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Characterization of NikR-responsive promoters of urease and metal transport genes of *Helicobacter mustelae*

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Abstract The NikR protein is a nickel-responsive regulator, which in the gastric pathogen *Helicobacter pylori* controls expression of nickel-transporters and the nickel-cofactored urease acid resistance determinant. Although NikR-DNA interaction has been well studied, the *Helicobacter* NikR operator site remains poorly defined. In this study we have identified the NikR operators in the promoters of two inversely nickel-regulated urease operons (*ureAB* and *ureA2B2*) in the ferret pathogen *Helicobacter mustelae*, and have used bioinformatic approaches for the prediction of putative NikR operators in the genomes of four urease-positive *Helicobacter* species. *Helicobacter mustelae* NikR bound to the *ureA2* promoter to a sequence overlapping with the −35 promoter region, leading to repression. In contrast, NikR binding to a site far upstream of the canonical σ^{80} promoter in the *H. mustelae ureA* promoter resulted in transcriptional induction, similar to the situation in *H. pylori*. Using *H. pylori* NikR operators and the newly identified *H. mustelae* NikR operators a new consensus sequence was generated (TRWYA-N_{15}-TRWYA), which was used to screen the genomes of four urease-positive *Helicobacter* species (*H. mustelae*, *H. pylori*, *H. acinonychis* and *H. hepaticus*) for putative NikR-regulated promoters. One of these novel putative NikR-regulated promoters in *H. mustelae* is located upstream of a putative TonB-dependent outer membrane protein designated NikH, which displayed nickel-responsive expression. Insertional inactivation of the *nikH* gene in *H. mustelae* resulted in a significant decrease in urease activity, and this phenotype was complemented by nickel-supplementation of the growth medium, suggesting a function for NikH in nickel transport accross the outer membrane. In conclusion, the *H. mustelae* NikR regulator directly controls nickel-responsive regulation of ureases and metal transporters. The improved consensus NikR operator sequence allows the prediction of additional NikR targets in *Helicobacter* genomes, as demonstrated by the identification of a new nickel-repressed outer membrane protein in *H. mustelae*.

Keywords Nickel-responsive gene regulation · Nickel uptake · NikR repressor · Helicobacter

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Introduction

The mammalian stomach is an inhospitable environment for bacteria, and therefore was long thought to be sterile, but this dogma was refuted by the discovery of the human pathogen *Helicobacter pylori* (Marshall and Warren 1984). This bacterium is able to colonize the environment of the mucus layer overlaying the gastric epithelial cells, and the resulting gastritis predisposes to peptic ulceration and gastric cancer (Kusters et al. 2006). Since the discovery of *H. pylori*, many other *Helicobacter* species have been identified, and it is now generally acknowledged that the gastric mucosa of most, if not all, mammals can be colonized by gastric *Helicobacter* species (Solnick and Schauer 2001). These non-*pylori Helicobacter* species may provide good animal models to study *Helicobacter* infection in their natural hosts (O'Rourke and Lee 2003) and comparative genomics may contribute in our understanding of host specificity (Eppinger et al. 2006).

The divalent metal nickel plays a central part in the gastric lifestyle of *Helicobacter* species. Nickel is the cofactor of the urease enzyme (Burne and Chen 2000), which is the key component that enables gastric *Helicobacter* species to survive the stressful acidic conditions in the gastric mucosa. Urease converts urea into ammonia and carbon dioxide, which results in a net increase in the local pH (Burne and Chen 2000), and urease-negative mutants of different *Helicobacter* species are unable to colonize the gastric environment (Tsuda et al. 1994; Andrutis et al. 1995; Stingl et al. 2002).

Metal ions like nickel pose a problem for bacteria. While they are essential for metabolism, they are also capable of generating toxic compounds like reactive oxygen species, and hence bacteria have developed finely tuned systems to control intracellular availability of metals (Mulrooney and Hausinger 2003). For nickel, a nickel-responsive regulatory protein represents the common mechanism of control in bacteria. This nickel-responsive regulatory protein is capable of repressing nickel import mechanisms once cytoplasmic nickel concentrations exceed a certain threshold. The most common nickel-responsive regulator in bacteria is the ribbon-helix-helix regulatory protein NikR (Chivers and Sauer 2000). The NikR protein was previously identified as a key regulator of urease expression in *H. pylori* (van Vliet et al. 2002). In addition to urease, NikR regulates expression of other genes involved in nickel homeostasis by binding to NikR operators in, or upstream of, the promoter regions (Delany et al. 2005; Ernst et al. 2005b, 2006; Davis et al. 2006; Danielli et al. 2009). *H. pylori* NikR binds with different affinities to NikR operators in vitro (Abraham et al. 2006; Benanti and Chivers 2007; Dosanjh et al. 2009), and the sequence variation in the NikR operators has made it difficult to define a consensus sequence which correctly predicts NikR-regulated genes.

Recently we described the presence (Pot et al. 2007) and characterization (Stoof et al. 2008) of two urease gene clusters (*ureABIEFGH* and *ureA2B2*) in three *Helicobacter* species (*H. mustelae*, *H. acinonychis* and *H. felis*) colonizing obligate carnivores (ferrets, big cats and cats, respectively). As with the *H. pylori* urease gene cluster, *H. mustelae* UreAB is positively regulated by the availability of nickel (van Vliet et al. 2001, 2002). In sharp contrast to the UreAB urease system, the expression of the *H. mustelae* UreA2B2 urease system is repressed upon nickel supplementation, but induced upon iron supplementation. Due to the regulatory pattern, independency of accessory proteins and the inactivation of the enzyme upon lyses, we speculated that this gene cluster may encode an iron cofactored urease. UreA2B2 may therefore be an evolutionary adaptation of carnivore colonizing *Helicobacter* species to the nickel-limited but iron-rich diet of their host (Stoof et al. 2008). Although nickel-dependent regulation of both ureases was absent in an *H. mustelae nikR* mutant, direct interaction of NikR with the *ureA* and *ureA2* promoters was not tested.

In this study we have investigated the role of NikR in nickel-responsive regulation of the UreAB and UreA2B2 urease systems of *H. mustelae*. We demonstrate that NikR directly interacts with both urease promoters of *H. mustelae*. Using the newly identified NikR binding sites of the *H. mustelae ureA* and *ureA2* promoters, a new NikR operator consensus sequence has been proposed, and this new consensus sequence has been used for the prediction of new NikR operators in complete genome sequences of *Helicobacter* species. All previously confirmed high-affinity NikR operators in *H. pylori* were recognized, and searching of the *H. mustelae* genome sequence allowed the identification of a new nickel-regulated outer membrane protein in *H. mustelae*, which contributes to urease activity.

Materials and methods

Bacterial strains and growth conditions

Helicobacter mustelae strain NCTC 12198 (ATCC 43772) was used for all experiments in this study and was cultured at 37°C in a microaerobic atmosphere of 5% O_2, 7.5% CO_2, 7.5% H_2 and 80% N_2. Dent agar plates consisting of Columbia agar (Oxoid) supplemented with 7% saponin-lysed defibrinated horse blood (BioTrading), 0.004% triphenyltetrazolium chloride (Sigma) and Dent Selective Supplement (Oxoid) were used for routine growth. Broth cultures of *H. mustelae* were grown in Ham's F-12 tissue culture medium (Kaighn's modification, Invitrogen) supplemented with 0.2% ß-cyclodextrin (Fluka) and Dent supplement. Since this medium may not contain ligands needed for nickel transport across the outer membrane, the urease assay was performed with *H. mustelae* grown in Brucella broth supplemented with 3% heat inactivated Newborn Calf Serum (Difco) and Dent supplement.

Broth cultures were shaken at 70 rpm and incubated at 37°C for a maximum of 24 h. Iron-restriction of Ham's F-12 medium (Kaighn's modification) was achieved by addition of deferoxamine (Sigma) to a final concentration of 4 μM, whereas iron-replete medium was obtained by supplementing iron-restricted Ham's F-12 medium (Kaighn's modification) with $FeCl_3$ to a final concentration of 10 μM (Stoof et al. 2008).

Escherichia coli strains DH5α and M147 were cultured in Luria-Bertani media (Biotrading) (Sambrook et al. 1989). When appropriate, growth media were supplemented with ampicillin (100 μg ml^{-1}), chloramphenicol (20 μg ml^{-1}), kanamycin (20 μg ml^{-1}) or erythromycin (250 μg ml^{-1}) for selection of *E. coli* transformants. *H. mustelae* mutants were selected on media 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 *H. mustelae fur, hm0418-1*, *hm0418-2* and *hm0418-3* mutants

Construction of the *H. mustelae nikR* mutant was described previously (Stoof et al. 2008). The *fur*, *hm0418-1*, *hm0418-2* and *hm0418-3* genes of *H. mustelae* strain NCTC 12198 were PCR amplified using the KO primers described (Table 1), and the amplicons were cloned in pGEM-T_{easy} vector (Promega). The sources of the chloramphenicol, kanamycin and erythromycin resistance cassettes were plasmids pAV35 (van Vliet et al. 1998), pJMK30 (van Vliet et al. 1998) and pDH20 (Haas et al. 1993), and these were inserted in the unique restriction sites *Bcl*I (*fur*), *Bam*HI (*hm0418-1*), *Eco47*III (*hm0418-2*) and *Eco47*III (*hm0418-3*) of the corresponding genes, in the same transcriptional orientation. The interrupted genes were subsequently introduced into *H. mustelae* strain NCTC 12198 by natural transformation (Croinin et al. 2007; Stoof et al. 2008) and correct replacement by homologous recombination of the genes with the interrupted version was confirmed by PCR using ORS primers described (Table 1) which are located outside of the recombination region.

Protein analysis

Liquid cultures were centrifuged for 10 min at 4,000×*g* and resuspended in phosphate-buffered saline to a final OD_{600} of 10. Bacteria were lysed by sonication for 15 s with an MSE Soniprep 150 set at amplitude 6. Whole-cell proteins (OD10) were separated on 6% (Hm0418 detection) or 8% (UreB detection) sodium dodecyl sulfate polyacrylamide gels (SDS–PAGE) (Sambrook et al. 1989). For Western immunoblot, proteins were subsequently transferred to a nitrocellulose membrane. Expression of urease subunits was monitored on immunoblots by using a 1:10,000 dilution of antibodies raised against *H. felis* urease (Belzer et al. 2005; Pot et al. 2007).

Urease assay

The enzymatic activity of urease was determined by measuring ammonia production from hydrolysis of urea, by using the Berthelot reaction as described previously (van Vliet et al. 2001). Briefly, cells lysed by sonication were 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 addition of phenol nitroprusside and alkaline hypochlorite (Sigma Diagnostics).

Table 1 Oligonucleotide primers used in this study

Primer	Sequence (5′ → 3′)
Hmus_fur_KO_F	AGGCCATCTCCCTGTATTGC
Hmus_fur_KO_R	TGCACGCAGTCTCTTTGTG
Hmus_fur_ORS_F	GCAAAAGCACAATCCCCTCTG
Hmus_fur_ORS_R	AGAATTGAGGGGAAAACGTG
Hmus_0418-1_KO_F	GAGGACGTGGATGGAGTTTG
Hmus_0418-1_KO_R	TGATAAGGAAGCGCGATGTG
Hmus_0418-1_ORS_F	GCAGAGCAAGGGCGAAATTC
Hmus_0418-1_ORS_R	TATGCCCACCACCTTATCCC
Hmus_0418-2_KO_F	GGATTTCAGGGCTGCATGTG
Hmus_0418-2_KO_R	CCCAGTGGCTCACCAAATTC
Hmus_0418-2_ORS_F	GGCGTGCCCACACCTATAAG
Hmus_0418-2_ORS_R	GAATGCCGCCAGCGTATAGG
Hmus_0418-3_KO_F	CCAAGAAGGCGATTCTTTGC
Hmus_0418-3_KO_R	CCCACTGGCTATTTAGAGTC
Hmus_0418-3_ORS_F	GCGCAATTCATGACGAATCC
Hmus_0418-3_ORS_R	TTATCCTGCCCATCACAACC
Hmus_NikR_BamHI_F	GGATCCATGCGGACTATGGAAAAGGA
Hmus_NikR_PvuII_R	CAGCTGTCAAAGATCTTTGGGGAAATGAC
Hmus_ureA1_prom_F	CAATCCAAGCGCGTTTTTCAT
Hmus_ureA1_prom_R-dig	CGGCATAATGCAACATCATC
Hmus_ureA2_prom_F	AGTTAAGACTTTTGCCGTGTAG
Hmus_ureA2_prom_R-dig	CTCTCCTGCATAATACAACAAGAAT
ureA2_GS_NikR[a]	AATTATTACTAAATAATACTTTTTTAAAAAAGTTAATAC AAAGTATCAAGA
ureA2_GS_NikRc[a]	CTTGATACTTTGTATTAACTTTTTTAAAAAAGTATTATTTA GTAATAATTA
ureA2_GS_NikR_pal12[a]	AATTATTACTAAACCCCCCCTTTTTAAAAAACCCCCCCCA AAGTATCAAGA
ureA2_GS_NikR_pal12_c[a]	CTTGATACTTTGGGGGGGGTTTTTTAAAAAGGGGGGGTT TAGTAATAATTA
pGEM_F	ACGCCAAGCTATTTAGGTGAC
pGEM_R-Dig	AAACGACGGCCAGTGAATTG

[a] Primer contains an additional 3′ A residue to facilitate cloning into the pGEM-T_{easy} plasmid

The absorbance of the samples was determined at 570 nm, and compared with a standard NH_4Cl concentration curve. Protein concentrations were determined with the bicinchoninic acid method (Pierce) using bovine serum albumin as standard. Urease enzyme activity was expressed as units representing μmol of urea hydrolysed per min, and is expressed as U mg^{-1} of total protein.

Electrophoretic mobility shift assay

Recombinant *H. mustelae* NikR protein was produced in *E. coli* using the StrepTag system, essentially as described for *H. pylori* NikR (Ernst et al. 2005b). Briefly, the *H. mustelae nikR* gene was amplified using primers HmusNikR_BamHI F and HmusNikR_PvuII R (Table 1), cloned into the pASK-IBA7 vector (IBA, Gottingen, Germany), and recombinant NikR protein was purified as previously described (Ernst et al. 2005b). The promoter regions of the *ureA* and *ureA2* genes were PCR amplified with primers Hmus_ureA1_prom_F, Hmus_ureA2_prom_F, and DIG-labeled primers Hmus_ureA1_prom_R-dig and Hmus_ureA2_prom_R-dig, respectively (Table 1). Two complementary 50 nt oligonucleotides (ureA2_GS_NikR and ureA2_GS_NikR_c, Table 1), containing the putative NikR binding sites of the *ureA2* promoter, were mixed in equimolar ratio and heated to 80°C, and were slowly

cooled to room temperature. As negative control the same oligonucleotides were synthesized with replacement of both halves of the NikR bindingsite by a stretch of C-residues (ureA2_GS_NikR_pal12 and ureA2_GS_NikR_pal12_c, Table 1). Subsequently the double stranded products were cloned into pGEM-T_{easy} (Promega) and checked for sequence integrity. A 250 bp fragment was amplified from these pGEM-T_{easy} clones using the primers pGEM_F and pGEM_R-dig (Table 1). Electrophoretic mobility shift assays were performed with recombinant NikR protein as described previously (Ernst et al. 2005b). Briefly, 32.5 pM of digoxygenin-labeled *ureA* of *ureA2* promoter fragment was mixed with recombinant NikR protein at concentrations ranging from 0 to 122 pM. Protein and DNA were mixed in binding buffer (24% glycerol, 40 mM Tris–Cl, pH 8.0, 150 mM KCl, 2 mM DTT, 600 μg/ml bovine serum albumin, 2.5 ng/μl herring sperm DNA, 200 μM $NiCl_2$) in a 20 μl (final volume) mixture and incubated at 37°C for 30 min. Samples were subsequently separated on a 5% polyacrylamide gel in running buffer (25 mM Tris, 190 mM glycine) for 30 min at 200 V. The gel was then blotted onto a nylon membrane (Roche Molecular Biochemicals), and this was followed by chemiluminescent detection of DIG-labeled DNA.

Purification and analysis of RNA

Total RNA was isolated using Trizol (Gibco) according to the manufacturer's instructions. The amount of RNA was determined spectrophotometrically using the Qubit Quantitation platform according to the manufacturers' instructions (Invitrogen). The transcription start site of both the *ureA* and the *ureA2* gene of *H. mustelae* strain NCTC12198 ware determined by primer extension analysis (Ernst et al. 2005a). Briefly, approximately 5–7 μg of total RNA isolated from *H. mustelae* NCTC 12198 was incubated with 50 pmol of 5′-DIG-labeled primer UreA_R1_dig and UreA2_R1_dig (Table 1) and avian myeloblastosis virus reverse transcriptase (Promega). The sequence reactions were performed using the fmol sequencing kit (Promega) with 5′-DIG-labeled primer UreA2_R2_dig. Primer extension products and sequence reactions were separated on an 8% polyacrylamide-8 M urea gel and blotted onto a nylon membrane (Roche), and this was followed by chemiluminescent DIG detection (van Vliet et al. 2001).

Quantitative reverse transcriptase-PCR (qRT-PCR) was essentially performed as described previously (Stoof et al. 2008), using primer combinations Hmus_ureB1_qpcr_F/Hmus_ureB1_qpcr_R, Hmus_ureB2_qpcr-F/Hmus_ureB2_qpcr_R and Hmus_16S_qpcr_F/Hmus_16S_qpcr_R (Table 1), with the IQ5 system (Bio-Rad) and SYBR-green. qRT-PCR assays were performed using RNA isolated from at least three independent growth experiments. Transcript levels were normalized against the levels of 16S rRNA in each sample [2(ΔΔC(T)) analysis] (Livak and Schmittgen 2001), and expressed as ratio to the mRNA level in cells grown in nickel- and iron-restricted Ham's F-12 medium (Kaighn's modification).

Bioinformatic prediction of NikR operators in *Helicobacter* genome sequences

The intergenic regions from −198 to +2 (relative to the first nucleotide of the annotated translation initiation codon, excluding overlaps with upstream coding sequences) of *H. pylori*, *H. acinonychis* and *H. hepaticus* were extracted using the regulatory sequence analysis tools (RSAT) website (http://rsat.ulb.ac.be/rsat/) (Thomas-Chollier et al. 2008). The *H. mustelae* genome sequence was obtained from the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/Projects/H_mustelae/) and first interrogated using *H. pylori* protein sequences from metal metabolism genes. Subsequently the upstream sequences of the corresponding coding sequences were extracted. The DNA-pattern module of the RSAT website was used to search for NikR operators using the TRWYA-N_{15}-TRWYA consensus sequence, after extraction of the −198 to +2 sequences of intergenic regions relative to the first nucleotide of the translation initiation codon. The Weblogo algorithm (Crooks et al. 2004) was used to represent sequence conservation in predicted NikR operators.

Results

Role of Fur and NikR in nickel-responsive expression of *H. mustelae* urease genes

We previously demonstrated that inactivation of the *H. mustelae nikR* gene results in nickel-independent expression of both ureases of *H. mustelae*, but did not

further investigate the mechanism governing iron-responsive induction of *ureA2B2* transcription (Stoof et al. 2008). The *H. mustelae* genome sequence contains a gene encoding an ortholog of the iron-responsive regulatory protein Fur (Bereswill et al. 1998), and to further define the role of the NikR and Fur metal-responsive regulators in nickel- and iron-responsive expression of *ureA2B2* transcription, an *H. mustelae fur* mutant was created using insertional mutagenesis. UreB2 expression and *ureB2* transcription were very high in the *nikR* mutant (Stoof et al. 2008), and independent of the iron and nickel concentration, whereas mutation of *fur* did not significantly affect the nickel-responsive pattern of UreB2 expression and *ureB2* transcription (Fig. 1a, b). The expression pattern of the UreB protein and *ureB* gene was similar to that of *H. pylori* (van Vliet et al. 2001, 2002), as mutation of *nikR* resulted in absence of nickel-responsive induction, whereas mutation of *fur* did not affect UreB expression or *ureB* transcription.

Identification of the *ureAB* and *ureA2B2* promoters

The transcription start site of both urease gene clusters of *H. mustelae* was identified using primer extension analysis (Fig. 2a). Primers specific for *ureA* and *ureA2* were used in equimolar concentrations or added separately (identical results, data not shown) for primer extension analysis with RNA isolated from *H. mustelae* NCTC12198 grown in media with either nickel- and iron-restricted or -replete conditions. The transcription start site of *ureA* is located at the G residue 52 nt upstream of the *ureA* ATG start codon. The transcription start site of *ureA2 is* located at the G residue 52 nt upstream of the *ureA2* ATG start codon. Both transcription start sites are preceded by a σ^{80} -10 sequence (Fig. 2b) at the correct distance from the transcription initiation site (Petersen et al. 2003), similar to what has been observed for the *H. pylori* urease promoter (Davies et al. 2002; van Vliet et al. 2002). Nickel- and iron-responsive

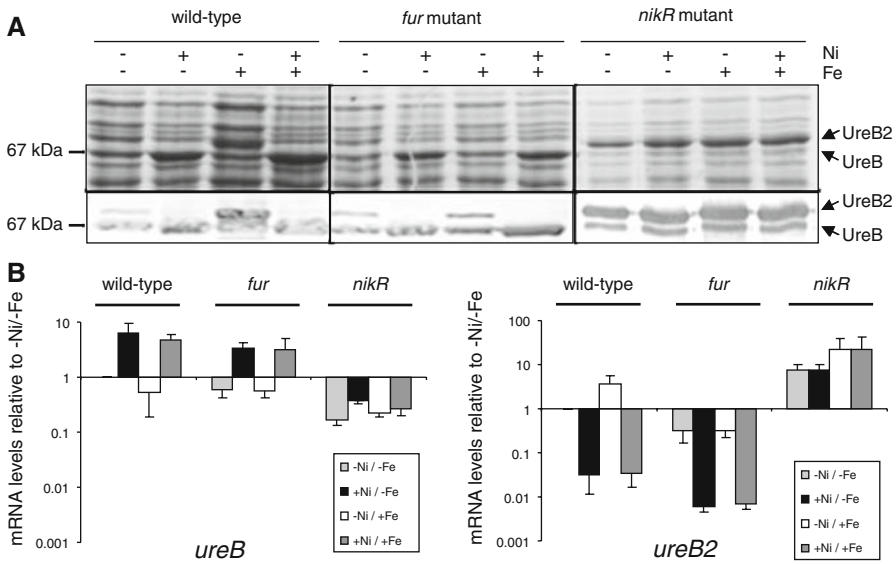


Fig. 1 NikR but not Fur controls nickel-responsive expression of the UreAB and UreA2B2 ureases of *Helicobacter mustelae*. **a** Regulation of the UreB and UreB2 protein in the *H. mustelae* wild-type strain and its isogenic *fur* and *nikR* mutants, grown in different combinations of nickel-restricted, nickel-replete, iron-restricted and iron-replete conditions (see *top panel*), as demonstrated by SDS–PAGE (*top panel*) and immunoblot using an antibody recognizing both the UreB and UreB2 subunits (*bottom panel*). The relevant marker size is indicated on the *left*, the position of the UreB and UreB2 proteins on the *right*. **b** Regulation of *ureB* and *ureB2* mRNA levels in the *H. mustelae* wild-type strain and its isogenic *fur* and *nikR* mutants, grown in the same combinations of nickel- and iron-conditions as shown in *panel A*, as determined using quantitative reverse transcriptase PCR. The levels of mRNA observed in the qRT-PCR were normalized (Livak and Schmittgen 2001) to the levels of 16S rRNA, and the -Ni/-Fe condition in the wild-type strain was set to 1. Normalization to the *ppk* gene gave similar results (data not shown) All other mRNA levels are expressed as compared to the -Ni/-Fe condition in the wild-type strain. *Error bars* represent standard deviation. Results shown are the average of three independent experiments

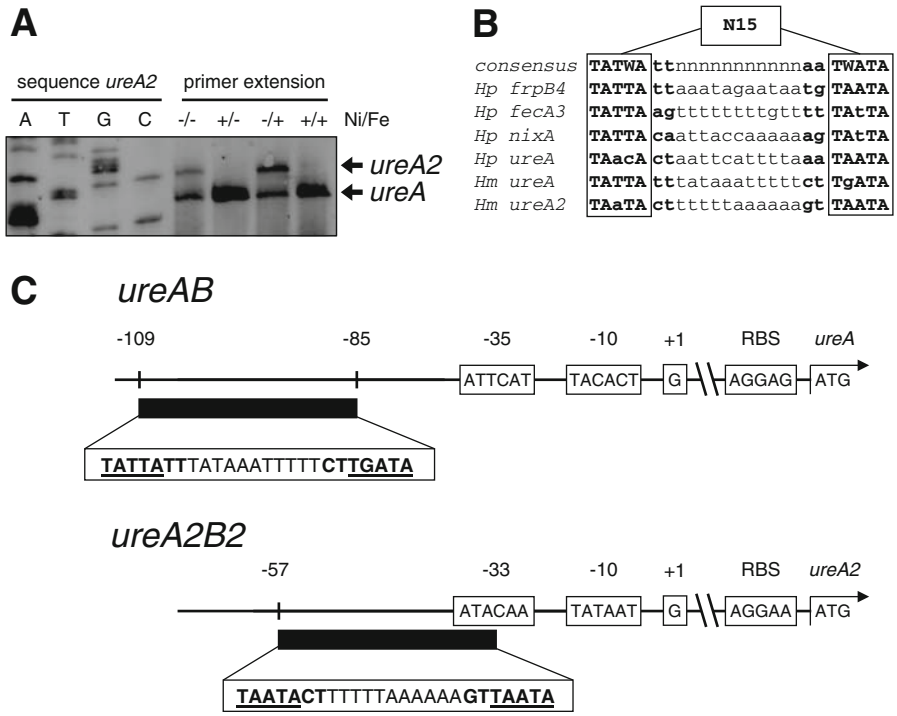


Fig. 2 Characterization of the *H. mustelae ureA* and *ureA2* promoter regions. **a** Determination of the transcriptional start site (TSS) of the *ureA* and *ureA2* genes by semi-quantitative primer extension analysis, with RNA from *H. mustelae* NCTC 12198 wild-type cells grown in different combinations of nickel-restricted, nickel-replete, iron-restricted and iron-replete conditions. The primer extension products representing the *ureA* and *ureA2* TSS are indicated on the right. The position of the TSS was determined using a sequencing reaction of the *ureA2* promoter as displayed on the left (with *lanes A*, *T*, *G*, *C*). **b** Prediction of the NikR operator sequence in the *H. mustelae* (Hm), based on the previously described consensus sequence (TATWA-N_{15}-TWATA) of *H. pylori* NikR (Delany et al. 2005; Ernst et al. 2006; Dosanjh et al. 2009). **c** Graphical representation of the *H. mustelae ureA* and *ureA2* promoter regions with the TSS, -10 and -35 regions, ribosomal binding site and ATG start codon of the *ureA* and *ureA2* genes. The predicted location and sequence of the NikR operators are indicated with a *black bar* and the sequence underneath

regulation of the transcript start site cDNA of both urease clusters is in accordance with the immunoblot and qRT-PCR data (Fig. 1a, b).

In *H. pylori*, NikR binds to palindromic sequence with as consensus sequence 5′-TATWATT-N_{11}-AATWATA, with one of the two half sites usually being less conserved (Delany et al. 2005; Ernst et al. 2006; Dosanjh et al. 2009). The *ureA* and *ureA2* promoters of *H. mustelae* and *H. acinonychis* were searched for such putative binding sites (adapted to 5′-TATWA-N_{15}-TWATA, Fig. 2b). A putative NikR box was detected from 140 to 164 nt upstream of the ATG start codon of *H. mustelae ureA* (Fig. 2c). Similar searches in the *ureA2* promoter region of *H. mustelae* allowed the identification of a putative NikR-binding site 88–112 nt (Fig. 2b) upstream of the ATG start codon of *ureA2*. In *H. mustelae* this binding sequence overlaps with the -35 region of the σ^{80} promoter upstream of the *ureA2* gene, whereas the binding sequence upstream of the *ureA* gene is located from −112 to −88, upstream of the canonical σ^{80} promoter (Fig. 2c).

NikR binds with different affinities to the *ureA* and *ureA2* promoter

To investigate whether the nickel-responsive regulation of *ureAB* and *ureA2B2* transcription is mediated by NikR binding to the *ureA* and *ureA2* promoters, an electrophoretic mobility shift assay was performed using both the *ureA* and *ureA2* promoter regions and recombinant *H. mustelae* NikR protein. In the absence of nickel, NikR neither bound the *ureA* promoter nor the *ureA2* promoter (data not shown). In the presence of nickel, NikR was able to bind to both promoters (Fig. 3a) albeit with different affinities.

Incubation of NikR with the *ureA2* promoter region led to two bands in the electrophoretic mobility shift assay (Fig. 3a), which is suggestive for the presence of multiple NikR binding sites. When the *ureA* and *ureA2* promoters were mixed in equimolar concentrations, NikR first shifted the *ureA2* promoter and subsequently the *ureA* promoter (Fig. 3a), which is consistent with the regulatory patterns of *ureAB* and *ureA2B2* transcription, where *ureA2B2* transcription is repressed at lower nickel concentrations than those where *ureAB* transcription is induced (Stoof et al. 2008).

To confirm the predicted NikR-binding site in the *H. mustelae ureA2* promoter (Fig. 2b, c), we performed electrophoretic mobility shift assays with cloned 50 nt fragments of the *H. mustelae ureA2* promoter region, one version representing the wild-type promoter including the putative NikR binding sequence, and one version where the putative NikR binding was replaced with a C stretch, as described previously (Dosanjh et al. 2009). Recombinant NikR did bind when the *ureA2* promoter with the putative NikR-binding site was present (Fig. 3b, first and second panel), but replacement of the NikR-binding site by a C-stretch resulted in absence of binding (Fig. 3b, third panel).

Prediction of NikR operators in *Helicobacter* complete genome sequences

The previously suggested consensus sequence for NikR operators (TATWA-N_{15}-TWATA) does not allow identification of several of the confirmed high-affinity NikR operators in *H. pylori*, due to mismatches with C or G residues. Using the previously described *H. pylori* high affinity NikR binding sites (Delany et al. 2005; Ernst et al. 2006; Dosanjh et al. 2009) and the *H. mustelae* NikR operators in the *ureA* and *ureA2* promoters, we redefined the consensus sequence to TRWYA-N_{15}-TRWYA. This consensus sequence was used to search the intergenic regions from −198 to +2 (relative to the first nucleotide of the annotated translational startcodon) of the *H. pylori*, *H. acinonychis* and *H. hepaticus* genome sequences (Tomb et al. 1997; Suerbaum et al. 2003; Eppinger et al. 2006). We also searched the

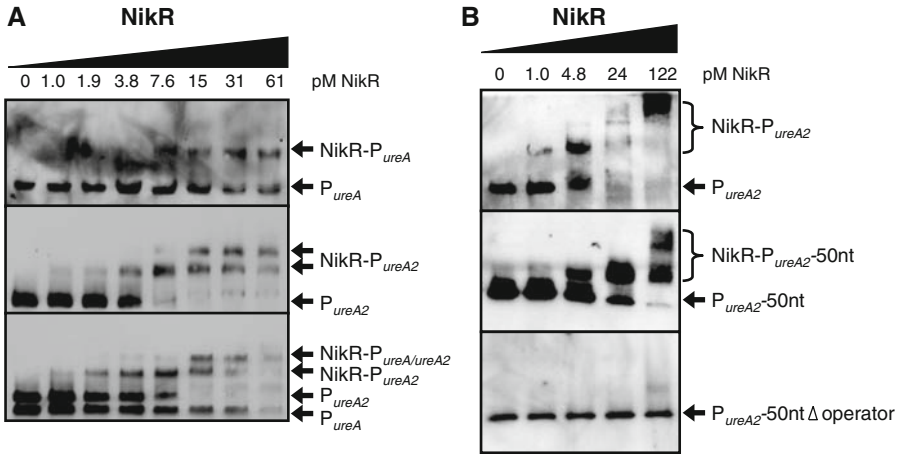


Fig. 3 NikR controls *ureAB* and *ureA2B2* transcription by sequence-specific direct binding to the *H. mustelae ureA* and *ureA2* promoter regions. **a** Electrophoretic mobility shift assays with recombinant *H. mustelae* NikR protein and the *ureA* (P_{ureA}) and *ureA2* promoters (P_{ureA2}) in the presence of $NiCl_2$. The NikR-complexed *ureA* and *ureA2* promoters are indicated as NikR-P_{ureA} and NikR-P_{ureA2}. The *first panel* shows NikR binding to the *ureA* promoter, the *second panel* to the *ureA2* promoter. The *third panel* shows that when the *ureA* and *ureA2* promoters are mixed at equimolar ratios, the *ureA2* promoter is shifted at lower concentrations of NikR and hence has a higher affinity for NikR. **b** NikR binds to the predicted operator in the *ureA2* promoter. The predicted operator in the *ureA2* promoter was reconstructed as a 50 nt fragment (P_{ureA2}-50nt) with the TAATACT-N_{11}-GTTAATA operator replaced by CCCCCCC-N_{11}-CCCCCCC (P_{ureA2}-50ntΔoperator). The *three panels* show electrophoretic mobility shift assays with recombinant *H. mustelae* NikR protein in the presence of $NiCl_2$. *Top panel*: full length *ureA2* promoter, *middle panel*: P_{ureA2}-50nt, *bottom panel*: P_{ureA2}-50ntΔoperator. The unbound and NikR-complexed versions are indicated on the right. NikR concentrations used (in pM) are indicated above the lanes; the DNA concentration was 32.5 pM of each promoter region

unannotated *H. mustelae* genome sequence for orthologs of metal transport and metal-regulatory genes, and included their predicted promoters in the search. Table 2 shows genes of all four *Helicobacter* species, which fulfilled two criteria: (1) a putative NikR operator in predicted promoter region (2) either a predicted function in metal homeostasis or previously shown to be controlled by NikR (Contreras et al. 2003; Delany et al. 2005; Ernst et al. 2005b, 2006; Abraham et al. 2006; Benanti and Chivers

Table 2 Prediction of NikR-operators within intergenic regions of *Helicobacter* genomes, upstream of genes putatively involved in metal homeostasis

Gene	Position[a]		Box sequence[b]	Notes
H.pylori	**26695**			
ureA (hp0073)	-143	-119	ata**TAACA**ctaattcattttaaa**TAATA**att	
nixA (hp1077)	-48	-24	ata**TATTA**caattaccaaaaaag**TATTA**ttt	
fecA3 (hp1400)	-112	-88	cat**TATTA**agtttttttgttttt**TATTA**ctt	
frpB4 (hp1512)	-91	-67	agg**TATTA**ttaaatagaataatg**TAATA**ata	
frpB4 (hp1512)	-88	-64	tat**TATTA**aatagaataatgtaa**TAATA**acc	
frpB2 (hp0916)	-75	-51	aaa**TAATA**ctttttagttataa**TAACA**att	
fecDE (hp0890)	-125	-101	gaa**TATTA**gaggaattttaaaaa**TAATA**aga	c
omp31(hp1469)	-49	-25	ata**TATTA**ttatttctttataag**TAATA**ctt	
omp32(hp1501)	-99	-75	cta**TAATA**aaataattaaaaaag**TAACA**ctt	
copAP (hp1067)	-99	-75	tgc**TATTA**tttggaacgatttat**TATTA**taa	c
copA2 (hp1502)	-160	-136	aag**TGTTA**ctttttaattattt**TATTA**tag	
H.acinonychis	**Sheeba**			
ureA (Hac_1532)	-140	-116	aga**TAACA**ctaatttgttacaaa**TAACA**ttc	
ureA2 (Hac_0448)	-81	-57	aag**TATTA**ctttcttaaaaaagt**TAATA**aca	
frpB (Hac_0072)	-89	-65	agg**TATTA**ttaaatagaatagtg**TAATA**ata	
frpB (Hac_0072)	-86	-62	tat**TATTA**aatagaatagtgtaa**TAATA**atc	
fecA (Hac_0865)	-112	-88	cgt**TATTA**aatttctttagtttt**TATTA**cct	
omp31 (Hac_0095)	-54	-30	tta**TAATA**aaataattcagaaag**TAATA**ctg	
omp32 (Hac_1718)	-77	-53	ata**TATTA**ttattccactataag**TAATA**ctt	
H.hepaticus	**ATCC 51449**			
nikA (hh0417)	-145	-121	cat**TATTA**ctctttaatattttaT**GTTA**tta	d
hh0418	-28	-4	taa**TAACA**taaaatattaaagag**TAATA**atg	d
arsRS (hh1608)	-77	-53	ttt**TATTA**tacaatcctcattttt**TAATA**cct	e
H. mustelae	**NCTC 12198**			
ureA	-164	-140	ttt**TATTA**tttataaatttttctT**GATA**aat	
ureA2	-142	-118	atc**TAATA**atttttgatataaat**TATTA**gtt	
ureA2	-132	-108	ttcT**GATA**taaattattactaaa**TAATA**ttt	
ureA2	-112	-88	aaa**TAATA**ctttttaaaaaagt**TAATA**caa	
0418-1 (nikH)	-73	-49	tag**TAATA**ttgcatcgcaaaaaa**TATTA**cat	

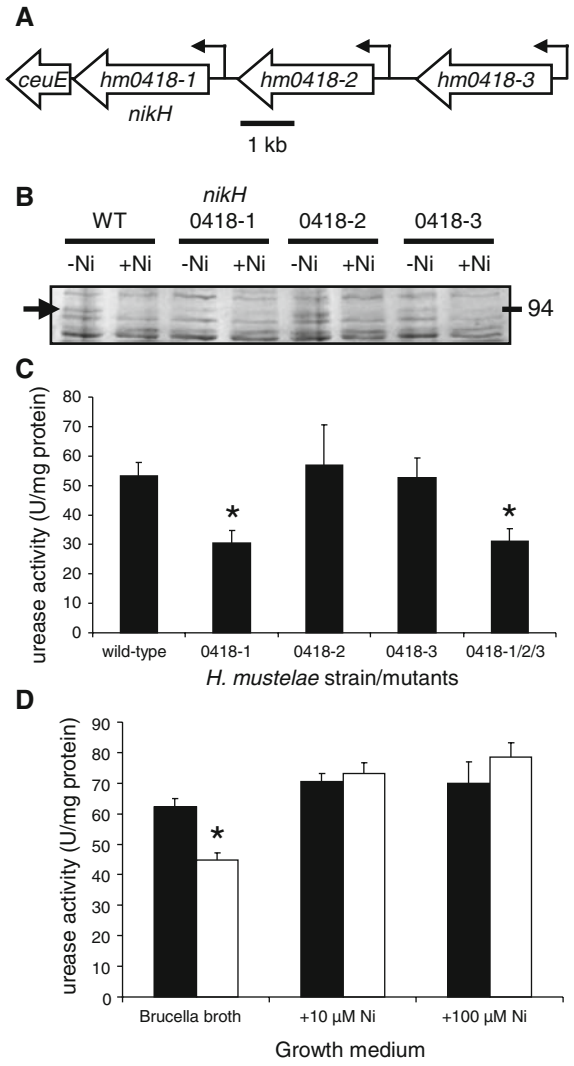
[a] Position of the TRWYA-N_{15}-TRWYA sequence relative to annotated or predicted translation start codon

[b] Residues in gray background are mismatches to the previously suggested TATWA-N_{15}-TWATA consensus sequence (Delany et al. 2005; Ernst et al. 2006; Dosanjh et al. 2009)

[c] Predicted to be in an operon. The predicted operator is present in the intergenic region upstream of the first gene of the predicted operon

[d] Overlaps with startcodon of *hh0418* gene, shared NikR operator in divergent *nikA-hh0418* promoter region

[e] The *hh1608-1607* genes encode a two-component regulatory system homologous to the *H. pylori* ArsRS acid-responsive regulatory system (Pflock et al. 2006)



◀ **Fig. 4** Nickel-responsive expression of the *H. mustelae* Hm0418-1 (NikH) protein and contribution of NikH to urease activity. **a** Schematic representation of the *H. mustelae* genomic region containing the *hm0418-1* (*nikH*), *hm0418-2*, *hm0418-3* and *ceuE* genes. **b** SDS–PAGE analysis of protein profiles of wild-type *H. mustelae* NCTC 12198 and its isogenic *hm0418-1*, *hm0418-2* and *hm0418-3* mutants, grown under either nickel-restricted (−Ni) or nickel-replete (+Ni) conditions. The wild-type strain shows one protein of approximately 92 kDa which displays expression under nickel-restricted conditions only, and is absent in the *hm0418-1* mutant but not in the *hm0418-2* and *hm0418-3* mutants. The *arrow* on the left indicates the Hm0418-1 (NikH) protein, the closest marker size (in kDa) is indicated on the right. **c** Urease activity of *H. mustelae* wild-type and isogenic *hm0418* mutants. Insertional mutagenesis of the nickel regulated *hm0418-1* (*nikH*) gene resulted in a significant decreased urease activity. Insertional mutagenesis of the *hm0418-2* and *hm0418-3* genes did not affect urease activity, whereas a triple mutant lacking all three *hm0418* genes gave the same phenotype as the *hm0418-1* mutant. **d** Supplementation of Brucella media with nickel restores urease activity in the *H. mustelae hm0418-1* mutant to levels comparable to the wild-type strain. *Black bars* represent the wild-type strain, white bars the *hm0418-1* (*nikH*) mutant strain. Results shown are the average of three independent growth experiments. *Error bars* represent standard deviation, an *asterisk* represents a significant difference in urease activity ($P \leq 0.05$, Mann–Whitney *U* test) when compared to the wild-type strain (*Panel C*) or unsupplemented Brucella media (*Panel D*)

2007; Dosanjh et al. 2009). Interestingly, many genes previously suggested to be NikR-regulated in *H. pylori* (Contreras et al. 2003; Ernst et al. 2005b, 2006; Abraham et al. 2006; Danielli et al. 2009) were independently identified in our predictive search (Table 2). The absence of a NikR operator in the urease promoter region of *H. hepaticus* is consistent with the lack of nickel-responsive regulation of urease transcription (Belzer et al. 2005).

Identification of a nickel-regulated outer membrane protein of *H. mustelae*

One of the potential NikR operators in *H. mustelae* is located upstream of a gene encoding a putative TonB-dependent outer membrane protein homologous to the HH0418 protein of *H. hepaticus* (Suerbaum et al. 2003; Belzer et al. 2007). In *H. hepaticus* the *hh0418* gene is located divergently to the *nikABDE* nickel-specific ABC transporter sytem (Beckwith et al. 2001; Suerbaum et al. 2003). In the unpublished *H. mustelae* genome, the *hm0418-1* gene is preceded upstream by two paralogs tentatively named *hm0418-2* and *hm0418-3*, and the three Hm0418 paralogs are 64% identical over the whole length of the proteins, with the Hm0418-2 and Hm0418-3 proteins showing higher identity. The *hm0418-1* gene is followed downstream by a *ceuE* gene encoding a putative periplasmic iron-binding protein (Fig. 4a). The *H. hepaticus* HH0418 protein was previously suggested to be involved in nickel-transport (Belzer et al. 2007), similar to the *H. pylori* FecA3 and FrpB4 proteins (Davis et al. 2006; Ernst et al. 2006; Schauer et al. 2007). To test whether the presence of a NikR operator upstream of the *hm0418-1* gene was indicative of NikR- and nickel-responsive regulation, we compared protein profiles of *H. mustelae* grown in nickel-restricted and nickel-replete conditions. One band corresponding to the predicted

molecular weight (92 kDa) of the Hm0418 proteins was repressed in nickel-replete conditions, but present in nickel-restricted conditions (Fig. 4b). The protein was absent in a *hm0418-1* negative mutant, but was present in *hm0418-2* and *hm0418-3* mutants of *H. mustelae*. This confirmed that *hm0418-1* is nickel-regulated.

To test whether Hm0418-1 could be contributing to nickel transport, analogous to the *H. pylori* FrpB4 protein (Schauer et al. 2007), we assessed the effect of the *hm0418* mutations on activity of the UreAB nickel-cofactored urease, since reduced nickel import will reduce urease activity (Schauer et al. 2007). To exclude any contribution of the UreA2B2 urease, cells were lysed by sonication which abolishes all UreA2B2 activity (Stoof et al. 2008). Inactivation of the *hm0418-1* gene significantly reduced urease activity, while inactivation of the *hm0418-2* and *hm0418-3* genes did not affect urease activity (Fig. 4c). Supplementation of Brucella media with nickel, which may enter the periplasm via outer membrane porins, restored urease activity of the *hm0418-1* mutant to wild-type levels (Fig. 4d), suggesting that the reduced urease activity in the *hm0418-1* mutant is caused by reduced nickel transport accross the outer membrane. Since the presence of the NikR operator, nickel-responsive regulatory pattern and the effect on urease activity all suggest a function of *hm0418-1* in nickel uptake by *H. mustelae*, and we propose the name of NikH for Hm0418-1.

Discussion

The nickel-cofactored urease enzyme is widespread among bacterial species, where it often has a role in the production of ammonia for cellular nitrogen metabolism. However, in several bacterial pathogens urease also contributes to resistance to low pH and pathogenesis of infection (Burne and Chen 2000). This is especially apparent in *Helicobacter* species colonizing the gastric mucosa of many mammals, as all gastric *Helicobacter* species display high-level expression of either one or two urease enzymes (Solnick and Schauer 2001; Kusters et al. 2006; Stoof et al. 2008). This high level of urease expression in gastric *Helicobacter* species has not only necessitated the development of high-affinity acquisition systems of the nickel cofactor (Eitinger and Mandrand-Berthelot 2000; Schauer et al. 2007), but handling of such potentially toxic metals also requires mechanisms controlling all aspects of nickel metabolism (Mulrooney and Hausinger 2003; Belzer et al. 2007; Maier et al. 2007).

We recently demonstrated that the three carnivore-colonizing *Helicobacter* species, *H. mustelae*, *H. acinonychis* and *H. felis* have a second, independent urease system designated UreA2B2 (Pot et al. 2007; Stoof et al. 2008), which in *H. mustelae* allows survival of acid shocks at pH 1.5 (Stoof et al. 2008). Expression of UreA2B2 and UreAB was inversely regulated in response to nickel, with UreA2B2 being nickel-repressed, and UreAB being nickel-induced (Stoof et al. 2008). Although nickel dependent regulation was absent in a *nikR* mutant, direct interaction of NikR with the urease promoters was not demonstrated. In this study we have further characterized this regulatory mechanism, by studying the role of NikR and Fur in nickel-responsive regulation of *H. mustelae* urease expression. From the studies with the *H. mustelae nikR* and *fur* mutants (Fig. 1), it was apparent that the NikR regulator has a dominant phenotype, as inactivation of the *nikR* gene resulted in constitutive, nickel- and iron-independent expression of both the UreAB and UreA2B2 ureases, whereas mutation of *fur* had no effect on nickel dependent regulation of the ureases (Fig. 1).

Further characterization of the promoters driving transcription of the *ureAB* and *ureA2B2* genes by primer extension showed that both promoters are transcribed from promoters recognised by the σ^{80}-cofactored RNA polymerase (Petersen et al. 2003), upstream of the *ureA* and *ureA2* genes. Bioinformatic searches for potential NikR-binding sites were based on those identified in *H. pylori* (van Vliet et al. 2001; Delany et al. 2005; Ernst et al. 2005b; Benanti and Chivers 2007; Zambelli et al. 2008; Dosanjh et al. 2009), and allowed the prediction of a binding sites in both the *H. mustelae ureA* and *ureA2* promoters, albeit in different locations. The *H. mustelae ureA* promoter was similar to the *H. pylori ureA* promoter (Delany et al. 2005; Ernst et al. 2005b), having a NikR-binding site far upstream of the canonical σ^{80} promoter, and shows the same nickel-induced expression pattern (van Vliet et al. 2001). The *ureA2* promoter was more similar to the *H. pylori nixA* promoter (Ernst et al. 2005b), although one of the predicted binding sites overlaps with the -35

sequence of the promoter (Fig. 2c). A secondary *ureA2* NikR operator is located directly upstream of the tested NikR operator (Table 2), and could also be involved in regulation, through multimerisation at the promoter analogous to what has been described for Fur in *E. coli* (Escolar et al. 1998). In fact the second shift at higher nickel concentrations (middle panel Fig. 3a) may represent NikR binding to a second (lower affinity) NikR binding site. Direct binding of NikR to the *ureA* and *ureA2* promoters was confirmed by gel-shift assays (Fig. 3a), and using site-directed mutagenesis we showed that the initial prediction of a NikR binding site in the *ureA2* promoter was correct, as replacement of these residues with a stretch of C residues abolished binding of NikR to this binding site (Fig. 3b).

When the *ureA* and *ureA2* promoters were mixed at equimolar concentrations, NikR first shifted the *ureA2* promoter (Fig. 3a). This suggests that NikR has a higher affinity for the *ureA2* promoter, and is consistent with the observed regulatory pattern of UreAB and UreA2B2 expression. In nickel-restricted conditions, UreA2B2 expression is high, and UreAB expression relatively low (Fig. 1; Stoof et al. 2008). At increasing nickel concentrations, UreA2B2 expression is first switched off, allowing pre-produced UreAB enzyme to be activated (van Vliet et al. 2002). When nickel concentrations increase further, preproduced UreAB is saturated with nickel, and *de novo* expression of the UreAB urease is increased (van Vliet et al. 2002, 2004; Stingl and De Reuse 2005; Dosanjh et al. 2009). These results suggest that the different promoters compete for available NikR protein, and that promoter affinity determines the order of regulation at increasing nickel concentrations. Interestingly, comparison of the NikR operators in the *ureA* and *ureA2* promoters did suggest differences in binding sequence (Fig. 2b) which may lead to differences in affinity. The *ureA2* promoter does contain a perfect palindromic repeat of the left and right arms of the operator sequence whereas the *ureA* promoter is an imperfect repeat which may lead to reduced binding affinity (Table 2). This *ureA*/*ureA2* promoter configuration is mirrored in *H. acinonychis* (Table 2). However, the exact role of these differences in binding sites remains to be elucidated, since also the presence of multiple binding sites and the sequences surrounding the binding site, may play a role in determining affinity of NikR for its operator sequences (Benanti and Chivers 2007; Zambelli et al. 2008; Dosanjh et al. 2009).

The NikR binding sites found in the *ureA* and *ureA2* promoter of *H. mustelae* were used to define a new consensus sequence (TRWYA-N_{15}-TRWYA) which was able to identify all confirmed high affinity binding sites in *H. pylori* (Dosanjh et al. 2009). Next to all the confirmed high affinity operators in the promoters of the *H. pylori fecA3*, *frpB4*, *ureA* and *nixA* genes, we also identified novel putative NikR operators (Table 2). Amongst these were the genes encoding the *H. pylori* outer membrane porins HopV (Omp31) and HopW (Omp32) which were previously reported as NikR-regulated by microarray analysis (Contreras et al. 2003). Surprisingly a perfect NikR operator was also found before an operon starting with the *hp0890* gene, which includes the genes encoding for the putative ferric citrate ABC transporter system (Tomb et al. 1997; Velayudhan et al. 2000). As for *frpB4* and *fecA3*, the *fecDE* genes were reported to be iron-independent (van Vliet et al. 2002), and inactivation of *fecD* gene did not affect iron transport in *H. pylori* (Velayudhan et al. 2000). The *fecDE* and upstream *hp0890* gene of *H. pylori* are acid-regulated by the ArsRS two-component regulatory system (Pflock et al. 2006), supporting a role of *fecDE* in acid-resistance of *H. pylori*, and a putative role for the *fecDE* genes in nickel metabolism of *H. mustelae* is currently under investigation.

Screening the genome sequences of other non-pylori *Helicobacter* species with the revised consensus sequence confirmed the presence of putative NikR operators in front of the nickel-responsive urease systems in *H. acinonychis* (Stoof et al. 2008), while such an operator is absent in the *H. hepaticus* urease promoter (Belzer et al. 2005). Similar to *H. pylori*, its close relative *H. acinonychis* also encodes homologs of *fecA3*, *frpB4*, *hopV* and *hopW* which all contain putative NikR boxes in their putative promoter region (Table 2). The *H. mustelae* and *H. hepaticus* genomes do not contain orthologs of the *H. pylori frpB4* gene, which mediates TonB-dependent nickel transport (Davis et al. 2006; Ernst et al. 2006; Schauer et al. 2007). However, both genomes do encode a different TonB-dependent outer membrane ortholog with no known homologs in other bacterial genera [HH0418 in *H. hepaticus* (Suerbaum et al. 2003; Belzer et al. 2007)]. In *H. mustelae* there are three orthologs of *hh0418*, arranged in tandem

(Fig. 4a). The *hh0418* and *hm0418-1* genes contain a putative NikR operator in their promoter sequence, whereas the *hm0418-2* and *hm0418-3* gene do not. In *Helicobacter hepaticus* this gene could potentially be involved in nickel transport since the location of the gene is divergent to NikABDE, the periplasmic and inner membrane transporters for nickel (Beckwith et al. 2001; Belzer et al. 2007). In *H. mustelae*, expression of Hm0418 is nickel-repressed (Fig. 4b), and inactivation of the *hm0418-1* gene but not *hm0418-2* or *hm0418-3* results in reduced urease activity (Fig. 4c). The decreased urease activity in the *hm0418-1* mutant could be restored to wild-type levels by nickel-supplementation of the growth medium (Fig. 4d). Taken together, this strongly suggests that Hm0418-1 functions as an outer membrane transporter for nickel, although further experiments are required to confirm this.

Comparison of all predicted NikR operators in promoters of *Helicobacter* metal metabolism genes using the Weblogo program suggests that the NikR operator in *Helicobacter* is surprisingly well conserved (Fig. 5). To reduce the number of false-positive hits in genomes it may be sufficient to use TRWTA-N_{15}-TRWTA as consensus sequence if both DNA strands are included in the search. The binding site TRWYA-N_{15}-TRWYA is quite similar to the *E. coli* NikR binding site *G*TATG*A*-N_{16}-T*C*ATA*G* and the predicted pseudo-NikR box TATTAC-N_{14}-GTAATA, present in front of urea carboxylase genes in α- and *β*-proteobacteria, where these represent an alternative, nickel independent urea degradation pathway (Rodionov et al. 2006). This supports our approach for the prediction of NikR operators in *Helicobacter* species, and this can potentially be extended to NikR regulators from other bacterial species.

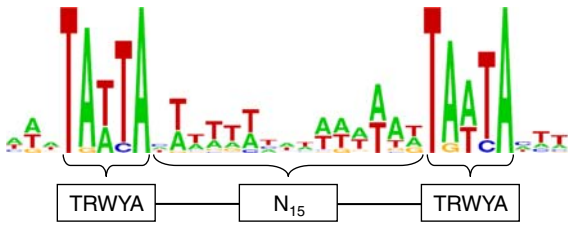


Fig. 5 Weblogo representation of *Helicobacter* NikR operators identified in the *H. pylori, H. mustelae, H. acinonychis* and *H. hepaticus* genomes. The Weblogo algorithm [http://weblogo.berkeley.edu/logo.cgi (Crooks et al. 2004)] was used to represent nucleotide conservation within the *Helicobacter* NikR operator sequences identified in the upstream regions of *Helicobacter* genes predicted to be involved in metal metabolism (Table 2), using the TRWYA-N_{15}-TRWYA consensus sequence

When the genome sequences of the first *Helicobacter* species were originally published (Berg et al. 1997; Tomb et al. 1997; Suerbaum et al. 2003), it was suggested that these bacterial species have a relatively limited capacity for gene regulation, which is consistent with their restricted ecological niches. However, ongoing studies on the nickel-regulatory protein NikR revealed more and more genes regulated by this protein, but lack of apparent sequence homology in the NikR operator, made it difficult to predict new targets. Here we present a study which demonstrates that the *ureA* and *ureA2* promoters of *H. mustelae* are directly regulated by NikR and using previously and newly identified NikR operators, we have been able to refine the NikR consensus sequence to one predicting new NikR targets in different *Helicobacter* species. Our prediction was validated by the identification of the *H. mustelae nikH* (*hm0418-1*) gene, a nickel-regulated outer membrane protein contributing to urease activity.

Acknowledgments The complete genome sequence of *H. mustelae* was determined at the Wellcome Trust Sanger Institute, Hinxton, UK, with funding of the Wellcome Trust. Urease antibodies were donated by the Department of Bacteriological R&D of Intervet International BV (Boxmeer, The Netherlands). AHMvV is supported in part by the BBSRC Institute Strategic Programme Grant to the Institute of Food Research.

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