at a resolution of 200 μm. The 2-D gel image analysis was performed with the DECODON Delta 2-D software (http://www.decodon.com), which is based on dual channel image analysis (5). Protein spots of interest were cut out from the 2-D gel after staining. Mass spectrometry was carried out using a MALDI-TOF MS (Voyager DE-STR; PerSeptive Biosystems). Peptide mass fingerprints were analyzed by using MS-Fit software (http://prospector.ucsf.edu).

Purification and analysis of RNA. Total RNA was isolated using Trizol (Gibco) according to the manufacturer's instructions. The amount of RNA was determined spectrophotometrically. RNA electrophoresis and blotting to nylon membranes were carried out as described previously (37). Immediately after transfer, the membranes were stained with methylene blue to confirm integrity of the RNA samples and to confirm loading of equal amounts of RNA based on the relative intensities of the 16S and 23S rRNA (37). A DIG-labelled *sodB*-specific RNA probe was synthesized by in vitro transcription using T7 RNA polymerase (Roche Diagnostics) and a PCR product amplified using primers SodB-F1 and SodB-R-T7 (Table 1). Hybridization of DIG-labeled probes was visualized using the DIG detection kit (Roche Molecular Biochemicals) and the chemiluminescent substrate CDP-Star.

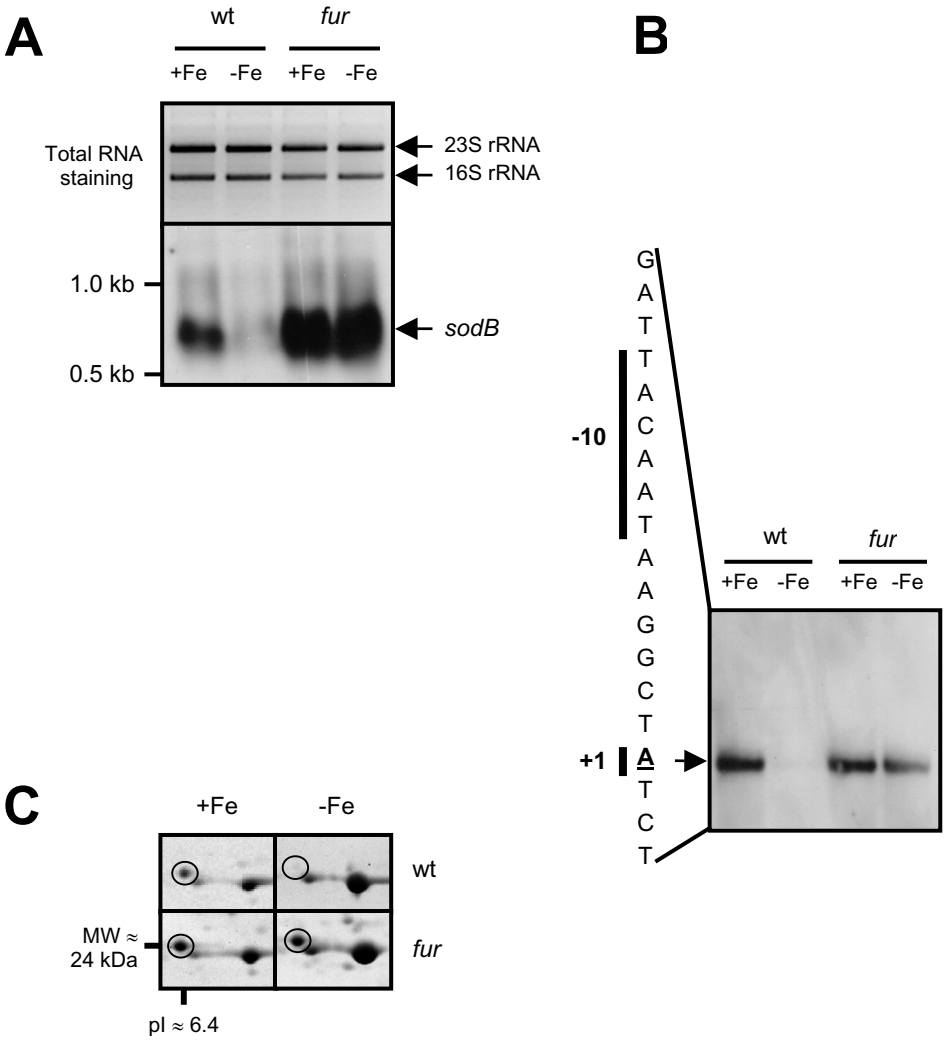

Fig. 1. Expression of the *sodB* gene in *H. pylori* strain 26695 is iron-induced and Fur-repressed. (A) Northern hybridization with a probe specific for the *sodB* gene and RNA purified from *H. pylori* wild-type strain 26695 (wt) and *fur* mutant (*fur*) cells grown in iron-restricted (-Fe) and iron-replete (+Fe) conditions. The specific mRNAs are indicated on the right, and the positions of the RNA size markers are indicated on the left. Staining of RNA by methylene blue is included for comparison of RNA amounts. (B) Identification of the *H. pylori sodB* transcription start site by primer extension analysis, using RNA purified from *H. pylori* wild-type strain 26695 (wt) and *fur* mutant (*fur*) cells grown in iron-restricted (-Fe) and iron-replete (+Fe) conditions. The sequence of the respective region is displayed on the left, with the +1 nucleotide and the -10 promoter sequence indicated. (C) Iron- and Fur-responsive regulation of SodB at the protein level. Lysates of *H. pylori* wild-type strain 26695 (wt) and *fur* mutant (*fur*) cells, grown in iron-restricted (-Fe) and iron-replete (+Fe) conditions, were separated on 2D-protein gels. Only the relevant part of the protein gels (MW 20-30 kDa, pI 6-7) is shown, and the position of the iron- and Fur-repressed SodB protein (as identified by MALDI-TOF mass spectometry) is circled.

The transcription start site of the *sodB* gene of *H. pylori* 26695 was determined using primer extension analysis (11). Briefly, approximately 5-7 µg of total RNA of *H. pylori* wild-type strain 26695 and the isogenic *fur* mutant was incubated with 50 pmol of 5'-DIG end-labeled primer SodB-R1 (Table 1) and AMV reverse transcriptase (Promega). Primer extension products were separated on an 8% polyacrylamide-8 M urea gel, blotted onto a nylon membrane (Roche), and this was followed by chemiluminescent DIG-detection.

Electrophoretic Mobility Shift Assay. Recombinant *H. pylori* Fur protein was produced in *E. coli* and purified as previously described (38, 40). The *sodB* promoter region was PCR amplified with primers TagD-R1 and DIG-labeled SodB-R1 (Table 1), which flank the 374 bp *tagD-sodB* intergenic region. Electrophoretic mobility shift assays were performed with two independent isolations of recombinant Fur protein as described previously (38). Briefly, 22 pM of *sodB* promoter DNA was mixed with recombinant Fur protein at concentrations ranging from 0 to 4500 nM. Protein and DNA were mixed in binding buffer (24% Glycerol; 40 mM Tris-Cl pH 8.0; 150 mM KCl; 2 mM DTT; 600 µg/ml BSA; 50 ng herring sperm DNA) to a final volume of 20 µl and incubated at 37°C for 30 min. As indicated below, manganese chloride ($MnCl_2$, Sigma) or EDTA was added to a final concentration of 200 µM. Samples were subsequently separated on a 5% polyacrylamide (37.5:1) gel in running buffer (25 mM Tris, 190 mM glycine) for 30 min at 200 V. The gel was then blotted onto a nylon membrane (Roche Molecular Biochemicals), and this was followed by chemiluminescent detection of DIG-labeled DNA. To calculate the binding affinity of Fur to the promoter region of *sodB*, the autoradiograph was digitalized using a Canon CanoScan 5200F scanner at 300 dots per inch and analyzed by densitometry using the Kodak 1D Image Analysis Software, version 3.5.

DNAse I footprinting. DNAse I footprinting was performed using 440 pM of DIG-labeled *tagD-sodB* intergenic region, which was mixed with 0, 2.3, 4.6, 6.9, 9.2 or 16 µM Fur protein in DNAse binding buffer (10 mM Tris-HCL, pH 8, 50 mM NaCl, 10 mM KCl, 1 mM DTT, 0.1% NP-40, 10% glycerol, 1 µg herring sperm DNA (13)). Reactions were carried out in the presence of 200 µM EDTA, and the mixtures were incubated for 30 min at 37ºC. Subsequently the DNA was digested with 0.25 U DNAseI (Promega) for 1 min, and the reaction was stopped as described previously (13). Subsequently the fragments were separated on a 7% polyacrylamide-8 M urea sequencing gel. Gels were blotted onto a nylon membrane (Roche), and this was followed by chemiluminescent DIG-detection (39).

RESULTS

Iron-responsive expression of SodB is mediated at the transcriptional level. Using DNA array-based transcriptional profiling, the *sodB* gene was recently identified as a member of a regulon comprising 15 *H. pylori* genes, which display Fur-dependent, iron-induced transcription (14), similar to the previously described regulation of the *pfr* gene (4, 13). This regulatory pattern was further characterized for the *sodB* gene using Northern hybridization with RNA purified from *H. pylori* wild-type strain 26695 and its isogenic *fur* mutant, both grown in iron-restricted and iron-replete conditions (Fig. 1A). The *sodB*-specific probe hybridized to a single approximately 0.7 kb monocistronic mRNA (29).

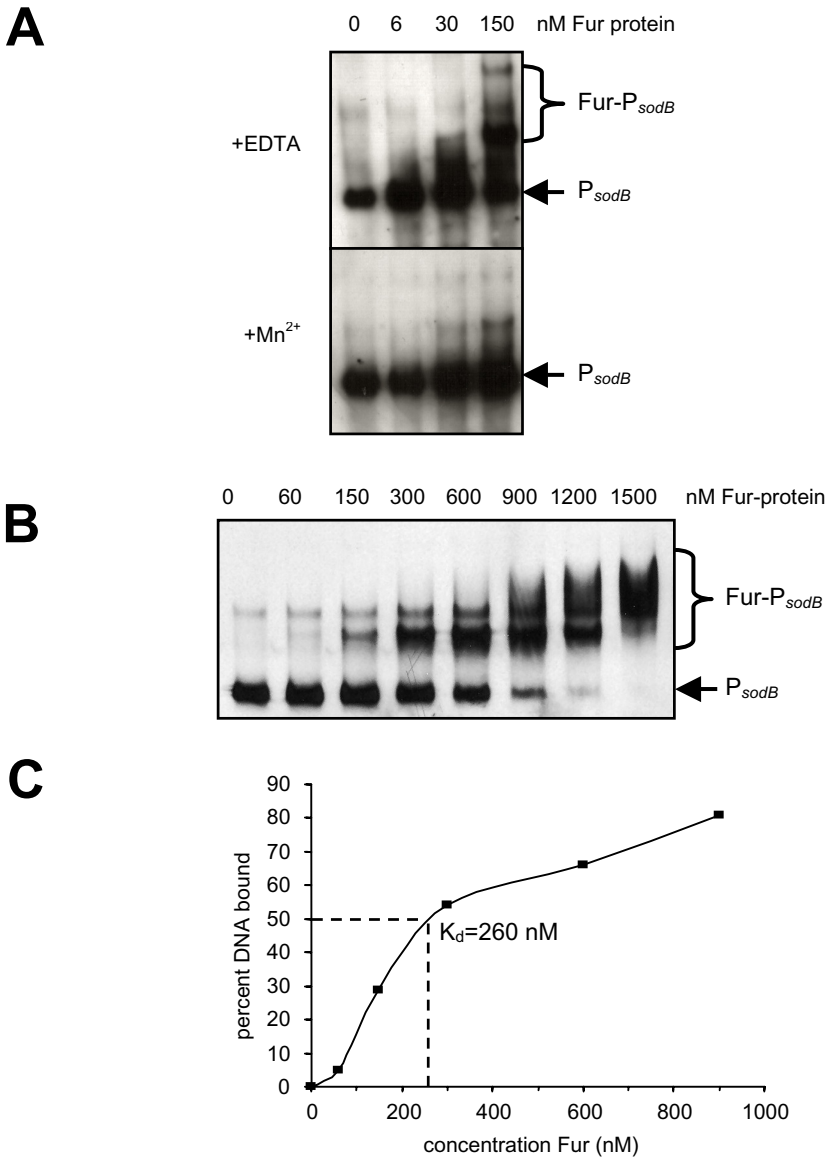

Fig. 2. Regulation of *sodB* transcription is mediated by direct binding of the *H. pylori* Fur protein to the *sodB* promoter. (A) Electrophoretic mobility shift assay using recombinant *H. pylori* Fur protein and DIG-labeled *sodB* promoter DNA (P_{sodB}) isolated from *H. pylori* strain 26695. In the presence of the iron-substitution manganese (+Mn; bottom panel), Fur is unable to complex with the *sodB* promoter region, and a shift is not observed. Only in the absence of manganese (+EDTA, top panel), apo-Fur is able to bind to the *sodB* promoter and cause a mobility shift (indicated as Fur-P_{sodB} complex). The concentration of Fur in nM is indicated above the lanes, the concentration of DNA was 22 pM. (B) Determination of the affinity of apo-Fur for the *sodB* promoter sequence. 22 pM of DIG-labeled P_{sodB} was mixed with increasing concentrations of Fur, with EDTA in the buffer. (C) Graphical representation of the determination of the K_d of purified *H. pylori* Fur for the *sodB* promoter, using the data presented in Fig. 2B. The relative amounts of P_{sodB} and Fur-P_{sodB} were quantified by densitometry.

In the wild-type strain, this mRNA was detected after growth in iron-replete conditions (+Fe), and not after growth in iron-restricted conditions (-Fe), whereas the *sodB* mRNA was present in the *fur* mutant independent of iron availability (Fig. 1A) at levels even higher than those of the wild-type strain.

The transcription start site of the *sodB* gene in *H. pylori* strain 26695 was identified using primer extension analysis (Fig. 1B). There was only a single primer extension product, located at the A residue 21 basepairs upstream of the ATG startcodon, and this matched the transcription start reported previously for *H. pylori* strain 60190 (29), despite the presence of three sequence differences in the *sodB* promoter sequences of both strains. In accordance with the Northern hybridization data (Fig. 1A), the primer extension product could be detected in the wild-type strain only after growth in iron-replete conditions, whereas it was constitutively present in the *fur* mutant (Fig. 1B).

Iron- and Fur-responsive regulation of the *sodB* gene was confirmed at the protein level by two-dimensional protein gel electrophoresis (Fig. 1C). The SodB protein migrated according to its predicted molecular mass of ~24 kDa and pI of ~6.4 (18, 31), and was positively identified using MALDI-TOF mass spectometry. The SodB protein was expressed in the wild-type strain only after growth in iron-replete conditions, and it was absent in iron-restricted conditions. Conversely, in the *fur* mutant strain the SodB protein was expressed independent of iron availability (Fig. 1C).

The Fur repressor mediates direct regulation of the *H. pylori sodB* gene. To investigate whether the iron-responsive regulation of *sodB* expression is directly or indirectly mediated by Fur, we performed an electrophoretic mobility shift assay (EMSA) with the *sodB* promoter region, using recombinant *H. pylori* Fur protein. Manganese was used as a substitute for the iron cofactor, as it is more stable under the assay conditions, and has been shown to function like iron under *in vitro* binding conditions (15). In the absence of manganese, Fur caused a shift of mobility of the *sodB* promoter (Fig. 2A). Addition of manganese to the binding reaction abolished the mobility shift (Fig. 2A), indicating that only the metal-free form of Fur (apo-Fur) is able to interact with the *sodB* promoter, which is consistent with the transcriptional regulatory pattern. The affinity of *H. pylori* apo-Fur for the *sodB* promoter region was low, and the K_d value was ~260 nM at a DNA concentration of 22 pM (Fig. 2B and 2C).

Fur binds to an operator sequence located at positions -5 to -47 in the *sodB* promoter. The Fur binding sequence in the *sodB* promoter region was localized using DNAse I footprinting (Fig. 3A). Addition of apo-Fur led to the protection of two regions in the *sodB* promoter, which were separated by a DNAse I hypersensitivity site located at position -24 (Fig. 3A). The two protected regions span the area from nucleotides –5 to nucleotice –47 relative to the transcription start site, overlapping the –10 and –35 region of the *sodB* gene (Fig. 3B). While the regulatory pattern of *sodB* regulation resembles that of the *pfr* gene, the apo-Fur binding site in the *sodB* promoter exhibits only poor sequence homology (Fig. 3C) with the two high-affinity binding sites present in the *pfr* promoter region (13), which may explain the relatively low affinity of apo-Fur for the *sodB* promoter.

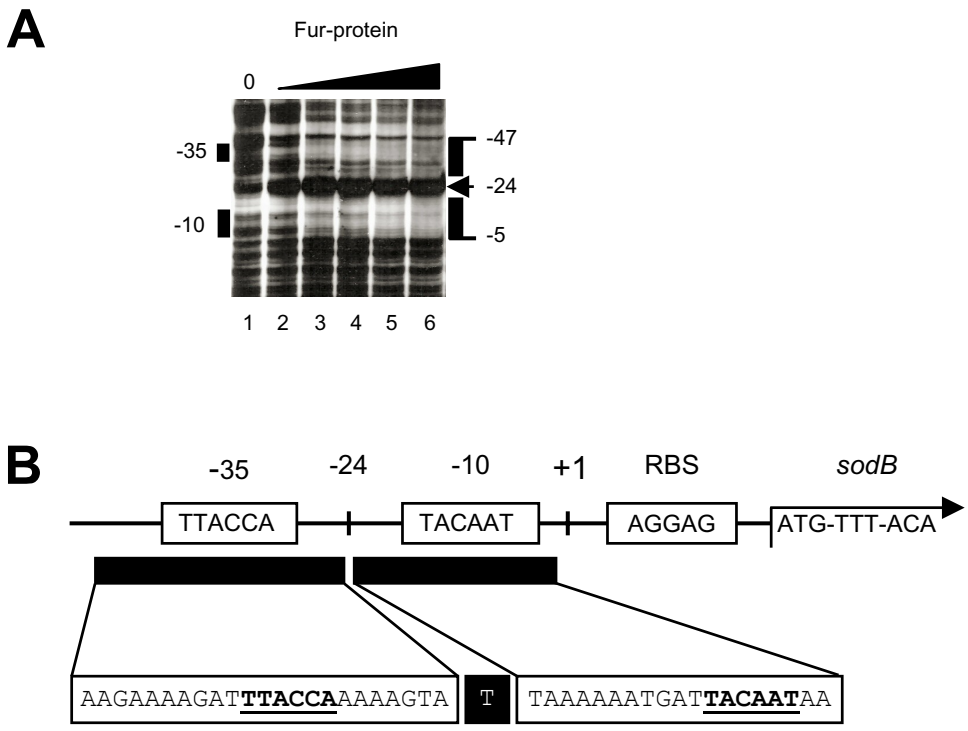

Fig. 3. Identification of the operator sequence for apo-Fur in the *H. pylori* 26695 *sodB* promoter. (A) DNAse I footprinting assay using 440 pM DIG-labeled *H. pylori* 26695 *sodB* promoter DNA and increasing concentrations apo-Fur. The positions of the -10 and -35 promoter sequences located in the *sodB* promoter are indicated on the left side, and the positions of the two protected regions (located at positions -5 to -23 and -25 to -47) and the DNAse I hypersensitive site (at position -24) are indicated on the right. The concentrations of Fur protein used in lanes 1 to 6 were 0, 2.3, 4.6, 6.9, 9.2 and 16 μM, respectively. (B) Graphic representation of the *sodB* promoter with the location and sequence of the apo-Fur binding site indicated. The DNAse I hypersensitivity residue is indicated between the two binding sites by a black background. The -10 and -35 promoter sequences are underlined in the binding sequence. (C) Alignment of the proposed binding sequence for apo-Fur in the *H. pylori* 26695 *sodB* promoter with the high affinity binding sequences in the *H. pylori pfr* promoter (*pfr* boxes I and II) (13). Residues in the *sodB* sequence identical in both *pfr* binding sequences are indicated by a black background, and residues identical to residues in only one of the *pfr* binding sites (13) are indicated by a grey background.

DISCUSSION

The versatility of iron in redox reactions has resulted in the extensive biological use of iron as a cofactor of enzymes, but iron also potentiates the formation of reactive oxygen species like superoxides. Therefore, cells contain mechanisms for protection against iron-associated oxidative stress, and these mechanisms include detoxifying enzymes like catalases and superoxide dismutases, as well as proteins which carefully control cytoplasmic iron homeostasis by balancing the availability of free iron through control of iron acquisition and storage. As the central regulator of iron homeostasis in many bacteria, the Fur repressor often acts both directly and indirectly as regulator of oxidative stress defense (2, 15, 33).

In *H. pylori*, Fur mediates iron-dependent repression of iron-uptake systems leading to expression of iron uptake proteins, only when iron is required (12, 39). Conversely, Fur also mediates repression of iron storage systems in iron-restricted conditions by repression of ferritin expression (4, 13). The switch between repression and induction of iron-uptake is coupled to the iron-availability in the cytoplasm; when iron is available, a Fur dimer forms a complex with ferrous iron, and binds to Fur binding sequences (Fur boxes) in the promoters of iron-uptake genes (15). However, the situation is not as clear for the switch in repression and induction of ferritin-mediated iron-storage; while iron-induction of ferritin expression is found in several bacteria, the role of Fur in this process is not universal. Since *H. pylori* colonizes the gastric mucosa, it is thought to encounter both severe iron restriction by lactoferrin and also periods of iron overload after release of iron from food sources (23). Thus, the ability to regulate genes in response to iron restriction and iron overload is an important feature thought to allow chronic colonization of the gastric niche.

SOD catalyses the dismutation of O_2^- to H_2O_2, which is subsequently removed by catalase. *H. pylori* expresses a single SOD (SodB), which is cofactored by iron (29, 30, 32), and is essential for gastric colonization by *H. pylori* in an animal model (30). An absence of SodB leads to cessation of growth in microaerobic conditions, and an increase in DNA mutation rate, probably caused by oxygen radicals formed by iron via the Haber-Weiss and Fenton reactions (30). In *E. coli*, two cytoplasmic SOD species are present: the manganese-cofactored SodA protein and the iron-cofactored SodB protein. Expression of both the *sodA* and *sodB* genes is regulated by iron: in iron-restricted conditions, only the SodA protein is expressed, whereas the SodB protein is expressed in iron-replete conditions (25). Iron-responsive repression of the *sodA* gene is mediated directly by Fur, while iron-responsive induction of the *sodB* gene is indirectly affected by Fur via the RyhB small RNA (22). Transcription of the RyhB small RNA is Fur-dependent, and once transcribed, RyhB can bind to complementary sequences in the 5'-end of the *sodB* mRNA, blocking translation and making the mRNA unstable (21, 22).

Regulation of oxidative stress defense in *H. pylori* has not been studied in detail, but recent studies indicated that expression of antioxidant genes is controlled by transcriptional regulation through an intricate regulatory network (3, 10, 16, 19, 40). This is exemplified by compensatory increase of expression of the antioxidant protein NapA upon mutation of the antioxidant enzyme alkyl hydroperoxide reductase (AhpC) (26). In contrast to *E. coli*, regulation by small RNAs has not been described for *H. pylori*, but it could also not explain the regulation of the *H. pylori* ferritin gene *pfr* (4, 13) or of the *sodB* gene (this study). As in *E. coli*, expression of both Pfr and SodB is iron-induced, but

in contrast to *E. coli*, the mRNA levels of *pfr* and *sodB* are constitutively high in the *fur* mutant (Fig. 1A) (4). This expression pattern of SodB suggested a direct role of apo-Fur in regulation of the *sodB* gene, as was previously described for the *pfr* gene (13, 40).

Direct and sequence-specific binding of *H. pylori* apo-Fur to the *H. pylori sodB* promoter region was confirmed using EMSA and DNAse I footprinting assays (Fig. 2 and 3). Apo-Fur bound to the region overlapping with the -10 and -35 promoter sequences present in the *sodB* promoter (Fig. 3A and B). The affinity of *H. pylori* apo-Fur for the *sodB* promoter was suprisingly low (K_d ~260 nM), as compared to the affinity of metal-cofactored *H. pylori* Fur for the *amiE* promoter (~10 nM, calculated from (38)). This low affinity may have biological significance. Genes involved in iron metabolism need to be tightly regulated to prevent iron surplus in the cell, and therefore creation of Haber-Weiss and Fenton reactions. In contrast, the SodB protein is the only defense of *H. pylori* to superoxide stress (30), and thus its expression should not be repressed, unless *H. pylori* encounters such severe iron restriction that even activating SodB enzyme is not feasible anymore. Low-affinity binding of Fur to the *sodB* promoter is one way of achieving such a regulation.

The DNAse I footprinting pattern obtained for the *sodB* promoter resembled those identified in the *pfr* promoter, as there was a DNAse I hypersensitivity site between the two protected regions (Fig. 3). However, the operator sequence present in the *sodB* gene displayed only very limited sequence homology to those identified in the *pfr* promoter. The limited availability of binding sequences of apo-Fur currently precludes the definition of a consensus sequence. This could be resolved by studying additional promoters, which are regulated by apo-Fur, and several candidate promoters have been recently described (14), including the *hydABC* operon encoding the iron- and nickel-cofactored hydrogenase enzyme (28).

Despite its small genome, *H. pylori* is a highly successful colonizer of the human gastric mucosa, persisting lifelong unless eradicated by antibiotic treatment (7). The Fur protein, which is well known for its central role in iron homeostasis in bacteria, affects the expression of different pathways involved in normal metabolism, stress resistance, motility and virulence (4, 8, 13, 36, 38, 39). In our study, we expanded the role of Fur in one of these aspects, the regulation of oxidative (superoxide) stress defense. Fur directly represses the expression of SodB when the iron cofactor is not available, thus not wasting valuable cellular resources. When iron is available, repression is terminated, allowing expression of iron-cofactored SodB in conditions, where the risk of formation of reactive oxygen species is high. This direct role of Fur contrasts with the indirect Fur-mediated regulation of iron-cofactored SOD in *E. coli* (22), and highlights the special aspects of *H. pylori* Fur, as compared to other eubacterial Fur proteins. In conclusion, this is the first description of a *sodB* gene that is directly regulated by apo-Fur, and thus the mechanism described here is a novel mechanism for regulation of expression of Fe-containing superoxide dismutases in prokaryotes.

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CHAPTER **5**

The nickel-responsive regulator NikR controls activation and repression of gene transcription in *Helicobacter pylori*

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The NikR protein is a nickel-dependent regulatory protein, which is a member of the ribbon-helix-helix family of transcriptional regulators. The gastric pathogen *Helicobacter pylori* expresses a NikR ortholog, which was previously shown to mediate regulation of metal metabolism and urease expression, but the mechanism governing the diverse regulatory effects had not been described until now. In this study it is demonstrated that NikR can regulate *H. pylori* nickel metabolism, by directly controlling transcriptional repression of NixA-mediated nickel-uptake and transcriptional induction of urease expression. Mutation of the nickel-uptake gene *nixA* in a *H. pylori* 26695 *nikR* mutant restored the ability to grow in Brucella media supplemented with 200 µM $NiCl_2$, but did not restore nickel-dependent induction of urease expression. Nickel-dependent binding of NikR to the promoter of the *nixA* gene resulted in nickel-repressed transcription, whereas nickel-dependent binding of NikR to the promoter of the *ureA* gene resulted in nickel-induced transcription. Subsequent analysis of NikR-binding to the *nixA* and *ureA* promoters showed that the regulatory effect was dependent on the location of the NikR-recognized binding sequence. NikR recognized the -13 to +21 region of the *nixA* promoter, encompassing the +1 and –10 region, and this binding resulted in repression of *nixA* transcription. In contrast, NikR bound to the -56 to -91 region upstream of the *ureA* promoter, resulting in induction of urease transcription. In conclusion, the NikR protein is able to function both as repressor and activator of gene transcription, depending on the position of the binding site.

The human gastric pathogen *Helicobacter pylori* colonizes the mucus layer covering the gastric epithelium. To colonize its acidic niche, *H. pylori* requires the activity of the nickel-containing urease and hydrogenase enzymes (21, 30), and thus it requires efficient acquisition of nickel from the environment. The main route for nickel uptake in *H. pylori* is via the NixA protein, which is a monomeric, high-affinity nickel transporter located in the cytoplasmic membrane (4, 24, 25, 27, 47). Expression of NixA is also required for efficient colonization of the gastric mucosa (29). Hence, the uptake and metabolism of nickel are of critical importance to *H. pylori*. When cytoplasmic nickel availability is insufficient, the urease and hydrogenase systems cannot be fully activated (39). This will impair survival of acid shocks, growth at acidic pH and colonization of the gastric mucosa (3, 10). However, high concentrations of nickel are also detrimental to the cell (28, 42). Nickel metabolism thus requires tight control to maintain cytoplasmic nickel concentrations within tolerable levels, by regulation of uptake, efflux, usage and storage (28). Adaptation to such changes in the conditions inside or outside the bacterial cytoplasm is often achieved through transcriptional regulation of effector genes.

The nickel-responsive regulatory protein NikR is a member of the ribbon-helix-helix (RHH) family of DNA binding proteins (12). The NikR protein consists of two different domains, an N-terminal DNA-binding domain homologous to the Arc/CopG/MetJ/Mnt family of RHH regulators, and a C-terminal domain that is required for binding of nickel and for tetramerization (8, 11-14, 35, 46). NikR was first identified in *Escherichia coli*, where it functions as transcriptional repressor of the Nik nickel-uptake system (18). NikR mediates its repressor function via nickel-dependent binding to a palindromic sequence in the promoter region of the *nik* operon (12, 14). The net result of this regulation is expression of the Nik system only when nickel is scarce in the cell (18).

NikR orthologs have been identified in other Gram-negative bacteria, including *H. pylori* (15, 42). In *H. pylori*, NikR mediates nickel- and acid-responsive gene regulation (10, 15, 40-42) and is predicted to affect different pathways involved in metal metabolism (15, 39). NikR has been suggested to function as the main nickel-responsive regulatory system in *H. pylori*, since absence of NikR results both in reduced growth at higher environmental nickel-concentrations, and the absence of nickel- and acid-responsive induction of urease expression (10, 15, 40, 42). However, these functions of NikR have mostly been demonstrated using *H. pylori* mutant strains (10, 15, 40, 42), while evidence of direct regulation by NikR was not presented.

Here, it is demonstrated that *H. pylori* NikR directly binds to specific sequences in the *nixA* and *ureA* promoters in a nickel-dependent fashion. This nickel-dependent binding of NikR to the *nixA* and *ureA* promoters results in repression and induction of transcription, respectively. The sequences recognized by *H. pylori* NikR are significantly different from the consensus sequence proposed for recognition by *E. coli* NikR. Based on these results, we hypothesize that the location of operator sequence in the promoter region determines, whether NikR represses or induces transcription in *H. pylori*.

MATERIALS AND METHODS

Bacterial strains, plasmids, media and growth conditions. *H. pylori* strains used in this study were reference strain 26695 (38), its isogenic *nikR*::Km^R mutant (42) and an isogenic *nikR*::Km^R *nixA*::Cm^R mutant constructed for this study (see below). *H. pylori* was routinely cultured on Dent agar (41) at 37°C under microaerobic conditions (10%

CO_2, 5% O_2 and 85% N_2). Broth cultures were grown in Brucella Broth (Difco, Sparks, MD) supplemented with 3% Newborn Calf Serum (Gibco Life technologies) (BBN). Cultures were started at an OD_{600} of 0.05 and shaken at 37°C with 40 rpm for a maximum of 24h. BBN medium, as used in this study, contains ~0.2 µM of Ni^{2+} (6). $NiCl_2$ (Sigma) was used to supplement BBN medium to final concentrations of 20 and 200 µM. *E. coli* strains were grown aerobically at 37°C in Luria-Bertani medium (34). When appropriate, BBN and Luria-Bertani media were supplemented with ampicillin, kanamycin, or chloramphenicol to a final concentration of 100 µg/ml, 20 µg/ml or 10 µg/ml, respectively.

Urease assay. The enzymatic activity of urease was determined in fresh *H. pylori* lysates by measuring ammonia production from hydrolysis of urea by using the Berthelot reaction, as described previously (41). The concentration of ammonia in the samples was inferred from a standard NH_4Cl concentration curve. Enzyme activity was expressed as micromoles of substrate hydrolyzed per minute, per milligram of protein. Protein concentrations were determined with the bicinchoninic acid method (Pierce) using bovine serum albumin as standard.

Cloning, expression, and purification of *H. pylori* NikR. The *nikR* gene was amplified from *H. pylori* 26695 using the primers NIKRSK7-L1 and NIKRSK7-R1 (Table 1). The resulting fragment was digested with *Bsa*I and ligated into *Bsa*I-digested pASK-IBA7 (IBA, Göttingen, Germany) to create pASK-IBA7-NikR. The wild-type sequence of the *nikR* gene was confirmed by DNA sequencing. *H. pylori* NikR was expressed with an N-terminal Streptag, which does not influence DNA-binding activity of the *H. pylori* Fur protein (23, 43, 45) and therefore was not removed prior to use. The recombinant protein was purified as described in the manufacturer's instructions (45), and designated Strep-NikR. The recombinant protein was over 90% pure, as determined by staining with Coomassie Brilliant Blue, following electrophoresis on 12% SDS-polyacrylamide gels (14). Purified protein was used directly for electrophoretic mobility shift and DNase I footprinting assays.

Construction of a *nikR-nixA* double mutant. The region containing the *nixA* gene was amplified by using the primers NixA-F2-mut and NixA-R2-mut (Table 1). The resulting 936 bp *nixA* PCR fragment was cloned into pGEMTeasy (Promega). The *nixA* coding region of this plasmid was interrupted by insertion of the chloramphenicol resistance gene from pAV35 (44) in the unique *Bgl*II site, resulting in plasmid pAHJNcat. Plasmid DNA was prepared using Wizard spincolumns (Promega) and used for natural transformation (7) of the *H. pylori* 26695 *nikR* mutant. Correct replacement of the *nixA* gene by the interrupted copy was confirmed using PCR (not shown).

Purification and analysis of RNA. Total RNA was isolated from *H. pylori* 26695 and its isogenic *nikR* mutant using Trizol (Gibco Life Technologies) (41). 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 (22, 41). Probes specific for *nixA* and *ureI* were synthesized by *in vitro* transcription using T7 RNA polymerase (Roche) and PCR products obtained with primers NixA-F1/NixA-R1-T7 and UreI-F2/UreI-R2-T7 (Table 1).

Electrophoretic mobility shift assays. The ureA-DFP-F and ureA-DFP-R-Dig primers (Table 1) were used to amplify a 430 bp fragment from plasmid pBJD3.3 (17), which contains the wild-type *ureA* promoter region from *H. pylori* strain 1061 (designated P*ureA*). These primers were also used to amplify a 390 bp fragment from plasmid pBJD

3.9 (17, 42), where the region encompassing nucleotides -50 to -90 is deleted from the *ureA* promoter (17, 42) (designated P*ureA*-del). The 514 bp *nixA* promoter region fragment (designated P*nixA*) was amplified with primers NixA-DFP-F and NixA-DFP-R-Dig (Table 1). An internal fragment of the *H. pylori amiE* gene was amplified with primers int-amiE-F1 and int-amiE-R1-Dig (Table 1), and was used as a negative control. Electrophoretic mobility shift assay was performed using 18, 20 and 16 pM of P*ureA*-wt, P*ureA*-del and P*nixA* promoter fragments, as well as with 43 pM of the negative control.

Table 1. Oligonucleotide primers used in this study

Primer	Sequence (5' → 3')
NixA-F2-mut	GCTGTGAAATTGTGGTTTCC
NixA-R2-mut	CAAATAAGCCCACCAAGTAA
NixA-F1	GATCGCTTGGGCTAAAGAAC
NixA-R1-T7[a]	ctaatacgactcactatagggagaCGATTTCACTAGCGGTATCA
UreI-F2	AAGCACTGCGGTGATGAACT
UreI-R2-T7[a]	ctaatacgactcactatagggagaACCAATCGCCTTCAGTGATG
NIKRSK7-L1	ATGGTAGGTCTCAGCGCATGGATACACCCAATAAAGACGATT
NIKRSK7-R1	ATGGTAGGTCTCATATCATTCATTGTATTCAAAGCTAGACGCC
UreA-DFP-F	GTGGGCGTTTTATTGTTGAA
UreA-DFP-R-Dig[b]	ACTCTTTTGGGGTGAGTTTC
NixA-DFP-F	TGATGGCGATTTAGAAACCC
NixA-DFP-R-Dig[b]	GAGCAACGCTAAACCCAATG
Int-amiE-F1	ACTCATTGTGCGCTGTCAAG
Int-amiE-R1-Dig[b]	CCCGCATTCGCCCAAAGTAT

[a] Lowercase letters indicate a 5´-extension with T7 promoter sequence for creation of an antisense RNA probe.
[b] primer is digoxigenin labelled at the 5' end.

DNA fragments were mixed with Strep-NikR protein to final concentrations of 0, 15, 30, 150 and 300 nM in binding buffer (consisting of 20 mM Tris (pH 7.6), 100 mM KCl, 3 mM $MgCl_2$, 0.1% Nonidet P-40, 5% glycerol and 100 μM of $NiCl_2$) and incubated for 30 min at 37°C. Subsequently, samples were loaded on nickel-containing 7% acrylamide gels (34). Gels were blotted onto a nylon membrane (Roche), followed by chemiluminescent DIG-detection (41).

DNase I footprinting. DNase I footprinting was performed using 360, 400 and 320 pM of P*ureA*, P*ureA*-del and P*nixA* fragment, respectively. DNA fragments were incubated without or with 2.86 μM of Strep-NikR protein in the presence or absence of 100 μM $NiCl_2$ in binding buffer (10 mM HEPES (pH 7.6), 100 mM KCl, 3 mM $MgCl_2$ and 1.5 mM $CaCl_2$) for 30 min at 37 °C. Subsequently, the DNA was digested with 0.25 U DNaseI (Promega) for 1 min, and the reaction was stopped, as described previously (20). Fragments were separated on a 7% acrylamide-8 M urea sequencing gel (Bio-Rad) (34). Gels were blotted onto a positively charged nylon membrane (Roche), followed by chemiluminescent DIG-detection (41).

RESULTS

Absence of NixA complements nickel sensitivity but does not restore urease regulation in a *H. pylori nikR* mutant. The main phenotypes of a *H. pylori* 26695 *nikR* mutant are reduced growth in BBN medium supplemented with $NiCl_2$ concentrations >100 µM, and absence of nickel-responsive induction of urease expression (42). To examine the role of the NixA nickel-transporter in these phenotypes, the *nixA* gene was interrupted in a *H. pylori* 26695 *nikR* mutant (42), thereby creating a *nikR-nixA* double mutant. The growth of wild-type *H. pylori*, *nikR* mutant and the *nikR-nixA* mutant did not

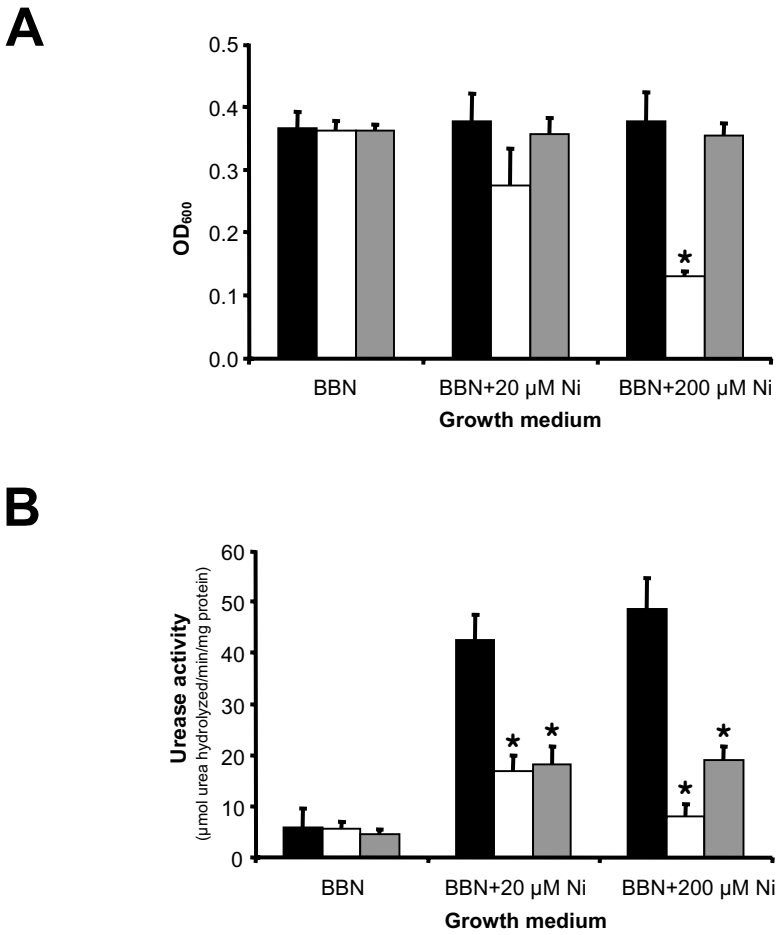

Fig. 1. Mutation of *nixA* complements nickel-sensitivity of an *H. pylori nikR* mutant, but does not restore nickel-dependent induction of urease activity. A) Growth of *H. pylori* 26695 wild-type (black bars), *nikR* mutant (white bars) and *nikR-nixA* double mutant (grey bars) in BBN medium supplemented with 0, 20 and 200 µM $NiCl_2$. Results show the average of three independent growth experiments after measuring the OD_{600} 24h after inocculation. B**)** Urease activity measurements of *H. pylori* 26695 wild-type (black bars), *nikR* mutant (white bars) and *nikR-nixA* double mutant (grey bars) grown in BBN medium supplemented with 0, 20 and 200 µM $NiCl_2$. Results show the average of three independent urease activity measurements. The error bars denote standard deviations, the asterisks indicate a significant difference in growth (panel A) or urease activity (panel B) of the mutants, as compared to the wild-type strain ($P < 0.05$, Mann-Whitney U test).

differ in unsupplemented BBN medium or in BBN medium supplemented with 20 µM $NiCl_2$ (Fig. 1A). Consistent with our earlier data (42), growth of the *nikR* mutant was significantly decreased, when BBN was supplemented with $NiCl_2$ to a final concentration of 200 µM (Fig. 1A). The decrease in growth of the *nikR* mutant after supplementation with $NiCl_2$ at concentrations of 40 µM or higher was accompanied by a significant decrease in viability (data not shown). In contrast, the *nikR-nixA* double mutant grew to similar levels to those of the wild-type strain at the non-permissive $NiCl_2$ concentration of 200 µM (Fig. 1A).

Mutation of *nixA* in the *nikR* mutant did not, however, restore nickel-responsive regulation of urease activity (Fig. 1B). Urease activity was relatively low in unsupplemented medium in all three strains (Fig. 1B). In medium supplemented with 20 and 200 µM $NiCl_2$, the wild-type strain displayed an increase in urease activity, which was not apparent in either the *nikR* mutant or the *nikR-nixA* double mutant. Urease activity even decreased in the *nikR* mutant, when grown in medium supplemented with 200 µM $NiCl_2$ (Fig. 1B), but this coincided with decreased growth of this strain at these conditions (Fig. 1A).

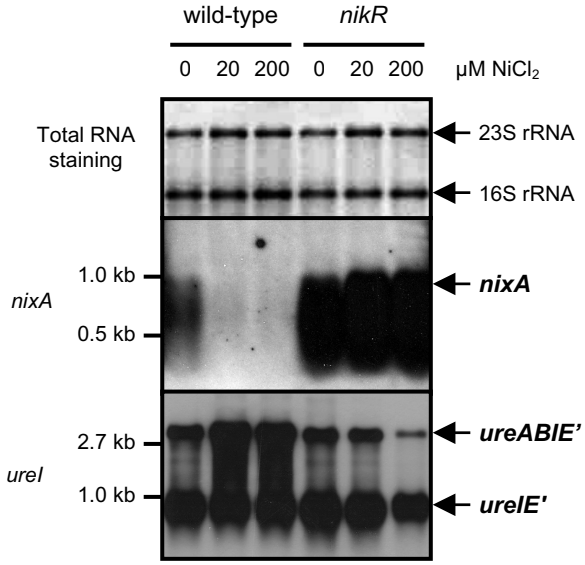

Fig. 2. Transcription of *nixA* is nickel- and NikR-repressed, whereas transcription of *ureA* is nickel-and NikR-induced in *H. pylori*. Northern hybridization of RNA from *H. pylori* 26695 wild-type and *nikR* mutant cells grown in BBN medium supplemented with 0, 20 and 200 µM $NiCl_2$. Staining of transferred RNA by methylene blue is included for comparison of RNA amounts (top panel). The position of the predicted *nixA*, *ureABIE'* and *ureIE'* transcripts (1, 41) is indicated on the right side, whereas probes used and relevant marker sizes are included on the left side.

Transcription of the *nixA* and *ureA* genes is regulated by NikR. Northern hybridization with probes specific for the *nixA* and *ureI* genes was used to assess whether transcription of *nixA* and the urease operon is regulated by nickel and NikR. RNA was isolated from cultures grown in BBN medium supplemented with 0, 20 or 200 µM $NiCl_2$ (Fig. 1). The *nixA* probe hybridized to a transcript with a size of approximately 1 kb in

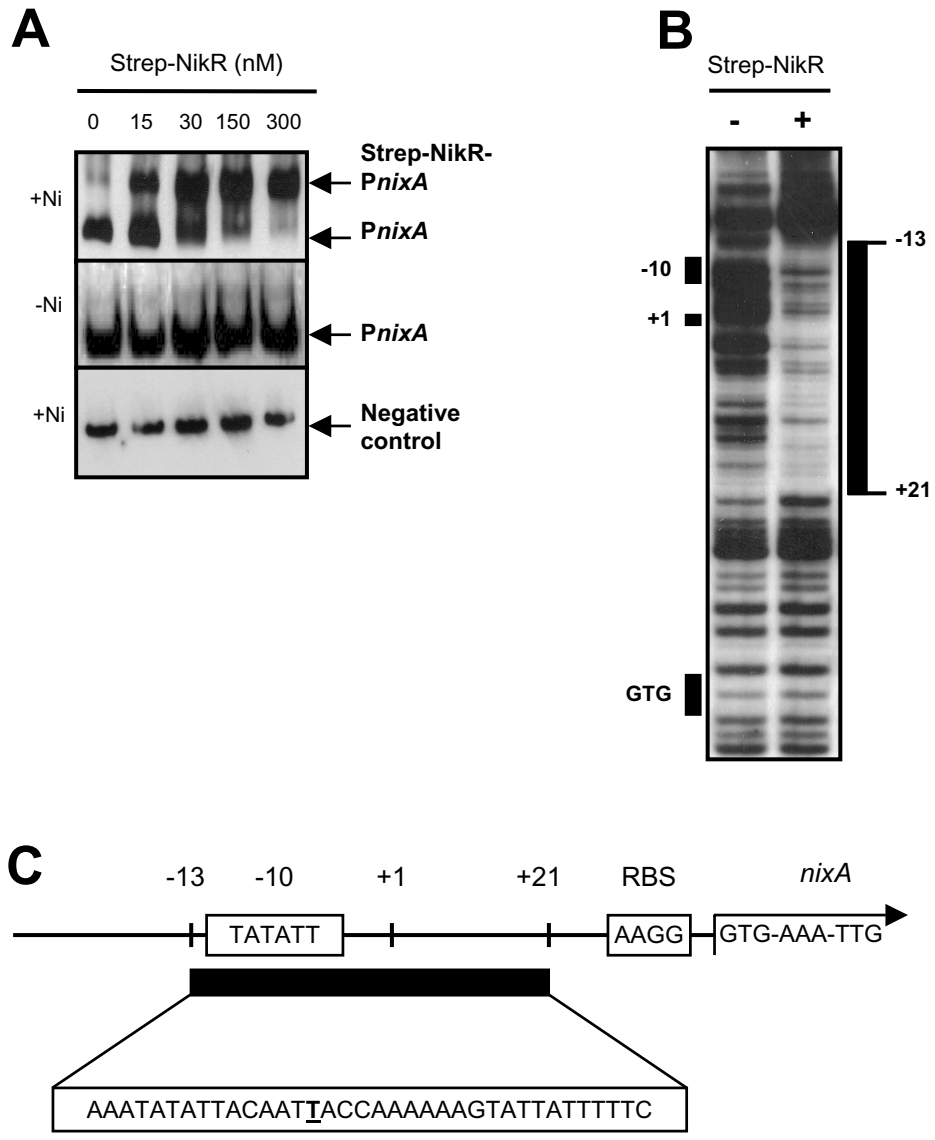

Fig. 3. NikR directly represses *nixA* transcription by nickel-dependent direct binding to a specific operator in the *H. pylori nixA* promoter region. A) Electrophoretic mobility shift assays with recombinant *H. pylori* Strep-NikR protein and the *nixA*-promoter (P*nixA*) in the presence (+Ni) and absence (-Ni) of $NiCl_2$. An internal fragment of the *amiE* gene was used as negative control only in the presence of $NiCl_2$. The Strep-NikR-complexed *nixA* fragment is indicated as Strep-NikR-P*nixA*. Strep-NikR concentrations used (in nM) are indicated above the lanes; the DNA concentration was 16 pM (P*nixA*) and 43 pM (negative control). B) Identification of the NikR operator sequence in the *nixA* promoter by DNAse I footprinting in the absence (-) and presence (+) of Strep-NikR protein, in the presence of nickel. The protected region is indicated by a black bar on the right side, while the positions of the GTG startcodon, the +1 transcriptional start site and the -10 promoter region are indicated on the left hand side. C) Schematic representation of the *nixA* promoter region with the location and sequence of the NikR binding site indicated, whereby -13 and +21 indicate the boundaries of the NikR binding site. The *nixA* transcriptional start site (16) is underlined in the binding sequence.

RNA, isolated from wild-type *H. pylori* grown in unsupplemented medium, but was not detected in RNA isolated from wild-type *H. pylori* grown in medium supplemented with 20 and 200 μM $NiCl_2$ (Fig. 2, middle panel). In contrast, in the *nikR* mutant, transcription of the *nixA* gene was constitutively high and independent of $NiCl_2$ supplementation (Fig. 2, middle panel). The size of the *nixA* mRNA is consistent with monocistronic transcription of *nixA*. The *ureI* specific probe hybridized to 2 fragments, which are predicted to represent the constitutively transcribed *ureIE'* mRNA (0.9 kb) and the nickel-responsive *ureABIE'* mRNA (3.4 kb) (1, 41). In wild-type *H. pylori* 26695, the amount of the 3.4 kb *ureABIE'* mRNA increased upon nickel supplementation, as compared to unsupplemented medium. In contrast, in the *nikR* mutant nickel-responsive induction of the *ureABIE'* mRNA was abolished (Fig. 2, bottom panel). Taken together, these findings suggest, that *H. pylori* NikR acts as a nickel-dependent repressor of *nixA* transcription and as a nickel-dependent activator of urease transcription.

NikR mediates repression of *nixA* transcription by nickel-dependent binding to the *nixA* promoter. A 514 bp fragment containing the *nixA* promoter region was amplified by PCR and incubated with Strep-NikR in the presence or absence of nickel (Fig. 3A). In the absence of nickel, addition of Strep-NikR protein did not result in an electrophoretic mobility shift (Fig. 3A). When nickel was present in the binding buffer, addition of the Strep-NikR protein resulted in an electrophoretic mobility shift (Fig. 3A). An internal fragment of the *H. pylori amiE* gene was used as a negative control, and did not display any shift in the presence of nickel and Strep-NikR (Fig. 3A).

The location of the binding sequence for NikR in the *nixA* promoter was identified using DNase I footprinting assay (Fig. 3B). In the presence of nickel, Strep-NikR protein blocked DNase I degradation of a single sequence (AAATATATTACAATTACCAAAAAAGTATTATTTTTC). Since the transcription start site of the *nixA* mRNA is the T residue 36 bp upstream of the GTG startcodon (16), this sequence is located from -13 to +21 relative to this transcription start site (Fig. 3C). The protected region includes the transcriptional start site and the putative -10 promoter region (Fig. 3C). The -13 to +21 region was not protected against DNAse I degradation by Strep-NikR in the absence of nickel (not shown).

NikR induces urease transcription by binding to an upstream operator sequence of *ureA*. A 430 bp fragment was amplified containing the wild-type *H. pylori ureA* promoter region (P*ureA*). In the presence of nickel, addition of Strep-NikR to P*ureA* resulted in an electrophoretic mobility shift, which was missing in the absence of nickel (Fig. 4A). Using DNase I footprinting assay, it was demonstrated that in the presence of nickel, Strep-NikR protein consistently blocked DNAse I degradation of a single binding sequence (CAAAGATATAACACTAATTCATTTTAAATAATAATT) located from -56 to -91 relative to the transcription start site (17) (Fig. 4B, 4C). The region bound by Strep-NikR was not protected against DNase I degradation in the absence of nickel or in the absence of NikR (Fig. 4B, left panel), consistent with the electrophoretic mobility shift assays (Fig. 4A).

It was previously suggested that the palindromic region present at positions –49 to –67 in the *ureA* promoter may be involved in the regulation of *ureA* transcription by NikR (17, 42). Using the *ureA* promoter deletion fragment P*ureA*-del, which lacks the sequence from positions -50 to -90, no mobility shift complex was observed in either the presence or absence of nickel (Fig. 4A). In addition, deletion of this region resulted in a complete absence of protection to DNase I digestion (Fig. 4B, right panel).

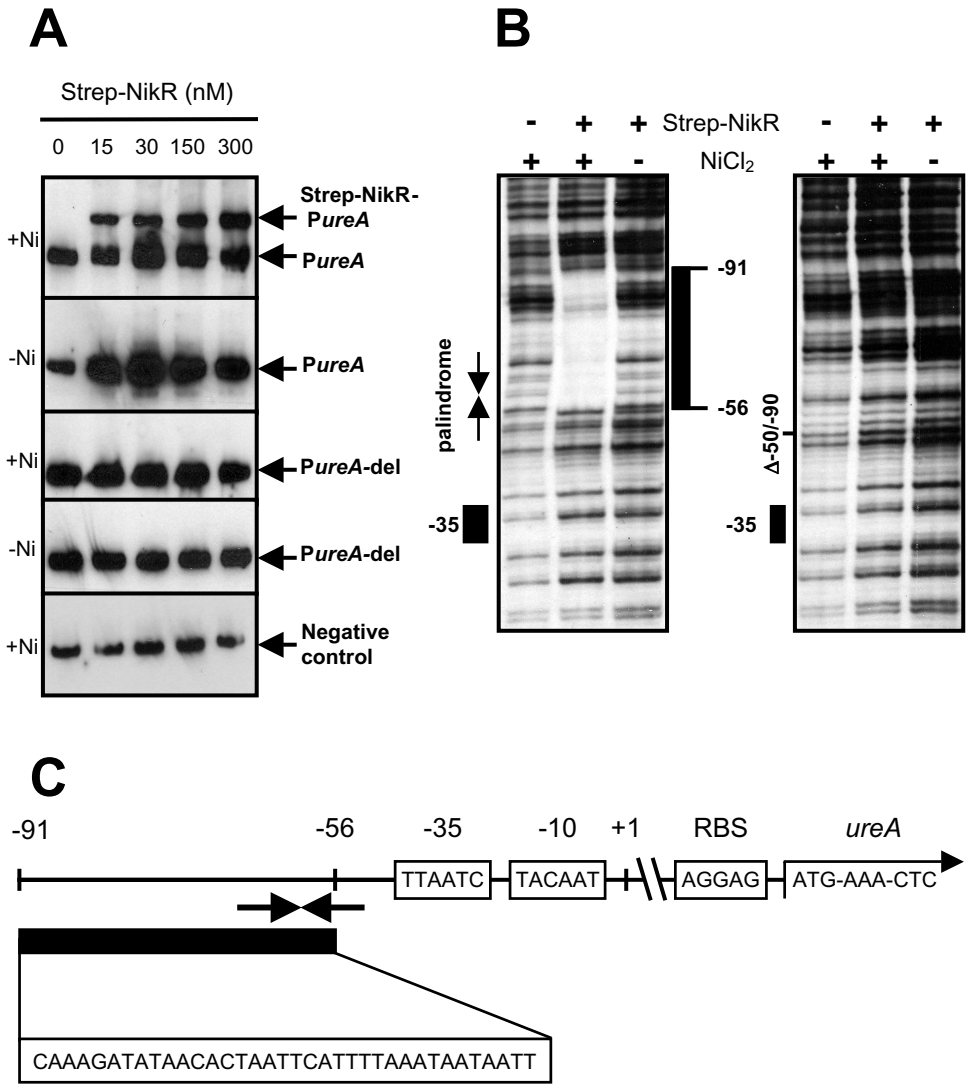

Fig. 4. NikR directly induces *ureA* transcription by nickel-dependent direct binding to a specific operator in the *H. pylori ureA* promoter region. A) Electrophoretic mobility shift assays with recombinant *H. pylori* Strep-NikR protein and the *ureA*-promoter (P*ureA*) and a -90/-50 deletion mutant (P*ureA*-del) in the presence (+Ni) and absence (-Ni) of $NiCl_2$. An internal fragment of the *amiE* gene was used as negative control only in the presence of $NiCl_2$. The Strep-NikR-complexed *ureA* fragment is indicated as Strep-NikR-P*ureA*. Strep-NikR concentrations used (in nM) are indicated above the lanes; the DNA concentration was 20 pM (P*ureA*), 22 pM (P*ureA*-del) and 43 pM (negative control). B) DNAse I footprinting assays with the P*ureA* promoter fragment (left panel) and the P*ureA*-del promoter fragment (right panel) in the absence (-) and presence (+) of Strep-NikR protein in the absence or presence of nickel. The protected region is indicated by a black bar on the right side of the P*ureA* panel. Convergent arrows indicate the position of the -49 to -67 palindrome in the *ureA* promoter, while Δ-50/-90 indicates the position of the deletion in the *PureA*-del promoter fragment. The location of the -35 promoter sequence is also indicated. C) Schematic representation of the *ureA* promoter region with the location and sequence of the NikR binding site indicated, whereby -91 and -56 indicate the boundaries of the NikR binding site. The two arrows represent the putative inverted repeat at position -67 to -49.

DISCUSSION

H. pylori expresses a NikR ortholog (HP1338), which is required for nickel-responsive induction of urease expression, nickel-resistance and acid-responsive gene regulation (10, 15, 40, 42). However, these effects were demonstrated mostly using mutational studies, and thus the possibility remained that these phenotypes were secondary or indirect effects of the *nikR* mutation. In this study, it is demonstrated that the *H. pylori* NikR protein is a DNA-binding protein that functions as activator of urease expression and repressor of NixA-mediated nickel-uptake. The role of NikR in regulation of *nixA* expression is consistent with the nickel-sensitivity of the *nikR* mutant (Fig. 1A), which is due to derepressed expression of the NixA nickel-uptake system (Fig. 1A and 2A). Next to its role in regulation of nickel-uptake, the NikR protein also controls the usage of nickel by regulation of urease expression (Fig. 1B and 2B). Both of these regulatory phenomena are mediated at the transcriptional level (Fig. 2) by nickel-dependent binding of the NikR protein to specific sequences in the *nixA* and *ureA* promoters (Fig. 3 and 4).

Nickel-responsive regulation by NikR had been in depth only studied for *E. coli*, where NikR regulates the expression of the Nik nickel transporter system (14, 18). Once the intracellular concentration of nickel exceeds a certain threshold (13), *E. coli* NikR binds to a palindromic sequence (GTATGA-N_{16}-TCATAC) that overlaps with the -10 region of the *nikA* promoter. This is thought to effectively block access of RNA polymerase to the promoter and results in cessation of transcription (14, 18). This process allows the cell to maintain control of the intracellular nickel concentration. Similar forms of metal-responsive regulation have been described for other metals, such as the control of iron metabolism by Fur (2).

The *H. pylori* NikR binding sequences in the *nixA* and *ureA* promoters were identified using DNase I footprinting. The NikR binding sequence in the *nixA* promoter consistes of a 36 bp sequence, which is located at positions -13 to +21 relative to the transcriptional start site. This region in the *nixA* promoter effectively overlaps with the -10 and +1 sequence, and this may prevent transcription upon binding of NikR. In contrast, the NikR-binding site in the *ureA* promoter is located upstream of the canonical σ^{80} promoter motifs (17, 36, 42), at positions -56 to -91, and partially overlaps with the putative palindrome previously suggested as possible binding sequence for NikR (42). Deletion of the region upstream from residue -50 in the *ureA* promoter was previously shown not to affect basal levels of urease expression (17) but prevented nickel-responsive induction of urease expression (42), and this is consistent with the position of the NikR binding site in the *ureA* promoter as identified in this study. The deletion of the -50/-90 region, indeed, abolished binding of NikR (Fig. 4B), indicating the importance of this region in NikR-binding and nickel-responsive regulation of urease transcription (17, 42). We hypothesize that binding of NikR to the *ureA* binding site allows RNA polymerase an easier access to the *ureA* promoter by a mechanism currently unknown.

The two binding sequences recognized by *H. pylori* NikR do not resemble the *E. coli* NikR binding sequence (GTATGA-N_{16}-TCATAC) (14), and thus exemplify the clear differences between the *E. coli* and *H. pylori* NikR systems. A single homolog of the *E. coli* sequence is present in the *H. pylori* genome, in the promoter of the *nikR* gene itself. Although binding of recombinant NikR to its own promoter was reported, this binding did not result in nickel-responsive regulation of the *nikR* gene (15). Taken together, this suggests that the sequences recognized by *H. pylori* NikR differ significantly from the *E.*

A

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nixA  -AAATATATTACAATTACCAAAAAAGTATTATTTTTC
ureA  CAAAGATATAACACTAATTTATTTTAAATAATAATT-
       *** **** *** * *   *      ** **  **
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B

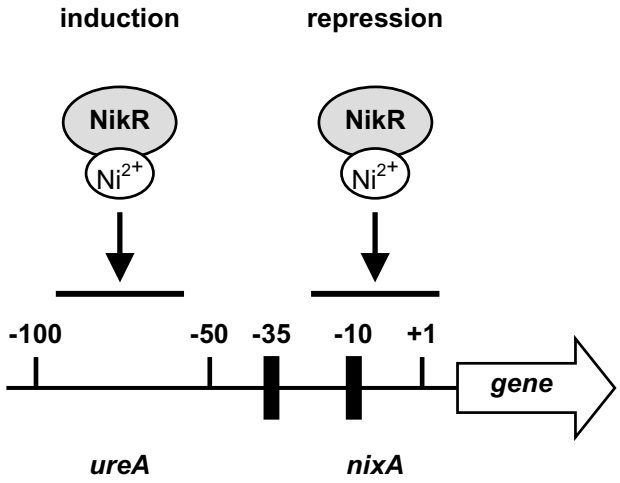

Fig. 5. Analysis and model of nickel-responsive gene regulation by *H. pylori* NikR. A) Comparison of the *H. pylori* NikR binding sites in the *nixA* and *ureA* promoters. The *H. pylori* NikR binding sites in the *nixA* and *ureA* promoters were aligned using the Clone Manager 7 Suite (Scientific and Educational Software, Cary, NC, USA). Asterisks indicate identical residues. B) Schematic overviews of NikR-mediated transcriptional regulation of *nixA* and *ureA* transcription in *H. pylori*. When the NikR-nickel complex binds to sequences upstream of the canonical promoter (like in the *ureA* promoter), this results in induction of transcription, whereas binding of the NikR-nickel complex to the canonical promoter results in repression of transcription (like in the *nixA* promoter).

coli NikR consensus sequence. Alignments of the NikR-binding sites *nixA* and *ureA* promoters revealed that they have only relatively limited homology to each other (19/36 residues, Fig. 5A). It is therefore not yet possible to define a consensus sequence for the *H. pylori* NikR binding site.

The NikR protein is a member of the RHH family of regulatory proteins, which function as transcriptional regulators. Members of this family include the Mnt (9) and Arc (32) repressors of bacteriophage P22 as well as the activator AlgZ of *Pseudomonas aeruginosa* (5). Dual regulation of transcription is already known from the Arc regulatory protein, which upon binding to a target promoter, can either slow down open-complex formation or accelerate promoter clearance, and thereby can act both as repressor and as

an activator of transcription (37). The regulator AlgZ of *P. aeruginosa* is necessary for activation of *algD* (5) and recently was demonstrated to display autorepression (31).

Comparison of the positions of the NikR binding sites in the *nixA* and *ureA* promoters with the regulatory response observed suggests that the position of the binding site determines, whether transcription of a NikR-controlled gene is nickel-repressed or nickel-induced (Fig. 5B). When the binding site overlaps with the promoter motifs, transcription is repressed, whereas binding of NikR upstream of the promoter motifs results in induction of transcription. A similar type of regulation was described recently for the ferric uptake regulator protein Fur in *N. meningitidis*, where transcription of the *tbp2* gene was iron- and Fur-repressed by binding of Fur to a sequence overlapping the -10 and +1 sequence, and transcription of three other genes is induced in an iron-dependent manner by binding of Fur to sequences upstream of the promoter region (19). A similar type of regulation has also been reported for *Mycobacterium tuberculosis* IdeR (26, 33).

In conclusion, the NikR protein of *H. pylori* functions as a repressor or an activator of nickel-responsive transcription, depending on the position of its binding site. Binding is dependent on nickel, and this mechanism allows *H. pylori* NikR to control both the uptake and the usage of nickel, dependent on intracellular nickel availability. Compared to the *E. coli* NikR system, which is currently only known to regulate nickel uptake, *H. pylori* NikR is a versatile regulatory protein that can control important aspects of nickel metabolism and virulence.

ACKNOWLEDGMENTS

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CHAPTER **6**

The NikR regulatory protein mediates nickel-responsive regulation of iron-uptake genes in *Helicobacter pylori*

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Intracellular homeostasis of the essential nutrient iron is a necessity for all living organisms, and requires careful control of iron-uptake from the environment. Iron uptake in Gram-negative bacteria is often mediated by specific outer membrane receptors, and is energized via the TonB-ExbB,D complex. The gastric pathogen *Helicobacter pylori* expresses three copies each of the FecA and FrpB outer membrane receptors, but also two copies of the TonB-ExbB,D complex. The iron-responsive regulator Fur controls expression of the FecA1,2 and FrpB1,2 proteins, but not the FecA3 and FrpB3 proteins. Here it is demonstrated that expression of the FecA3 and FrpB3 proteins and one of the TonB-ExbB,D systems is regulated by nickel and the nickel-responsive regulator NikR. Transcription and expression of the *fecA3* and *frpB3* genes was nickel-repressed in wild-type *H. pylori* 26695, but constitutive in an isogenic *nikR* mutant. Similarly, NikR also mediated nickel-responsive regulation of the *hp1339-1341* operon encoding a TonB-ExbB,D complex. Both the *frpB3* and *fecA3* genes were transcribed from a promoter directly upstream of the genes. NikR displayed nickel-dependent binding to specific sequences overlapping the +1 or -10 sequences in both the *fecA3* and *frpB3* promoters. Finally, mutation of the *frpB3* gene did not affect growth in nickel-supplemented growth medium, compared to the wild-type. The *H. pylori* NikR protein thus controls the expression of a several proteins required for iron-uptake. While the exact function of this regulatory process is not yet known, it does allow *H. pylori* to mediate concerted regulation of iron-uptake in a diverse set of environmental conditions, and thus may contribute to the chronicity of *H. pylori* infection in humans.

The human gastric pathogen *Helicobacter pylori* is the causative agent of gastritis and peptic ulcer disease, and infection with *H. pylori* is associated with the development of adenocarcinoma of the distal stomach. About half of the worlds' population is infected with *H. pylori*, making it a very successful pathogen. The bacterium colonizes the mucus layer overlaying the gastric epithelial cells(5). In its niche *H. pylori* is exposed to hostile environmental conditions, caused by acid and changes in nutrient availability.

Iron is an essential nutrient for almost all living organisms (2, 7). However, pathogenic bacteria like *H. pylori* are subjected to iron-restriction in host tissues, as almost all free iron is complexed into heme compounds, lactoferrin or transferrin (31). These iron complexes are too large to enter the periplasm of bacteria cells by diffusion through porin channels (29). Therefore bacteria require specific, high-affinity iron uptake outer membrane proteins (2, 7, 8, 31) to mediate transport of iron compounds over the outer membrane. The analysis of the genome sequence of *H. pylori* allowed the identification of six genes encoding outer membrane proteins predicted to be involved in the uptake of ferric iron (1, 33). Interestingly, these genes can be divided into two groups, each containing three copies of a specific class of iron-uptake genes (39). Three copies display similarities to the ferric citrate receptor FecA of *Escherichia coli* (45), and the other three genes encode homologs of the *Neisseria meningitidis* FrpB protein (11).

Uptake of iron through the outer membrane is an energy consuming process. The energy necessary is created by the proton motive force of the cytoplasic membrane and transferred to the iron-transporters via the TonB system consisting of the cytoplasmic TonB-ExbB,D protein complex (2, 30). The genome of *H. pylori* contains three genes encoding homologs of the ExbB and ExbD proteins, as well as two genes encoding homologs of the TonB protein (1, 33).

As both metal-restriction and metal-overload are deleterious for bacteria, maintaining metal homeostasis is of critical importance (2, 34, 39). The process of metal homeostasis is often controlled by metalloregulatory proteins, which are able to repress or induce transcription of effector genes dependent on the intracellular metal concentration. However, only two metal-regulatory proteins have been identified in *H. pylori*, the ferric uptake regulator Fur (3) and the nickel-uptake regulator NikR (38). Fur is a regulatory protein that can sense intracellular ferrous ions, and subsequently displays iron-dependent binding to conserved sequences (Fur boxes) in the promoters of its target genes (2, 23). Iron uptake genes are often repressed in iron-replete conditions, and this regulation is usually mediated by Fur (16, 23, 39). NikR is a dual regulator that can sense intracellular nickel availability, and is involved in the regulation of nickel metabolism by nickel-dependent repression of the nickel uptake system *nixA* (22, 35) and acid resistance by nickel-dependent induction of the urease gene *ureA* (22, 35). Furthermore, NikR was previously demonstrated to mediate regulation of Fur (10, 36), and thus is able to indirectly influence iron-uptake via Fur.

Expression of the *frpB*1, *frpB*2, *fecA*1 and *fecA*2 genes was previously demonstrated to be classically iron- and Fur-dependent regulated (16, 39). In contrast, expression of *frpB*3 and *fecA*3 was not regulated by iron nor by Fur, and therefore it was concluded that *frpB*3 and *fecA*3 are constitutively transcribed (39). In the present study, it is demonstrated that expression of the *frpB3* and *fecA3* genes is nickel-regulated, and that this is mediated by NikR. NikR is also shown to regulate the transcription of the *exbB2-exbD2-tonB2* operon, and thus NikR is able to control iron uptake both directly and indirectly.

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids, media and growth conditions. *H. pylori* strains (Table 1) were routinely cultured on Dent agar (37) at 37°C under microaerophilic conditions (10% CO_2, 5% O_2 and 85% N_2). Broth cultures were grown in Brucella broth (Difco, Sparks, MD) supplemented with 0.2% β-cyclodextrin (Fluka) (BBC) and shaken at 37°C with 40 rpm for a maximum of 24 h. $NiCl_2$ (Sigma) was used to supplement BBC medium to achieve various nickel concentrations. Iron-restriction was achieved by supplementing Brucella broth with desferal (deferoxamine mesylate, Sigma) to a final concentration of 20 μM (39) before adding β-cyclodextrin. Iron-replete conditions were achieved by supplementing desferal-treated BBC with ferric chloride (Sigma) to a final concentration of 100 μM (39). *E. coli* strains were grown aerobically at 37°C in Luria-Bertani medium (32). When needed, growth media were supplemented with ampicillin, kanamycin, or chloramphenicol to a final concentration of 100 μg/ml, 20 μg/ml and 20 μg/ml, respectively.

Table 1. Bacterial strains used in this study

H. pylori strain	Relevant characteristics	Source / Reference
26695	Reference strain	(33)
26695 *nikR*	26695 *nikR*::Km^R	(38)
26695 *frpB3*	26695 *frpB3*::Cm^R	This study

Recombinant DNA techniques. Restriction enzymes and DNA-modifying enzymes were used according to the manufacturer's instructions (New England Biolabs, Promega). Standard protocols were used for the manipulation of DNA and the transformation of *E. coli* (32) and *H. pylori* (4). Plasmid DNA was prepared using Qiaprep spincolumns (Qiagen) and PCR was carried out using Taq polymerase (Roche).

Construction of an *frpB3* mutant. The region containing a part of the *frpB3* gene was amplified from *H. pylori* strain 26695 by using the primers frpB3-mut-for and frpB3-mut-rev (Table 2). The resulting *frpB3* PCR fragment was cloned into pGEMTeasy (Promega). The *frpB3* coding region of this plasmid was interrupted by insertion of the chloramphenicol resistance gene from pAV35 (40) in the unique *Ava*I restriction site. This plasmid was subsequently used for natural transformation of *H. pylori* 26695. Correct replacement of the *frpB3* gene by the interrupted copy was confirmed using PCR.

Purification and analysis of RNA. Total RNA was isolated from 4×10^9 *H. pylori* cells using Trizol (Gibco), according to the manufacturer's instructions. For Northern hybridization experiments RNA was separated on 2% formaldehyde-1.5% agarose gels in 20 mM sodium phosphate buffer (pH 7), transferred to positively charged nylon membranes (Roche), and covalently bound to the membrane by cross-linking with 0.120 J/cm^2 of UV-light of 254 nm wavelength (37). Directly after transfer, the membranes were stained with methylene blue to confirm integrity of the RNA samples, and to confirm loading of equal amounts of RNA based on the relative intensities of the 16S and 23S rRNA. The sizes of the hybridizing RNA species were calculated from comparison with a digoxigenin-labelled marker (RNA marker I, Roche). The DIG-labelled specific RNA probes were synthesized by *in vitro* transcription using T7 RNA polymerase (Roche) and PCR products amplified using primers listed in Table 2. Detection of RNA was carried out, as described previously (20, 37).

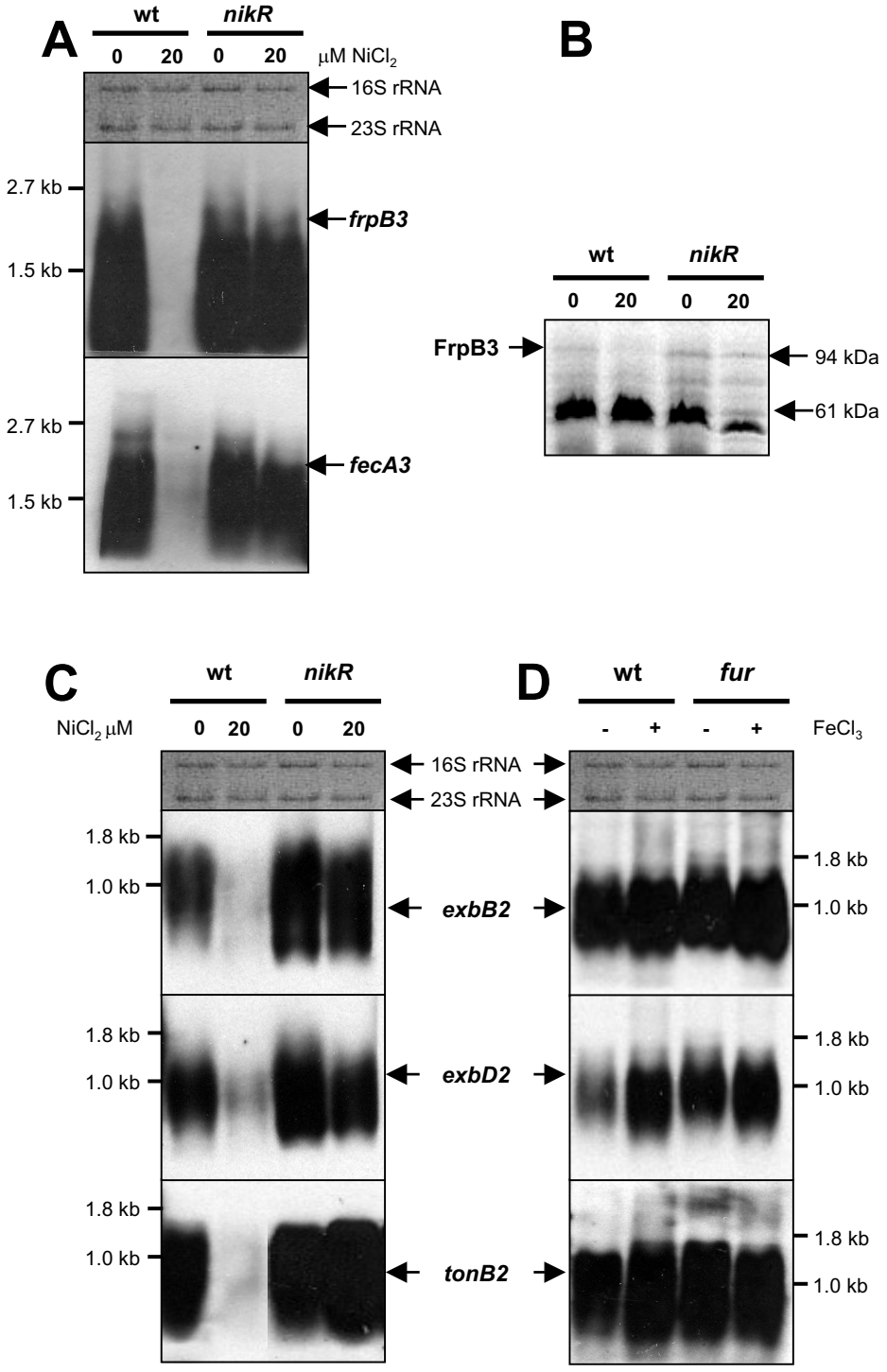
A
wt
nikR
0
20
0
20
μM NiCl2
16S rRNA
23S rRNA
2.7 kb
1.5 kb
frpB3
2.7 kb
1.5 kb
fecA3
B
wt
nikR
0
20
0
20
FrpB3
94 kDa
61 kDa
C
wt
nikR
NiCl2 μM
0
20
0
20
16S rRNA
23S rRNA
1.8 kb
1.0 kb
exbB2
1.8 kb
1.0 kb
exbD2
1.8 kb
1.0 kb
tonB2
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Fig. 1. In *H. pylori*, transcription of *fecA3* and *frpB3* is nickel-and NikR-dependent repressed. (A) Northern hybridization of RNA from *H. pylori* 26695 wild-type and *nikR* mutant cells grown in medium supplemented with 0 and 20 μM $NiCl_2$. (B) FrpB3 is nickel-and NikR-dependent regulated in *H. pylori* 26695. SDS-PAGE of whole cell protein from *H. pylori* 26695 wild-type and *nikR* mutant cells grown in medium supplemented with 0 and 20 μM $NiCl_2$. Protein is stained with Coomassie blue. The FrpB3 protein is indicated on the left side, the size on the right side. Transcription of the *tonB2* operon is nickel-and NikR-dependent repressed, but not iron- and Fur regulated. (C) Northern hybridization of RNA from *H. pylori* 26695 wild-type and *nikR* mutant cells grown in medium supplemented with 0 and 20 μM $NiCl_2$. (D) Northern hybridization of RNA from *H. pylori* 26695 wild-type and *fur* mutant cells grown in medium in the absence or presence of iron. Staining of transferred RNA by methylene blue is included for comparison of RNA amounts. The *frpB3*, *fecA3*, *exbB2*, *exbD2* and *tonB2* transcripts are indicated between pannels C and D.

Primer extension. To map the transcriptional start site of the *H. pylori frpB3* and *fecA3* genes, primer extension was carried out, as described previously (21). The digoxygenin-labelled primers frpB3-DFP-rev or fecA3-DFP-rev were annealed stepwise to 10 μg of total RNA from strain 26695, and was followed by extension with 5 U of AMV-reverse transcriptase (Promega) for 1h at 42˚C. Nucleotide sequencing reactions were carried out with the f-mol® DNA Cycle Sequencing System (Promega) using primer frpB3-DFP-rev on a fragment created with primers frpB3-DFP-for and frpB3-DFP-rev, as well as using primer fecA3-DFP-rev on a fragment created with primers fecA3-DFP-for and fecA3-DFP-rev (Table 2). Sequence reactions were separated on a 7% acrylamide-8 M urea sequencing gel, and then blotted onto a nylon membrane (Roche), followed by chemiluminescent DIG-detection (21).

Expression and purification of *H. pylori* NikR. The recombinant NikR protein was overexpressed and purified, as described previously (22). The recombinant protein was over 90% pure as determined by staining with Coomassie Blue following electrophoresis on 12% polyacrylamide-SDS gels (13). Purified protein was used directly for DNase I footprinting assays.

Table 2. Oligonucleotide primers used in this study

Primer	Sequence (5' → 3')
FecA3-F3	GATTACCGCGCCTAAGAGTT
FecA3-R4-T7[a]	ctaatacgactcactatagggagaCTGCCTCCACCCTTGATCAC
FrpB3-F4	AGCCGTCTCTTAAGGGTAAC
FrpB3-R-T7[a]	ctaatacgactcactatagggagaTCGCTATTGCTTGGATCTTG
UreA-F1	ATGAAACTCACCCCAAAAGA
FecA1-R1-T7[a]	ctaatacgactcactatagggagaGGAAGTGTGAGCCGATTTGA
FrpB3-DFP-for	TGCTTGATTCAGCCGCTCAG
FrpB3-DFP-rev[b]	TGCTAGCGACAATACAAGAG
FecA3-DFP-for	GCGTCAAAGAGTGTCTTGTG
FecA3-DFP-rev[b]	TCCTTAGCGAACAAAGACTC
Hp1339-F	AGCTTTGTGGTTTGCGATTG
Hp1339-R-T7[a]	ctaatacgactcactatagggagaGTGGGAATCGCCACAGCAAG
Hp1340-F	AGCATCAGAAGAGGCGATGG
Hp1340-R-T7[a]	ctaatacgactcactatagggagaCTGAGCTTGCGTGGAGATGG
Hp1341-F	AATGCTGAGTCGGCTAAACC
Hp1341-R-T7[a]	ctaatacgactcactatagggagaGTCCGTAACGCTCCCATCAG

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

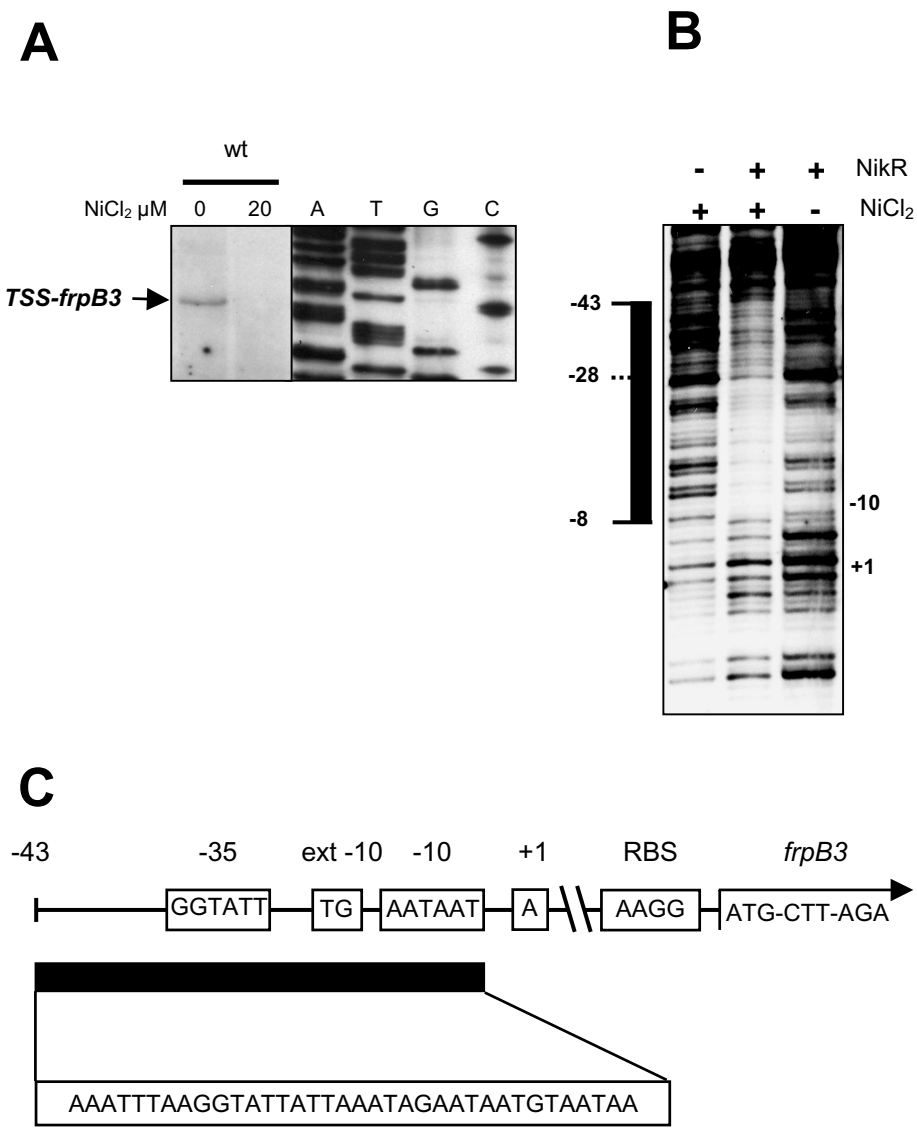

Fig. 2. Characterization of the promoter region of the *frpB3* gene. (A) Transcriptional start site (TSS) of *frpB3* determined by primer extension with RNA from *H. pylori* 26695 wild-type cells grown in medium supplemented with 0 and 20 µM $NiCl_2$. The primer extension fragment of *frpB3* is marked with an arrow, the sequence reaction is displayed in lanes A, T, G, C. (B) Identification of the NikR operator sequence in the *frpB3* (P*frpB3*) promoter by DNase footprinting in the absence (-) and presence (+) of NikR protein in the absence (-) or presence (+) of $NiCl_2$. The protected region is indicated by a black bar on the left side of the panel. The location of the TSS and -10 region is also indicated. (C) Graphical representation of the *frpB3* promoter region with the TSS, –10, extended -10 and -35 box, ribosomal binding site and ATG start codon of the *frpB3* gene. The location and sequence of the NikR binding site is indicated with a black bar and the sequence underneath. -43 indicates the boundary of the NikR binding site.

DNase I footprinting. Primers frpb3-DFP-for and frpB3 DFP-rev (Table 2) were used to amplify a 228 bp digoxigenin-labelled fragment of the promoter region of the *frpB3* gene (P*frpB3*), and primers fecA3-DFP-for and fecA3 DFP-rev (Table 1) were used to create a 351 bp digoxigenin-labelled fragment of the promoter region of the *fecA3* gene (P*fecA3*). DNase I footprinting was performed using 721 pM and 469 pM of P*frpB3* and P*fecA3*, respectively. DNA fragments were incubated without or with 2.86 μM of NikR protein in the presence or absence of 100 μM $NiCl_2$ in binding buffer (10 mM HEPES (pH 7.6), 100 mM KCl, 3 mM $MgCl_2$ and 1.5 mM $CaCl_2$) for 30 min at 37°C. Subsequently the DNA was digested with 0.25 U DNaseI (Promega) for 1 min, and the reaction was stopped as described previously (17). Fragments were separated on a 7% acrylamide-8 M urea sequencing gel (Bio-Rad) (32). Gels were blotted onto a positively charged nylon membrane (Roche), followed by chemiluminescent DIG-detection (21).

Protein analysis. Approximately 20 μg of protein from wild-type and *nikR*-mutant cells were separated by sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue. Protein lanes of interest were cut out from the SDS-PAGE gel after staining. Protein sequencing was performed by Theo Hogenboezem at the Department of Pediatrics, Erasmus-MC-University Medical Center.

RESULTS

NikR regulates transcription of iron-uptake genes in *H. pylori*. It was previously reported that NikR is involved in regulation of iron-uptake genes of *H. pylori* (14), but it was unclear, whether this was direct or indirect regulation. To examine the role of NikR and nickel in the regulation of iron-uptake genes in *H. pylori*, RNA from *H. pylori* wild-type and *nikR* mutant strains was isolated, and hybridized to probes specific for the iron-uptake genes *fecA*3, *frpB*3, *tonB*, *exbB* and *exbD* (Fig.1A and B and C). Transcription of all these genes was nickel-repressed in the wild-type strain, since transcription was absent when the wild-type strain was grown in nickel-supplemented medium (Fig. 1A and B). In contrast, in the *nikR* mutant transcription of the all five genes was constitutively high and independent of $NiCl_2$ supplementation (Fig 1A and B). Since this transcriptional pattern is identical to that of the NikR-regulated *nixA* gene (22), this suggests that NikR may directly mediate regulation of all five iron-uptake genes. As a control, RNA was also isolated from the wild-type and an isogenic *fur* mutant strain grown in iron-restricted and iron-replete medium (Fig. 1C). As expected, the *exbB*, *exbD* and *tonB* genes were constitutively transcribed in these conditions (Fig. 1C), which resemble unsupplemented BBC medium with regard to nickel-availability (Fig. 1A and B).

Nickel- and NikR-dependent regulation was confirmed on the translational level for the FrpB3 protein. A protein band was detected at 94 kDa, which was expressed only in the wild-type cells grown in unsupplemented medium and was nickel-independent expressed in the *nikR* mutant (Fig. 1D). This protein band was subsequently identified as the FrpB3 protein with the help of MALDI-TOF mass spectrometry.

The promoter of the *frpB3* gene is nickel- and NikR-dependent regulated. The transcription start site (TSS) of the *frpB3* gene was identified with the help of primer extension (Fig. 2A). Transcription of *frpB3* started at the A-residue 54 bp upstream of the ATG start codon of the *frpB3* gene. The +1 position is preceded by a suitable –10 promoter region (AATAAT), and an extended -10 region (TG at position -14) (9, 42), whereas the -35 sequence does not resemble the *E. coli* consensus sequence (Fig. 2C).

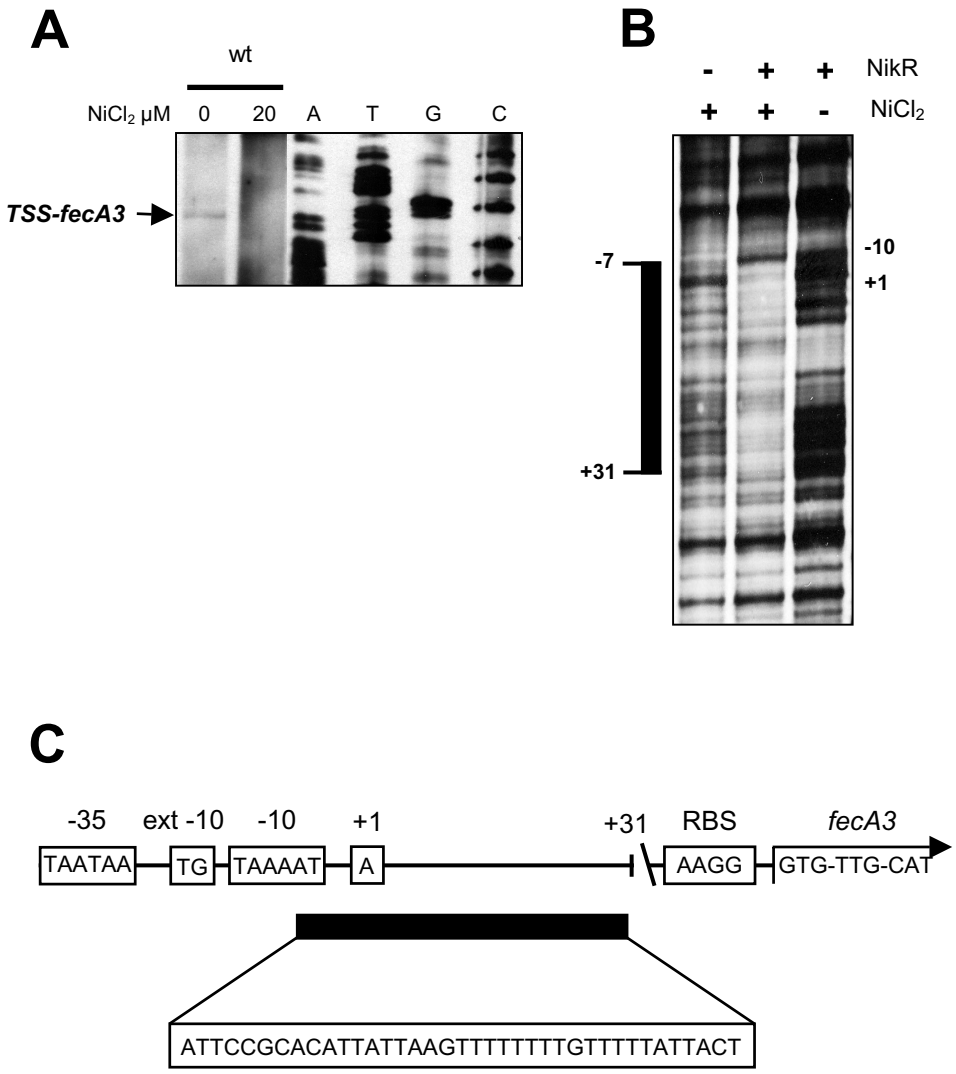

Fig. 3. Characterization of the promoter region of the *fecA3* gene. (A) Transcriptional start site (TSS) of *fecA3* determined by primer extension with RNA from *H. pylori* 26695 wild-type cells grown in medium supplemented with 0 and 20 µM $NiCl_2$. The primer extension fragment of *fecA3* is marked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

Direct binding of the NikR protein to the *frpB3* promoter was demonstrated using DNase I footprinting assay (Fig. 2B). In the presence of nickel, NikR protein blocked DNase I degradation of a single sequence (AAATTTAAGGTATTATTAAATAGAATAATGTAATAA). This sequence is located from -43 to -8 relative to the transcription start site (Fig. 2B and C) and is overlapping with the putative -10 promoter region (Fig. 2B, C). Interestingly, the NikR protein seems to bind tighter in the region from -8 to -28, as compared to region -29 to -43 (Fig. 2B). The -43 to -8 region was not protected against DNAse I degradation by NikR in the absence of nickel (Fig. 2B, C).

Nickel- and NikR-dependent regulation of the *fecA3* gene. The transcription start site (TSS) of the *fec3* gene was mapped at the A-residue 113 bp upstream of the GTG start codon (Fig. 3A). Again the +1 position is preceded by a suitable –10 promoter region (TAAAAT, Fig. 3A, C) and an extended -10 region (TG at position -14), but no discernable -35 sequence. In the presence of nickel, NikR protein blocked DNase I degradation of a single sequence (ATTCCGCACATTATTAAGTTTTTTTTGTTTTTATTACT) in the promoter of the *fecA3* gene (Fig. 3B). This sequence is located from -7 to +31 relative to the transcription start site (Fig. 3B and C). Again, NikR did not bind to this region in the absence of nickel (Fig. 3B and C).

Mutation of *frpB3* does not result in nickel-sensitivity. The effect of an *frpB3* mutation on nickel-resistance of *H. pylori* was determined in medium supplemented with 0, 20, 200 and 750 μM $NiCl_2$. Mutation of the *frpB3* gene did not affect growth in nickel-supplemented media, as compared to the growth of the wild-type cells under the same conditions (Fig. 4).

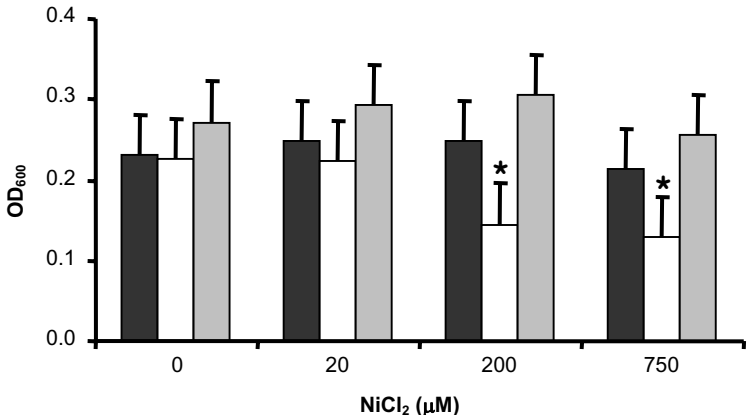

Fig.4. Mutation of *frpB3* has no effect on nickel resistance of *H. pylori*. Growth of *H. pylori* 26695 wild-type (black bars), *nikR*- (white bars) and *frpB3* mutant (dark grey bars) in medium supplemented with 0, 20, 200 and 750 μM $NiCl_2$. The results show the average of three independent growth experiments after measuring the OD_{600} 24h after inoculation.

DISCUSSION

In the present study, it is demonstrated that the putative iron-uptake genes *frpB3* and *fecA3* are directly and nickel-dependently repressed by the nickel uptake regulator NikR. NikR belongs to the Ribbon-Helix-Helix family of transcriptional regulators that bind to the DNA as tetramers (12). It was recently demonstrated that NikR functions as a nickel-dependent repressor of transcription of the nickel uptake system *nixA*, as well as an activator for transcription of the urease (22) by directly binding to the promoter region of both genes. Activation of transcription occurred, when NikR bound upstream of the *ureA* promoter at position -56 to -91, whereas repression occurred, when NikR bound a region in the *nixA* promoter at overlapping with the -10 and +1 region (22). Binding to this region is believed to prevent transcription due to competition of the regulator with the RNA polymerase (13, 15). The nickel- and NikR-dependent regulation of *frpB3* and *fecA3* is similar to the *nixA* gene, as it is demonstrated here that the NikR protein binds at position -43 to -8 in the promoter region of the *frpB3* gene (Fig. 2) and at position -7 to +31 in the promoter region of the *fecA3* gene (Fig. 3). Therefore, in both genes either the -10 or the +1 sites are blocked by NikR from binding of the RNA polymerase.

Even with the NikR binding sequences in the *frpB3*, *fecA3* and *nixA* promoters known, it is still not possible to define a consensus sequence for NikR binding: only 6/36 to 10/36 bases are similar in all three promoters (Fig. 5). However, more homology seems to be present at the 3'-end site of the protected region (Fig. 5). This might be the explanation, why the protected region in the *frpB3* gene is divided in two parts, the first one from position -8 to -28, where binding seems to be stronger, as compared to the second part from position -29 to -43 (Fig. 2B and C).

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nixA        -AAATATATTACAATTACCAAAAAAGTATTATTTTTC--
frpB3       -AAATTTAAGGTATTATTAAATAGAATAATGTAATAA--
fecA3       -ATTCCGCACATTATTAAGTTTTTTTTGTTTTTATTACT
              *           *         *  *  *  *
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Fig.5. Alignment of the NikR binding sites in the *nixA* (22), *frpB3* and *fecA3* promoter. Residues with black background are identical in all three genes at the same position (also indicated with asterixes below the alignment), whereas residues with gray background represent A/T and T/A substitutions.

Previous studies demonstrated that *H. pylori* can utilize only a limited set of iron compounds including ferrous iron, ferric citrate, heme compounds and lactoferrin, but not siderophores (18, 19, 25, 44). This may have to do with the narrow host range of *H. pylori*, and thus be a result of adaptation to the human host. Nevertheless, several homologs of iron uptake systems are present in *H. pylori*, of which only FeoB (41), a ferrous iron transporter, had been described in *H. pylori* in detail. Apart from FeoB, at least six putative ferric iron uptake genes are described in the genome of *H. pylori*, which can be clustered in two groups of three genes each. Three genes are putative homologs of *fecA*, a TonB-dependent ferric citrate receptor in *E. coli* (45), and the other three display homology to *frpB*, a low-affinity, TonB-dependent enterobactin receptor in *Neisseria gonorrhoeae* (11). Previously, it was demonstrated that both *frpB1,2* and *fecA1,2* but not

frpB3 and *fecA3* are iron- and Fur-dependent regulated, and it was concluded that *frpB3* and *fecA3* are not regulated but constitutive transcribed (39). Furthermore, in a transcriptome analysis study, it was suspected that both *frpB3* and *fecA3* are nickel- and NikR-dependent repressed (14). An explanation for the surprising regulation of the putative iron uptake genes via nickel and NikR may be found in the niche of *H. pylori*. In general, free iron is scarcely available in nature, and therefore an iron-dependent uptake system is necessary (2). However, the pH of the human stomach is most of the time <4 and it is well-known that ferrous iron is better solubilized under acidic pH (2). The low pH of the lumen may lead to increased iron toxicity in the human stomach. *H. pylori* is thought to detect the pH of the environment by the bioavailability of nickel ions (35). Therefore, under acidic pH *fecA3* and *frpB3* may be nickel- and NikR-dependent repressed to decrease the risk of iron toxicity (Fig. 1B). The genes *fecA1,2* and *frpB1,2* in contrast continue to be iron-dependent regulated by Fur to assure that enough iron is available in the *H. pylori* cell (Fig. 1C).

A second finding of this study is that the *exbB2*, *exbD2* and *tonB2* genes are transcribed as an operon (Fig. 1B and C). Transport of iron complexes through the outer membrane into the periplasm is an energy-dependent process (2, 8, 30). As the outer membrane cannot build a proton motive force due to its permeability through pores, the proton motive force of the inner membrane is used to power many outer membrane transporters (8, 30). The energy is transduced by the TonB system. This is a cytoplasmic complex consisting of 1 TonB: 2 ExbD: 7 ExbB proteins that interact with each other. ExbB contains three transmembrane sequences and is mostly oriented in the cytoplasm, ExbB and TonB have one transmembrane domain each at the N-terminal end, and are mostly situated in the periplasm (2, 7, 8, 30). It is thought that TonB is responding to the proton motive force by binding a proton and thereby changes conformation in an energized form. Energized TonB is than transporting the energy to the outer membrane transporter by binding to a TonB box (8, 30).

The *exbB2*, *exbD2* and *tonB2* genes are nickel- and NikR-dependent regulated (Fig. 1B), which is consistent with the finding that a *nikR* mutant with a gentamycine resistance cassette in the *exbB* gene is more resistant to gentamycine, as compared to the wild-type (14). However, direct nickel- and NikR-dependent regulation of the *tonB2* operon has to be proven by DNase footprint experiments with the *exbB* promoter.

A second explanation for the nickel- and NikR-dependent regulation of the *frpB3* and *fecA3* genes might be that both genes are not involved in iron-uptake at all. They rather might be involved in nickel-uptake. Several findings support this hypothesis. So far, the function of these putative iron-uptake genes has purely been described by homology to other known iron-uptake systems (1, 33), therefore it is not known what is transported by Frpb3 and FecA3. Secondly, a high concentration of nickel ions is of importance for the acid resistance of *H. pylori* as up to 10 % of the whole cell protein consists of the nickel-cofactored urease (24), but only one high affinity nickel uptake system (NixA) has been described so far (28). Thirdly, *H. pylori* possesses two sets of three homologs of putative iron-uptake systems, which is quite a lot, as compared to the limited iron sources that can be used by the bacterium. Furthermore, the *fecA* genes have homology with metal-citrate uptake genes, and in *Bacillus subtilis* it was demonstrated that the metal-citrate transporter CitM imports Ni^{2+}-citrate, but also Ni^{2+}-isocitrate complexes (26, 43). However, the CitM transporter catalyzes electrogenic proton/substrate import and does not use a *tonB* system (6). Nevertheless, further experiments are necessary to determine the exact function of

FrpB3 and FecA3.

This is to our knowledge the first description of putative iron uptake genes being directly regulated by nickel and the nickel uptake regulator NikR. It was demonstrated earlier for the ferric uptake regulator Fur that the paucity of regulators in *H. pylori* broadens the adaptive capacity of the few regulators *H. pylori* possesses (20, 27). To also broaden the capacity of the nickel-uptake regulator NikR further increases *H. pylori*'s possibility in long-term colonisation of hostile environmental niches like the human stomach.

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CHAPTER 7

NikR-mediated regulation of *Helicobacter pylori* acid-adaptation

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Nickel is the cofactor of the *Helicobacter pylori* urease enzyme, a factor essential for the chronic colonization of the acidic, hostile environment in the human stomach. The NikR regulatory protein directly controls urease expression and regulates the uptake of nickel, and is also able to regulate the expression of other regulatory proteins including the iron-responsive regulator Fur. Through regulatory crosstalk and overlapping regulons, the NikR protein controls the expression of many systems important for colonization and acid-adaptation. Despite the paucity of regulatory proteins, this enables *H. pylori* to optimally adapt to conditions in the stomach, making it one of the most successful human pathogens.

The human gastric pathogen *Helicobacter pylori* is responsible for the large majority of peptic ulcer disease in humans. Infection with *H. pylori* also significantly increases the risk of developing gastric carcinoma and MALT-lymphoma (8). Despite displaying optimal growth at neutral pH, *H. pylori* is able to withstand the acidic conditions of its only known natural habitat, which consists of the mucus layer overlaying the epithelial cells of the human stomach. If left untreated, colonization is usually lifelong, indicating that *H. pylori* is well-adapted to the variable and hostile conditions found in its niche (8).

Bacterial adaptation to environmental changes is often mediated by stress-responsive regulatory systems. Originally, it was thought that the gastric mucosa is a relatively stable environment with little need for stress-responses, and the relative paucity of regulatory genes identified in the *H. pylori* genome sequence appeared to confirm this concept (35, 42). However, recent studies using transcriptional profiling of *H. pylori* stress responses have indicated that many *H. pylori* genes are up- or downregulated by multiple stresses, indicating that extensive gene regulation must occur in *H. pylori* (27, 28). This suggests that (i) individual *H. pylori* regulatory proteins have adapted to sense and respond to multiple environmental signals, (ii) *H. pylori* uses overlapping regulons resulting in significant regulatory crosstalk, or (iii) *H. pylori* uses novel regulatory mechanisms, to be able to adequately respond to multiple stresses with a limited repertoire of environmental sensor systems.

A major candidate for the role as master-regulator of the protective response to acid in *H. pylori* is the HP1338 protein (35, 42), which is an ortholog of the *Escherichia coli* NikR nickel-regulatory protein (15). The HP1338 protein displays 30% identity and 68% similarity to the *E. coli* NikR protein, and was originally identified as the regulator required for nickel-responsive induction of urease expression (47). In contrast to the *E. coli* NikR protein, which only functions in the regulation of nickel uptake (12, 15), the *H. pylori* NikR ortholog is involved in regulating many other cellular functions (10, 13, 45). We propose here that *H. pylori* uses NikR to sense the environmental pH via nickel bioavailability and subsequently activates *H. pylori* acid resistance both directly and indirectly, the latter through interaction with other regulatory systems. The coupling of different signals through one regulatory system obviates the need for many separate sensors and regulators, albeit with a loss of versatility in adapting to alternative host environments.

Acid adaptation and acid resistance of *H. pylori*

Although the transmission route of *H. pylori* remains unknown, the bacterium has to pass the lumen of the stomach, where the pH is mostly below 2. The lower pH limit for growth of *H. pylori* is 5.0-5.5, depending on the isolate tested (7, 10), but *H. pylori* is also able to survive more severe acid shocks ($pH \leq 3.0$) for short periods of time (40). However, the survival of acid shocks is only a part of *H. pylori* acid-resistance. In the gastric mucus layer, the pH is also acidic, varying between ~4 and 6.5, and might occasionally drop, when the mucus layer is damaged (36). Therefore, *H. pylori* is continuously exposed to acidic pH, requiring mechanisms to survive acid shocks and mechanisms to allow growth in acidic conditions.

The resistance of *H. pylori* to acid shocks centers around its nickel-cofactored urease enzyme, which is expressed to very high levels (up to 10% of the soluble protein fraction of *H. pylori* (2)). Urease enzyme converts urea into ammonia and bicarbonate (9), and the ammonia produced buffers acid, although it is still under debate, as to whether this

happens in the periplasm or cytoplasm of *H. pylori* (40). Sudden exposure of *H. pylori* to acid results in the rapid activation of urease apo-enzyme, which requires the bioavailability of nickel in the environment (37, 46), and the rapid entry of urea into the cell via the acid-activated urea transporter UreI (50). Urease activity is essential for *H. pylori* colonization of the stomach in several animal models, indicative of the important role of this enzyme in *H. pylori* colonization (43).

Expression and activity of urease are dependent on the enzyme cofactor nickel, which is inserted in the active site of the enzyme (20). The *H. pylori* urease apo-enzyme consists of 12 UreA and 12 UreB subunits (20), and is activated by the accessory proteins UreE, UreF, UreG and UreH (4). Activation requires the insertion of 24 nickel atoms per urease enzyme (20), and thus it is clear that *H. pylori* requires significant amounts of nickel to satisfy its requirement for urease activity. The expression of urease is coupled to nickel availibility, because supplementation of Brucella media with intermediate to high $NiCl_2$ concentrations results in a significant increase of urease expression and activity (13, 46, 47). This nickel-responsive induction is initially mediated at the post-translational level, and then at the transcriptional level; the latter process is dependent on the NikR regulator (13, 46, 47).

The adaptation of *H. pylori* to growth at acidic pH is multifactorial. Growth at acidic pH induces changes in LPS composition (29), increases expression of chaperone-like proteins (22), and results in the induction of ammonia-producing pathways (see below) (10, 27, 45). The urease enzyme also plays an important role in this process, because urease-negative mutants of *H. pylori* are unable to colonize the stomach in animal models (43), even when the gastric pH is increased after use of acid-inhibitory drugs (17). In addition to the urease-based acid resistance, there is also a urease-independent acid resistance in *H. pylori*. This is apparent, when *H. pylori* is grown in acidic conditions (pH~5.5) in the absence of urea (6). Mutation of several independent loci resulted in acid-sensitivity, but did not affect growth at neutral pH (6, 7). These included the iron-responsive regulator Fur, which is involved in acid resistance in several bacteria (7, 21). In many bacterial species, Fur is the regulator of iron homeostasis (1), and iron bioavailability and pH are linked (1). However, the acid-sensitivity of *fur* mutants is not linked to iron metabolism in either *Salmonella typhimurium* or *H. pylori* (7, 21, 49). There are further links between acid resistance and gene regulation.

Growth of *H. pylori* in acidic conditions results in changes in the expression of many genes (10, 26-28, 45). Included in these acid-regulated genes, there are several genes encoding components of alternative pathways for the production of ammonia. These included the two paralogous amidases AmiE and AmiF, the four amino acid deaminases, and the arginase enzyme of *H. pylori* (10, 25, 27, 28, 38). The amidases are under the control of the Fur regulator (48), which autoregulates its own expression (16), but is also nickel- and acid-repressed via the NikR regulator (10, 13, 45). Thus the NikR protein can control the expression of different pathways for ammonia production directly via urease and indirectly through the Fur regulator and possibly other regulators.

NikR as pleiotropic regulator in *H. pylori*

Metals, such as nickel, pose problems for the cell, because they are required for growth, but when present in excess they can cause toxicity through the formation of radicals or the displacement of metal cofactors from enzymes (31). To control cellular homeostasis of metals, cells require transport systems to acquire sufficient quantities of

the metal, sensors to measure the intracellular metal concentration, and effector proteins to prevent and/or combat deleterious effects of metal overload (30, 31). In general, the NikR protein is thought to function as a nickel sensor, which changes its conformation upon binding nickel that is free intracellularly, and only the nickel-bound protein is able to bind to and repress transcription from its target promoters (Fig. 1).

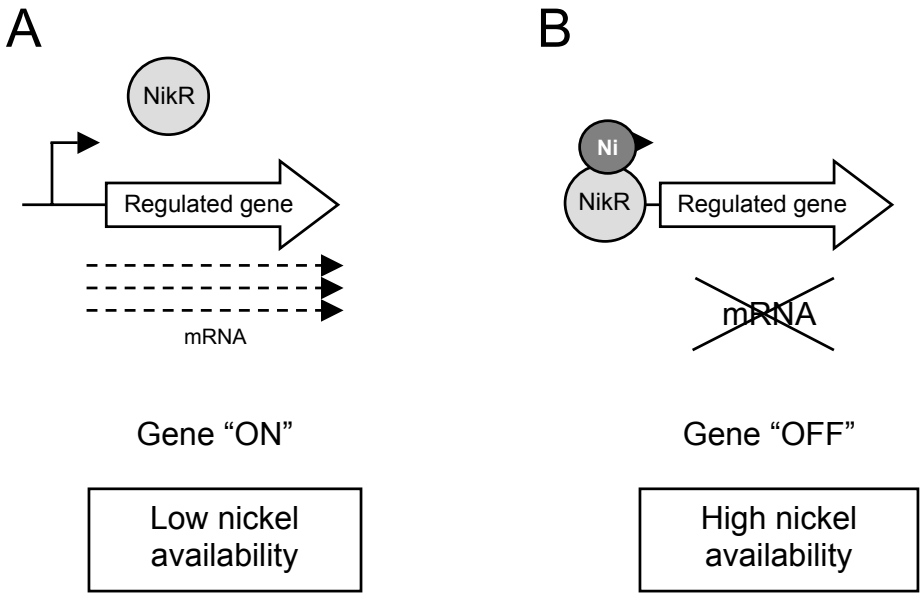

Fig. 1. The mechanism of nickel-dependent repression of gene expression by NikR. The NikR protein is a nickel-binding protein that is only able to bind to its target promoters, when complexed to nickel. (A) Nickel (depicted by Ni) availability is low; consequently, there is no free nickel in the cytoplasm. The NikR protein cannot complex with nickel and cannot bind to the operator sequence in the promoter of its target gene (depicted by arrow combination), from which mRNA is freely transcribed (indicated by the dashed arrows). The net result is that the gene is switched "ON". (B) Nickel availability is high; therefore, free nickel is present in the cytoplasm. This can form complexes with the NikR protein, which changes its conformation and is able to bind the target promoter. Binding of the target promoter blocks access of RNA polymerase, therefore, mRNA is not transcribed. The net result is that the gene is switched "OFF".

NikR was first identified in *E. coli*, where its sole function is the regulation of nickel uptake, which is mediated by the Nik ABC-transporter (12, 15). NikR is a member of the ribbon-helix-helix family of DNA-binding proteins, and the *E. coli* NikR protein binds to a 28-bp palindromic sequence (GTATGA-N_{16}-TCATAC) in the promoter region of the *nikABCDE* nickel-uptake operon (12). Binding results in occupation of the *nikA* promoter region and blocks access of RNA polymerase to this promoter (Fig. 1). The net result of this binding is the repression of transcription of nickel uptake genes, when nickel becomes available in the cytoplasm, a regulation analogous to the Fur-based regulation of iron acquisition (1).

In *H. pylori*, nickel metabolism has always been of interest owing to its role as cofactor of the important colonization factors urease and hydrogenase (9, 30, 32, 33). Other bacterial ureases are subject to a varied repertoire of regulatory signals, such as urea

availability, nitrogen status, or growth phase (9), but these stimuli are not involved in regulation of the *H. pylori* urease system. To date, the *H. pylori* urease enzyme is the only bacterial urease, for which regulation by nickel at the transcriptional level has been demonstrated (46). In contrast, the urease system of *Streptococcus salivarius* also displays nickel-dependent activation of urease enzyme activity, but this induction is mediated at the post-translational level (11).

Comparitive genome analyses have revealed that NikR orthologs are present in both gastric and non-gastric *Helicobacter* species (41, 42). In *H. pylori*, NikR is encoded by the HP1338 gene (42), whereas in the non-gastric *Helicobacter* species *H. hepaticus*, it is encoded by the HH0352 gene (41). It is unclear what function NikR serves in the non-gastric *Helicobacter* species, as only *H. pylori* NikR has been studied in detail. The *H. pylori* NikR protein mediates the nickel-responsive transcriptional induction of urease via the promoter upstream of the *ureA* gene (14, 47). *H. pylori* mutant strains with an interrupted *nikR* gene were unable to induce urease expression, although basal levels of urease were still expressed (13, 47). Deletion and mutational analysis of the nickel-responsive *ureA* promoter indicated that this promoter is modular, because sequences upstream of the -35 sequence were not required for basal levels of transcription from the *ureA* promoter (14), but abolished nickel-responsive induction in a similar vein, as demonstrated in the *nikR* mutant (47). The absence of nickel-responsive induction was pinpointed to an imperfect 19-bp palindromic sequence (TTAAaTAAT-A-ATTAgTTAA), located at positions -49 to -67 to the transcriptional start site of the *ureA* gene (14, 47). This sequence is clearly distinct from the *E. coli* NikR recognition sequence (GTATGA-N_{16}-TCATAC) (12), and to date the exact role of the *ureA* palindrome remains unclear.

H. pylori nikR mutants are unable to grow at high $NiCl_2$ concentrations, indicating that NikR is involved in other aspects of nickel homeostasis in *H. pylori* (10, 13, 47). Nickel uptake in *H. pylori* is mediated by the NixA protein (3), which is a member of the HoxN high-affinity metal-permease family (30). *H. pylori* NikR mediates nickel-dependent repression of the *nixA* gene (44), although NikR-mediated induction of *nixA* transcription has also been suggested (13). However, the latter possibility is unlikely, as this would result in continuous influx of nickel in wild-type *H. pylori*, and cannot explain the increased nickel-sensitivity of the *nikR* mutant (13, 47). Further characterization of the *H. pylori* NikR regulon by transcriptional profiling showed that mutation of NikR leads to alterations in expression of genes involved in metabolism of nickel, and also of other metals (10, 13). The nickel- and iron-cofactored hydrogenase operon was repressed in a *nikR* mutant, as was expression of the nickel-binding protein HP1432 (13). Other genes with altered expression in the *nikR* mutant are associated with iron metabolism (i.e. iron uptake and storage), metal metabolism (i.e. copper export), respiration (i.e. cytochrome oxidase and pyruvate ferredoxin oxidoreductase), stress response (i.e. heat shock proteins), motility, as well as chemotaxis (i.e. flagellin A and B, and CheV) and porin function (13). Expression of regulatory proteins was also altered in the *nikR* mutant, as expression of the heat shock regulator HspR and the iron-responsive regulator Fur is repressed by NikR (10, 13, 45). Also, the NikR protein was subject to autoregulation, repressing its own transcription (13).

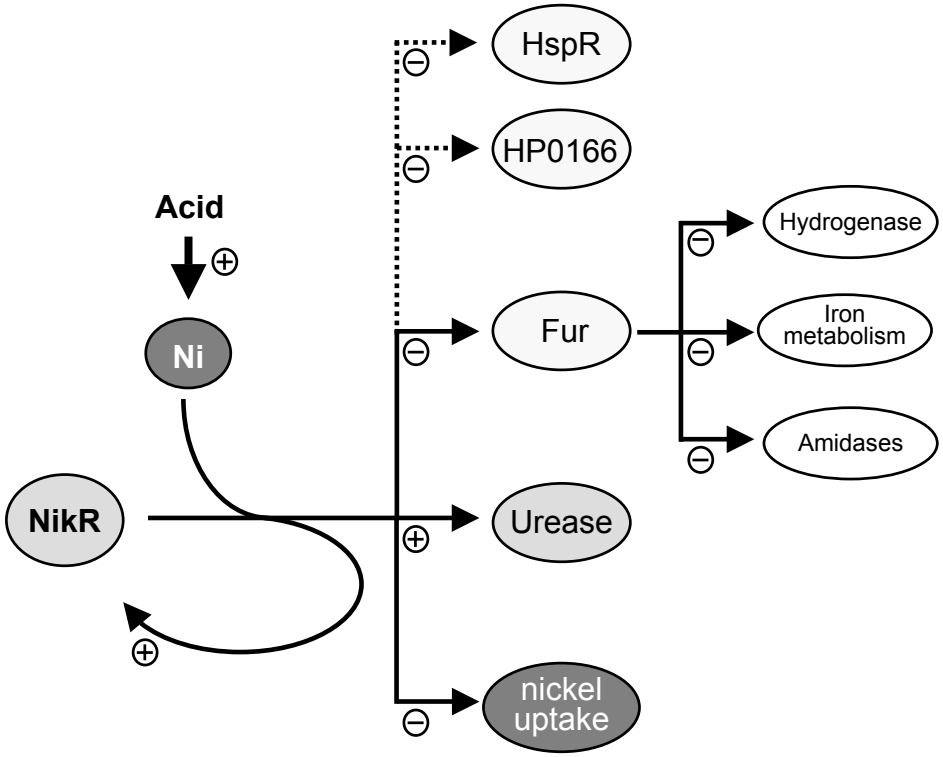

Fig. 2. The NikR protein is a master regulator of acid-responsive gene regulation in *H. pylori* via nickel availability and a repressor cascade that includes the Fur regulator and possibly the HP0166 regulator, and both their regulons. When the extracellular pH decreases, the NikR regulator senses the increased availability of nickel (Ni) and is able to regulate different aspects of acid adaptation and metabolism of *H. pylori*. Through autoregulation NikR increases its own expression, and increases expression of the urease virulence factor. To avoid further amplification of the response to acid, NikR represses the expression of NixA-mediated nickel uptake (44). NikR also represses the expression of the Fur regulator, which allows NikR to indirectly regulate the expression of iron metabolism, amidase-mediated ammonia production and hydrogenase-based energy production (32). Whether NikR regulates the HP0166 response regulator and HspR stress-response regulator directly or indirectly, remains to be investigated; this is indicated using a dashed line. Using such repressor cascade or regulatory crosstalk, NikR can control many aspects of *H. pylori* acid adaptation and metabolism.

Further evidence supporting an important role of NikR in acid resistance and virulence of *H. pylori* was obtained during two independent studies on the effect of mildly acidic conditions (pH ~5) on the transcription of genes encoding ammonia-producing enzymes (10, 45). The pH of approximately 5 was independently selected by both groups, as this is thought to represent the average pH in the gastric mucosa, and thus mimics the conditions, where *H. pylori* normally resides. During growth at this pH, the expression and activity of urease and both amidases was induced significantly (10, 45). NikR was subsequently shown to directly mediate the induction of urease, whereas the induction of both amidases was mediated via an indirect route involving the Fur regulator (Fig. 2). NikR was also shown to mediate nickel- and acid-responsive regulation of the *fur* gene (7, 10, 13, 45).

Interestingly, although acid-responsive regulation of the amidases was mediated via an indirect route, transcription of the *nikR* gene itself was acid-induced (45). Because expression of the *nikR* gene is also autoregulated (13), NikR might mediate its own acid-responsive regulation. Finally, *nikR* and *fur* mutants of *H. pylori* are attenuated in a mouse model for *H. pylori* colonization (10). Taken together, this suggests an important role for NikR in the response to acidic conditions.

NikR as master regulator of *H. pylori* acid adaptation

The extensive regulation of acid resistance genes in *H. pylori* suggests that expression of virulence factors in *H. pylori* is governed by an intricate interplay of different environmental signals and sensors, despite the limited number of regulatory proteins present in the *H. pylori* genome (35, 42). To date, three *H. pylori* regulators have been directly implicated as being involved in acid-adaptation: the NikR protein (10, 45), the Fur protein (7, 10) and the HP0164/0165-HP0166 two component regulatory system (10, 34). Expression of the Fur and HP0166 proteins is acid-repressed (10), and expression of HP0166 is absent in a *fur* mutant background (23). This is suggestive of a NikR > Fur > HP0166 regulatory cascade, and therefore it is tempting to propose that NikR acts as a master regulator of acid adaptation in *H. pylori* (Fig. 2 and 3).

In our current working model, *H. pylori* uses the increased intracellular availability of nickel as a signal that the extracellular environment has become more acidic (Fig. 3). When compared with neutral pH, more nickel accesses the cytoplasm in acidic conditions via the NixA transporter and probably additional transporters. This might occur as a result of (i) increased bioavailability of nickel in acidic conditions, or (ii) increased expression of nickel transporters, or (iii) increased efficiency of nickel transporters. Initially this increased influx of nickel is used to activate the preformed pool of urease apo-enzyme, but once the complete pool of this high affinity cytoplasmic apo-enzyme is activated, nickel is no longer immediately removed from the cytoplasm resulting in formation of NikR-nickel complexes. This NikR-nickel complex can bind to target promoters with different binding affinities (F.D. Ernst, E.L. Benanti, P.D. Chivers, A.H.M. van Vliet, unpublished results). The promoter of the nickel uptake gene *nixA* is already repressed at low $NiCl_2$ concentrations (44). The repression of NixA expression will eventually cause a decrease and finally the cessation of NixA-mediated nickel influx. Another target of NikR is the urease operon (*ureAB*), as transcription of the urease subunit genes is induced, leading to additional formation of urease and thus an increase in nickel utilization. A further decrease of external pH and the coupled increase in nickel availability results in even more influx of nickel, and this leads to increased expression of NikR and to formation of extra NikR-nickel complexes. The increased levels of NikR-nickel complexes bind to other target promoters regions that have lower binding affinities, such as the *fur* promoter and possibly the *hspR* and *hp0166* promoter regions (Fig. 3). The decrease in *fur* transcription will lead to changes in expression of Fur-regulated genes, including the downregulation of iron storage and increased expression of iron-uptake systems (5, 49), leading to an increase in the cytoplasmic availability of iron. All the latter steps are probably required to decrease the risks of nickel toxicity to *H. pylori* (5).

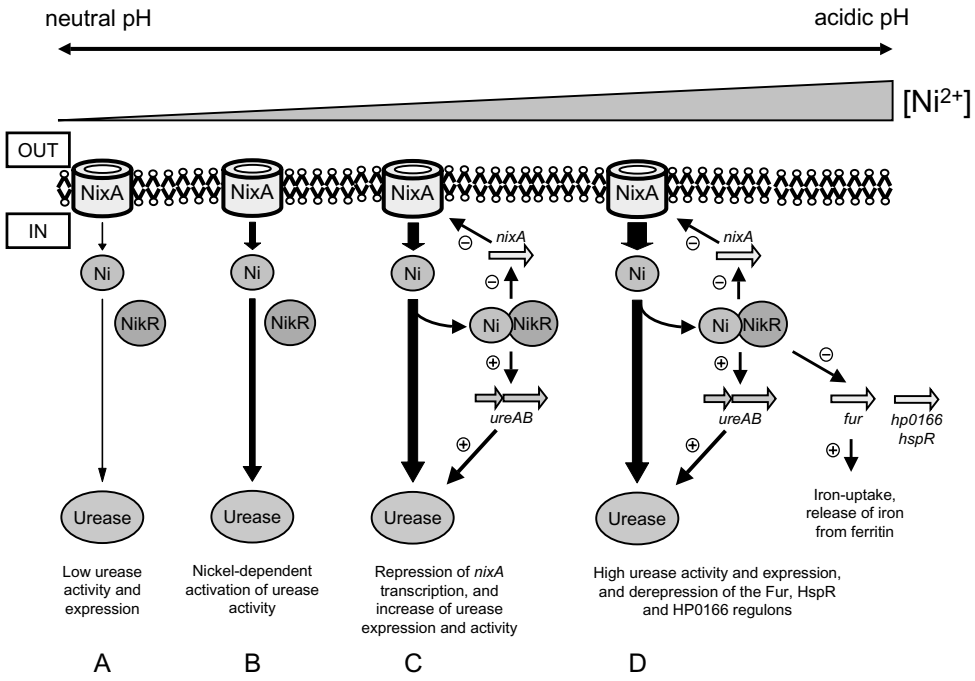

Fig. 3. Model depicting the NikR- and nickel-mediated response of *H. pylori* to decreasing environmental pH. The boxed IN indicates the cytoplasm, and is separated from the external space (periplasmic and extracellular compartment, represented by the box OUT). The extracellular pH ranges from neutral (A) to acidic (D), and increasing acidity is coupled to an increased bioavailibility of nickel (Ni). (A) At neutral pH, nickel influx is low and as a consequence, urease is only partially activated, leading to low urease activity. (B)-(D) When the pH decreases, nickel influx increases. (B) At first, the increased pool of cytoplasmic nickel is used to activate urease apo-enzyme, leading to an increase in urease activity only. (C) Further decrease of the pH leads to further increase in the cytoplasmic nickel pool, which is now also able to complex with the NikR protein. This complex binds to the *nixA* promoter, which will eventually lead to cessation of nickel uptake, and to the promoter of the *ureAB* operon, leading to increased production of urease apo-enzyme, which can subsequently be activated. Therefore, urease expression and urease activity are both increased. (D) Finally, when the cytoplasmic nickel concentration further increases, the NikR-nickel complex is also capable of binding to the promoter regions of *fur* and possibly of the *hspR* and *hp0166* regulator genes, reducing the amount of Fur and other regulatory proteins, and thus derepresses iron metabolism and other regulons. The net result is an increase in cytoplasmic iron availability, which may assist in avoiding nickel toxicity.

This model is supported by the effect of NikR on other acid-responsive systems. Transcription of hydrogenase is altered in a *nikR* mutant (13), but induced in a *H. pylori fur* mutant in a similar vein to the *H. pylori* amidases (10, 48), suggesting that hydrogenase is subject to regulation via the same NikR-Fur repressor cascade as the amidases. Similarly, expression of the *H. pylori* nickel-binding, histidine- and glutamine-rich protein Hpn (HP1432) is altered in both *nikR* and *fur* mutants (10, 18), but is also altered, when the HP0165-HP0166 two-component regulatory system it is absent (10, 19, 34). Finally, urease expression and activity is also affected in *hspR*, *hp0166* and *flbA* isogenic mutants (24, 34, 39).

CONCLUDING REMARKS

H. pylori is a human pathogen that is optimally adapted to the conditions within the gastric mucosa, as is evident from its ability to colonize a niche that only few bacteria can colonize successfully. Despite the paucity of regulatory proteins that it possesses, *H. pylori* is able to adequately respond to stresses occuring in the gastric mucosa, and an important part of this adaptation is mediated via a NikR-controlled regulatory cascade. Consequently, NikR controls many important cellular processes and virulence factors either directly or indirectly. The latter is mediated via the Fur regulatory protein (Fig. 2), and possibly also via other regulatory proteins such as HP0166 and HspR. This makes the NikR regulatory protein an attractive target for further exploration of the role of gene regulation in chronic colonization by *Helicobacter* species.

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Summary and Conclusions,
Zusammenfassung,
Samenvatting,
Acknowledgments,
Curriculum Vitae,
List of Publications

Up to 50 % of the world's population is infected with *Helicobacter pylori*. Colonization of the mucus layer of the human stomach by *H. pylori*, is lifelong unless treated with antibiotics (26). *H. pylori*, which is a neutralophilic bacterium, survives in the mucus layer of the human stomach with the help of the enzyme urease. Urease is an enzyme that converts urea into ammonium and carbon dioxide, thereby keeping the intracellular and periplasmic pH at neutral. It is estimated that up to 10% of the whole cell protein consists of this nickel-cofactored enzyme (19). The nickel necessary to activate the urease is thought to come from foodsources, such as nuts, tea and cereals, which are rich in nickel (1).

Metal ions like nickel or iron can be dangerous for bacteria, as they can react with oxygen in order to create reactive oxygen species that in turn can destroy macromolecules like nucleic acids, proteins and cell wall components (27). Therefore, the bacterial metal metabolism has to be tightly regulated. In *H. pylori*, regulatory proteins are scarce. Only two metal-regulatory proteins are known, the ferric uptake regulator Fur (4), and the nickel responsive regulator NikR (31). Fur is a regulatory protein that can sense and bind intracellular ferrous ions, and subsequently displays iron-dependent binding to conserved promoter sequences (Fur boxes) of its target genes (17). The classical regulation is repression of iron uptake genes in iron-replete conditions (15, 17, 33). Unlike all other Fur homologs known so far, *H. pylori* Fur can also bind to Fur-boxes in an iron free form (apo-Fur), as was shown for *pfr* (16) and *sodB* (Chapter 3).

The second metal-dependent regulatory protein is NikR, the nickel responsive regulator, which belongs to the family of Ribbon-Helix-Helix regulatory proteins (9). NikR is directly involved in the regulation of acid resistance via urease and nickel-uptake (Chapter 4), and was previously demonstrated to mediate regulation of the ferric uptake regulator Fur (7, 12, 29).

The aim of this thesis was to gain further insight into the transcriptional regulation the ferric uptake regulator Fur and the nickel responsive regulator NikR.

The Ferric Uptake Regulator Fur and Iron Metabolism

Apart from being involved in the regulation of the iron metabolism (3, 16, 33) in *H. pylori*, the ferric uptake regulator Fur has also been implicated to be involved in regulation of acid resistance (5, 7, 29), nitrogen metabolism (30, 32), and oxidative stress resistance (2, 13, 18). The aim of Chapter 2 was to identify and characterize the complete Fur regulon. Array hybridization analysis was carried out using *H. pylori wild*-type and isogenic *fur* mutant cells grown in the presence and absence of iron. A set of 32 genes was found to be Fur-dependently regulated. 16 genes are classical iron- and Fur-dependently repressed. These genes are, among others, involved in metal-metabolism (*fecA1/2*, *frpB1*), nitrogen metabolism (*amiE*), motility (*fliP*), cell wall biosynthesis (*murB*) and cofactor synthesis (*bioB*, *pdxA*). Furthermore, 16 genes were found to be iron-induced and Fur-dependently expressed. These genes are, among others, involved in iron storage (*pfr*), respiration energy metabolism (*hydABC*), chemotaxis (*cheV*), and oxygen scavenging (*sodB*). These findings indicate that the ferric uptake regulator Fur has broadened its regulatory potential from regulating expression of only iron homeostasis to also controlling several other pathways involved in normal metabolism, motility, virulence and stress resistance. This adaptive capacity can explain why *H. pylori*, a bacterium that possesses only few regulators and has a small genome, is such a highly successful coloniser of the human gastric mucosa (6).

Array hybridization analysis is a powerful tool to identify complete regulons, however,

each finding should be verified by additional experiments, as detection differences cannot be completely eliminated. A thorough analysis was performed on *sodB*, a gene that codes for the single, iron-cofactored superoxide dismutase (SodB) (23-25). Superoxide dismutases catalyse the dismutation of O_2^- to H_2O_2, which is subsequently removed by catalase.

With the help of transcriptional and translational studies, it is demonstrated in Chapter 3 that expression of SodB is iron-induced in the wild-type, but is constitutively high in a *fur* mutant, which suggests a direct role of apo-Fur in regulation of the *sodB* gene. This regulation is in contrast to the regulation of the superoxide dismutase in *Escherichia coli*, where *sodB* is regulated by the small RNA RyhB (20). Transcription of the RyhB small RNA in *E. coli* is Fur-dependent, and once transcribed, RyhB can bind to complementary sequences in the 5'-end of the *sodB* mRNA, blocking translation and making the mRNA unstable (20, 21). A possible direct regulation of *sodB* by Hp-Fur, similar to the one previously described for the *pfr* gene (16, 35), was examined using electrophoretic gelshift and DNase footprinting assays. Recombinant *H. pylori* Fur protein bound to the *sodB* promoter region with surprisingly low affinity, when iron was present. This low affinity may have biological significance, as the SodB protein is the only defense of *H. pylori* to superoxide stress (24) and thus its expression should not be repressed unless *H. pylori* encounters such severe iron-restriction that even activating SodB enzyme is not feasible anymore. The operator sequence of apo-Fur, as identified by DNAse I footprinting, was located directly upstream of the *sodB* gene at positions -5 to -47 from the transcription start site, overlapping with the -10 and -35 region of the *sodB* promoter region. Binding of Fur to the *sodB* promoter region possibly interferes with binding of the RNA polymerase. The electrophorectic gelshift and DNase footprint assays proved that the *sodB* gene is directly regulated by apo-Fur in *H. pylori*, and thus constitutes a novel mechanism for regulation of expression of Fe-containing superoxide dismutases in prokaryotes.

The Nickel Responsive Regulator NikR and Nickel Metabolism

Nickel-responsive regulation by NikR has sofar only been studied in depth in *E. coli*, where NikR regulates the expression of the Nik nickel transporter system (11, 14). Once the intracellular concentration of nickel exceeds a certain threshold (10), *E. coli* NikR binds to a palindromic sequence (GTATGA-N_{16}-TCATAC) that overlaps with the -10 region of the *nikA* promoter. This is thought to effectively block access of RNA polymerase to the promoter, and results in cessation of transcription (11, 14). This process allows the cell to maintain control of the intracellular nickel concentration.

In earlier studies, it was demonstrated that the *H. pylori* NikR ortholog is required for nickel-responsive induction of urease and that a palindromic region at position -48 to -68 seems to be involved in this regulation (30, 31). *H. pylori* also possesses a high affinity uptake system for nickel (NixA, (22)), but in contrast to *E. coli*, its regulation had never been truly investigated. In Chapter 4, it is demonstrated that NikR of *H. pylori* functions both as repressor and inducer of gene transcription. It is involved in the regulation of nickel metabolism by direct nickel-dependent repression of the nickel uptake system *nixA* (28) by binding to the -13 to +21 region of the *nixA* promoter, overlapping with the +1 region and possibly interfering with binding of the RNA poloymerase.

NikR is also involved in the acid resistance by nickel-dependent induction of the urease gene *ureA* by binding to the -56 to -91 region, which is partially overlapping with the palindromic region at position -48 to -68 upstream of the *ureA* promoter. Whether Hp NikR

functions as repressor or activator of gene transcription seems therefore to dependent on the position of the NikR binding site.

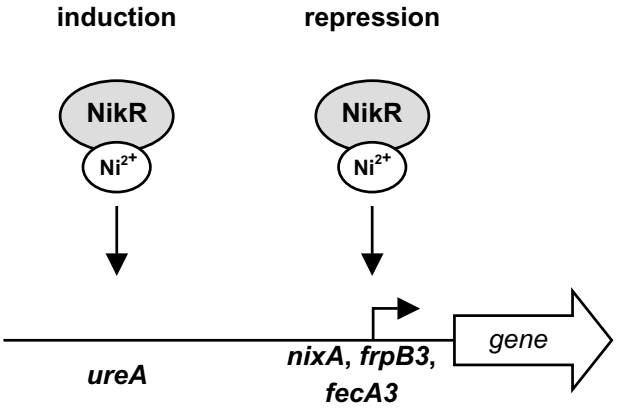

Fig. 1. NikR as a repressor and activator of gene transcription

Apart from regulation of the urease and nickel uptake via *nixA*, NikR was previously demonstrated to mediate regulation of Fur (7, 29), thereby indirectly regulates the iron metabolism of *H. pylori*. In Chapter 5, it is demonstrated that, next to this indirect route, NikR is also able to regulate putative iron-uptake genes by direct nickel-dependent binding. *H. pylori* possesses three homologs of *fecA*, coding for the TonB-dependent ferric citrate receptor FecA of *E. coli* (36), and three homologs of *frpB*, coding for the TonB-dependent *Neisseria meningitidis* FrpB protein (8), of which only *frpB1/2* and the *fecA1/2* genes are iron- and Fur-dependent regulated (33). It was previously suggested that *frpB3* and *fecA3* are constitutive transcribed (33). With the help of DNase footprint analysis, it is demonstrated in Chapter 5 that NikR binds nickel-dependent to the region -43 to -8 in the promoter of the *frpB3* gene and to the region -7 to +31 in the promoter of the *fecA3* gene. These positions are similar to that of the NikR binding sites in the *nixA* promoter, and thus confirm the model suggested there. It is also demonstrated in Chapter 5 that regulation of the *tonB2* operon, consisting of the genes *exbB2* (*hp1339*), *exbD2* (*hp1340*) and *tonB2* (*hp1341*), is nickel- and NikR-, but not iron- and Fur-dependent. Two obvious explanations can be given for the observed regulatory pattern: One is that nickel- and iron metabolism are linked, thus preventing damage through reactive oxygen species that would be generated if free metal ions occur in the cytoplasm. The second explanation might be that *frpB3* and *fecA3* are not iron-uptake genes but are involved in the uptake of nickel. To solve this problem, further experiments are necessary to determine the exact function of FrpB3 and FecA3.

Chapter 6 is a review and an opinion chapter on the nickel- and NikR-dependent regulation in *H. pylori*. The main conclusions of this chapter are presented in a model depicting the NikR- and nickel-mediated response of *H. pylori* to decreasing environmental pH. The model is based on the assumption that acidity and bioavailability are linked, i.e. an increasing acidity is coupled to an increased bioavailability of nickel ions. The most importent components in this system are the nickel responsive regulator NikR, the high-affinity nickel uptake system NixA and the nickel-cofactored urease. At neutral pH, nickel

influx via NixA is low, and as a consequence urease is only partially activated, leading to low urease activity. When the pH decreases, nickel influx in the cytoplasm increases. At first, the increased pool of cytoplasmic nickel is used to activate urease apo-enzyme, leading to an increase in urease activity only. Further decrease of the pH leads to further increase in the cytoplasmic nickel pool, which is now also able to complex with the NikR protein. This complex binds to the *nixA* promoter, which will eventually lead to cessation of nickel uptake, and to the promoter of the *ureAB* operon, leading to increased production of urease apo-enzyme, which can subsequently be activated. Thus urease expression and urease activity are both increased. Finally, when the cytoplasmic nickel concentration further increases, the NikR-nickel complex is also capable of binding to the promoter regions of *fur* and possibly of the *hspR* and *hp0166* regulator genes, and thus regulating iron metabolism and other regulons.

General Conclusions

The only known habitat of *H. pylori* is the mucus layer of the human stomach. This limited niche may explain why this neutralophilic bacterium only possesses a small genome and uses few regulators. To cope with changing nutritional concentrations or pH, regulators like Fur or NikR have broadened their range of regulation onto several pathways. Fur is classically involved in regulation of iron metabolism, but in *H. pylori* it is also involved in general metabolism, motility, virulence and stress resistance (Chapter 3 and Chapter 4), whereas the nickel responsive regulator NikR is not only regulating nickel uptake (Chapter 5), but also acid resistance (Chapter 5), and even directly (Chapter 6) and indirectly the iron metabolism.

The metal-specific binding sites for Fur or NikR in the promoter region of its target genes in *H. pylori* seem to differ from the ones found in many other bacterial species. For example, the Fur box of *E. coli* has been used to identify various Fur-regulated genes in several other bacterial species (17). In contrast to *E. coli*, it is still not possible to define a template binding sequence for both NikR- and Fur-regulated promoter sequences enabling the search for other putative Fur- or NikR-regulated genes in *H. pylori*. Even though the binding sequences in *H. pylori* can be exactly mapped with the help of DNase footprinting assays, the overlap of sequences in the AT-rich genome of *H. pylori* leads to an inconclusive template binding sequence in this bacterium.

So far, it is not known, whether nickel is a nutrient for humans or not. In case it is used as a nutrient, then only in minute amounts (1). Therefore, it is surprising that *H. pylori*, whose only habitat is the stomach of humans, requires this metal in relatively large quantities, in order to survive the acidic surrounding. By using a metal its host does not use, other than perhaps in limited amounts, *H. pylori* avoids competition with the host, however it is dependent on the nutritional status of the host that enough nickel will be available.

A surprising finding is the nickel- and NikR-dependent regulation of the putative iron-uptake genes *frpB3* and *fecA3*. However, only little is known about the function and substrate-specificity of iron uptake systems of *H. pylori*, as only the FeoB system had been analyzed so far (34). The exact function of FrpB3 and FecA3 is still unknown. It is possible that some putative iron uptake systems are uptake systems for anything but iron.

The work described in this PhD thesis has broadened our knowledge on the bacterial iron- and nickel- metabolism in bacteria. Fur and NikR mediate transcriptional regulation of a wide variety of cellular processes by regulatory cascades and cross-talk in *H. pylori*, which is in

contrast to many other bacteria, where both proteins only regulate processes related to iron- or nickel- metabolism. The use of overlapping and crossresponding regulons by *H. pylori* may allow the finetunning of the multifactorial response to unique environmental stresses encountered in the gastric mucosa.

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Zusammenfassung

Bis zu 50% der Menschheit ist mit *Helicobacter pylori* infiziert, einem Bakterium, welches die Magenschleinhaut des Menschen kolonisiert. Eine Infektion mit *Helicobacter pylori* führt zu Magengeschwüren, und kann Magenkrebs zur Folge haben. Um in der Magenschleimhaut zu überleben, nutzt *Helicobacter pylori* das Enzym Urease, welches Harnstoff in Ammonium und Kohlendioxid umwandelt, und so das Bakterium gegen die Säure des Magens schützt. Die aktive Urease benötigt Nickel als Kofaktor, um aktiv zu sein. Da etwa 10% aller intrazellulären Proteine Ureaseproteine sind, benötigt *Helicobacter pylori* eine grosse Menge Nickelionen.

Metallionen, wie etwa Eisen und Nickel, sind für alle Organismen notwendig, da diese bei der Atmung oder als Kofaktor für Enzyme genutzt werden. Allerdings ist die Ionenkonzentration von entscheidender Bedeutung: Bei zu wenigen Metallionen sind viele Enzyme nicht mehr aktiv; sind zu viele freie Metallionen in der Zelle vorhanden, können diese mit Sauerstoff zu Radikalen reagieren, welche dann Makromoleküle der Zelle, wie etwa Proteine oder Nukleinsäuren, zerstören. Um dies zu vermeiden, ist die Regulation des Metallstoffwechsels von zentraler Bedeutung. *Helicobacter pylori* besitzt vergleichsweise wenige Regulatorproteine, und nur zwei metallabhängige Regulatoren sind bekannt: das eisenabhängige Regulatorprotein Fur (Ferric Uptake Regulator) und das nickel-abhängige Regulatorprotein NikR (Nickel-responsive Regulator).

Das Ziel dieser Doktorarbeit war es, weitere Einlicke in die transkriptionelle Regulation dieser beiden Regulatorproteine zu erhalten.

Der eisenabhängige Regulator Fur erkennt und bindet Fe^{2+}-Ionen um daraufhin an spezifische Promoterregionen (sogenannte Fur Boxes) von durch Fur regulierten Genen zu binden. Die klassische Regulation durch Fur ist Unterdrückung von Eisenaufnahmegenen in Eisenüberflusssituationen. Verschiedene Studien zeigten, dass *Helicobacter pylori* Fur nicht nur den Eisenmetabolismus reguliert, sondern auch bei der Resistenz gegen Säure und gegen Sauerstoffradikale beteiligt ist. Eine Charakterisierung aller eisen- und Fur-abhängigen Gene wird in Kapitel 3 mit Hilfe von Arrayanalysen beschrieben. Es wurden 32 Fur-abhängig regulierte Gene identifiert; darunter befanden sich Gene des Eisen- und Nitratstoffwechsels, der Chemotaxis und Beweglichkeit, sowie der Zellwand- und Kofaktorbiosynthese. Dadurch dass Fur gleichzeitig verschiedene Aufgaben übernimmt, kann *Helicobacter pylori*, trotz der geringen Anzahl an Regulatorproteinen und trotz eines relativ kleinen Genoms, die unwirtliche Umgebung der Magenschleimhaut so erfolgreich kolonisieren.

Einzigartig für *Helicobacter pylori* ist, dass auch eine eisenfreie Form von Fur an spezifische Fur Boxes binden kann; dies wurde zuerst für das Eisenspeichergen Ferritin *pfr* gezeigt. Ein weiteres Gen dieses eisenfreien Furregulons ist das *sodB* Gen, dessen genaue Regulation in Kapitel 4 beschrieben ist. Superoxiddismutasen bauen Sauerstoffradikale in der Zelle ab. Mit Hilfe von DNA-Protein Interaktionsassays konnte gezeigt werden, dass *sodB* durch Fur direkt reguliert wird, allerdings bindet Fur nur mit geringer Affinität. Da SodB die einzige Superoxiddismutase in *Helicobacter pylori* ist, und deshalb nur unter extremem Eisenmangel abgeschaltet werden sollte, ist die geringe Bindungsaffinität von Fur an die Promoterregion von *sodB* sinnvoll. Die direkte Regulation von *sodB* in *Helicobacter pylori* unterscheidet sich von der indirekten Regulation in *Escherichia coli* und stellt somit eine weitere Möglichkeit dar, Superoxiddismutasen zu regulieren.

Die Regulation durch NikR wurde bisher nur in *Escherichia coli* auf molekularer Ebene charakterisiert. Abhängig von der intrazellulären Nickelkonzentration bindet NikR freie Nickelionen, und bindet daraufhin an die Promoterregion des Nickelaufnahmesystems *nikABCDE*,

welches dann abgeschaltet wird.

Auch *Helicobacter pylori* besitzt ein NikR Regulatorprotein. Es wurde gezeigt, dass NikR bei Aktivierung des Ureaseenzyms beteiligt ist. Um den Bedarf an Nickel zu decken, besitzt *Helicobacter pylori* das Aufnahmeprotein NixA, welches Nickel schon in kleinen Konzentrationen aufnehmen kann. In Kapitel 5 wird gezeigt, dass NikR sowohl Aktivator als auch Repressor der Transkription in *Helicobacter pylori* sein kann. NikR unterbindet die Transkription von *nixA*, wenn die intrazelluläre Nickelkonzentration (zu) hoch ist. Dabei bindet NikR an die Region -10 bis +1 (der Bindungsstelle für die RNA-Polymerase; es wird angenommen, dass NikR mit der RNA-Polymerase um die gleiche Bindungstelle konkurriert und die Transkription dadurch unterbindet). Weiterhin aktiviert NikR die Transkription der Ureasegengruppe. Ist die Nickelionenkonzentration hoch in der Zelle, bindet NikR an Sequenzen oberhalb der Promoterregion des *ureA* Gens, zwischen den Nukleotiden -50 bis - 100, und aktiviert so die Transkription der Urease. Je nachdem, an welcher Stelle des Promoters NikR bindet, kann NikR so die Tanskription unterbinden oder aktivieren.

Interessanterweise ist NikR auch an der Regulation des Eisenmetabolismus beteiligt (Kapitel 6). Die Gene für die Eisenaufnahmeproteine FrpB3 und FecA3 sind nickel-abhängig reguliert, durch direktes Binden von NikR an die +1 oder -10 Region in beiden Genen. Da Eisenaufnahme durch die äussere Membran energieabhängig ist, dort aber keine Möglichkeit vorhanden ist, Energie zu gewinnen, erhalten FrpB3 und FecA3 die benötigte Energie durch den Proteinkomplex TonB-ExbB-ExbD, welcher sich in der inneren Membran befindet. Weiterhin wird gezeigt, dass auch die Gene dieses TonB-Komplexes nickel- und NikR-abhängig reguliert werden. Dies steht im Gegensatz zur Regulation der *frpB1/2* und *fecA1/2* Gene, welche Eisen- und Fur-abhängig reguliert werden. Eine mögliche Erklärung der nickel-abhängigen Regulation von Eisenaufnahmegenen ist, dass dies eine Feinregulierung des Eisen- und Nickelstoffwechsels darstellt. Eine andere Erklärung wäre, dass FrpB3 und FecA3 gar keine Eisen-, sondern Nickelaufnahmesysteme sind, da die genaue Funktion von FrpB und FecA nur durch Homologie, nicht aber experimentell beschrieben worden ist.

Kapitel 7 ist ein Review, in dem ein Model, welches die nickel- und NikR-abhängige Regulation gegen Säurestress beschreibt, zusammenfasst ist. Dabei wird davon ausgegangen, dass die Konzentration an Nickelionen mit dem pH-Wert korreliert: je mehr verfügbare Nickelionen vorhanden sind, desto saurer ist das Medium. Bei neutralem pH werden nur wenige Nickelionen durch NixA in die Zelle transportiert; die nickel-abhängige Urease ist nur teilweise aktiviert. Bei veringertem pH-Wert steigt die Konzentration durch NixA transportierter Nickelionen in der Zelle an, dadurch werden alle verfügbaren Ureaseenzyme aktiviert. Bei einem weiteren Abnehmen des pH Wertes, also einer weiteren Aufnahme von Nickelionen, bindet NikR freie Nickelionen. Dieser NikR-Nickel-Komplex reguliert dann seine Zielgene: die Transkription von *nixA* wird gestoppt, um einen weiteren Anstieg der Nickelionenkonzentration zu verhindern, und die Transkription von *ureA* wird aktiviert, um die Zelle gegen die Säure zu beschützen. Bei einem weiteren Anstieg der intrazellulären Nickelkonzentration bindet der NikR-Nickel-Komplex an den Promoter des *fur*-Gens und wahrscheinlich noch an die Promotoren der *hspR* and *hp0166* Regulator Gene, um dadurch den Eisenmetabolismus und andere Stoffwechselreaktionen zu regulieren.

Der Gebrauch von wenigen Regulatorproteinen, die überlappende Aufgaben erledigen, steht im Gegensatz zu vielen anderen Bakterien, in denen Fur und NikR nur Aufgaben des Eisen- und Nickelmetabolismus regulieren. Der Gebrauch dieser Strategie trägt dazu bei, dass *Helicobacter pylori* lebenslang die menschliche Magenschleimhaut kolonisieren kann.

Samenvatting

Ruim de helft van de mensheid is besmet met *Helicobacter pylori*, een bacterie die het maagslijmvlies van de mens koloniseert. Een besmetting met *Helicobacter pylori* leidt tot ontsteking van het maagslijmvlies, welke zich kan doorontwikkelen tot maagzweren of maagkanker. Om in het maagslijmvlies te kunnen overleven maakt *Helicobacter pylori* gebruik van het enzym urease, dat ureum omzet naar ammonium en kooldioxide, en deze stoffen stellen de bacterie in staat zich tegen het zuur van de maag te beschermen. Aktief urease heeft nikkel nodig als cofactor. Omdat 5-10% van alle intracellulaire geproduceerde eiwitten uit urease bestaat, heeft *Helicobacter pylori* een grote hoeveelheid nikkel ionen nodig.

Alle levende organismen hebben metaal ionen nodig, zoals b.v. ijzer of nikkel. Deze metalen zijn betrokken bij de respiratie of worden gebruikt als cofactor voor enzymen. Echter, de concentratie van ionen in de cel luistert heel nauw: als er te weinig metaalionen zijn, zijn veel enzymen niet meer actief; maar zijn er te veel metaalionen in de cel dan kunnen deze met zuurstof reageren tot radicalen, en deze kunnen op hun beurt macromolekulen als eiwitten en DNA in de cel beschadigen. Daarom is de regulatie van het metaalmetabolisme van groot belang. *Helicobacter pylori* heeft weinig eiwitten die betrokken zijn bij genregulatie, en maar twee daarvan zijn metaalafhankelijke regulatoren: het ijzer-afhankelijke regulatoreiwit Fur (Ferric Uptake Regulator) en het nikkel-afhankelijke regulatoreiwit NikR (Nikkel-responsive Regulator).

Het doel van dit proefschrijft was om een meer inzicht in de regulatiemechanismen van *Helicobacter pylori* Fur en NikR te krijgen.

De ijzer-afhankelijke regulator Fur herkent en bindt ijzer ionen, om daarna aan specifieke promoter regios (zogenaamde Fur boxen) van zijn doelwitgenen te binden. De klassieke regulatie door Fur is een repressie van ijzeropname genen in condities waar ijzer voldoende beschikbaar is, waarmee een verdere toename van de ijzerconcentratie voorkomen wordt. Verschillende onderzoeken hebben laten zien dat *Helicobacter pylori* Fur niet alleen maar het ijzer metabolisme reguleert, maar ook betrokken is bij resistentie tegen zuur en bescherming tegen zuurstofradikalen. Een karakterisatie van alle ijzer- en Fur-gereguleerde genen is in Hoofdstuk 3 met behulp van array-analyses beschreven. Er zijn 32 Fur-afhankelijke gereguleerde genen geïdentificeerd waaronder genen van het ijzer- en stikstofmetabolisme, de chemotaxis en beweeglijkheid, maar ook van de celwand- en cofactorbiosynthese. Door het Fur regulator eiwit in te zetten bij de controle van verschillende taken, kan *Helicobacter pylori* ondanks de kleine hoeveelheid van regulatoreiwitten de ongastvrije omgeving van het menselijke maagslijmvlies toch succesvol koloniseren.

Het is uniek dat in *Helicobacter pylori* Fur ook in staat is om in de ijzervrije vorm aan specifieke Fur boxen te binden, wat blijkt uit onderzoek van het ferritin gen *pfr* en in hoofdstuk 4 voor het superoxide dismutase gen *sodB*. Superoxide dismutase is een enzym dat zuurstofradikalen in de cel onschadelijk maakt. Experimenten op transcriptioneel en translationeel niveau tonen aan dat het *sodB* gen door de ijzervrije vorm van Fur wordt gereguleerd (hoofdstuk 4). Met behulp van DNA-eiwitinteractie assays was het mogelijk om te laten zien dat Fur het *sodB* gen direct reguleert. De affiniteit van Fur voor de promoter regio van het *sodB* gen is zeer laag. Deze regulatie heeft mogelijk zin omdat SodB het enige superoxide dismutase in *Helicobacter pylori* is, en daarom mag het alleen in een extreem tekort aan ijzer worden uitgeschakeld. De directe regulatie van *sodB* in *Helicobacter pylori*

verschilt van de indirecte regulatie beschreven in de veel bestudeerde darmbacterie *Escherichia coli*, en zo ontstaat een verdere mogelijkheid om superoxidedismutase te reguleren.

De regulatie door NikR was tot nu toe alleen maar gekarakteriseerd op moleculair niveau in *Escherichia coli*. In deze bacterie controleert NikR de opname van nikkel, doordat NikR vrije nikkelionen kan binden. Zodra dit NikR-nikkel complex is gevormd, kan NikR aan de promoterregio van het nikkel opname systeem *nikABCDE* binden, en daarmee de transcriptie blokkeren.

Helicobacter pylori bezit ook een NikR regulator eiwit. Eerder is aangetoond dat *Helicobacter pylori* NikR betrokken is bij de activering van het urease enzym. Om aan de behoefte voor nikkel te voorzien heeft *Helicobacter pylori* het opnameeiwit NixA, welke nikkel al in zeer lage concentraties kan opnemen. In hoofdstuk 5 is aangetoond, dat in *Helicobacter pylori* NikR zowel activator als repressor van transcriptie kan zijn, een eigenschap die nog niet eerder voor dit type eiwit is beschreven. NikR onderbreekt de transcriptie van *nixA*, als de intracellulaire nikkelconcentratie (te) hoog is. Deze blokkade vindt plaats als NikR bindt aan de regio van -10 tot +1 in de promoter, want dit is ook de plek waar het RNA-polymerase bindt. Daardoor concurreren NikR en het RNA-polymerase voor dezelfde bindingspositie, als NikR eenmaal gebonden heeft dan wordt de transcriptie onderbroken. Verder activeert NikR de transcriptie van het urease systeem. Als de nikkel concentratie in de cel hoog genoeg is, dan bindt NikR aan sequenties in de promoterregio van het *ureA* gen. Deze positie is voor de *ureA* promoter gelegen, namelijk tussen de nucleotiden -50 tot - 100. Binding van NikR activeert zo de transcriptie van het urease systeem. Dit suggereert dat afhankelijk van de promoterpositie, NikR de transcriptie kan onderbreken of activeren.

Verder maakt in *Helicobacter pylori* NikR ook deel uit van de regulatie van het ijzer metabolisme (hoofdstuk 6). De genen die coderen voor de ijzeropname eiwitten FrpB3 en FecA3 worden gereguleerd door NikR, afhankelijk van nikkel. Dit gebeurt door directe binding van NikR aan de -10 promoter regio van allebei de genen, een systeem vergelijkbaar met het *nixA* gen (Hoofdstuk 5). IJzeropname door de buiten membraan vereist energie, en dat wordt aan FrpB3 en FecA3 geleverd via het TonB-ExbB-ExbD eiwitcomplex. Ook deze *tonB-exbB-exbD* genen zijn nikkel- en NikR-afhankelijk gereguleerd. Dit is een in bacteriën niet eerder beschreven situatie, daar deze componenten meestal door het Fur eiwit worden gereguleerd. Een mogelijke verklaring van de nikkel afhankelijke regulatie van de FecA3 en FrpB3 ijzer opname genen is, dat dit een fijnregulering van het ijzer- en nikkelmetabolisme weerspiegelt. Een andere verklaring is dat FrpB3 en FecA3 geen ijzer opname systemen zijn, maar nikkel opname systemen zijn.

Hoofdstuk 7 is een overzichtsartikel waarin een model wordt gepresenteerd waar NikR de regulator is van de respons tegen zuur. Uitgangspunt is een verband tussen de concentratie van nikkel ionen en de zuurgraad: hoe meer vrije nikkel ionen, hoe zuurder het medium. Bij een neutrale pH worden maar weinig nikkel ionen door NixA in de cel getransporteerd; het nikkel afhankelijke urease is maar ten dele geactiveerd. Bij een lagere pH stijgt de concentratie van de door NixA getransporteerde nikkel-ionen in de cel aan, en inactief (reserve) urease enzym wordt geactiveerd. Neemt de pH-waarde verder af, dan bindt het NikR vrije nikkel ionen in de cel. Dit NikR-nikkelcomplex reguleert dan zijn doelgenen: de transcriptie van *nixA* wordt gestopt om een verdere stijging van de nikkel ionen concentratie te vermijden. De transcriptie van het urease systeem wordt geactiveerd om de cel tegen het

zuur te kunnen beschermen. Bij een verdere stijging van de intracellulaire nikkel concentratie bindt het NikR-nikkel complex ook aan de promoter van het *fur* gen, en waarschijnlijk ook aan de promotoren van de *hspR* en *hp0166* regulator genen. Daardoor wordt het ijzer metabolisme en andere stofwisseling reacties gereguleerd.

Helicobacter pylori gebruikt relatief weinig regulator eiwitten, die overlappende opdrachten uitvoeren, wat een groot verschil is met verschillende andere bacteriën waar Fur en NikR alleen maar opdrachten van het ijzer- en nikkel metabolisme reguleren. Beide regulatoreiwitten zijn noodzakelijk voor kolonisatie van de maag, en het gebruik van overlappende regulatie systemen levert dus een belangrijke bijdrage aan de levenslange kolonisatie van het maagslijmvlies door *Helicobacter pylori*.

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Curriculum Vitae

Florian Ernst was born in 1975 in Lörrach, situated in the complete south-western part of Germany, next to Swizerland and France. There he also completed his school career and performed one year of civil service St Katarina nursing home. Subsequently, from 1997-2002, he studied biology with the main subjects molecular biology, biochemistry and ecology at the Ernst-Moritz-Arndt University, Greifswald, situated in the north-eastern part of Germany. His diploma thesis on transcriptional regulation of iron metabolism in *Helicobacter pylori* was supervised by Dr. Georg Homuth and Prof. Dr. Michael Hecker. After his diploma the author continued his research on *Helicobacter pylori*, however moved to Rotterdam at the Deparment of Gastroenterology and Hepatology of the Erasmus MC-University Medical Center to complete this PhD thesis under supervision of Prof Dr. Ernst Kuipers, Dr. Arnoud H. M. van Vliet and Dr. Johannes G. Kusters.

List of Publications

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