Helicobacter hepaticus NikR controls urease and hydrogenase activities via the NikABDE and HH0418 putative nickel import proteins

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Helicobacter hepaticus open reading frame HH0352 was identified as a nickel-responsive regulator NikR. The gene was disrupted by insertion of an erythromycin resistance cassette. The *H. hepaticus* *nikR* mutant had five- to sixfold higher urease activity and at least twofold greater hydrogenase activity than the wild-type strain. However, the urease apo-protein levels were similar in both the wild-type and the mutant, suggesting the increase in urease activity in the mutant was due to enhanced Ni-maturation of the urease. Compared with the wild-type strain, the *nikR* strain had increased cytoplasmic nickel levels. Transcription of *nikABDE* (putative inner membrane Ni transport system) and *hh0418* (putative outer membrane Ni transporter) was nickel- and NikR-repressed. Electrophoretic mobility shift assays (EMSAs) revealed that purified HhNikR could bind to the *nikABDE* promoter (P_{nikA}), but not to the urease or the hydrogenase promoter; NikR- P_{nikA} binding was enhanced in the presence of nickel. Also, qRT-PCR and EMSAs indicated that neither *nikR* nor the *exbB-exbD-tonB* were under the control of the NikR regulator, in contrast with their *Helicobacter pylori* homologues. Taken together, our results suggest that HhNikR modulates urease and hydrogenase activities by repressing the nickel transport/nickel internalization systems in *H. hepaticus*, without direct regulation of the Ni-enzyme genes (the latter is the case for *H. pylori*). Finally, the *nikR* strain had a two- to threefold lower growth yield than the parent, suggesting that the regulatory protein might play additional roles in the mouse liver pathogen.

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INTRODUCTION

Maintenance of nickel (Ni) homeostasis is crucial for many bacteria because high nickel levels can be toxic, whereas limiting levels can compromise important cellular functions. A proper balance is often achieved via the nickel-responsive regulator NikR, which modulates the expression of key genes involved in metal homeostasis or metal enzyme function (Eitinger & Mandrand-Berthelot, 2000; Mulrooney & Hausinger, 2003; Dosanjh & Michel, 2006). NikR is a ribbon-helix-helix protein belonging to the CopG/MetJ/ArcA family of DNA-binding transcriptional activators (Chivers & Sauer, 1999). NikR contains an N-terminal region involved in DNA binding and a C terminus involved in metal binding. NikR was first described in *Escherichia coli* (EcNikR) as a repressor of the nickel uptake

operon *nikABCDE* (De Pina *et al.*, 1999). The ABC-type transporter is the only known NikR target in *E. coli* and a palindromic sequence has been characterized in the corresponding promoter region (Chivers & Sauer, 2000). In contrast, the other well-characterized orthologue of this family, *Helicobacter pylori* NikR (HpNikR), is a pleiotropic regulator; depending on the regulated genes, it can act as an activator or a repressor (for a review, see Danielli & Scarlato, 2010). A recent study showed that HpNikR utilizes a two-tiered mode of DNA recognition by binding to some gene promoters/operators with high affinity and to others with low affinity (Dosanjh *et al.*, 2009). Based on binding studies with various HpNikR target sequences (Delany *et al.*, 2005; Romagnoli *et al.*, 2011; Evans & Michel, 2012) and sequence alignments, partially conserved palindromic sequences such as TATTATT-N₁₁-AATAATA (Evans & Michel, 2012) or TRWYA-N₁₅-TRWYA (Stoof *et al.*, 2010b) have been proposed as HpNikR DNA binding motifs.

While Ni homeostasis begins to be well understood in *H. pylori*, not much is known about it in the murine pathogen *Helicobacter hepaticus*. *H. hepaticus* also possesses both the hydrogenase and urease Ni-containing proteins (Beckwith

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Abbreviations: EMSA, electrophoretic mobility shift assay; ICP-MS, inductively coupled plasma emission mass spectrometer.

Two supplementary figures are available with the online version of this paper.

et al., 2001; Mehta *et al.*, 2005). *H. hepaticus* hydrogenase mutants cause much less liver damage (liver lesions) than the parent strain in mice (Mehta *et al.*, 2005), while urease plays a crucial role in hepatic disease but is not required for caecal colonization (Ge *et al.*, 2008). Genome sequence analysis and previous studies suggest that nickel transport and urease expression differ significantly among the two *Helicobacter* (*pylori* and *hepaticus*) species (Benoit & Maier, 2008). Indeed, *H. hepaticus* urease gene expression does not respond to nickel level fluctuations (Belzer *et al.*, 2005), and Fur, not NikR, is proposed to be the major transcriptional regulator of *H. hepaticus* urease (Belzer *et al.*, 2007b). The nickel transport systems also seem to differ among the two *Helicobacter* strains, with *H. pylori* having a high affinity nickel-specific permease (NixA) (Mobley *et al.*, 1995) and *H. hepaticus* having the *E. coli*-like ATP-dependent ABC-type nickel transporter, NikABDE (Beckwith *et al.*, 2001; Belzer *et al.*, 2005; Benoit & Maier, 2008). Likewise, *H. hepaticus* lacks FecA3 and FrpB4 outer membrane Ni receptor homologues; instead it seems to rely on a different outer membrane receptor HH0418 (Belzer *et al.*, 2007a; Benoit & Maier, 2008) that is homologous to a protein shown to be involved in nickel acquisition in *Helicobacter mustelae* (Stoof *et al.*, 2010a). Finally, *H. hepaticus* also lacks the putative nickel storage proteins Hpn and Hpn-like, proteins shown to affect mature urease expression in *H. pylori* (Seshadri *et al.*, 2007). Besides, *H. hepaticus* HspA lacks the C-terminal his-rich nickel-binding domain shown to be important for full hydrogenase activity in *H. pylori* (Schauer *et al.*, 2010).

In the present study, the functions of NikR in *H. hepaticus* were determined by studying the phenotype of a *nikR* mutant strain and by analysing the interactions between recombinant purified HhNikR and various DNA promoter sequences. The *nikR* mutant is viable, though it has a lower growth yield than the wild-type. HhNikR does not modulate urease and hydrogenase enzyme activities through transcriptional regulation of their respective genes; instead, HhNikR represses genes involved in importing nickel into the cell, such as *nikA* or *hh0418*; therefore internal nickel levels in the cell are modulated to affect enzyme levels. This indirect regulation of urease or hydrogenase activity by NikR in *H. hepaticus* is in sharp contrast with the direct activation of urease and hydrogenase structural genes by NikR in *H. pylori*.

METHODS

Bacterial strains, plasmids and primers. *H. hepaticus* ATCC 51449 was used as the wild-type strain and for mutant strain construction (Table 1). Plasmids used to generate *H. hepaticus* mutants or to overexpress T7-HhNikR in *E. coli* were sequenced at the Sequencing and Synthesis Facility of the University of Georgia, Athens, GA, USA, and compared to DNA sequences from strain 51449 (Suerbaum *et al.*, 2003) to ensure that no errors had been introduced following PCR amplification.

Growth conditions. *H. hepaticus* cells were grown on Brucella agar (Difco) plates supplemented with 10% defibrinated sheep blood (BA

plates) at 37 °C. The plates were supplemented with 5 µg erythromycin ml⁻¹ when needed. Cultures were grown under micro-aerobic conditions with 5% CO₂, 2% O₂ and 93% N₂. Broth experiments were performed in triplicate in Mueller–Hinton broth (MHB) with 5% fetal bovine serum (FBS) in bottles with a gas-controlled environment (10% H₂, 5% CO₂, 2% O₂ and 83% N₂). Increase in growth was measured spectrophotometrically (OD₆₀₀) at 24, 48 and 72 h and expressed as c.f.u. ml⁻¹ using a standard curve based on plate counting. From this curve an OD₆₀₀ of 1.0 is approximately equal to 1.5 × 10⁹ c.f.u. ml⁻¹ within the medium. The initial cell concentration (at t₀) was 2.5 × 10⁷ c.f.u. ml⁻¹. *E. coli* strain TOP10 and BL21DE3 Rosetta/pLysS were grown aerobically at 37 °C on Luria–Bertani (LB) medium supplemented with ampicillin (100 µg ml⁻¹) or chloramphenicol (30 µg ml⁻¹) when required or on brain heart infusion (BHI) agar plates supplemented with 150 µg erythromycin ml⁻¹, when required. IPTG (0.2 mM) and nickel chloride (Sigma) were added when needed.

Construction of *H. hepaticus* hh0352 (*nikR*) and hh0351 mutants. Genomic DNA from *H. hepaticus* strain 51449 and primers nikR-Mut1 and nikR-Mut2 (Table 2) were used to amplify a 917 bp long PCR product, containing *nikR* (hh0352) (Suerbaum *et al.*, 2003; and J. Craig Venter Institute, <http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi>) along with some flanking sequence. This PCR product was cloned into the pGEM-T vector to yield pGEM-*nikR* (Table 1). A 1140 bp long erythromycin resistance cassette (*ery*) was inserted into a unique *Hind*III restriction site within *nikR* in the pGEM-*nikR* vector to yield pGEM-*nikR*::*ery*. A similar strategy was followed to construct the hh0351::*ery* mutant; primers hh0351-Mut1 and hh0352-Mut2 (Table 2) were used to amplify the 657 bp open reading frame hh0351 along with 500 bp flanking each side and the resulting PCR product was cloned into the pGEM-T vector to yield plasmid pGEM-hh0351. The *ery* cassette was then inserted into a unique *Nde*I restriction site (within hh0351) in the pGEM-hh0351 vector to generate plasmid pGEM-hh0351::*ery*. Each plasmid (pGEM-*nikR*::*ery* or pGEM-hh0351::*ery*) was introduced into *H. hepaticus* by electroporation. Erythromycin resistance was used to select colonies. Dozens of clones appeared for the *nikR*::*ery* mutant construction, whereas none could be obtained for the hh0351::*ery* mutation despite several attempts. PCR using DNA from the mutant as template and primers nikR-Mut1 and nikR-Mut2 confirmed the insertion of the *ery* cassette within the *H. hepaticus* *nikR* gene (Fig. S1, available with the online version of this paper).

Urease assays. Urease activity was assessed by measuring ammonia production (Weatherburn, 1967) and is expressed as (µmol urea hydrolysed) min⁻¹ (mg total protein)⁻¹.

Hydrogenase assays. Hydrogenase activity was assessed by using a previously described amperometric method (Maier *et al.*, 1996) and is expressed as (nmol H₂) min⁻¹ (10⁹ cells)⁻¹.

Cloning, expression and purification of *H. hepaticus* NikR. The NikR (HH0352) protein was expressed in *E. coli* and purified as a T7-tagged fusion protein. Briefly, primers NikR-OP1 and NikR-OP2 (Table 2) and genomic DNA from *H. hepaticus* strain 51449 were used to amplify a 450 bp-long DNA sequence containing the whole hh0352 (*nikR*) ORF. Primers NikR-OP1 and NikR-OP2 were designed to incorporate a 5' *Bam*HI and a 3' *Eco*RI restriction site, respectively. The PCR product was digested with *Bam*HI and *Eco*RI, gel-purified and cloned into similarly digested pET21b plasmid, generating pET-T7-NikR. This plasmid was used to transform *E. coli* BL21DE3 Rosetta /pLysS. Cells were grown at 37 °C with shaking until cultures reached OD₆₀₀ 0.3 and expression of T7-HhNikR was induced by adding 0.2 mM IPTG to the medium and leaving for another 4 h at 37 °C. The overexpressed T7-NikR protein localized in the soluble fraction and was purified to near homogeneity using the T7 tag

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics*	Source or reference
Strains		
<i>H. hepaticus</i>		
51449	Parent strain	ATCC
<i>nikR</i> mutant	<i>nikR::ery</i> ; Er ^r	This study
<i>E. coli</i>		
TOP10	Cloning strain	Invitrogen
BL21 Rosetta/pLysS	BL21 derivative. Host for protein overproduction; Cm ^r	Novagen
Plasmids		
pGEM-T	Cloning vector; Ap ^r	Promega
pGEM- <i>nikR</i>	pGEM-T with a 917 bp sequence containing <i>hh0352(nikR)</i> ; Ap ^r	This study
pGEM- <i>nikR::ery</i>	pGEM- <i>nikR</i> with <i>ery</i> gene inserted within <i>HindIII</i> of <i>nikR</i> ; Ap ^r Er ^r	This study
pGEM- <i>hh0351</i>	pGEM-T with a 1600 bp sequence containing <i>hh0351</i> ; Ap ^r	This study
pGEM- <i>hh0351::ery</i>	pGEM- <i>hh0351</i> with <i>ery</i> gene inserted within <i>NdeI</i> of <i>hh0351</i> ; Ap ^r Er ^r	This study
pET21b	Expression vector; Ap ^r	Invitrogen
pET-T7-NikR	<i>H. hepaticus nikR</i> cloned into <i>BamHI</i> – <i>EcoRI</i> sites of pET21b; Ap ^r	This study
pSLB167	pBC-KS with <i>ery</i> (<i>EcoRV</i>); Cm ^r Er ^r ; source of <i>ery</i> cassette	Mehta <i>et al.</i> (2007)

*Ap^r, Ampicillin resistance; Cm^r, chloramphenicol resistance; Er^r, erythromycin resistance.

affinity purification kit (Novagen). The protein purity was assessed by SDS-PAGE (Fig. S2) and the protein concentration was determined using the BCA protein assay kit (Thermo Fisher Pierce). The

T7-HhNikR protein was used 'as isolated' (West *et al.*, 2012) for the Ni-supplemented EMSA. For EDTA-supplemented EMSA, Ni-free, apo-HhNikR was obtained by dialysing for 24 h the protein

Table 2. Primers used in this study

All primers were purchased from Integrated DNA Technology (IDT), Coralville, IA.

Name	Sequence (5'–3')*	Use
nikR-Mut1	TTGGCATAAGGATAATCCGC	Construction of <i>nikR</i> mutant
nikR-Mut2	ACTGTGCCTAAAAGCCCCAC	Construction of <i>nikR</i> mutant
hh0351-Mut1	CGATTTAGTGTATCTCTCCCGCAG	Construction of <i>hh0351</i> mutant
hh0351-Mut2	AAGCACAACAAAAAACGC	Construction of <i>hh0351</i> mutant
nikR-OP1	<u>ggtcgcgatcc</u> GAAGTCTAGTATTATCCGATTTAG	Expression of T7-HhNikR
nikR-OP2	<u>ggtcaggaattc</u> TTACTCAAATCGGCTTGCACG	Expression of T7-HhNikR
nikA-EMSA1	GTTATTATGACATAGGAT	Amplification of <i>nikA</i> promoter for EMSA
nikA-EMSA2	CATTTTCTCCTTCCTC	Amplification of <i>nikA</i> promoter for EMSA
nikR-EMSA1	CTGCATATTATAAAAAATCATTCT	Amplification of <i>nikR</i> promoter for EMSA
nikR-EMSA2	AATACTAGACTTCATTATGTCTCC	Amplification of <i>nikR</i> promoter for EMSA
hyaA-EMSA1	CTCTGTATCAAAGTTATCATAG	Amplification of <i>hyaA</i> promoter for EMSA
hyaA-EMSA2	CCCTTTCCTTATGATTTTTG	Amplification of <i>hyaA</i> promoter for EMSA
ureA-EMSA1	GATTTTACTTATTTCTTTTGG	Amplification of <i>ureA</i> promoter for EMSA
ureA-EMSA2	GTTAATCTCATACTCTCTCC	Amplification of <i>ureA</i> promoter for EMSA
nikA-RT1	AGGGGAAAATTGTTCCAAGC	qRT-PCR (<i>nikA</i> mRNA transcripts)
nikA-RT2	CCACGAATGCCTTGCTCTAT	qRT-PCR (<i>nikA</i> mRNA transcripts)
nikR-RT1	CGTTCTGAAATTGTGCGAGA	qRT-PCR (<i>nikR</i> mRNA transcripts)
nikR-RT2	TTAAGCTCACGCTGATGGTG	qRT-PCR (<i>nikR</i> mRNA transcripts)
exbB-RT1	CACCCTATGTGGGGCTTTTA	qRT-PCR (<i>exbB</i> mRNA transcripts)
exbB-RT2	ACCAAGCCAAGAGCAGTAGC	qRT-PCR (<i>exbB</i> mRNA transcripts)
hh0418-RT1	GGTGTGCGCGTAGATAATGA	qRT-PCR (<i>hh0418</i> mRNA transcripts)
hh0418-RT2	TAGAATCACGCCATCAAGCA	qRT-PCR (<i>hh0418</i> mRNA transcripts)
gyrA-RT1	TTATTGTGGGGAGAGCCTTG	qRT-PCR (<i>gyrA</i> mRNA transcripts)
gyrA-RT2	GGACGACGTGGTGAGAGATT	qRT-PCR (<i>gyrA</i> mRNA transcripts)

*Upper-case letters indicate *H. pylori*-derived sequences. Restriction sites are underlined.

twice against 1 l of buffer containing 20 mM Tris/HCl (pH 7.6), 100 mM KCl, 3 mM MgCl₂, 10 mM EDTA, 1 mM DTT.

Immunoblotting. Anti-UreB immunoblotting was done as previously described (Benoit *et al.*, 2007).

Electrophoretic mobility shift assays (EMSAs). The ability of the purified T7-HhNikR to bind *in vitro* to various promoters (P_{nikA} , P_{nikR} , P_{hyaA} or P_{ureA}) was investigated by EMSA following a previously described protocol (Bloom & Zamble, 2004). Primers nikA-EMSA1 and nikA-EMSA2 (Table 2) were used to amplify a 120 bp PCR product containing the *nikA* (*hh0417*) promoter (Fig. 1). Primers nikR-EMSA1 and nikR-EMSA 2 were used to amplify a 131 bp PCR product containing the *nikR* (*hh0352*) promoter (Fig. 1). Primers hyaA-EMSA1 and hyaA-EMSA2 were used to amplify a 400 bp PCR product containing the *hyaA* (*hh0056*) promoter. Primers ureA-EMSA1 and ureA-EMSA2 were used to amplify a 173 bp PCR product containing bases -115 to +57 relative to the transcription start site of the *ureA* (*hh0406*) gene as determined by Belzer *et al.* (2007b). Each PCR product was gel purified using the QIAquick gel extraction kit (Qiagen) and end-labelled with [γ -³²P]ATP and T4 polynucleotide kinase. Unincorporated nucleotides were removed using the QIAquick DNA purification kit (Qiagen). Each PCR product with DNA concentration ranging from 200 to 250 pM (5000 to 6250 c.p.m.) was incubated with purified T7-HhNikR (0.1–4 μ M) in the presence of 0.8 mM NiSO₄ or 1 mM EDTA for at least 30 min at room temperature in a final volume of 50 μ l before loading on a 7% polyacrylamide gel running at 200 V, 4 °C. NiSO₄ or EDTA was also added to the binding buffer, running buffer and the polyacrylamide gel before polymerization. Gels were exposed to a phosphor screen, scanned using a Typhoon phosphorimager (GE Healthcare) and analysed with ImageQuant 5.0 software.

Determination of nickel levels in *H. hepaticus* cells. *H. hepaticus* wild-type and *nikR* mutant cells were grown for 2 days on BA plates with the indicated nickel supplement (0–50 μ M). Cells were washed twice in 10 mM Tris/HCl, pH 7.4, resuspended in the same buffer, broken by sonication and the lysate was centrifuged at 15 000 g for

20 min to remove the cell debris and unbroken cells. The protein concentration of the cell-free supernatant was determined (BCA assay) and the amount of nickel in 1 ml of the cell-free supernatant was assayed by an inductively coupled plasma emission mass spectrometer (ICP-MS). The amount of nickel in each strain and for each condition is expressed in (ng nickel) (mg total protein)⁻¹.

RNA isolation and cDNA synthesis. Total RNA was isolated from *H. hepaticus* grown for 36–48 h on BA plates (with or without supplementary NiCl₂) by using the Aurum isolation kit (Bio-Rad) and the Turbo DNA-free kit (Ambion). RNA (200 ng) was used to synthesize cDNA (iScript cDNA kit, Bio-Rad). The cDNA generated was used as a template for qPCR.

Real-time quantitative PCR. Primers were designed to amplify 100–150 bp regions of *hh0352* (*nikR*), *hh0353* (*exbB*), *hh0417* (*nikA*), *hh0418* (putative outer membrane Ni transporter) or *hh1633* (*gyrA*; internal control) genes (see Table 2). A reaction mixture containing the master mix with SYBR green (Bio-Rad) and each set of primers was added to a 96-well plate, along with diluted cDNA samples for a final reaction volume of 20 μ l per well. Negative controls contained the reaction mixture with only mRNA. Samples (in triplicate) were incubated in the iCycler (Bio-Rad) for 45 cycles (15 s at 95 °C, 30 s at 58 °C and 30 s at 72 °C). Gene expression was quantified by the comparative C_t (cycle threshold) method and normalized to the C_t of the housekeeping *gyrA* gene for each sample (ΔC_t). Finally, normalized transcript signals obtained for *H. hepaticus* wild-type or *nikR* mutant grown with Ni were compared to those of the wild-type grown on BA without supplementary Ni ($\Delta\Delta C_t$), using the $2^{-\Delta\Delta C_t}$ formula (fold changes). Fold change means are from two independent experiments and three to five measurements.

RESULTS

H. hepaticus possesses a NikR homologue

Analysis of the sequenced genome of *H. hepaticus* strain 51449 (Suerbaum *et al.*, 2003) revealed the presence of an

Fig. 1. Nucleotide sequence of the *nikA*–*hh0418* and *nikR*–*exbB* intergenic regions. Putative open reading frames are shown in bold. *hh* numbers refer to annotated genes from strain *H. hepaticus* 51449 (Suerbaum *et al.*, 2003). PCR products used for EMSAs are underlined. Putative NikR binding boxes for both *nikA* and *hh0418*, as defined by Stoof *et al.* (2010b), are highlighted in grey. Putative alternative NikR binding motifs are indicated by boxes.

open reading frame (HH0352) that shows 66 % identity and 78 % similarity with the well characterized *H. pylori* nickel-responsive regulator NikR; 61 % identity and 77 % similarity with *Helicobacter mustelae* NikR (HmNikR); and 33 % identity and 60 % similarity with the other well-studied NikR orthologue, EcNikR. Amino acid sequence comparison between these four NikR sequences suggests a putative role for N-terminal residues of HhNikR in DNA binding (Fig. 2), as previously shown for HpNikR and EcNikR (Benanti & Chivers, 2007). The 9-residue long N-terminal arm of HpNikR (MDTPNKDDSD) has been shown to be required to avoid non-specific/low affinity DNA binding and for binding to the *nixA* promoter (Benanti & Chivers, 2007); however, HhNikR possesses only four of the nine important residues. The C terminus has conserved residues that are involved in nickel binding, similar to EcNikR, HmNikR and HpNikR (Chivers & Sauer, 2002; Dosanjh *et al.*, 2007).

The genomic region containing *H. hepaticus* hh0352 (herein referred to as *nikR*) is organized in a similar way as in other sequenced species of the genus *Helicobacter* (Benoit & Maier, 2008) (Fig. 1). The gene convergently transcribed downstream of *nikR*, hh0351, is homologous to a conserved gene downstream of *nikR* in *H. pylori* or *H. acinonychis* species (Tomb *et al.*, 1997; Alm *et al.*, 1999; Eppinger *et al.*, 2006; Oh *et al.*, 2006); similarly, the genes divergently transcribed with respect to *H. hepaticus* *nikR* (hh0353/0354/0355) share significant homology with the genes located upstream of *nikR* in *H. pylori*; those genes encode the ExbB-ExbD-TonB complex which provides energy for nickel transport through the outer membrane (Schauer *et al.*, 2007).

Construction of *H. hepaticus* *nikR* mutant

A *nikR* mutant strain was constructed by inserting an erythromycin resistance gene (*ery*) in the hh0352 locus. The disruption of the 432 bp-long putative *nikR* gene was confirmed by PCR as an increased size of the PCR product on an agarose gel (917 bp for the wild-type versus 2057 bp for the *nikR* mutant; Fig. S1). The absence of *nikR* mRNA transcripts was confirmed by quantitative real-time PCR (data not shown). The fact that *H. hepaticus* *nikR* is part of a bi-cistronic operon hh0352–hh0351 prompted us to construct an hh0351::*ery* mutant as a control for

downstream polar effects. Attempts to disrupt this region always resulted in loss of viability, as we were unable to recover any transformant. Homology searches revealed that *H. hepaticus* hh0351 encodes a putative nicotinic acid mononucleotide adenylyltransferase known to be essential in several bacteria, including *Salmonella enterica* serovar Typhimurium (Hughes *et al.*, 1983). Therefore we concluded that the disruption of *nikR* with the erythromycin cassette does not cause a polar effect, as the *nikR* mutant is viable. This is in good agreement with previous results suggesting that the *ery* cassette is a safe tool to construct targeted mutants in *H. hepaticus* (Benoit *et al.*, 2007).

Urease activity, but not urease expression, is increased in the *nikR* mutant

To determine whether the NikR regulator controls urease activity in *H. hepaticus*, urease activity was measured in cell-free extracts from both the wild-type and the *nikR* mutant strains grown in media with or without supplementary nickel chloride. Without added nickel, urease activity was five- to sixfold higher in the *nikR* mutant strain in comparison to the wild-type (Table 3). This result suggested that NikR acts as a transcriptional repressor of urease and/or that NikR affects urease activity via (Ni-dependent) maturation of the apoenzyme. To address whether the increased urease activity was due to an increased amount of urease apoenzyme, immunoblotting was performed on cell-free extracts from the wild-type and the mutant grown on unsupplemented medium. There was no difference in the amount of urease apoprotein between the wild-type and the *nikR* mutant (data not shown), indicating that (i) *nikR* does not regulate transcription of urease and (ii) the increase in urease activity in the mutant is probably due to enhanced Ni-maturation of the urease.

Addition of nickel to the medium dramatically increased the urease activity in the wild-type (see Table 3), as previously reported (Belzer *et al.*, 2005; Benoit *et al.*, 2007). Interestingly, the same activation trend was observed in the *nikR* mutant, although levels of urease activity were always higher in the mutant than the parental strain. These results suggest a role for HhNikR in modulating urease activity, regardless of the amount of nickel present in the medium.

Hh NikR	-----MKSSIIIRFSVSLPQNLLDRLTHKGYSSRSEIVRDMIREKLNEEIWSSGAEN	55
Hp NikR	-MDTPNKDDSIIRFSVSLQNNLLDELNDRIKNGYSSRSELVRDMIREKLVEDNWAEDNPN	60
Hm NikR	MRTMEKEKNSLMRFSVSLQNNLLDRLTNQGYASRSEIVRDLIREKIVQESWEKDDN-	60
Ec NikR	-----MQRVITILLDDLLDRLTSLQRRGYNRSEAIRDLRSALAEATQQHG--	49
Hh NikR	TQG-VAVLTIIYDHHQRELNRQMIDIQTSTHKGNEVEILCNTHVLDQHNLETIILRGN	114
Hp NikR	DESKIAVLVVIYDHHQRELNRQMIDIQHAS----GTHVLCTTHIMDEHNCLETIIILQGN	116
Hm NikR	SEDKVAVFVIVYDHHQRELNRQMIDIQHAS----DTEILCTTHVLDHHCLETIIFRGK	116
Ec NikR	TQG-FAVLSSVYVEHEKRDLSRIVSTQHHS----HDLVATLHVIHNDDELEIAVLKGD	104
Hh NikR	GVHIEDLSIEIGGLKGKFSKLTASRFE---	143
Hp NikR	SFEIQLRLQLEIGGLRGVKFAKLTAKSFYNE	148
Hm NikR	SADIHQIALEIAGLKGKFSKLTQTGHFPKDL	148
Ec NikR	MGDVQHEADVDIAQRGVRHGHQLQCLPKED---	133

Fig. 2. NikR protein sequences of *H. hepaticus*, *H. pylori*, *H. mustelae* and *E. coli* NikR. The residues shaded in grey and located toward the N-terminal region are conserved residues previously identified to be involved in DNA binding in *H. pylori* or *E. coli*. The H and C residues shaded in grey and located towards the C terminus have been shown to be involved in nickel binding in *E. coli* (Schreiter *et al.*, 2003).

Table 3. Urease and hydrogenase activities of wild-type and *nikR* mutant as a function of nickel added to the growth media

Results are the mean $\pm$ SD of three independent experiments, each performed in duplicate. Results are statistically different between the two strains at all nickel levels: $P < 0.005$, as determined by Student's *t*-test for every nickel supplement condition tested, except for hydrogenase activity, 1 μ M nickel supplement, where $P < 0.01$.

Nickel added (μ M)	Urease activity [μ moles urea min^{-1} (mg total protein) $^{-1}$]		Hydrogenase activity [nmoles H_2 min^{-1} (10^9 cells) $^{-1}$]	
	WT 51449	<i>nikR</i>	WT 51449	<i>nikR</i>
0	0.05 $\pm$ 0.02	0.31 $\pm$ 0.08	0.28 $\pm$ 0.18	0.40 $\pm$ 0.13
1	0.35 $\pm$ 0.17	1.11 $\pm$ 0.54	1.26 $\pm$ 0.30	1.68 $\pm$ 0.18
5	1.56 $\pm$ 0.23	3.14 $\pm$ 0.83	1.32 $\pm$ 0.44	4.10 $\pm$ 0.66
10	3.27 $\pm$ 0.96	9.14 $\pm$ 3.34	2.26 $\pm$ 0.98	6.91 $\pm$ 1.56
50	11.27 $\pm$ 2.22	23.56 $\pm$ 7.76	2.42 $\pm$ 0.60	17.86 $\pm$ 5.47

H. hepaticus *nikR* mutant strain shows increased hydrogenase activity

Hydrogen uptake abilities of the *nikR* mutant and the wild-type strains were measured by whole cell amperometry and compared: the hydrogenase activity was consistently at least twofold higher in the *nikR* mutant strain than in the wild-type strain (Table 3). When the growth medium was supplemented with increasing concentrations of nickel, hydrogenase activity in the wild-type increased, as previously shown (Mehta *et al.*, 2005; Benoit *et al.*, 2007). Likewise, nickel supplementation resulted in increased hydrogenase activity in the *nikR* mutant, but levels were consistently two- to fourfold higher in the mutant than in the wild-type, for any given nickel concentration (Table 3). This suggested that HhNikR either directly (transcriptional repression) or indirectly (maturation) affects the unique hydrogenase of *H. hepaticus*. Since anti-hydrogenase antiserum is not available at this time, it is not known whether the increase in hydrogenase activity can be linked to an increase in hydrogenase synthesis in the mutant.

Increased intracellular nickel pools in a *nikR* strain

As stated above, the *nikR* mutant had increased urease activities although it has similar levels of urease apoenzyme. This suggested a difference in Ni-maturation, linked to an increase in Ni import into the mutant strain cell. To test this hypothesis, the amount of intracellular nickel in wild-type and mutant strains grown under different Ni concentrations was determined by ICP-MS. As expected, the intracellular nickel levels in the wild-type cells increased when nickel chloride was added to the medium (Fig. 3). Interestingly, there was a consistent increase in the internal nickel levels in the *nikR* mutant strain compared with the wild-type strain, with a significant difference in nickel levels at 5–50 μ M nickel supplementation. This result suggested that HhNikR could play a role with respect to Ni transport in the cell.

HhNikR represses transcription of *nikA* and *hh0418*, but not of *exbB* and *nikR*

Analysis of the genome sequence suggests that *H. hepaticus* relies on the NikABDE transport system (*hh0417–hh0414*), which displays homologies to the *E. coli* nickel-specific ABC NikABCDE transport system (Beckwith *et al.*, 2001; Belzer *et al.*, 2005; Benoit & Maier, 2008). In *E. coli*, NikR has been shown to repress the transcription of the *nikABCDE* genes in presence of nickel (De Pina *et al.*, 1999; Chivers & Sauer, 2000). To determine whether NikR would play a similar role in *H. hepaticus*, we used qRT-PCR to compare *nikA* transcript levels in the wild-type and the *nikR* mutant strains, when grown on medium not supplemented with nickel or supplemented with 1, 10 or 100 μ M NiCl_2 (Fig. 4). In *H. hepaticus* 51449 wild-type strain, *nikA* transcript levels decreased in a step-wise fashion as nickel concentrations increased. The Ni-dependent

Fig. 3. Amount of nickel detected in cells by ICP-MS. *H. hepaticus* wild-type (white bars) and *nikR* mutant (black bars) cells were grown with the indicated nickel supplement (0–50 μ M). Total protein and nickel content were determined by BCA assay and ICP-MS, respectively. Bars show mean $\pm$ SD and are from one experiment and six measurements. The experiment was performed three other times and the same trend was observed. The mutant values were greater than the parent for all nickel supplementation levels: $P < 0.005$, as determined by Student's *t*-test.

downregulation of *nikA* was abolished in the *nikR* mutant, suggesting that NikR represses (directly or indirectly) *nikA* transcription in *H. hepaticus*. The same approach was used to study the transcription of *hh0418*, which is divergently transcribed with respect to *nikA* (Fig. 1). The *hh0418* gene was hypothesized to be NikR-regulated because the HH0418 protein sequence shares partial homology with outer membrane Ni transporters FecA3 and FrpB4, which have been shown to be regulated by NikR in *H. pylori* (Davis *et al.*, 2006; Ernst *et al.*, 2006). Indeed, our results demonstrate that expression of *hh0418* is Ni-repressed in the wild-type strain but not in the *nikR* mutant (Fig. 4), indicating that this gene is also under the control of the Ni-dependent regulator. Finally, we measured transcript levels of the divergently transcribed *nikR* and *exbB* genes (Fig. 1). Both genes have been shown to be under the control of NikR in *H. pylori* (Contreras *et al.*, 2003; Delany *et al.*, 2005). However neither *exbB* nor *nikR* expression was significantly repressed in the presence of increasing Ni concentrations; in addition *exbB* transcripts levels in the *nikR* mutant strain were not altered compared with those in the wild-type strain (Fig. 4). Taken together, our results suggest that *H. hepaticus nikA* and *hh0418* are NikR-regulated, while *exbB* and *nikR* are not. Therefore the increased urease and hydrogenase activities observed in the *H. hepaticus nikR* mutant are thought to be the consequence of de-repression of the NikABDE and the HH0418 Ni transporters, leading to better Ni maturation.

HhNikR directly binds to the P_{nikA} promoter, but does not bind to P_{ureA} , P_{hyaA} or P_{nikR}

EMSAs were carried out either with 'as isolated' (West *et al.*, 2012) T7-HhNikR protein (Fig. S2) in the presence

of Ni^{2+} or with EDTA-dialysed HhNikR in presence of EDTA. There was no binding of HhNikR to either P_{hyaA} or P_{ureA} in presence of Ni^{2+} (Fig. 5a) or EDTA (data not shown). In contrast, binding of HhNikR to the P_{nikA} promoter was observed in the presence of EDTA (Fig. 5b) or Ni^{2+} (Fig. 5c). In the presence of EDTA, a weak HhNikR- P_{nikA} complex was observed; there was always a significant amount of free P_{nikA} probe remaining, even in the presence of high (8 μM) NikR concentrations (Fig. 5b). In the presence of Ni^{2+} , NikR bound to the P_{nikA} promoter with nanomolar affinity (Fig. 5c). Two additional bands were also observed, suggesting the presence of multiple binding sites. In contrast, no binding of NikR to the P_{nikR} promoter was observed, even when micromolar concentrations of protein were used, in the presence of EDTA or nickel (data not shown). In conclusion, these results are in agreement with qPCR results and suggest that *H. hepaticus* NikR (i) represses *nikABDE* by direct binding to its promoter and (ii) does not auto-regulate itself, in sharp contrast with *H. pylori* NikR.

Growth deficiency in a *nikR* strain

Both the wild-type and *nikR* mutant were grown in triplicate in Mueller–Hinton broth supplemented with fetal calf serum (no added nickel), and the growth was estimated in each bottle after 24, 48 and 72 h. For every time point, the growth yield of the *nikR* mutant strain was two- to threefold lower than that of the wild-type. For example, c.f.u. counts at 48 h were $2.36 (\pm 0.14) \times 10^8$ c.f.u. ml^{-1} for the wild-type, compared with $1.05 (\pm 0.20) \times 10^8$ c.f.u. ml^{-1} for the *nikR* mutant. This suggests that NikR plays other roles in *H. hepaticus* besides its proposed involvement in nickel metabolism.

Fig. 4. Transcript levels of *nikA*, *hh0418*, *exbB* and *nikR* as a function of nickel and NikR. The level of transcripts after culture on BA without added $NiCl_2$ or with supplement of 1, 10 or 100 μM $NiCl_2$ was determined relative to that of the level of the housekeeping gene *gyrA* (*hh1633*) under the same conditions. For each gene, fold changes are relative to transcript levels of the wild-type grown on BA without added Ni, using the $2^{-\Delta\Delta C_t}$ formula. The average fold changes are from two independent experiments, each with three to five measurements.

Fig. 5. EMSAs. Increasing concentrations of purified T7-HhNikR protein (in μM, indicated above the lanes) were incubated with (a) the *P_{ureA}* promoter (220 pM) or the *P_{hyaA}* promoter (240 pM) in presence of 0.8 mM NiSO₄, or the *P_{nikA}* promoter (220 pM) in presence of 1 mM EDTA (b) or 0.8 mM NiSO₄ (c). Free or bound ³²P-labelled DNA probes are indicated on the side of each panel.

DISCUSSION

In this study, we report the construction and characterization of the first *nikR* mutant in the mouse pathogen *H. hepaticus*. Disruption of the gene led to increased activities of two Ni-requiring enzymes, urease and hydrogenase but urease levels between the mutant and the wild-type (by immunoblotting against UreB) were unchanged, suggesting that the difference in urease activity is solely due to a difference in post-translational maturation. This conclusion is supported by (i) increased transcription of the putative *nikABDE* Ni import operon and the putative outer membrane Ni receptor *hh0418* in the mutant, (ii) higher levels of Ni²⁺ detected in the *nikR* mutant and (iii) failure of the purified HhNikR to bind to the *ureA* promoter. The interpretation is in agreement with the results of Belzer *et al.* (2005), who showed that the increase in urease activity of *H. hepaticus* was not associated with elevation of

urease mRNA or protein levels. This type of urease regulation (exclusively at the post-translational level) is similar to what was reported for *Streptococcus salivarius* (Chen & Burne, 2003); those researchers observed that mutational elimination of part of the Ni transporter (UreM) abolished both nickel accumulation and urease activity. Likewise, it is tempting to speculate that the increase in hydrogenase activity observed in the *H. hepaticus* *nikR* mutant is solely due to increased maturation of the hydrogenase, rather than to transcriptional regulation. Indeed, EMSA revealed that purified *H. hepaticus* NikR failed to bind to the *P_{hyaA}* promoter even when high amounts of protein (up to 2 μM) were used in the shift assay.

In contrast, HhNikR-*P_{nikA}* complexes were observed, confirming that NikR can regulate the synthesis of *NikABDE* by direct binding to its promoter. This binding

was observed even with EDTA-treated apo-NikR. When using 'as isolated' (West *et al.*, 2012) HhNikR in the presence of nickel, the binding affinity of HhNikR for P_{nikA} increased and several Ni²⁺-bound NikR–DNA complexes were observed, suggesting multiple binding sites. Ni²⁺-HhNikR bound to P_{nikA} with nanomolar affinity, similar to what has been reported for EcNikR–P_{nik} (Wang *et al.*, 2010) or for *Geobacter uraniireducens* NikR and the putative nikMN nickel transport (Benanti & Chivers, 2010). Interestingly, the nikABDE operon and the divergently transcribed hh0418 gene (encoding a putative outer membrane Ni receptor) share the same intergenic region (see Fig. 1). Since hh0418 is under the control of NikR (as shown by qPCR in the present study), this raises the possibility that one or several NikR–DNA complexes observed would in fact be NikR–P_{hh0418} complexes instead of or overlapping with NikR–P_{nikA} complexes. Such an overlap was suggested by Stoof *et al.*, (2010b), whose bioinformatics predictions for putative NikR binding boxes upstream of nika (TATTA-N₁₅-TGTTA) fully overlap with those predicted upstream of hh0418 (TAACA-N₁₅-TAATA) (Fig. 1). However the DNA sequences used in the present EMSA studies only include part of the first motif (Fig. 1). This suggests that additional motifs might be involved in HhNikR recognition. Based on our own analysis of the intergenic region upstream of nika, one alternative HhNikR binding site is proposed for P_{nikA} (and possibly P_{hh0418}): a 5'-TATCA-N₁₅-TCATA-3' motif can be found upstream of the nikABDE operon (Fig. 1).

Genes located upstream and divergently transcribed with respect to *H. hepaticus* nikR hh0353/hh0354/hh0355 are homologues of *exbB/exbD/tonB* genes shown to provide energy for Ni import through the outer membrane in *H. pylori* (Schauer *et al.*, 2007). Since these genes, as well as nikR itself, are repressed by NikR in *H. pylori*, we expected that this was also the case in the murine pathogen. However analysis of nikR and exbB mRNA does not support this hypothesis. In addition, EMSA studies showed no binding of apo-NikR or Ni²⁺-bound NikR to its own promoter. Finally, sequence analysis of the nikR–exbB intergenic region did not reveal any putative HhNikR binding box. Taken together, these results suggest that *H. hepaticus* neither regulates its own transcription nor controls hh0353/hh0354/hh0355 genes, a striking difference from *H. pylori* NikR. Since additional tonB and exbB–D homologues can be found in the *H. hepaticus* genome sequence (hh1579 and hh0431/0432, respectively), future studies will be aimed at determining whether those genes are involved in providing the energy for Ni transport.

Besides their difference in urease regulation and Ni transport, *H. hepaticus* and *H. pylori* also differ with respect to accessory proteins and Ni storage. Insertion of Ni into the catalytic site of urease or hydrogenase is a complex process that requires accessory proteins. In *H. pylori* as well as in *H. hepaticus*, hydrogenase accessory proteins are needed for hydrogenase maturation. In addition, HypA and HypB are needed for full urease

maturation in both species (Benoit & Maier, 2008). Upon addition of NiCl₂ in the medium, urease activity can be restored to wild-type levels in *H. pylori* hypA or hypB mutants; this is not the case for *H. hepaticus* hypA and hypB mutants (Benoit *et al.*, 2007). These results suggest different urease maturation mechanisms for the two species; the stringent requirement for HypA or HypB proteins in *H. hepaticus* might reflect the inability of the mouse pathogen to modulate urease expression in response to Ni levels. Indeed, since *H. hepaticus* urease activity can only be post-translationally modulated, its dependence on accessory proteins is probably greater than for *H. pylori*.

H. pylori possesses two histidine-rich proteins termed Hpn and Hpn-like, which have been shown to play dual roles as Ni storage and metal detoxification, depending on the exogenous nickel levels in the micro-organism (Ge *et al.*, 2006; Seshadri *et al.*, 2007). Genes for both proteins are transcriptionally activated by NikR (Contreras *et al.*, 2003). Analysis of the genome sequence of *H. hepaticus* indicated that these proteins are not present in the mouse pathogen. Unless other mechanisms are involved, this likely means that *H. hepaticus* is not capable of storing intracellular Ni pools, and thus cannot respond well to transient nickel needs; it apparently relies primarily on its Ni transport system for immediate supplies of Ni when the metal is needed, in a fashion similar to *E. coli*. It might be expected that cytoplasmic ionic Ni overload would pose a problem for *H. hepaticus*, so excretion of excess Ni out of the cell might be more critical for *H. hepaticus* than it is for *H. pylori*. *H. hepaticus* HH0623/0624/0625 gene products are homologues of the *H. pylori* CznABC Ni export system (Stähler *et al.*, 2006). For the reason stated above, this operon is likely to be regulated by NikR in *H. hepaticus*, although that remains to be demonstrated.

Even though *H. hepaticus* and *H. pylori* share the same genus in the epsilonproteobacteria lineage, their preferred environment is quite different: *H. hepaticus* is an enterohepatic colonizer of mice while *H. pylori* has to adapt to the acidic conditions of the human (or primate) stomach. Therefore one can expect their nickel homeostasis to be different. Further studies of *H. hepaticus* NikR, specifically with respect to its DNA recognition motif, nickel binding affinities, factors providing specificity in DNA binding and structure elucidation would enrich our knowledge on comparative nickel homeostasis systems. This becomes more important as more pathogens are shown to use H₂ or urea in the host. Comparative knowledge on the roles of NikR would also seem to be important to understand microbial diversity from environmental and specifically metal sensing perspectives.

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