

Nickel-Responsive Induction of Urease Expression in *Helicobacter pylori* Is Mediated at the Transcriptional Level

ARNOUD H. M. VAN VLIET,^{1,2*} ERNST J. KUIPERS,² BARBARA WAIDNER,³ BEVERLY J. DAVIES,⁴ NICOLETTE DE VRIES,^{1,5†} CHARLES W. PENN,⁴ CHRISTINA M. J. E. VANDENBROUCKE-GRAULS,¹ MANFRED KIST,³ STEFAN BERESWILL,³ AND JOHANNES G. KUSTERS^{1,2}

Department of Medical Microbiology, Faculty of Medicine, Vrije Universiteit,¹ and Department of Gastroenterology, Vrije Universiteit Academic Hospital,⁵ Amsterdam, and Department of Gastroenterology and Hepatology, Academic Hospital Dijkzigt, Rotterdam,² The Netherlands; Department of Microbiology, Institute of Medical Microbiology and Hygiene, University of Freiburg, Freiburg, Germany³; and School of Biosciences, University of Birmingham, Edgbaston, Birmingham, United Kingdom⁴

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The nickel-containing enzyme urease is an essential colonization factor of the gastric pathogen *Helicobacter pylori*, as it allows the bacterium to survive the acidic conditions in the gastric mucosa. Although urease can represent up to 10% of the total protein content of *H. pylori*, expression of urease genes is thought to be constitutive. Here it is demonstrated that *H. pylori* regulates the expression and activity of its urease enzyme as a function of the availability of the cofactor nickel. Supplementation of brucella growth medium with 1 or 100 μM NiCl_2 resulted in up to 3.5-fold-increased expression of the urease subunit proteins UreA and UreB and up to 12-fold-increased urease enzyme activity. The induction was specific for nickel, since the addition of cadmium, cobalt, copper, iron, manganese, or zinc did not affect the expression of urease. Both Northern hybridization studies and a transcriptional *ureA::lacZ* fusion demonstrated that the observed nickel-responsive regulation of urease is mediated at the transcriptional level. Mutation of the HP1027 gene, encoding the ferric uptake regulator (Fur), did not affect the expression of urease in unsupplemented medium but reduced the nickel induction of urease expression to only twofold. This indicates that Fur is involved in the modulation of urease expression in response to nickel. These data demonstrate nickel-responsive regulation of *H. pylori* urease, a phenomenon likely to be of importance during the colonization and persistence of *H. pylori* in the gastric mucosa.

Helicobacter pylori is a gram-negative, microaerophilic human pathogen, which colonizes the gastric mucosa. Infection with *H. pylori* leads to gastritis and is associated with the development of peptic ulcer disease and gastric cancer (16, 33). Since approximately half of the world population is infected with *H. pylori* (16), it constitutes a major public health problem.

H. pylori expresses large quantities of the enzyme urease (3), an essential colonization factor of *H. pylori* (17, 18, 56). This enzyme converts urea, which is present in millimolar concentrations in the gastric mucosa, into ammonia and bicarbonate. The ammonia protects *H. pylori* against the acidic microenvironment (31, 44, 47, 58), causes damage to the gastric epithelium (50), is essential for chemotactic behavior (39), and serves as a nitrogen source (23). The bicarbonate protects *H. pylori* against the bactericidal activity of peroxynitrite, a nitric oxide metabolite (34).

Urease is a multimeric, nickel-containing enzyme which consists of six UreA and six UreB subunits (15, 25, 28). The UreA and UreB subunits have molecular masses of 27 and 62 kDa, respectively, and are encoded by the *ureA* and *ureB* genes which are organized in an operon structure (35). The gene

products of a second operon, containing the *ureIEFGH* genes and located downstream of the *ureAB* genes, are required for the production of active urease. The UreEFGH accessory proteins probably function in subunit assembly and in the incorporation of nickel in the active sites of urease (8, 11, 38). This second operon also contains the *ureI* gene, which encodes a putative acid-activated urea transporter (44, 46, 49, 58). Transcription of the *H. pylori* urease gene cluster occurs from two promoters: one upstream of the *ureA* gene (P_{ureA}) (1, 48) and one in the intergenic region between *ureB* and *ureI* (P_{ureI}) (1). Transcription from these two promoters, followed by pH-dependent differential mRNA decay, leads to the formation of mRNAs containing *ureAB*, *ureABIE'*, *ureIE'*, and *ureF'GH* (1).

Urease is expressed by a wide variety of bacteria but not usually at the very high levels found in *H. pylori* (8, 38). Urease activity in other bacteria is seldom constitutive but is regulated in response to environmental changes, such as changes in pH, urea availability, nitrogen availability, or growth phase (8, 9, 12, 38, 40). *H. pylori* produces large amounts of urease, and it has been estimated that up to 10% of the total protein content of *H. pylori* consists of urease (3). The production of such large amounts of urease must constitute a heavy metabolic burden, and therefore it is likely that *H. pylori* regulates the expression of urease. However, the published *H. pylori* genome sequences lack homologs of the urease regulators UreR and NtrC (2, 8, 40, 55), and urease expression in *H. pylori* is not transcriptionally regulated by urea availability or pH (3). The activity of urease is increased at low pH, but expression of the UreA and

* Corresponding author. Mailing address: Department of Gastroenterology and Hepatology, L448, Academic Hospital Dijkzigt, Dr. Molewaterplein 40, 3015 GD Rotterdam, The Netherlands. Phone: 31-10-4635946. Fax: 31-10-4634682. E-mail: vanvliet@mdl.azr.nl.

† Present address: Department of Gastroenterology and Hepatology, Academic Hospital Dijkzigt, Rotterdam, The Netherlands.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
<i>H. pylori</i>		
1061	Wild-type strain	7
26695	Wild-type strain	55
NCTC 11638	Wild-type strain	National Collection of Type Cultures
ATCC 43504	Wild-type strain	American Type Culture Collection
N6	Wild-type strain	22
J99	Wild-type strain	2
AV433	1061 <i>ureA::lacZ</i> Km ^r	This study
1061 <i>fur</i>	1061 <i>fur::Cm</i> ^r	J. J. E. Bijlsma et al. ^a
<i>E. coli</i> ER1793	Host for pBW-derived vectors	43
Plasmids		
pBW	Insertional vector, Km ^r , containing a promoterless <i>lacZ</i> gene in pBCα3 (7)	N. de Vries et al. ^b
pBJD3.3	pBW containing an <i>H. pylori ureA::lacZ</i> transcriptional fusion	This study

^a J. J. E. Bijlsma, S. Bereswill, A. H. M. van Vliet, B. Waidner, C. M. J. E. Vandenbroucke-Grauls, M. Kist, and J. G. Kusters, unpublished data.

^b N. de Vries, E. J. Kuipers, N. E. Kramer, A. H. M. van Vliet, J. J. E. Bijlsma, M. Kist, S. Bereswill, C. M. J. E. Vandenbroucke-Grauls, and J. G. Kusters, unpublished data.

UreB subunits is unchanged (1, 44, 46, 49, 58). Several loci have been demonstrated to affect either urease expression or activity: the nickel transporter genes *nixA* and *abcCD* (4, 26), the heat shock gene *hspA* (30) and the heat shock regulator gene *hspR* (51), the heavy metal P-type ATPase gene *cadA* (27), the RNA helicase gene *deadD* (7) and helicase genes *hp0511* and *hp0548* (36), the flagellar biosynthesis gene *flbA* (36), and the hydrogenase subunit genes *hypA* and *hypB* (41). However, these loci have not been demonstrated to directly regulate *ureAB* transcription.

Although urease is essential for gastric colonization (17, 18, 56) and plays a central role in the pathogenesis of *H. pylori* infection, the expression of urease has so far not been recognized to be regulated by environmental stimuli. In this study, nickel-responsive induction of expression and activity of *H. pylori* urease is described and is shown to be mediated at the transcriptional level via the *ureA* promoter.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The *H. pylori* and *Escherichia coli* strains and the plasmids used in this study are listed in Table 1. *H. pylori* was routinely cultured on Dent plates (14), consisting of Columbia agar (Oxoid) supplemented with 7% saponin-lysed defibrinated horse blood, 0.004% triphenyltetrazolium chloride (Sigma), and Dent Selective Supplement (Oxoid), at 37°C under microaerophilic conditions (10% CO₂, 5% O₂, and 85% N₂). *H. pylori* was grown in broth cultures in brucella broth (Oxoid) supplemented with 3% Newborn Calf Serum (BBN, Gibco). Metal chlorides (ACS quality) were purchased from Sigma, dissolved in distilled water, filter sterilized, and used at the indicated concentrations. The total nickel content of BBN was determined with inductively coupled plasma-mass spectrometry (53) at the Chemische Landesuntersuchungsanstalt, Freiburg, Germany, and was approximately 0.2 μM. *E. coli* was grown aerobically in Luria-Bertani medium (45) at 37°C. When appropriate, growth media were supplemented with kanamycin or chloramphenicol to final concentrations of 20 or 10 μg/ml, respectively.

Recombinant DNA techniques. Restriction enzymes and modifying enzymes were purchased from New England Biolabs and were used according to the manufacturer's instructions. Standard protocols were used for manipulation of DNA and the transformation of *E. coli* (45) and *H. pylori* (7). Plasmid DNA was prepared using Qiaprep spin columns (Qiagen).

Protein analysis. *H. pylori* cultures were grown in unsupplemented or NiCl₂-supplemented BBN for 20 to 24 h with moderate shaking to an optical density at 600 nm (OD₆₀₀) of 0.4 to 0.8, centrifuged for 10 min at 4,000 × *g* at 4°C, and resuspended in ice-cold phosphate-buffered saline to a final OD₆₀₀ of 10. *H. pylori* cells were lysed by sonication for 15 s on ice with an MSE Soniprep 150 set at amplitude 10. Protein concentrations were determined with the bicinchoninic

acid method (Pierce) using bovine serum albumin as the standard. Samples containing 15 μg of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% polyacrylamide gel (45) and stained with Coomassie brilliant blue or subjected to Western immunoblotting with a urease-specific antiserum (7). Protein gels were scanned at 300 dots per inch using a Microtek scanner and analyzed by densitometry using the Kodak 1D Image Analysis Software, version 3.5. Samples were normalized using a total protein content of between 35 and 60 kDa, and expression patterns of the urease subunits were compared to an internal control consisting of the 26-kDa *H. pylori* TsaA protein (29, 42) (indicated in Fig. 1).

Urease activity. Urease activity of fresh lysates was determined by measuring ammonia production from urea hydrolysis with the Berthelot reaction (11). Briefly, lysates of freshly sonicated cells (0.6 to 1.0 μg of protein) were incubated for 10 min at room temperature in 1 ml of PEB buffer (100 mM sodium phosphate, 10 mM EDTA; pH 7.5) containing 50 mM urea. Subsequently, 90-μl aliquots were mixed with 150 μl of phenol-nitroprusside (Sigma Diagnostics 640-1), 150 μl of alkaline hypochlorite (Sigma Diagnostics 640-3), and 750 μl of distilled water and then incubated for 10 min at 37°C. The ODs of the samples were determined at 570 nm. The amount of ammonia present in the 90-μl samples was then inferred from a standard NH₄Cl concentration curve. Urease activity was expressed as micromoles of urea hydrolyzed per minute per milligram of protein.

RNA analysis. Total RNA was isolated from 4 × 10⁹ freshly grown bacteria with RNeasy spin columns (Qiagen), according to the manufacturer's instructions. RNA was separated on 2% formaldehyde–1.5% agarose gels in 20 mM sodium phosphate buffer (pH 7), transferred to positively charged nylon membranes (Roche Diagnostics), and covalently bound to the membrane by cross-linking with 0.120 J of UV light of 254-nm wavelength per cm². RNA was visualized by methylene blue staining (1), and RNA samples were normalized based on 16S and 23S rRNA band intensities. The sizes of the hybridizing RNA species were calculated from comparison with a digoxigenin-labeled RNA marker (RNA Marker I; Roche Diagnostics). Internal fragments of the *ureA*, *ureI*, and *ureG* genes were PCR amplified with the primers listed in Table 2. The resulting PCR fragments contained a T7 promoter sequence and were used for the production of antisense RNA probes labeled with digoxigenin by in vitro transcription using T7 RNA polymerase (Roche Diagnostics). Northern hybridization and stringency washes were done at 68°C, and bound probe was visualized with the DIG-Detection Kit (Roche Diagnostics) and the chemiluminescent substrate CPD-Star (Amersham Pharmacia).

Construction and characterization of a *H. pylori ureA::lacZ* transcriptional fusion. Plasmid pBJD3.3 (Table 1) was constructed by PCR amplification of the *H. pylori* 1061 *ureA* promoter with primers BJD3.3-F1 and BJD3.3-R1 (Table 2), subsequent digestion with *Bam*HI, and cloning into the unique *Bgl*II site upstream of the promoterless *lacZ* gene of vector pBW (Table 1). The *ureA* PCR fragment was sequenced to verify the fidelity of the amplification. pBJD3.3 was transformed to *H. pylori* 1061 as described previously (7), resulting in the kanamycin-resistant *H. pylori* strain AV433 (Table 1). The β-galactosidase activity in *H. pylori* AV433 grown in either unsupplemented or in nickel-supplemented BBN medium was determined in lysates from freshly sonicated cells. A portion

TABLE 2. Oligonucleotide primers used in this study

Primer	Sequence (5'→3')
UreA-F1	ATGAAACTCACCCCAAAGA
UreA-R1-T7 ^a	ctaatagactcactataggagaGGAAGTGT GAGCCGATTTGA
UreI-F2	AAGCACTGCGGTGATGAACT
UreI-R2-T7 ^a	ctaatagactcactataggagaACCAATCG CCTTCAGTGATG
UreG-F1	TGACATGGCGGTCATCACTA
UreG-R-T7 ^a	ctaataagactcactataggagaCAAGTC TGAGCCGCGTGATTC
BJD3.3-F1 ^b	cgcggatccTTTGAAGGGCATTGTGTC
BJD3.3-R1 ^b	cgcggatccAACTTGTCTAACTCTTTTGG

^a Primers contained a 5' extension with T7 promoter sequence (in lowercase letters) for the creation of an antisense RNA probe.

^b Primers contained a 5' extension for cloning purposes, indicated in lowercase letters. A *Bam*HI restriction site is underlined.

(0.1 ml) of lysate (corresponding to approximately 10^9 cells) was mixed with 0.9 ml of Z-buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, 50 mM 2-mercaptoethanol, 0.002% SDS) and warmed to 37°C. Reactions were started by the addition of 0.2 ml of *o*-nitrophenyl-β-D-galactopyranoside (4 mg/ml), and terminated by the addition of 0.5 ml of Na₂CO₃ (1 M). The OD₄₂₀, the OD₅₅₀, and the time required for the solution to become yellow were recorded. The β-galactosidase activity was expressed in Miller units (45).

RESULTS

Nickel supplementation of growth medium increases urease expression and activity. We tested the effect of medium supplementation with the urease cofactor nickel on protein expression patterns in one clinical isolate and one reference strain of *H. pylori* (1061 [7] and 26695 [55], respectively). Strains were grown either in unsupplemented BBN or in BBN supplemented with NiCl₂ to final concentrations of 1 and 100 μM. These NiCl₂ concentrations represent a significant increase in the total nickel content of BBN, which is approximately 0.2 μM, but are well below the nickel toxicity levels for *H. pylori*, which are between 1 and 6 mM (27, 37, 57). Supplementation of BBN with 1 or 100 μM NiCl₂ did not have a significant effect on the growth of *H. pylori* (not shown) but did result in the increased expression of two proteins with molecular masses of approximately 30 and 67 kDa, respectively (Fig. 1A). The molecular mass, amount, and recognition of these two proteins by antibodies to *H. pylori* urease (Fig. 1B) verified that these proteins were the urease subunit proteins UreA and UreB. BBN supplementation with 500 μM NiCl₂ did not give rise to higher levels of urease expression, and BBN supplementation with 1 and 5 mM NiCl₂ started to adversely affect growth of *H. pylori* 1061 and 26695 (not shown).

Supplementation of BBN with 1 and 100 μM NiCl₂ gave a significant induction of up to 3.5-fold of UreA and UreB expression in *H. pylori* 1061 (Table 3, Fig. 1A). *H. pylori* 26695 did not significantly induce UreA and UreB expression in response to supplementation with 1 μM NiCl₂ (Table 3, Fig. 1A) but did give a significant 3.3-fold induction at 100 μM NiCl₂ (Table 3, Fig. 1A). Expression of the 26-kDa TsaA protein (29, 42), which was used as an internal control, was not affected by nickel supplementation (Table 3, Fig. 1A). Four other reference strains (NCTC11638, ATCC43504, J99, and N6) also showed two- to threefold-induced expression of UreA and UreB upon medium supplementation with 100 μM NiCl₂. Nickel supplementation of BBN also led to increased urease

activity in strains 26695 and 1061, albeit to different levels (Fig. 1C). Urease activity increased approximately 5-fold in *H. pylori* 1061 and almost 12-fold in *H. pylori* 26695 (Fig. 1C). The induction of urease expression was specific for nickel, since medium supplementation with 25 μM CoCl₂ or with 100 μM CdCl₂, CuCl₂, FeCl₃, MnCl₂, or ZnCl₂ did not affect urease expression in *H. pylori* 26695 (data not shown).

Nickel induction of urease expression is mediated at the transcriptional level. The levels of the mRNA species coding for urease subunit or accessory genes in *H. pylori* strain 1061 were analyzed with probes specific for the *ureA*, *ureI*, and *ureG* genes (Fig. 2A). The *ureA*-specific probe hybridized with a *ureAB*-containing mRNA of approximately 2.7 kb (1) and a *ureABIE'*-containing mRNA of 3.4 kb (1). The amounts of

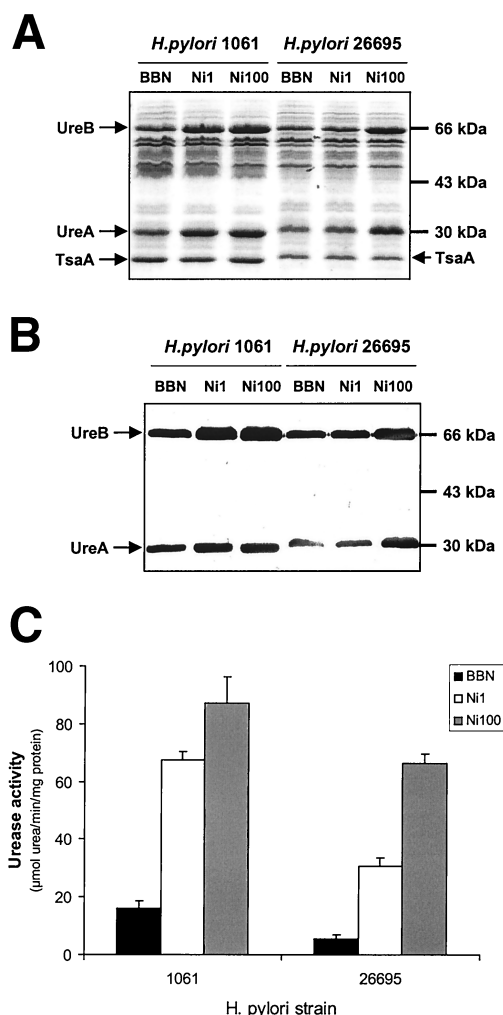


FIG. 1. Expression and activity of *H. pylori* urease upon supplementation of growth medium with nickel. *H. pylori* strains were grown in unsupplemented medium (BBN) or in BBN supplemented with NiCl₂ to final concentrations of 1 μM (Ni1) and 100 μM (Ni100). Changes in urease expression were monitored by SDS-PAGE followed by Coomassie brilliant blue staining (A), immunoblotting with *H. pylori* urease-specific antiserum (B), or quantitative urease enzyme assay (C). *H. pylori* strains, UreA and UreB proteins, relevant molecular mass markers, and the 26-kDa TsaA protein used as an internal control for densitometry are indicated.

TABLE 3. Quantitative determination of nickel-responsive induction of *H. pylori* urease expression

Strain and protein	Change in relative level of protein expression \pm SD ^a after supplementation with:	
	1 μ M NiCl ₂	100 μ M NiCl ₂
<i>H. pylori</i> 26695		
UreA	1.3 \pm 0.4	3.3 \pm 0.9*
UreB	1.4 \pm 0.3	3.4 \pm 0.2*
TsaA	1.0 \pm 0.1	1.0 \pm 0.1
<i>H. pylori</i> 1061		
UreA	2.8 \pm 0.6*	3.5 \pm 0.6*
UreB	2.8 \pm 0.6*	3.4 \pm 0.4*
TsaA	1.1 \pm 0.2	1.1 \pm 0.2
<i>H. pylori</i> 1061 <i>fur</i>		
UreA	1.9 \pm 0.1*	2.0 \pm 0.2*
UreB	1.7 \pm 0.2*	1.9 \pm 0.5*
TsaA	1.1 \pm 0.1	1.0 \pm 0.1

^a Relative levels of protein were determined by densitometry as described in Materials and Methods and were compared to the expression in unsupplemented BBN medium. The expression in unsupplemented BBN was set to 1. Samples were derived from three or four independent growth experiments. Asterisks indicate a significant change in expression compared to the level in unsupplemented medium ($P < 0.01$; Student's *t* test). TsaA served as an internal control, as indicated in Fig. 1A and Fig. 4.

both mRNAs were clearly increased upon nickel supplementation of the medium (Fig. 2B). The *ureI*-specific probe hybridized with mRNAs of approximately 3.4 kb (*ureABIE'*) and 0.9 kb (*ureIE'*) (1). The amount of the 3.4-kb mRNA was clearly induced upon nickel supplementation, while the amount of the 0.9-kb mRNA did not change significantly upon nickel supplementation (Fig. 2B). Finally, the *ureG*-specific probe hybridized with a single mRNA, with a length of approximately 1.7 kb (*ureF'GH*) (1), whose amount did not change significantly upon nickel supplementation (Fig. 2B). In summary, only transcripts originating from the promoter upstream of the *ureA* gene were induced upon nickel supplementation.

Nickel induction of urease expression is mediated via the *ureA* promoter. A chromosomal *ureA::lacZ* fusion was used to measure the effect of nickel supplementation on *ureAB* transcription. This transcriptional fusion would also allow us to determine whether the increase in *ureAB* mRNA resulted from true transcriptional induction (increased de novo mRNA synthesis) or increased mRNA stability. To construct the chromosomal *ureA::lacZ* fusion, plasmid pBJD3.3, which contains the *ureA* promoter in front of a promoterless *lacZ* gene (Table 1, Fig. 3A), was transformed to *H. pylori* 1061. The integration of the pBJD3.3 vector into the *H. pylori* 1061 chromosome by single homologous recombination leads to kanamycin resistance (7). The resulting transformant, named AV433, contains a duplicated *ureA* promoter (Fig. 3A). One *ureA* promoter is fused to the promoterless *lacZ* gene, whereas the other copy is still preceding the intact urease operon (Fig. 3A). Correct chromosomal integration of pBJD3.3 and the presence of the *ureA::lacZ* and wild-type urease operons in *H. pylori* AV433 was confirmed by PCR, sequencing, and Southern hybridization. The insertion of the pBW vector did not have a major effect on the expression, activity, and nickel induction of ure-

ase, since there were no significant differences in urease activity between strain AV433 and its parent strain 1061 (Fig. 3B). The β -galactosidase activity in *H. pylori* AV433 increased approximately threefold when the growth medium was supplemented with NiCl₂ compared to unsupplemented growth medium (Fig. 3C).

The *H. pylori* Fur homolog modulates nickel-responsive induction of urease. The role of the *H. pylori* metal-responsive regulator Fur (HP1027) (6) in the nickel-responsive induction of urease was established by insertional mutagenesis of the *fur* gene in *H. pylori* 1061. Mutation of *fur* did not affect basal levels of urease expression (Fig. 4), nor did it affect transcrip-

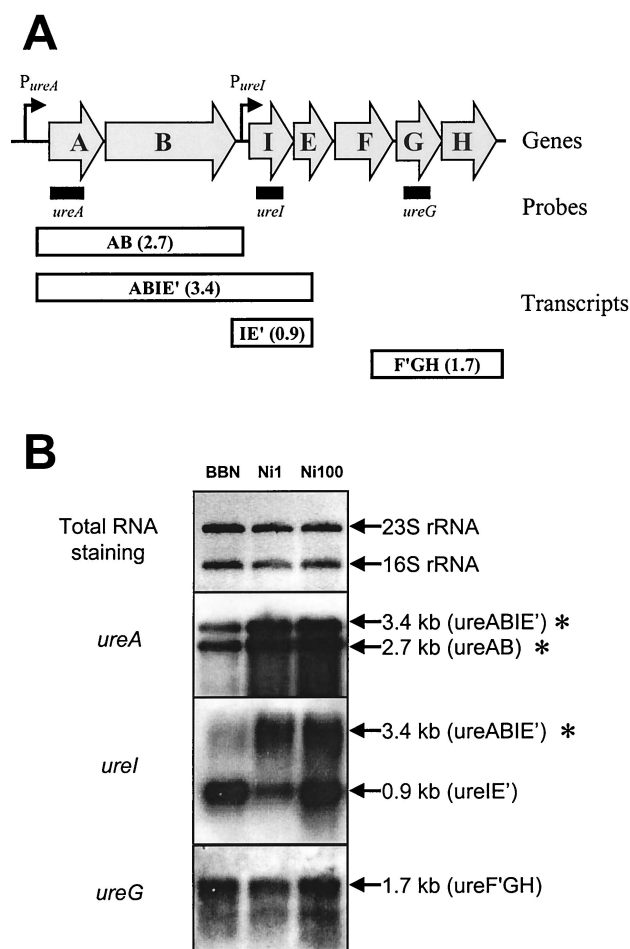


FIG. 2. Nickel induction of urease expression is mediated at the transcriptional level. (A) Transcriptional organization of the *H. pylori* urease operon, as modified from Akada et al. (1). The position of the probes used in this study are indicated, together with the different mRNA transcripts of the urease operon. The size (in kilobases) and the genes present on the respective mRNA species are indicated. An apostrophe indicates a truncated transcript; genes and the direction of transcription are indicated by arrows. P_{ureA} , *ureA* promoter; P_{ureI} , *ureI* promoter. (B) Analysis of urease transcription using Northern hybridization. *H. pylori* strains were grown in unsupplemented medium (BBN) or in BBN supplemented with NiCl₂ to final concentrations of 1 μ M (Ni1) and 100 μ M (Ni100). Staining of transferred RNA by methylene blue is included for comparison of the RNA amounts. The probes used are indicated on the left, whereas the rRNA and the sizes and content of the hybridizing mRNA species are indicated on the right. Nickel-induced mRNA species are marked with an asterisk.

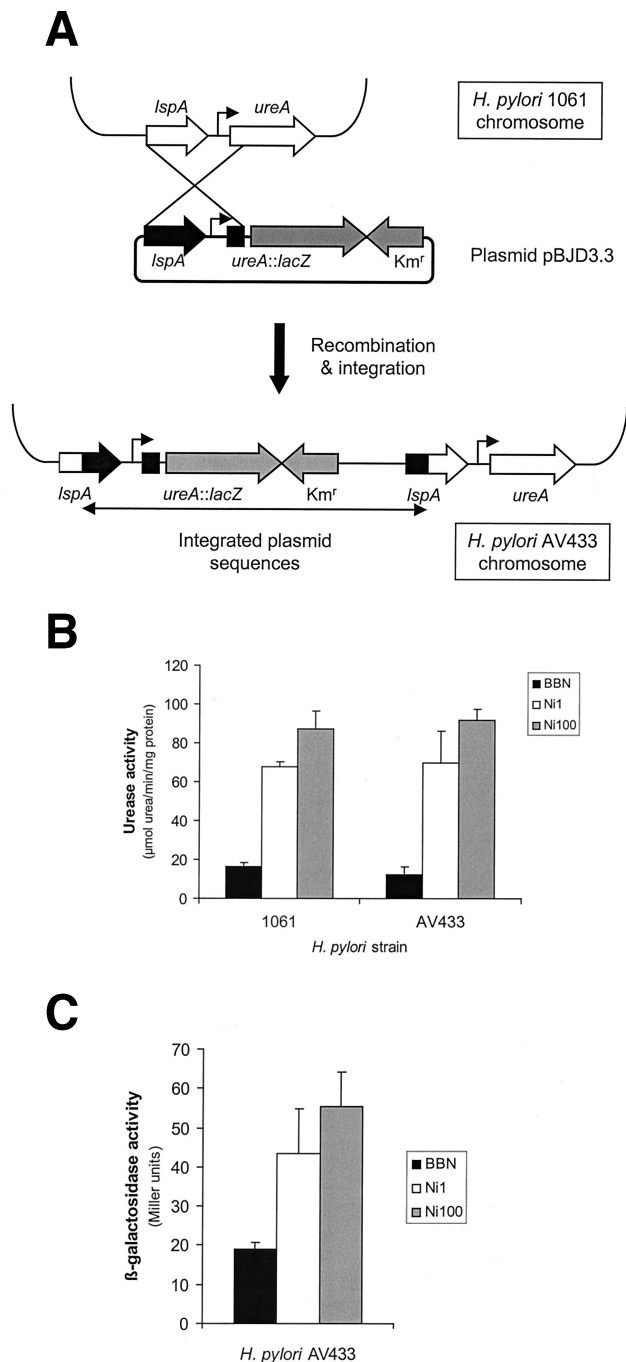


FIG. 3. Nickel induction of urease transcription is mediated via the *ureA* promoter, as determined by using a transcriptional *ureA::lacZ* fusion. (A) Schematic representation of *H. pylori* AV433, containing a single genomic copy of both the *ureA::lacZ* transcriptional fusion and the wild-type *ureA* promoter and downstream urease operon. *lspA* is the gene upstream of *ureA*, and *Km^r* indicates the kanamycin resistance determinant. (B) Activity and nickel-responsive induction of urease in *H. pylori* AV433 is not altered by duplication of the *ureA* promoter, compared to the parent strain 1061. (C) Induction of the *ureA::lacZ* transcriptional fusion in *H. pylori* AV433, as determined by β-galactosidase activity. BBN, unsupplemented medium; Ni1, medium supplemented with NiCl₂ to a final concentration of 1 μM; Ni100, medium supplemented with NiCl₂ to a final concentration of 100 μM. β-Galactosidase and urease activities were determined from three to eight independent cultures.

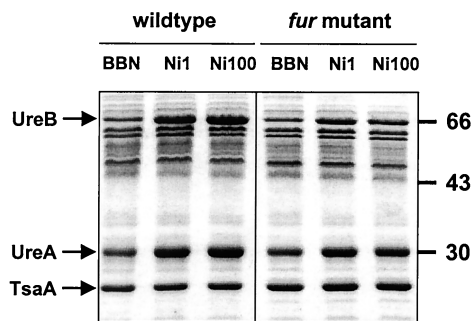


FIG. 4. Effect of a *fur* mutation in *H. pylori* 1061 on nickel-responsive induction of urease expression. *H. pylori* 1061 (wild type) and its isogenic *fur* mutant (*fur* mutant) were grown in unsupplemented medium (BBN) or in BBN supplemented with NiCl₂ to final concentrations of 1 μM (Ni1) and 100 μM (Ni100). Changes in UreA and UreB expression were monitored by SDS-PAGE, followed by Coomassie brilliant blue staining. *H. pylori* strains, UreA and UreB proteins, relevant molecular mass markers, and the 26-kDa TsaA protein used as internal control for densitometry are indicated.

tion of the different mRNA species of the urease operon (not shown). However, nickel-responsive induction of urease was clearly diminished in the *H. pylori fur* mutant. Where wild-type *H. pylori* 1061 showed up to 3.5-fold induction of UreA and UreB expression (Table 3), in the *fur* mutant this was only to a maximum of 2-fold induction at 100 μM NiCl₂ (Table 3). This difference was significant ($P < 0.05$) at both 1 and 100 μM NiCl₂. Expression of the 26-kDa TsaA protein was again independent from the NiCl₂ supplementation or the *fur* mutation (Fig. 4, Table 3). Increasing the NiCl₂ concentration to 500 μM did not result in an additional induction of urease expression in the *fur* mutant, indicating that maximum levels of expression of UreA and UreB are lower in the *H. pylori* 1061 *fur* mutant than in wild-type *H. pylori* 1061.

DISCUSSION

Urease activity is an essential factor in the colonization of the gastric mucosa by *H. pylori* (17, 18, 56). It is therefore not surprising that *H. pylori* expresses high levels of urease, up to 10% of its total protein content (3). This must constitute a heavy metabolic burden for this fastidious pathogen, but since none of the regulatory mechanisms described for other bacterial ureases apply to *H. pylori* urease, the expression of *H. pylori* urease was thought to be constitutive (3, 8, 38). As demonstrated here, *H. pylori* regulates urease transcription via the availability of the urease cofactor nickel, representing a novel type of transcriptional regulation for bacterial ureases.

Supplementation of growth media with nickel resulted in increased expression of the urease subunit proteins UreA and UreB and increased urease activity (Fig. 1). There were differences in the induction levels quantified in the two strains tested, since *H. pylori* 26695 expressed lower amounts of urease subunits and had a lower urease activity than strain 1061 but demonstrated much higher levels of induction in response to nickel supplementation of the growth medium (Fig. 1). The increase in urease expression and activity was accompanied by increased amounts of mRNA species containing the *ureA* and *ureB* genes (Fig. 2). The increase in *ureA*- and *ureB*-containing mRNAs is likely to be mediated exclusively through increased

transcription from the *ureA* promoter (Fig. 3), since the observed threefold increase in β -galactosidase activity in *H. pylori* AV433 (*ureA::lacZ*) upon nickel supplementation seems to match the increase in the amounts of urease proteins and transcripts. In contrast to our findings, Olson et al. (41) did not find induction of urease activity when *H. pylori* strain ATCC43504 was grown with medium supplementation of 1 or 5 μ M NiCl₂. This is likely to be due to differences in medium composition and growth conditions. Olson et al. (41) used brucella agar plates supplemented with 10% blood, and agar and blood components are likely to chelate nickel. Also, plate-grown cultures usually contain a mix of cells in different growth phases, and this might also influence urease activity and nickel availability. We have tested strain ATCC 43504 in BBN medium supplemented with 100 μ M NiCl₂ and, under these conditions, it demonstrated a clear nickel-responsive induction of urease expression, to similar levels as *H. pylori* 1061.

The increase in urease activity resulting from nickel supplementation is higher than the observed increase in UreA and UreB subunit proteins (Fig. 1A and C, Table 3). This suggests that not only the amount of urease protein but also the amount of the cofactor nickel is a limiting factor for urease activity of *H. pylori*. Apparently, when grown in unsupplemented growth medium, *H. pylori* contains inactive urease resulting from the limited availability of nickel. Assuming that in the natural niche of *H. pylori* (i) the bioavailability of nickel is limited and (ii) the availability of nickel is positively affected by a decrease in pH (32), as is, for example, the case for iron, then the increased availability of nickel at the low pH would provide a physiological mechanism for a rapid increase of urease activity. This would result in an instantaneous increase in acid resistance resulting from activation of inactive urease apoenzyme due to increased nickel availability, without the need for de novo synthesis of the urease apoenzyme. While the concentration of nickel in the gastric lumen or gastric mucosa is unknown, the daily nickel intake in industrialized countries averages 150 μ g/day but can vary significantly since some food sources, such as coffee, tea, nuts, and chocolate, are rich in nickel (52).

The nickel-responsive induction of urease expression observed in this study seems to be mediated through more than one regulatory system. The iron-regulatory protein Fur is involved in the nickel-responsive regulation of urease expression, since urease expression is only induced to a maximum of two-fold in *H. pylori* 1061*fur*, whereas in wild-type *H. pylori* 1061 it is induced more than threefold. Fur is not involved in the basal levels of urease expression, however, since urease expression in wild-type and *fur* mutant strains is very similar (Fig. 4, Table 3). Whether Fur exerts its regulatory function directly at the urease promoter or through other pathways is unknown. Although the *ureA* promoter region contains a stem-loop structure (48) that somewhat resembles the structure used by Fur (20), it does not contain significant sequence homology to the Fur binding sequence (20) and was not identified in screening of *H. pylori* DNA in the original and *H. pylori*-optimized Fur titration assays (21). This indicates that the Fur protein plays an important role in metal-responsive gene regulation in *H. pylori*, since it is also involved in metal-responsive repression of ferritin synthesis in *H. pylori* (5).

Nickel-responsive regulation has only been characterized at

the molecular level in a few bacterial species (19). Nickel induces the *cnr* cobalt and nickel resistance determinant of *Ralstonia* sp. via the CnrY, CnrX, and CnrH proteins (24, 54), whereas it represses the *E. coli* nickel acquisition system *nik* via the NikR protein (10, 13). *H. pylori* does not contain clear homologs of the CnrY, CnrX, or CnrH proteins, but it does contain a NikR homolog, designated HP1338 (55). We are currently assessing the role of the HP1338 protein in metal-responsive regulation of *H. pylori*.

In conclusion, *H. pylori* regulates the transcription and production of its virulence factor urease in response to nickel, and the *H. pylori* Fur homolog is involved in this regulation. Since urease expression is essential for colonization by *H. pylori*, it might be of interest to investigate the effect of a low-nickel diet on the colonization ability of *H. pylori* in animal models. Nickel-responsive induction of urease levels may also play a role in other urease-producing bacteria. If so, this may allow the development of new or improved strategies to prevent or control infection with urease-positive bacteria.

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