Iron-Responsive Repression of Urease Expression in *Helicobacter hepaticus* Is Mediated by the Transcriptional Regulator Fur[▽]

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Persistent colonization of mucosal surfaces by bacteria in the mammalian host requires concerted expression of colonization factors, depending on the environmental conditions. *Helicobacter hepaticus* is a urease-positive pathogen that colonizes the intestinal and hepatobiliary tracts of rodents. Here it is reported that urease expression of *H. hepaticus* is iron repressed by the transcriptional regulator Fur. Iron restriction of growth medium resulted in a doubling of urease activity in wild-type *H. hepaticus* strain ATCC 51449 and was accompanied by increased levels of urease subunit proteins and *ureA* mRNA. Insertional inactivation of the *fur* gene abolished iron-responsive repression of urease activity, whereas inactivation of the *perR* gene did not affect iron-responsive regulation of urease activity. The iron-responsive promoter element was identified directly upstream of the *H. hepaticus ureA* gene. Recombinant *H. hepaticus* Fur protein bound to this *ureA* promoter region in a metal-dependent manner, and binding resulted in the protection of a 41-bp, Fur box-containing operator sequence located at positions –35 to –75 upstream of the transcription start site. In conclusion, *H. hepaticus* Fur controls urease expression at the transcriptional level in response to iron availability. This represents a novel type of urease regulation in ureolytic bacteria and extends the already diverse regulatory repertoire of the Fur protein.

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

Helicobacter species colonize the mammalian gastrointestinal and hepatobiliary tracts, resulting in chronic inflammation, which may progress to ulceration and carcinogenesis (21, 25). The murine pathogen *Helicobacter hepaticus* colonizes the intestine, bile ducts, and liver, and this can result in hepatitis, hepatic malignancies, and possibly cholesterol gallstones (22, 29, 50, 51). The urease enzyme is an important virulence factor in gastric *Helicobacter* species such as *Helicobacter pylori* and *Helicobacter mustelae* (2, 15, 34), but a contribution of urease in the development of *H. hepaticus*-associated disorders is yet to be demonstrated. We have previously demonstrated that urease expression in *H. hepaticus* is neither nickel or pH responsive nor growth phase regulated (5). This contrasts with the situation in *H. pylori*, where urease expression is controlled by nickel and pH at the transcriptional, posttranscriptional, and translational levels (1, 39, 43, 44). Although urease expression in

H. hepaticus is lower than in *H. pylori* (5, 30), it is still relatively high compared to many other urease-positive pathogens (10). In view of the metabolic cost of urease expression and the importance of the enzyme in other bacteria, we hypothesized that urease expression in *H. hepaticus* is also regulated but may be responsive to a different environmental signal.

Iron is an important micronutrient and is required by almost all organisms (37). Iron is used as a cofactor of enzymes and participates in redox reactions. In the mammalian host, iron is mostly complexed into host proteins such as hemoglobin and ferritin, whereas at mucosal surfaces, iron availability is restricted due to chelation of iron by lactoferrin (37). Conversely, pathogenic bacteria often use iron restriction as an environmental cue for the expression of virulence factors such as hemolysins, toxins, and iron acquisition systems (38). Many of these iron-responsive virulence factors are under the control of the ferric uptake regulator (Fur) protein. Fur acts as a transcriptional repressor of iron-regulated promoters via iron-dependent DNA-binding activity (20). Under iron-replete conditions, Fur complexes with Fe²⁺ can recognize and bind to operator sequences (Fur boxes) in promoters. When iron is scarce, Fur dissociates from the DNA, allowing access of RNA polymerase to the promoter and transcription of the iron-regulated genes. The functions of Fur even extend outside iron metabolism, as the protein has also been reported to regulate oxidative stress resistance, acid resistance, and virulence factors, suggesting a key role for this protein in chronic colonization by pathogenic bacteria (7, 14, 16, 17, 23, 28).

In this study it is demonstrated that *H. hepaticus* uses iron as a signal for the regulation of urease expression and that this iron-responsive regulation of urease is mediated by the binding of the Fur protein to an operator sequence in the *H. hepaticus*

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TABLE 1. Oligonucleotide primers used in this study

Primer	Sequence (5'→3') ^a
HhureA-F1	TGCATTATGCTGGGGCACTA
HhureA-DIG	GTGCCCCAGCATAATGCAAC
Hhfur-mutF1	AGAATCGCAATCCTAAAAGC
Hhfur-mutR1	TATTGCCACAAAGCGGTGTC
Hhfur-outF1	GTGCTTTGCTGGAACCAAC
Hhfur-outR1	CCAAGAATGGCGACTACTAC
HhperR-mutF1	TTGGAGAATCTTTAGAG
HhperR-mutR1	GGAGCATTTACATATTCTG
HhperR-outF1	ATCTTGGAGCGCATTTATGA
HhperR-outR1	CTCACGCGAGATGATTGTAG
Hhfur-overF1	GGATCCATGAATGAATGCAAATCA
	TCGCTGGAA
Hhfur-overR1	CTGCAGCCTTTAGAGTCCTTGACATT
HhksgA-F1	GCGTGATAGATTCTGTCGTG
HhksgA-DIG	TCGCTTTACCGAACTTTCAAC

^a Primer sequences were derived from the *H. hepaticus* ATCC 51449 genome sequence (41).

ureA promoter. To our knowledge, this is the first demonstration of a direct role of Fur and iron in the regulation of urease expression; thus, this represents a novel function for Fur in the regulation of metabolic pathways and colonization factors.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *H. hepaticus* strain ATCC 51449 (22, 41) and its isogenic *fur* and *perR* mutants (this study) were routinely cultured at 37°C under microaerobic conditions (10% CO₂, 5% O₂, and 85% N₂) on Dent agar (Oxoid) (44). Liquid growth was performed in brucella broth (Difco) supplemented with 0.2% (wt/vol) β-cyclodextrins (Fluka) (BBC). Cultures were inoculated with a starting optical density at 600 nm (OD₆₀₀) of 0.05 and were shaken at 40 rpm. For growth under iron-restricted conditions, 20 μM desferoxamine mesylate (Desferal; Sigma) was added to brucella broth before addition of the cyclodextrins. Iron-replete media were prepared by the addition of 100 μM FeCl₃ (Sigma) to the iron-restricted medium. In case of transcriptional analyses (primer extension analysis), iron restriction was achieved by the addition of 2,2'-dipyridyl (Sigma) or 2,2'-dipyridyl and FeCl₃ to final concentrations of 100 μM to an overnight culture of *H. hepaticus*, followed by incubation for 30 min at 37°C (14). The reason for the use of different chelators was that *H. hepaticus* RNA isolated after overnight growth in the presence of Desferal was of lower quality than RNA isolated from cells incubated with dipyridyl (data not shown) and could not be used for primer extension analysis, whereas incubation with 2,2'-dipyridyl was too short to result in phenotypic changes (protein profile and urease activity). Both chelators have been used previously for iron restriction in *H. pylori* and gave similar results for iron-responsive regulation of iron uptake and iron storage genes (6, 14, 16). Incubation of *H. hepaticus* with 2,2'-dipyridyl resulted in the absence of growth, probably due to the toxicity of the chelator (data not shown). *Escherichia coli* strains ER1793 and DH5α were grown aerobically in Luria-Bertani medium (36) at 37°C. When indicated, growth media were supplemented with chloramphenicol and ampicillin to final concentrations of 20 μg/ml and 100 μg/ml, respectively.

Urease enzyme assay. Urease enzyme activity was determined in freshly sonicated lysates by measuring ammonia production from the hydrolysis of urea, as described previously (44). The concentration of ammonia in the samples was inferred from a standard NH₄Cl concentration curve. Enzyme activity was expressed as micromoles of urea substrate hydrolyzed per minute per milligram of protein.

Primer extension analysis. RNA was isolated from *H. hepaticus* with TRIzol reagent (Invitrogen), according to the manufacturer's instructions. Primer extension analyses were performed with the reverse primer HhureA-DIG (Table 1) essentially as described previously (17). The digoxigenin-labeled primer was annealed to 10 μg of total RNA from *H. hepaticus* strain ATCC 51449, and cDNA was synthesized after the addition of 5 units of avian myeloblastosis virus reverse transcriptase (Promega) and incubation for 1 h at 42°C. The cDNA product was separated on a 7% acrylamide–8 M urea sequencing gel and then blotted onto a nylon membrane (Roche), followed by chemiluminescent digoxi-

genin (DIG) detection (16). A sequencing reaction made with the same primer was used to determine the transcriptional start site.

Construction of *H. hepaticus fur* and *perR* mutants. The *fur* gene of *H. hepaticus* strain ATCC 51449 was amplified with primers Hhfur-mutF1 and Hhfur-mutR1 (Table 1) and cloned in pGEM-T Easy (Promega), resulting in plasmid pCB1. The *fur* gene was subsequently interrupted by insertion of the chloramphenicol resistance gene from pAY35 (47) in the unique BsmI site, resulting in plasmid pCB2. The *perR* gene of *H. hepaticus* strain ATCC 51449 was amplified with primers HhperR-mutF1 and HhperR-mutR1 (Table 1) and cloned in pGEM-T Easy (Promega), resulting in plasmid pCB3. The *perR* gene was subsequently interrupted by insertion of the chloramphenicol resistance gene from pAV35 (47) in the unique HindIII site, resulting in plasmid pCB4. Both pCB2 and pCB4 were first introduced into *E. coli* ER1793, and plasmids isolated from *E. coli* ER1793 were subsequently used for natural transformation of *H. hepaticus* 51449 (5). Chloramphenicol-resistant colonies isolated were designated 51449*fur* and 51449*perR*, respectively. Two colonies derived from independent transformations were tested, and both colonies gave identical results in all experiments. Correct allelic replacement of the wild-type *fur* and *perR* genes with the interrupted version was confirmed by PCR using combinations of the primers Hhfur-outF1/Hhfur-outR1 and HhperR-outF1/HhperR-outR1, respectively (Table 1).

Protein analysis. *H. hepaticus* ATCC 51449 was grown for 24 h in iron-restricted and iron-replete BBC, centrifuged at 4,000 × g for 10 min at room temperature, and resuspended in phosphate-buffered saline, pH 7.4, to a final OD₆₀₀ of 10. *H. hepaticus* cells were subsequently lysed by sonication for 15 s on ice with an MSE Soniprep 150 at amplitude 6. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% (wt/vol) polyacrylamide gel and stained with Coomassie brilliant blue. Immunoblotting was performed after electrotransfer of proteins from a 10% SDS-polyacrylamide gel to a nitrocellulose membrane (Roche). The blot was subsequently probed with antibodies raised in rabbits to *Helicobacter felis* UreA or UreB (Intervet International BV, Boxmeer, The Netherlands) (5). Bound antibodies were visualized with swine anti-rabbit antibodies labeled with alkaline phosphatase (Promega), with BCIP (5-bromo-4-chloro-3-indolylphosphate) and Nitro Blue Tetrazoleum (Promega) used as a substrate (5).

Recombinant expression of *H. hepaticus Fur* protein. The *fur* gene was amplified from *H. hepaticus* ATCC 51449 with primers Hhfur-overF1 and Hhfur-overR1 (Table 1). The resulting fragment was digested with BamHI and PstI and ligated into pASK-IBA7 (IBA, Gottingen, Germany) to create pCB10. The wild-type sequence of the *fur* gene was confirmed by DNA sequencing. *H. hepaticus Fur* was expressed with an N-terminal Streptag, which has been used with the *H. pylori* Fur, NikR, and CrdR proteins and which does not influence DNA-binding activity (17, 18, 48, 49); therefore, the Streptag was not removed prior to use. The recombinant protein was purified as described in the manufacturer's instructions (48). The recombinant protein was over 90% pure, as determined by staining with Coomassie brilliant blue following electrophoresis on 12% SDS-polyacrylamide gels. Purified protein was dialyzed and stored in 50 mM Tris (pH 8.0), 100 mM NaCl, 1 mM MgCl₂, 2 mM dithiothreitol (DTT), and 50% glycerol and was used without further purification for electrophoretic mobility shift and DNase I footprinting assays.

Electrophoretic mobility shift assays. DIG-labeled *ureA* promoter DNA was amplified with primers HhureAF1 and HhureA-DIG (Table 1). As a negative control, the promoter of the *ksgA* gene (HH1174) (41) was amplified with primers HhksgA-F1 and HhksgA-DIG (Table 1). The binding buffer used for the gel shift assays contained 24% glycerol, 40 mM Tris (pH 8.0), 150 mM KCl, 2 mM DTT, 600 μg/ml bovine serum albumin, and 1 μg denatured salmon sperm DNA. Gel shift assays were performed with 0.5 nM DIG-labeled *ureA* or *ksgA* promoter DNA, which was mixed with increasing concentrations of recombinant Fur protein and was subsequently incubated for 30 min in binding buffer at 37°C. Reactions were separated for 35 min at 200 V on an 8% polyacrylamide gel. The assay was performed in the presence of either 200 μM MnCl₂ or 200 μM EDTA in binding buffer and PAGE buffer (2.5 M Tris and 0.19 M glycine). Gels were blotted onto a positively charged nylon membrane (Roche), followed by chemiluminescent DIG detection (44).

DNase I footprinting assays. DNase footprint assays were performed with 500 nM recombinant Fur protein and 50 nM DIG-labeled *ureA* promoter DNA or different concentrations of recombinant Fur in combination with 20 nM DIG-labeled *ureA* promoter DNA. Binding buffer consisted of 50 mM Tris (pH 8.0), 250 mM NaCl, 50 mM KCl, 5 mM DTT, 0.5% NP-40, 50% glycerol, and 100 μg/ml denatured salmon DNA in the presence or absence of 200 μM MnCl₂. DNase I digestion was carried out by the addition of 0.25 units of DNase I (Promega) for 1 minute at room temperature, and the reaction was stopped by the addition of 2.5 M sodium acetate, 20 mM EDTA,

FIG. 1. Induction of *H. hepaticus* urease transcription, expression, and activity under iron-restricted conditions. (A) *H. hepaticus* ATCC5 1449 was grown in standard BBC (gray squares), iron-restricted medium (white diamonds), and iron-replete medium (black triangles), and the OD₆₀₀ was monitored over a time period of 36 h. The arrow indicates the 24-h time point, representing late log phase, which was chosen for comparison of iron-restricted and iron-replete conditions. (B) Urease activity in *H. hepaticus* is iron repressed, as measured after 24 h of growth in iron-restricted medium (white bar) or iron-replete medium (black bar). Each bar represents data from seven independent experiments; error bars denote standard deviations. An asterisk indicates a significant increase in urease activity after growth under iron-restricted conditions compared to growth under iron-replete conditions ($P < 0.05$ [Mann-Whitney U test]). (C) The increase in *H. hepaticus* urease activity under iron-restricted conditions is associated with increased expression of the UreA and UreB enzyme subunits, as shown by SDS-PAGE (left panel) and immunoblotting (right panel) with antibodies to *H. felis* urease. The left panel displays the relevant section of the protein profile of the lysates used for immunoblots, stained with Coomassie brilliant blue. Relevant marker sizes are indicated on the left. (D) Identification of the *H. hepaticus* ureA transcription start site by primer extension analysis using RNA purified from *H. hepaticus* ATCC 51449 grown under iron-restricted (–Fe) and iron-replete (+Fe) conditions. The sequence of the corresponding promoter region is displayed on the right, with the +1 residue and the –10 promoter sequence indicated. Note that the primer extension product displays iron-responsive repression of transcription.

and 10 μ g denatured salmon DNA. Subsequently the DNA was ethanol precipitated and resuspended in 6 μ l of loading buffer (0.05% bromophenol blue [Sigma] and 0.05% xylene cyanol [Merck] in deionized formamide [Sigma]), denatured, and separated on a 7% polyacrylamide gel containing 8 M urea. Gels were blotted onto a positively charged nylon membrane (Roche), followed by chemiluminescent DIG detection (44).

RESULTS

Induction of *H. hepaticus* urease expression and activity by iron restriction. The effect of iron on *H. hepaticus* growth was determined by growing bacteria in BBC (standard con-

ditions) or BBC supplemented with either desferoxamine mesylate (iron-restricted conditions) or desferoxamine mesylate and 100 μ M FeCl₃ (iron-replete conditions). Iron restriction resulted in reduced growth of *H. hepaticus*, and cells entered late log phase between 16 and 24 h of growth (Fig. 1A). In contrast, growth under standard conditions and iron-replete conditions saw cells in late log phase after approximately 24 h. At 24 h, cells were still viable, and examination of cells by Gram staining indicated that after 24 h in iron-restricted and iron-replete conditions the cultures still

contained >95% bacillary forms of *H. hepaticus*. Changes in gene expression after 24 h are therefore likely to be a result of changes in iron availability rather than being growth phase dependent (16, 31); thus, we decided to use the 24-h time point for phenotypic analyses.

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

Fur, but not PerR, mediates iron-responsive regulation of *H. hepaticus* urease. Since iron-responsive regulation of urease expression is mediated at the transcriptional level, this suggested the involvement of an iron-responsive regulatory protein. The *H. hepaticus* genome encodes two orthologs of such regulators: the peroxide stress regulator PerR (HH0942) and the iron-responsive regulator Fur (HH0893) (41). Both genes are predicted to be transcribed as a monocistronic mRNA (41); thus, mutation of these genes is unlikely to result in polar effects on surrounding genes. To determine whether Fur or PerR was involved in the iron-responsive regulation of urease expression, isogenic *H. hepaticus fur* and *perR* mutants were created by insertion of a chloramphenicol resistance gene.

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

Fur binds to the *H. hepaticus ureA* promoter in a metal-dependent manner. To determine whether the Fur-mediated

FIG. 2. Fur, but not PerR, mediates iron-responsive regulation of urease expression in *H. hepaticus*. Urease activity of *H. hepaticus* strain ATCC 51449 and the *fur* and *perR* mutants was assessed after 24 h of growth in iron-restricted medium (white bars) or iron-replete medium (black bars). Each bar represents data from three independent experiments for each strain; error bars denote standard deviations. An asterisk indicates a significant increase in urease activity after growth under iron-restricted conditions compared to growth under iron-replete conditions ($P \leq 0.05$ [Mann-Whitney U test]); NS, not significant.

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

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

DISCUSSION

Urease is an important colonization and virulence factor for many pathogenic bacteria (8). In view of both the possible deleterious effects of uncontrolled ammonia production and

FIG. 3. *H. hepaticus* Fur displays metal-dependent binding to the *ureA* promoter. Shown is an electrophoretic mobility shift assay using recombinant *H. hepaticus* Fur protein and DIG-labeled *ureA* promoter DNA (P_{ureA}). In the absence of the iron substitute manganese (+EDTA, middle panel), Fur is unable to complex with the *ureA* promoter region and a shift is not observed. Only in the presence of manganese (+MnCl₂, top panel) is Fur able to bind to the *ureA* promoter region and cause a mobility shift (indicated as P_{ureA} + Fur). No shift is observed when the Fur protein is incubated with the promoter region of the *ksgA* gene (P_{ksgA}) in the presence of manganese (+MnCl₂, bottom panel). The concentration of Fur is indicated above the lanes; the concentration of DNA was 0.5 nM *ureA* or *ksgA* promoter DNA.

the metabolic cost of urease production, expression and activity of urease are often controlled by specific environmental signals. These include substrate availability, nitrogen status of the cell, growth phase, and pH (1, 8, 11, 33). *Helicobacter* species are known to have some of the highest urease activities among ureolytic bacteria studied. This can be accompanied by urease protein expression reaching up to 10% of the soluble protein fraction in *H. pylori* (3). With the exception of pH, the aforementioned regulatory mechanisms of urease expression are absent in the few tested *Helicobacter* species (5, 9, 39). To date, only the molecular mechanisms governing urease activity in *H. pylori* have been reported, where urease expression is regulated at the transcriptional and posttranscriptional levels by nickel and pH (1, 44), with transcriptional regulation being controlled via the NikR and HP0166 (ArsR) regulatory proteins (13, 18, 35, 43, 45).

Urease activity of *H. hepaticus* is lower than that of *H. pylori* but still reaches high levels compared to many ureolytic bacterial species (5, 8, 10, 30). We previously demonstrated that the urease system in *H. hepaticus* is nickel responsive at the enzyme activity level (5) but not at the transcriptional level. The *H. hepaticus* genome encodes three orthologs of metal-responsive regulatory proteins that are all at different locations

FIG. 4. Identification of the Fur operator sequence in the *H. hepaticus* ATCC 51449 *ureA* promoter. (A) DNase I footprinting assay performed in the presence and absence of the iron substitute MnCl₂, using 50 nM DIG-labeled *H. hepaticus* ATCC 51449 *ureA* promoter DNA and 500 nM Fur. On the left side are indicated the positions relative to the *ureA* transcription start site. On the right-hand side is indicated the position of the protected region (located from -35 to -75 relative to the transcription start site). Note that a protected region is observed only in the presence of both Fur and MnCl₂. (B) Binding of *ureA* promoter DNA is dependent on the Fur concentration. Increasing concentrations of Fur were mixed with 20 nM DIG-labeled *H. pylori* ATCC 51449 *ureA* promoter in the presence of 200 μM MnCl₂. The concentration of Fur protein used is indicated above the lanes. Positions relative to the *ureA* transcription start site are indicated on the left; the protected region is indicated on the right.

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

in the genome: the nickel-responsive regulator NikR (HH0352), the peroxide stress regulator PerR (HH0942), and the iron-responsive regulator Fur (HH0893) (41). The absence of nickel-responsive transcriptional regulation of urease in *H. hepaticus* (5) already suggested that NikR is not involved in urease regulation. Since the regulatory repertoire of *H. hepaticus* is relatively limited (41), we hypothesized that one of the other metal-regulatory proteins could be responsible for urease regulation in *H. hepaticus*. Here it is reported that *H. hepaticus* uses iron restriction as a signal for the induction of urease transcription, expression, and activity (Fig. 1); that PerR is not involved in urease regulation (Fig. 2); and that the iron-responsive transcriptional regulator Fur directly mediates this process (Fig. 2 to 4). Both *fur* and *perR* are predicted to be transcribed as a monocistronic mRNA; thus, the observed phenotype of the *fur* mutant is unlikely to result from polar effects of the insertion of the chloramphenicol resistance cassette. In addition, both the *fur* and *perR* genes are surrounded by genes annotated as hypothetical proteins, without any predicted function in urease activity or metal metabolism (41).

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

The Fur protein has also been described to be involved in

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

H. hepaticus urease activity is not completely repressed by iron, as even under iron-replete conditions there is urease activity (Fig. 1A), and the *fur* mutant does not show a complete derepression of urease activity (Fig. 2). This could be due to a role of other regulatory systems in urease expression, as in *H. pylori* (35). Alternatively, binding of Fur may result in only incomplete or intermediate repression. The region protected from DNase I digestion in the footprint assay is located from -35 to -75 and thus is located just on the edge of the canonical σ^{80} promoter sequence of the *ureA* promoter. This may be an indication of the presence of a binding site for an additional regulatory system upstream of the *ureA* promoter, as has been described for NikR and ArsR in the *H. pylori ureA* promoter (13, 18, 35). Alternatively, binding of Fur may only reduce but not completely block access of RNA polymerase. However, it should be noted that urease activity is only an indirect readout and is subject to regulation at the posttranscriptional or post-translational level (5). The demonstration of sequence-specific binding of Fur to the *ureA* promoter (Fig. 3 and 4) confirms the direct role of Fur in urease regulation in *H. hepaticus*. The *ureA* operator sequence bound by Fur contains a putative Fur box (Fig. 5B) which matches well with the proposed consensus sequence (20, 26, 42).

The known environmental signals used for regulation of urease expression or activity are all somehow involved in the urease reaction or downstream processes, be it enzyme cofactor, substrate availability, nitrogen status, or pH. The link between iron and urease is not directly apparent, as iron is not involved in any of these pathways, with the exception of iron-responsive regulation of ammonia-producing enzymes in *H. pylori* (46). However, iron restriction is often used by pathogenic bacteria as a signal for entering the host and is a well known signal for regulation of virulence gene expression (32).

The sites of colonization of *H. hepaticus* are the murine intestine and liver, and both environments are thought to be iron limited (22). In the intestine, *H. hepaticus* has to compete for iron with many other bacteria, including the resident flora. In contrast, the liver is thought to be a rather sterile environment containing high concentrations of bile and urea (4). Bile components can chelate iron, and this may make the hepatobiliary tract also an iron-restricted site of colonization for *H. hepaticus* (4). Increased urease expression may help *H. hepaticus* to overcome such detrimental conditions through as-yet-unknown mechanisms. Similarly, iron restriction functions as a signal for increased expression of the ammonia-producing enzyme amidase of *H. pylori* (46), but the physiological role of this regulatory response is also still unknown. It is therefore conceivable that a low level of iron can serve as an indirect signal for encountering unfavorable conditions requiring additional urease activity. Such increases in urease activity may help the bacteria to colonize their niche, for instance, through competition for nitrogen sources with other bacteria.

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

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