An ABC Transporter and a TonB Ortholog Contribute to *Helicobacter mustelae* Nickel and Cobalt Acquisition[†]

Jeroen Stoof,^{1,‡} Ernst J. Kuipers,^{1,2} Gerard Klaver,³ and Arnoud H. M. van Vliet^{1,4*}

Department of Gastroenterology and Hepatology, Erasmus MC University Medical Center, 's Gravendijkwal 230, 3015 CE Rotterdam, Netherlands¹; Department of Internal Medicine, Erasmus MC University Medical Center, 's Gravendijkwal 230, 3015 CE Rotterdam, Netherlands²; TNO, P.O. Box 80.015, 3508 TA Utrecht, Netherlands³; and Institute of Food Research, Foodborne Bacterial Pathogens Programme, Colney Lane, Norwich NR4 7UA, United Kingdom⁴

Received 11 April 2010/Returned for modification 9 May 2010/Accepted 8 July 2010

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

The genus *Helicobacter* comprises bacterial pathogens that colonize the alimentary tract of mammals, with the best-known example being the human gastric pathogen *Helicobacter pylori* (17). Other examples include *Helicobacter* species colonizing the gastric mucosa of big cats (*Helicobacter acinonychis*), cats and dogs (*Helicobacter felis*), and ferrets (*Helicobacter mustelae*) (21, 31). Gastric colonization by *Helicobacter* species can illicit a strong immune response, which may develop into pathologies like peptic ulcer disease and precancerous lesions (17). The lifelong colonization of the gastric mucosa suggests that these *Helicobacter* species are well adapted to this harsh environment and are able to combat the diverse antimicrobial activities employed by the host within the gastric mucosa, such as iron restriction and acidity (43).

Transition metals like iron and nickel are both essential for gastric *Helicobacter* species. Iron is involved in redox reactions and functions as a cofactor of many enzymes, whereas nickel is the cofactor of two important enzymes in gastric *Helicobacter*

species: urease and hydrogenase. The urease enzyme is the main factor allowing acid resistance, whereas hydrogenase is important for energy production, and both enzymes are essential for gastric colonization by *Helicobacter* species (2, 20, 32, 37). Analyses of complete genome sequences of gastric *Helicobacter* species allowed the prediction of many genes putatively involved in iron acquisition but surprisingly few predicted nickel acquisition genes (12, 22, 35).

Ferric iron acquisition in Gram-negative bacteria is usually mediated by a TonB-dependent outer membrane receptor coupled to an ABC transporter for transport in the periplasm and over the inner membrane. Ferrous iron acquisition requires only an FeoB-like single-component system for inner membrane transport (1, 41). As for ferrous iron, the transport of nickel was until recently thought not to require an outer membrane component but only an ABC transporter or single-component NiCoT transporter (18, 19). However, it has become apparent that there is much more variation in these themes and that TonB-dependent outer membrane proteins, annotated as putative iron acquisition systems, may be involved in the transport of a range of other metals or compounds (10, 14, 28, 29, 34). Examples of these new insights stem from recent work with *H. pylori*, where two out of three FecA and FrpB orthologs were shown to be regulated by iron and Fur, whereas the third FecA and FrpB ortholog is NikR and nickel repressed (9, 14, 39) and has been proposed to function in nickel acquisition (10, 28). Similarly, the *H. mustelae* putative TonB-dependent outer membrane protein NikH contributes to urease activity, probably by mediating nickel acquisition (34). Caution

* Corresponding author. Mailing address: Institute of Food Research, Colney Lane, Norwich NR4 7UA, United Kingdom. Phone: 44-1603-255250. Fax: 44-1603-507723. E-mail: arnoud.vanvliet@bbsrc.ac.uk.

[†] Supplemental material for this article may be found at <http://iai.asm.org/>.

[‡] Present address: Molecular Bacteriology and Immunology Group, School of Molecular Medical Sciences, Centre for Biomolecular Sciences, University of Nottingham, University Park, Nottingham NG7 2RD, United Kingdom.

[§] Published ahead of print on 19 July 2010.

also needs to be taken when annotating ABC transporters, as the proposed ferric citrate permease and ATPase FecD and FecE, respectively, were shown not to contribute to iron acquisition in *H. pylori* (41), suggesting a role for these *H. pylori* genes in the acquisition of other metals or compounds. Hence, the currently available genome annotations are potentially unreliable, and functional and mutational data are required to validate or correct the annotation of genome sequences.

Here we present a study of some of the putative iron and nickel acquisition systems identified in the *H. mustelae* genome sequence (22) (listed in Table S1 in the supplemental material). We have determined their contribution to iron and nickel acquisition by using regulatory studies, growth promotion assays, urease activity, and intracellular metal content and demonstrate that the two TonB systems play differential roles in iron and nickel acquisition. We also show that the CeuE and the FecDE ABC transporter components are likely to be misannotated, as they function in nickel and cobalt acquisition.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *H. mustelae* strain NCTC 12198 (ATCC 43772) and its isogenic mutants (see Tables S1 and S2 in the supplemental material) were routinely grown at 37°C in a microaerobic atmosphere of 5% O₂, 7.5% CO₂, 7.5% H₂, and 80% N₂ on Dent agar plates consisting of BBL Columbia agar (Becton Dickinson) supplemented with 7% saponin-lysed defibrinated horse blood (BioTrading), 0.004% triphenyltetrazolium chloride (Sigma), and Dent selective supplement (Oxoid). Broth cultures of *H. mustelae* for urease assays, Northern hybridization, and metal content determinations were grown in brucella broth (Becton Dickinson) supplemented with 3% newborn calf serum (Biotrading) and Dent supplement. Iron restriction of brucella broth was achieved by the addition of deferoxamine B (Desferal; Sigma) to a final concentration of 20 µM, whereas iron-replete medium was obtained by supplementing deferoxamine B-treated medium with FeCl₃ to a final concentration of 100 µM. The effect of metals on *H. mustelae* growth was assessed by using CoCl₂ and NiCl₂ supplementation of defined Ham's F-12 tissue culture medium (Kaighn's modification; Invitrogen) with 0.2% β-cyclodextrin (Fluka) and Dent supplement. Broth cultures were shaken at 70 rpm and incubated at 37°C for a maximum of 24 h. *Escherichia coli* strains DH5α and M147 were cultured in Luria-Bertani medium (Biotrading) (27). When appropriate, growth medium was supplemented with chloramphenicol (20 µg ml⁻¹), kanamycin (20 µg ml⁻¹), or erythromycin (250 µg ml⁻¹) for the selection of *E. coli* transformants. *H. mustelae* mutants were selected by growth on medium containing chloramphenicol (10 µg ml⁻¹), kanamycin (10 µg ml⁻¹), or erythromycin (10 µg ml⁻¹). Concentrations of antibiotics given represent their final concentration in the growth medium.

Construction of isogenic *H. mustelae* mutants. The *H. mustelae* *nikR* and *fur* mutants were described previously (33, 34). For the insertional mutagenesis of the other genes studied here (see Tables S1 and S2 in the supplemental material), a ~1-kb fragment of the gene of interest was PCR amplified from DNA from *H. mustelae* strain NCTC 12198 by using knockout (KO) primers (Table S2). The amplicons were cloned into the pGEM-T Easy vector (Promega), and the cloned sequences were interrupted by the insertion of an antibiotic resistance cassette obtained from plasmids pJMK30 (kanamycin), pAV35 (chloramphenicol), and pDH20 (erythromycin) (16, 40). The antibiotic resistance cassettes were inserted into unique restriction sites in the cloned fragments: BglII for *tonB1*, *tonB2*, *nixA*, and *ceuE*; BclI for *frpB1*; EcoRV for *frpB2* and *fecA*; KpnI for *cfrA*; HindIII for *feoB*; and Eco47III for *fecD*. The constructs were then introduced into *H. mustelae* strain NCTC 12198 and selected mutant strains by natural transformation (8), and the correct replacement by homologous recombination of the gene was confirmed by PCR using primers (Table S2) located outside the recombination site (ORS).

Protein analysis. Cells obtained from cultures grown overnight were centrifuged for 10 min at 4,000 × g and resuspended in phosphate-buffered saline to a final optical density at 600 nm (OD₆₀₀) of 10. Bacteria were lysed by sonication for 15 s with an MSE Soniprep 150 set at an amplitude of 6. Whole-cell proteins were separated on a 6% or 15% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) (27). The assessment of UreB and UreB2 urease subunit expres-

sion was based on immunoblots incubated with a 1:10,000 dilution of antibodies raised against *H. felis* urease (3, 25, 33).

Purification and analysis of RNA. Total RNA was isolated by using Trizol (Gibco) according to the manufacturer's instructions. RNA was separated on 2% formaldehyde-1.5% agarose gels in 20 mM sodium phosphate buffer (pH 7), transferred onto positively charged nylon membranes (Roche Diagnostics), and covalently bound to the membrane by UV cross-linking (33, 34). RNA was visualized by methylene blue staining, and RNA concentrations were normalized based on 16S and 23S rRNA band intensities. Probes were amplified with the primers listed in Table S2 in the supplemental material. The PCR fragments contained a T7 promoter sequence and were used for the production of an antisense RNA probe labeled with digoxigenin by transcription with T7 RNA polymerase (Roche Diagnostics). Northern hybridization and stringency washes were performed at 68°C, and bound probe was visualized with the digoxigenin (DIG) detection kit (Roche Diagnostics) and the chemiluminescent substrate CPD-Star (Amersham Pharmacia) as described previously (33, 34).

Growth promotion assay. Medium was prepared by mixing 7 g brucella broth (Becton Dickinson) and 3.75 g Bacto agar (Becton Dickinson) in 250 ml water, followed by sterilization by autoclaving. The medium was cooled down to 56°C, and a solution containing 7.5 ml heat-inactivated newborn calf serum (Biotrading), 250 µl 20 mM deferoxamine B (Sigma), and 250 µl Dent selective supplement (Oxoid) was added, together with triphenyl tetrazolium chloride (Sigma) to a final concentration of 0.004%. The medium was distributed over 9-cm petri dishes, at 17.5 ml per dish. *H. mustelae* strain NCTC 12198 and isogenic mutants (see Tables S1 and S2 in the supplemental material) were grown for 24 h at 37°C on Dent agar, harvested by centrifugation, and diluted in phosphate-buffered saline (PBS) to an OD₆₀₀ of 0.2. This suspension was spread equally across the agar plate by using a cotton swab. Blank paper discs (Becton Dickinson) were saturated with 30 µl of either 1% heme (Sigma) solution in 0.1 N NaOH or 0.5 mM FeCl₃. PBS was used as a negative control. The discs were placed onto the agar plate and incubated for 48 h at 37°C in a microaerobic atmosphere. The diameter of the zone of growth was measured, and each experiment was performed at least three times independently.

Urease assay. The enzymatic activity of the *H. mustelae* UreAB urease was determined by measuring ammonia production from the hydrolysis of urea by using the Berthelot reaction as described previously (33, 34). Cells were lysed by sonication and incubated for 30 min at 37°C in buffer consisting of 100 mM sodium phosphate (pH 7.5), 10 mM EDTA, and 50 mM urea, and the ammonia produced was measured after the addition of phenol nitroprusside and alkaline hypochlorite (Sigma Diagnostics) by measuring the OD₅₇₀. Protein concentrations were determined by the bicinchoninic acid method (Pierce) using bovine serum albumin as a standard. Urease enzyme activity was expressed as units representing mmol of urea hydrolyzed per min and is expressed as U mg⁻¹ of total protein. The average data from three independent experiments are shown. Only the activity of the *H. mustelae* UreAB urease was measured, since sonication inactivates the UreA2B2 urease of *H. mustelae* (33).

Determination of cellular metal content. *H. mustelae* strains were grown as broth cultures for 24 h, and cells were harvested by centrifugation. The cell pellet was washed three times with 1 ml PBSE solution, consisting of PBS supplemented with EDTA to a final concentration of 1 mM. Cell density was assessed by measurement of the OD₆₀₀, and cells were subsequently lysed by using 500 µl HNO₃ (65% Suprapur; Merck) and 250 µl of a 30% H₂O₂ solution (Suprapur; Merck). Metal concentrations were determined by high-resolution inductively coupled plasma mass spectrometry (HR-ICPMS) (23, 36). Levels of iron, cobalt, and nickel in the wild-type strain were 4.31×10^6 ($\pm 1.21 \times 10^6$), 2.08×10^3 ($\pm 3.19 \times 10^3$), and 5.94×10^6 ($\pm 0.98 \times 10^6$) atoms/cell, respectively, and values for the respective mutants are expressed as a percentage of the levels found in the wild-type strain. The number of cells was calculated by using the following conversion formula: OD₆₀₀ = 1 equals 5×10^8 cells/ml. Results shown are the averages of data from three independent experiments.

The HR-ICPMS equipment used for quantification was an Element XR (Thermo Scientific) equipped with PC³ (Elemental Scientific [ESI]) and nickel interface cones. The following operating conditions for HR-ICPMS were used: radio frequency (RF) power of 12.5 kW and gas flow rates of 16 liter min⁻¹, 0.7 liter min⁻¹ (auxiliary), and 1.1 liter min⁻¹ (nebulizer). PC³ consists of a Peltier cooled inlet system, which incorporates the ESI cyclonic spray chamber, the ESI PFA-ST nebulizer, and the ESI Fast introduction system. A 0.25-ml loop was used on the Fast introduction system, and the PFA-ST nebulizer was fed by an ESI syringe system at a constant flow rate of 112 µl/min. The analytical masses (*m/z*) used for the analysis were *m/z* 56 for Fe, *m/z* 59 for Co, and *m/z* 60 for Ni. The medium resolution (MR) (greater than 4,000 *m/z*) was used because these elements were heavily interfered with by polyatomic ion species produced by a combination of isotopes coming from plasma, matrix, and reagents. Samples

were diluted 10 times before analysis, and only ultrapure acids and ultrahigh-quality (UHQ) water (18.2 M Ω) were used. The calibration range was adapted to the element concentrations in the unknown samples. In order to correct for eventual instrumental drifts and matrix effects, Ge 73 was used as an internal standard. In order to check the quality of the analysis, the certified reference samples SPS-SW1, SPS-SW2, and TMDA-52.3 were analyzed in between the *H. mustelae* samples and were within their certified values.

RESULTS

The *fecD*, *ceuE*, and *tonB2* genes are unlikely to encode iron acquisition proteins. We first assessed whether the transcription of the proposed ferric citrate and enterochelin uptake genes *fecD* and *ceuE* is regulated by Fur and/or iron, as is commonly observed for iron acquisition systems (1). RNA was isolated from *H. mustelae* wild-type and *fur* mutant strains cultured under iron-restricted and iron-replete conditions. As in *H. pylori* (13, 15, 39), the transcription of the *ceuE* and *fecD* genes was not affected by iron or the absence of Fur (Fig. 1A), suggesting a role in the acquisition of metals other than iron. As a control we included the *frpB2* gene, which displayed the characteristic iron-repressed transcription in the wild-type strain but displayed derepressed and iron-independent transcription in the *fur* mutant (Fig. 1A).

We also investigated whether the two *H. mustelae* TonB orthologs are involved in iron acquisition by growth promotion assays with heme and ferric chloride as the sole iron sources. Iron was chelated from serum-supplemented brucella medium by using deferoxamine B (39), resulting in the absence of growth without an external iron source, whereas the addition of ferric chloride restored the growth of wild-type *H. mustelae* (Fig. 1B). *H. mustelae* was capable of using heme as the sole iron source; however, an inhibition zone around the heme-supplemented disc indicated heme toxicity at higher concentrations, as reported recently (30). The inactivation of the *tonB1* gene prevented the utilization of heme as the sole iron source (Fig. 1B) but did not affect growth using iron chloride. In contrast, the inactivation of *tonB2* did not affect the utilization of heme as the single iron source (Fig. 1B). The absence of both TonB orthologs gave the same results as those with the *tonB1* mutant for heme and the *tonB2* mutant for iron chloride (Fig. 1B). The inactivation of any of the seven putative TonB-dependent outer membrane transporter genes (see Table S1 in the supplemental material) did not affect the ability to use heme or iron chloride as the sole iron source (data not shown). Overall, this suggests that the *tonB2*, *fecD*, and *ceuE* genes are not involved in iron acquisition but may function in the acquisition of other metals or compounds.

Inactivation of the *nikH*, *tonB2*, *fecDE*, and *ceuE* genes results in reduced urease activity and a reduced cellular nickel content. We subsequently investigated the possible role of the two *tonB* genes and the *fecDE* and *ceuE* genes in nickel acquisition, similar to what was shown previously for the *H. pylori* TonB2 system (28) and the *H. mustelae* *nikH* gene (34). Urease enzyme activity was initially used as a reporter system for the intracellular nickel concentration (34, 45). As a positive control we included the *H. mustelae* *nikH* gene, since the inactivation of *nikH* results in a ~50% reduction of urease activity in unsupplemented brucella medium (34). The inactivation of *tonB2*, *fecD*, and *ceuE* resulted in a ~50% reduction of urease activity compared to that of the wild-type strain, whereas the

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

inactivation of *tonB1* and *feoB* did not affect urease activity (Fig. 2A). Surprisingly, the inactivation of the *nixA* gene, encoding the *H. mustelae* ortholog of the *H. pylori* inner membrane transporter for nickel (19, 46), did not result in a reduction of urease activity, suggesting the presence of an alternative pathway for nickel transport across the inner membrane. The inactivation of the *fecD* gene in combination with *nixA*, *nikH*, or *tonB2* or the inactivation of the *ceuE* gene in combination with *nixA* or *nikH* resulted in a virtual absence of urease activity (Fig. 2A). In contrast, the inactivation of either *tonB2* or *nikH* in combination with a *nixA* mutation did not show a further reduction of urease activity. This finding suggests that NikH and NixA are involved in the same nickel acquisition pathway. Finally, the supplementation of brucella broth with nickel to a final concentration of 100 μ M fully restored urease activity in the *fecD* mutant, similar to what was described

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

previously for an *H. mustelae* *nikH* mutant (34), but only partially restored urease activity in the *nixA fecD* double mutant (see Fig. S1 in the supplemental material).

High-resolution induction-coupled mass spectrometry (HR-ICPMS) (23, 36) was used to determine the cellular levels of nickel and cobalt in the wild-type strain and eight of the *H. mustelae* mutants previously tested for urease activity (Fig. 2B). There was a very good match between cellular nickel content and urease activity, since reduced urease activity in the *nikH*, *tonB2*, *fecD*, *ceuE*, and *fecD nixA* mutants was reflected in similar decreases in nickel contents (Fig. 2B). Iron levels were not significantly different between the tested strains, ranging from 95% to 128% of the levels in the wild-type strain (see Fig. S2 in the supplemental material). We also used the regulation of the expression of the two *H. mustelae* urease systems to confirm the effect of mutations on nickel levels in the cell, since the UreAB urease is nickel induced and the UreA2B2 urease is nickel repressed, both via the NikR regulator (33, 34). When grown in nickel-supplemented Ham's F-12 tissue culture medium, only the UreB subunit was detected in the wild-type

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

Overlap between nickel and cobalt acquisition pathways in *H. mustelae*. To assess whether the putative nickel acquisition systems identified here are specific for nickel, we also determined cellular cobalt levels by HR-ICPMS. Relative cobalt levels matched the trend in cellular nickel levels, since the inactivation of *fecD*, *ceuE*, *tonB2*, and *nikH* resulted in a significant decrease in cobalt levels, whereas the inactivation of *nixA* did not affect cobalt levels (Fig. 2B). We also noticed that cobalt supplementation of Ham's F-12 medium resulted in growth inhibition, already at low concentrations (Fig. 3A), and hence also assessed the cobalt resistance of the *nixA* and *fecD* mutants and the *nixA fecD* double mutant. The inactivation of the *fecD* gene resulted in a significant increase in cobalt resis-

FIG. 3. The inactivation of the *fecD* gene increases the cobalt resistance of *H. mustelae*, and the inactivation of both *fecD* and cobalt deregulate the expression of urease subunits. (A) Cobalt sensitivity of wild-type *H. mustelae* NCTC 12198 and its isogenic *nixA*, *fecD*, and *nixA fecD* mutants, grown in Ham's F-12 medium, assessed by the optical density at 600 nm after 24 h of growth. Results shown are the averages of data from three independent experiments. Error bars represent standard deviations, and asterisks represent a significant difference in the OD₆₀₀ ($P \leq 0.05$ by a Mann-Whitney U test) compared to that of the same strain grown without the addition of cobalt. Mutants are shown on the x axis; dark gray bars represent no addition of cobalt, whereas white, black, and light gray bars represent final concentrations of 1, 10, and 100 μ M cobalt in the medium, respectively. (B) Cobalt supplementation at a final concentration of 1 μ M deregulates urease expression in wild-type *H. mustelae* NCTC 12198 (wt) and its isogenic *nixA* mutant but does not affect urease expression patterns in the isogenic *fecD* and *fecD nixA* mutants. (Top) Relevant part of an SDS-PAGE gel. (Bottom) Immunoblot using an antibody recognizing both the UreB and UreB2 subunits. Relevant marker sizes are indicated on the left, and the positions of the UreB2 and UreB proteins are indicated on the right.

tance, with growth unaffected at 10 μ M cobalt, where the wild type and *nixA* mutant showed virtually no increase in the OD₆₀₀ compared to that of the inoculum at 10 μ M cobalt (Fig. 3A). The overall growth rate of the *nixA* mutants was reduced, but the pattern of growth resistance was not altered by the *nixA* mutation compared to the wild-type strain and the *fecD* mutant background (Fig. 3A). In contrast, there was no difference between the mutants in nickel sensitivity, as a decrease in the final OD₆₀₀ was observed only at 1,000 μ M nickel but not at 10 and 100 μ M nickel (see Fig. S3 in the supplemental material).

Cobalt also affected the expression of the two *H. mustelae* ureases (Fig. 3B), since growth in Ham's F-12 medium supplemented with 1 μ M cobalt resulted in the repression of UreB2 expression, similar to the effect of nickel supplementation (33). The inactivation of the *fecD* gene abolished the cobalt-dependent repression of UreB2 expression (Fig. 3B), similar to what was observed for an *H. mustelae nikR* mutant (data not shown), supporting a role for the FecD protein in cobalt acquisition.

DISCUSSION

Iron and nickel are two essential metal ions for gastric *Helicobacter* species, since iron participates in many redox reactions and in respiration, whereas nickel functions as a cofactor for the urease and hydrogenase enzymes, responsible for acid resistance, nitrogen metabolism, and energy production (20, 32). Many studies of the role of iron and nickel in *Helicobacter* species have focused on the regulation of metal acquisition systems (24, 38, 44), while relatively few studies have investigated the putative metal acquisition systems themselves (10, 11, 28, 41). In this study we have inactivated genes annotated as iron and nickel acquisition systems of *H. mustelae* and demonstrate that several of the proposed iron acquisition genes have been misannotated and should be reannotated as probable nickel/cobalt transporters. Furthermore, our data support a role for *H. mustelae* TonB2 in facilitating the transport of metals other than iron, like nickel (28, 29).

Since heme is a predominant iron source in the host, it was not surprising that both *H. pylori* and *H. mustelae* are able to utilize heme as the sole iron source (11). We were able to confirm these results with our growth promotion assay (Fig. 1B). Interestingly, the genome sequences of *H. pylori* and *H. mustelae* lack genes encoding clear candidates for heme transporters (22, 35), and although our studies did not allow us to identify a heme transporter, we were able to demonstrate that a *tonB1* mutant was unable to grow on heme as the sole iron source (Fig. 1B), suggesting a role for a TonB-dependent outer membrane receptor.

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

A comparison of the complete genome sequences of *Helicobacter* species previously suggested that only the *H. hepaticus* genome contains a complete ABC transporter system for nickel (4), while the *H. pylori*, *H. acinonychis*, and *H. mustelae* genome sequences lack a clear candidate for such an ABC transporter (12, 22, 35), with only the predicted ferric citrate permease and ATPase FecDE and a CeuE periplasmic binding protein for enterochelin being annotated (12, 22, 35). Our findings that FecD and CeuE are involved in nickel acquisition are in agreement with the absence of Fur- and iron-responsive regulation of these genes in *H. pylori* (39) and *H. mustelae* (Fig.

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

1A), the location of the *H. mustelae* *ceuE* gene directly downstream of the *nikH* gene (22, 34), the nickel-responsive regulation of *ceuE* in *H. pylori* (7), and the predicted NikR operators in the *H. pylori* *fecDE* promoter (34).

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

Nickel and cobalt acquisition pathways often overlap; this is likely due to the similarities between these two transition metals (26, 48). It was demonstrated for *H. pylori* that a *nikR* mutant was affected in cobalt resistance, and nickel was able to rescue cobalt toxicity (5, 7), which is suggestive of competition in transport. The increased cobalt resistance observed for *fecD* mutants (Fig. 3B) suggests that FecD is also involved in cobalt acquisition, and this was confirmed by the decreased cellular cobalt levels in this mutant. The nickel-regulatory protein NikR seemed not to be able to fully discriminate between cobalt and nickel, since the addition of 1 μ M cobalt in the absence of nickel resulted in the repression of UreB2 expres-

sion (Fig. 3B), similar to what happens under nickel-sufficient conditions (33, 34). These results are in agreement with the finding that cobalt-cofactored NikR from *H. pylori* and *E. coli* is able to bind target DNA *in vitro* although with less affinity than nickel-cofactored NikR (42, 47). Similarly, the specificity of the *Mycobacterium tuberculosis* NmtR repressor not only is determined by the protein itself but also depends on the metals imported (6).

In conclusion, the available genome sequences of gastric *Helicobacter* species contain multiple genes annotated as being putatively involved in iron acquisition, of which the *H. mustelae* *ceuE* and *fecDE* genes are likely to encode a novel nickel and cobalt acquisition system. This redresses the apparent imbalance between iron and nickel acquisition genes in the *H. mustelae* genome sequence (4, 22, 48), since several genes previously annotated as being involved in iron acquisition can now be reclassified.

ACKNOWLEDGMENTS

The complete genome sequence of *H. mustelae* was determined at the Wellcome Trust Sanger Institute, Hinxton, United Kingdom, with funding of the Wellcome Trust. A.H.M.V.V. is supported in part by a BBSRC Institute Strategic Programme grant to the Institute of Food Research.

We thank Christopher Rice for the helpful discussions on HR-ICPMS experiments.

REFERENCES

- Andrews, S. C., A. K. Robinson, and F. Rodriguez-Quinones. 2003. Bacterial iron homeostasis. *FEMS Microbiol. Rev.* 27:215–237.
- Andruttis, K. A., J. G. Fox, D. B. Schauer, R. P. Marini, J. C. Murphy, L. Yan, and J. V. Solnick. 1995. Inability of an isogenic urease-negative mutant strain of *Helicobacter mustelae* to colonize the ferret stomach. *Infect. Immun.* 63:3722–3725.
- Belzer, C., J. Stoof, C. S. Beckwith, E. J. Kuipers, J. G. Kusters, and A. H. M. van Vliet. 2005. Differential regulation of urease activity in *Helicobacter hepaticus* and *Helicobacter pylori*. *Microbiology* 151:3989–3995.
- Belzer, C., J. Stoof, and A. H. M. van Vliet. 2007. Metal-responsive gene regulation and metal transport in *Helicobacter* species. *Biometals* 20:417–429.
- Bruggraber, S. F., G. French, R. P. Thompson, and J. J. Powell. 2004. Selective and effective bactericidal activity of the cobalt (II) cation against *Helicobacter pylori*. *Helicobacter* 9:422–428.
- Cavet, J. S., W. Meng, M. A. Pennella, R. J. Appelhoff, D. P. Giedroc, and N. J. Robinson. 2002. A nickel-cobalt-sensing ArsR-SmtB family repressor. Contributions of cytosol and effector binding sites to metal selectivity. *J. Biol. Chem.* 277:38441–38448.
- Contreras, M., J. M. Thiberge, M. A. Mandrand-Berthelot, and A. Labigne. 2003. Characterization of the roles of NikR, a nickel-responsive pleiotropic autoregulator of *Helicobacter pylori*. *Mol. Microbiol.* 49:947–963.
- Croinin, T. O., A. McCormack, A. H. M. van Vliet, J. G. Kusters, and B. Bourke. 2007. Random mutagenesis to identify novel *Helicobacter mustelae* virulence factors. *FEMS Immunol. Med. Microbiol.* 50:257–263.
- Danielli, A., S. Romagnoli, D. Roncarati, L. Costantino, I. Delany, and V. Scarlato. 2009. Growth phase and metal-dependent transcriptional regulation of the *fecA* genes in *Helicobacter pylori*. *J. Bacteriol.* 191:3717–3725.
- Davis, G. S., E. L. Flannery, and H. L. Mobley. 2006. *Helicobacter pylori* HP1512 is a nickel-responsive NikR-regulated outer membrane protein. *Infect. Immun.* 74:6811–6820.
- Dhaenens, L., F. Szczepara, S. Van Nieuwenhuysse, and M. O. Husson. 1999. Comparison of iron uptake in different *Helicobacter* species. *Res. Microbiol.* 150:475–481.
- Eppinger, M., C. Baar, B. Linz, G. Raddatz, C. Lanz, H. Keller, G. Morelli, H. Gressmann, M. Achtman, and S. C. Schuster. 2006. Who ate whom? Adaptive *Helicobacter* genomic changes that accompanied a host jump from early humans to large felines. *PLoS Genet.* 2:e120.
- Ernst, F. D., S. Bereswill, B. Waidner, J. Stoof, U. Mader, J. G. Kusters, E. J. Kuipers, M. Kist, A. H. M. van Vliet, and G. Homuth. 2005. Transcriptional profiling of *Helicobacter pylori* Fur- and iron-regulated gene expression. *Microbiology* 151:533–546.
- Ernst, F. D., J. Stoof, W. M. Horrevorts, E. J. Kuipers, J. G. Kusters, and A. H. M. van Vliet. 2006. NikR mediates nickel-responsive transcriptional repression of the *Helicobacter pylori* outer membrane proteins FecA3 (HP1400) and FecB4 (HP1512). *Infect. Immun.* 74:6821–6828.

15. Gancz, H., S. Censini, and D. S. Merrell. 2006. Iron and pH homeostasis intersect at the level of Fur regulation in the gastric pathogen *Helicobacter pylori*. *Infect. Immun.* **74**:602–614.
16. Haas, R., A. F. Kahrs, D. Facius, H. Allmeier, R. Schmitt, and T. F. Meyer. 1993. TnMax—a versatile mini-transposon for the analysis of cloned genes and shuttle mutagenesis. *Gene* **130**:23–31.
17. Kusters, J. G., A. H. M. van Vliet, and E. J. Kuipers. 2006. Pathogenesis of *Helicobacter pylori* infection. *Clin. Microbiol. Rev.* **19**:449–490.
18. Mulrooney, S. B., and R. P. Hausinger. 2003. Nickel uptake and utilization by microorganisms. *FEMS Microbiol. Rev.* **27**:239–261.
19. Nolan, K. J., D. J. McGee, H. M. Mitchell, T. Kolesnikow, J. M. Harro, J. O'Rourke, J. E. Wilson, S. J. Danon, N. D. Moss, H. L. Mobley, and A. Lee. 2002. In vivo behavior of a *Helicobacter pylori* SS1 *nixA* mutant with reduced urease activity. *Infect. Immun.* **70**:685–691.
20. Olson, J. W., and R. J. Maier. 2002. Molecular hydrogen as an energy source for *Helicobacter pylori*. *Science* **298**:1788–1790.
21. O'Rourke, J. L., and A. Lee. 2003. Animal models of *Helicobacter pylori* infection and disease. *Microbes Infect.* **5**:741–748.
22. O'Toole, P. W., W. J. Snelling, C. Canchaya, B. M. Forde, K. R. Hardie, C. Josenhans, R. L. Graham, G. McMullan, J. Parkhill, E. Belda, and S. D. Bentley. 2010. Comparative genomics and proteomics of *Helicobacter mustelae*, an ulcerogenic and carcinogenic gastric pathogen. *BMC Genomics* **11**:164.
23. Outten, C. E., and T. V. O'Halloran. 2001. Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. *Science* **292**:2488–2492.
24. Pflock, M., S. Kennard, N. Finsterer, and D. Beier. 2006. Acid-responsive gene regulation in the human pathogen *Helicobacter pylori*. *J. Biotechnol.* **126**:52–60.
25. Pot, R. G., J. Stoof, P. J. Nuijten, L. A. de Haan, P. Loeffen, E. J. Kuipers, A. H. M. van Vliet, and J. G. Kusters. 2007. UreA2B2: a second urease system in the gastric pathogen *Helicobacter felis*. *FEMS Immunol. Med. Microbiol.* **50**:273–279.
26. Rodionov, D. A., P. Hebbeln, M. S. Gelfand, and T. Eitinger. 2006. Comparative and functional genomic analysis of prokaryotic nickel and cobalt uptake transporters: evidence for a novel group of ATP-binding cassette transporters. *J. Bacteriol.* **188**:317–327.
27. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
28. Schauer, K., B. Gouget, M. Carriere, A. Labigne, and H. de Reuse. 2007. Novel nickel transport mechanism across the bacterial outer membrane energized by the TonB/ExbB/ExbD machinery. *Mol. Microbiol.* **63**:1054–1068.
29. Schauer, K., D. A. Rodionov, and H. de Reuse. 2008. New substrates for TonB-dependent transport: do we only see the 'tip of the iceberg'? *Trends Biochem. Sci.* **33**:330–338.
30. Senkovich, O., S. Ceaser, D. J. McGee, and T. L. Testerman. 2010. Unique host iron utilization mechanisms of *Helicobacter pylori* revealed using iron-deficient chemically defined media. *Infect. Immun.* **78**:1841–1849.
31. Solnick, J. V., and D. B. Schauer. 2001. Emergence of diverse *Helicobacter* species in the pathogenesis of gastric and enterohepatic diseases. *Clin. Microbiol. Rev.* **14**:59–97.
32. Stingl, K., and H. De Reuse. 2005. Staying alive overdosed: how does *Helicobacter pylori* control urease activity? *Int. J. Med. Microbiol.* **295**:307–315.
33. Stoof, J., S. Breijer, R. G. Pot, D. van der Neut, E. J. Kuipers, J. G. Kusters, and A. H. M. van Vliet. 2008. Inverse nickel-responsive regulation of two urease enzymes in the gastric pathogen *Helicobacter mustelae*. *Environ. Microbiol.* **10**:2586–2597.
34. Stoof, J., E. J. Kuipers, and A. H. M. van Vliet. 2010. Characterization of NikR-responsive promoters of urease and metal transport genes of *Helicobacter mustelae*. *Biometals* **23**:145–159.
35. Tomb, J. F., O. White, A. R. Kerlavage, R. A. Clayton, G. G. Sutton, R. D. Fleischmann, K. A. Ketchum, H. P. Klenk, S. Gill, B. A. Dougherty, K. Nelson, J. Quackenbush, L. Zhou, E. F. Kirkness, S. Peterson, et al. 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature* **388**:539–547.
36. Tree, J. J., S. P. Kidd, M. P. Jennings, and A. G. McEwan. 2005. Copper sensitivity of *cueO* mutants of *Escherichia coli* K-12 and the biochemical suppression of this phenotype. *Biochem. Biophys. Res. Commun.* **328**:1205–1210.
37. Tsuda, M., M. Karita, M. G. Morshed, K. Okita, and T. Nakazawa. 1994. A urease-negative mutant of *Helicobacter pylori* constructed by allelic exchange mutagenesis lacks the ability to colonize the nude mouse stomach. *Infect. Immun.* **62**:3586–3589.
38. van Vliet, A. H. M., F. D. Ernst, and J. G. Kusters. 2004. NikR-mediated regulation of *Helicobacter pylori* acid-adaptation. *Trends Microbiol.* **12**:489–494.
39. van Vliet, A. H. M., J. Stoof, R. Vlasblom, S. A. Wainwright, N. J. Hughes, D. J. Kelly, S. Bereswill, J. J. Bijlsma, T. Hoogenboezem, C. M. Vandenbroucke-Grauls, M. Kist, E. J. Kuipers, and J. G. Kusters. 2002. The role of the ferric uptake regulator (Fur) in regulation of *Helicobacter pylori* iron uptake. *Helicobacter* **7**:237–244.
40. van Vliet, A. H. M., K. G. Wooldridge, and J. M. Ketley. 1998. Iron-responsive gene regulation in a *Campylobacter jejuni* *fur* mutant. *J. Bacteriol.* **180**:5291–5298.
41. Velayudhan, J., N. J. Hughes, A. A. McCollm, J. Bagshaw, C. L. Clayton, S. C. Andrews, and D. J. Kelly. 2000. Iron acquisition and virulence in *Helicobacter pylori*: a major role for FeoB, a high-affinity ferrous iron transporter. *Mol. Microbiol.* **37**:274–286.
42. Wang, S. C., A. V. Dias, and D. B. Zamble. 2009. The "metallo-specific" response of proteins: a perspective based on the *Escherichia coli* transcriptional regulator NikR. *Dalton Trans.* **2009**:2459–2466.
43. Weinberg, E. D. 2007. Antibiotic properties and applications of lactoferrin. *Curr. Pharm. Des.* **13**:801–811.
44. Whitmire, J. M., H. Gancz, and D. S. Merrell. 2007. Balancing the double-edged sword: metal ion homeostasis and the ulcer bug. *Curr. Med. Chem.* **14**:469–478.
45. Wolfram, L., and P. Bauerfeind. 2009. Activities of urease and nickel uptake of *Helicobacter pylori* proteins are media- and host-dependent. *Helicobacter* **14**:264–270.
46. Wolfram, L., and P. Bauerfeind. 2002. Conserved low-affinity nickel-binding amino acids are essential for the function of the nickel permease NixA of *Helicobacter pylori*. *J. Bacteriol.* **184**:1438–1443.
47. Zambelli, B., A. Danielli, S. Romagnoli, P. Neyroz, S. Ciurli, and V. Scarlato. 2008. High-affinity Ni²⁺ binding selectively promotes binding of *Helicobacter pylori* NikR to its target urease promoter. *J. Mol. Biol.* **383**:1129–1143.
48. Zhang, Y., D. A. Rodionov, M. S. Gelfand, and V. N. Gladyshev. 2009. Comparative genomic analyses of nickel, cobalt and vitamin B12 utilization. *BMC Genomics* **10**:78.