The Nickel-Responsive Binding and Regulation of Two Novel *Helicobacter pylori* NikR–Targeted Genes

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

Graduate Department of Chemistry

University of Toronto

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Abstract

Nickel is an essential transition metal for the virulence and survival of *Helicobacter pylori* in the acidic human stomach. The nickel– and proton– dependent transcriptional regulator HpNikR is important for maintaining nickel homeostasis inside the cytosol by regulating multiple *H. pylori* genes. A previous ChIP-sequencing experiment with *H. pylori* G27 and HpNikR identified two novel genes currently annotated as putative iron-transporters, HpG27_866 and HpG27_1499. *In vitro* DNA-binding assays with the promoter sequences of the two genes revealed nickel-dependent HpNikR binding with an affinity of \( \sim 10^{-7} \) M. The recognition site of HpNikR was identified on HpG27_1499 by footprinting assays, which loosely correlates with the HpNikR pseudo-consensus sequence. Furthermore, HpG27_1499 transcription showed nickel-dependent repression in WT *H. pylori*, and no changes in an isogenic \( \Delta \)nikR strain. These data suggest that HpG27_1499 could be a nickel importer that is regulated by HpNikR in a nickel-responsive manner.
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List of Abbreviations

ChIP-seq: chromatin immunoprecipitation sequencing
DBD: DNA-binding domain
DEPC: diethylpyrocarbonate
DTNB: 5,5'-dithiobis-(2-nitrobenzoic acid)
DTT: dithiothreitol
E. coli: Escherichia coli
EDTA: ethylenediaminetetraacetic acid
EMSA: electrophoretic mobility shift assay
H. pylori: Helicobacter pylori
IGEPAL: octylphenoxypolyethoxyethanol
IPTG: isopropyl-β-D-thiogalactopyranoside
LB: lysogeny broth
MBD: metal-binding domain
NTA: nitrilotriacetic acid
PDB: protein data bank
qPCR: quantitative polymerase chain reaction
RHH: ribbon-helix-helix
SDS PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
TB: tris-borate
TBE: tris-borate-EDTA
TNB: 5-thio-2-nitrobenzoic acid
Tris: tris(hydroxymethyl)aminomethane
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1. Introduction

1.1 Nickel in the environment

The transition metal nickel plays an important role in the survival of various organisms such as *Helicobacter pylori* (*H. pylori*), by acting as a catalytic co-factor for important enzymatic activities (1–4). Nickel can be acquired nutritionally but it can also be toxic to *H. pylori* if it accumulates inside the cell at high concentrations (5). Hence, *H. pylori* have developed intricate mechanisms to tightly regulate nickel uptake and release from the cell, which include the nickel-responsive transcription factor NikR (6).

1.2 *Helicobacter pylori* and metalloregulation

*H. pylori* are gram-negative pathogenic bacteria that colonize the gastric mucosa lining the human stomach (7). Infections by the bacteria cause peptic ulcers and gastric cancers if left untreated with antibiotics (8). *H. pylori* are able to survive the harsh conditions of the stomach in which the pH fluctuates between 7.2 and 1.2 (8, 9). The bacteria have developed many acid-resistance mechanisms, mainly using urease to produce ammonia in order to neutralize the cytoplasm and periplasm upon acid shock (2, 4, 9–11). *H. pylori*’s survival in the stomach is also due to the presence of hydrogenase, which catalyzes the reversible oxidation of hydrogen gas to electrons and protons that are used by the bacteria as an energy source (12, 13). The activity of both of these enzymes is dependent upon the presence of metal ions in their active site. Urease contains a dinuclear nickel cluster at the active site while hydrogenase contains a [NiFe] bimetallic center (2, 11, 14). Intracellular metal concentrations are tightly controlled in *H. pylori* through the
efforts of metalloproteins that function in uptake, efflux, storage, and metalloenzyme assembly (2). Regulation of metal homeostasis is mainly at the transcriptional level, controlled by a nickel-responsive transcription factor called NikR (6, 15). HpNikR belongs in the ribbon-helix-helix (RHH) family of DNA-binding proteins with two domains: one N-terminal DNA-binding domain (DBD) and one C-terminal metal-binding domain (MBD), which also acts as a tetramerization domain (Figure 1.1) (16). Each asymmetric MBD binds to one nickel ion (4 ions per tetramer), either coordinated in a square planar geometry or in a 5-/6- coordinate geometry (17).

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**Figure 1.1 Crystal structure of Holo-HpNikR.**
Holo-HpNikR (Protein Data Bank (PDB) 2CAD) consists of a tetrameric core of four MBD and two flanking DBD. Two of the monomers are in yellow, while the other two are in purple. Nickel ions are shown as green spheres. The ligands coordinating to the square planar nickel are H88’, H99, H101 and C107 (not shown). The ligands coordinating in 5-/6- geometry are H88, H74’ and H101’. The image is generated using Pymol.
1.3 *Helicobacter pylori* NikR

Unlike its homologue, EcNikR, which regulates only the *nik* operon, HpNikR is a pleiotropic regulator, acting both as a repressor and an activator of a variety of genes (Figure 1.2) (18, 19). A nickel and proton-dependent transcription factor, HpNikR binds to a variety of genes important in nickel import (*nixA, fecA*), storage (*hpn*), enzymatic activity (*ureAB*), autoregulation (*nikR*), and also genes involved in iron homeostasis (*fur, pfr*), motility of *H. pylori* (*flaA, flab*), stress response (*grpE, dnaK*) and outer membrane proteins (*omp11*) (6, 20–23). The mechanism by which DNA binding is activated by nickel or protons remains an unresolved puzzle (19, 20, 23–25). It is proposed that HpNikR uses a two-tiered mode of recognition to selectively target certain DNA sequences with either high affinity (*K*_d* values of nM) and low affinity (*K*_d* values of µM) (23). The tightly-regulated genes generally encode proteins that utilize nickel, while the genes regulated with low affinity encode other types of proteins. Although the mechanism by which HpNikR distinguishes between the genes that it regulates is not completely understood, a number of different factors are speculated to play a role. The nine-residue N-terminal DNA-binding arm exhibits different conformations when making electrostatic interactions with various DNA target sequences, playing a role in the selective gene regulation (24). Furthermore, it is believed that nickel binding in two distinct coordination spheres may provide conformational diversity and promote DNA binding to multiple sequences (17, 26). Although a strong consensus DNA-binding sequence for HpNikR has not been identified, a putative AT-rich asymmetric sequence recognized by HpNikR has been suggested by Delany *et al.* to be a weak consensus (27).
**Figure 1.2 H. pylori NikR regulon.**
Nickel-bound HpNikR activates (right) nickel storage genes (shades of purple) and ureA (yellow) and represses (left) the transcription of nickel import genes (shades of blue) together with other genes either depicted or not in the figure, such as ammonia production genes. The genes under investigation are potential HpNikR targets that may encode nickel importers.
1.4 Purpose of Study

Understanding the physical basis of DNA recognition by HpNikR, as well as a complete picture of its physiological function, requires further work to identify additional HpNikR targets. To do so, our lab performed a chromatin immunoprecipitation experiment followed by sequencing (ChIP-seq) (28, 29), which revealed a number of putative novel promoter genes targeted by HpNikR. To verify these potential targets, the in vitro binding interaction of HpNikR on the promoters of two genes: HpG27_866 and HpG27_1499 were examined. These genes are annotated as possible iron-regulated outer membrane protein and the periplasmic component of an ATP-binding cassette (ABC) transporter predicted to be an Fe(III)-binding protein, respectively (30). However, the proposed functions of several genes previously assigned as iron-regulated, based on sequence homology, have been incorrect in the past (31). The physiological metal selectivity of previously studied genes (e.g. fecA3 and frpB4) is now assigned to nickel and they are thought to be regulated by HpNikR in a nickel-dependent fashion (32, 33). We further examined whether HpG27_1499 and HpG27_866 genes are regulated by HpNiKR in vivo in a nickel-dependent manner. This information will give us a better insight on the role of HpNikR as a global regulator of multiple genes in H. pylori and the HpNikR mechanism of action in nickel homeostasis.
2. Experimental

2.1 Materials

Polymerases, restriction endonucleases and kinases were purchased from New England Biolabs, except where noted. Primers were purchased from Integrated DNA Technologies. Metal salts and other reagents were purchased from Sigma Aldrich, except where noted. The ΔnikR H. pylori G27 strain was generously donated by Dr. Peter Chivers (Department of Chemistry and Biochemistry, Oberlin College, Oberlin, OH) (34) All samples were prepared using 18.2 MΩ-cm resistance (Millipore) water, and the pH of all buffers was adjusted using either HCl or NaOH at room temperature.

2.2 Methods

Vector Construction:

The pET24bhpnikr vector was generated previously (35). HpG27_866 was PCR amplified from the G27 genomic DNA using the oligonucleotide forward and reverse primers p866F and p866R, which are listed in Table 2.1, using an an annealing temperature of 57 °C. The PCR fragment was cut with EagI and SalI to be inserted into pBS-SK(+) plasmid cut with the same enzymes. Following digestion, the plasmid was treated with calf intestinal phosphatase to prevent self-ligation. Ligation was performed with T4 ligase (Fermentas) at room temperature for 30 minutes at an ideal molar ratio of plasmid to insert of 1:16. The newly ligated pBS-SK866 plasmid was transformed by heat shock into NEB-Turbo E. coli cells and plated on ampicillin-containing LB agar plates. The plasmid was isolated with GeneJet Plasmid Miniprep Kit (Fermentas) following growth of colonies in 50 mL of LB media supplemented with 50 µg/ml
ampicillin. The correct insertion was detected via sequencing (The Centre for Applied Genomics, TCAG, Sickkids Hospital) and by restriction enzyme digests, with a combination of *NheI, ScaI, XhoI* and *EagI* to generate fragments that were resolved on a 1.5% (w/v) agarose gel.

HpG27_1499, was previously PCR amplified in our lab (M. Jones) from the G27 genomic DNA using p1499F and p1499R primers (Table 2.1). Following digestion of pBS-SK(+) plasmid with an *EcoRV* enzyme, the plasmid was treated with calf intestinal phosphatase to prevent self-ligation. Ligation with the PCR fragment was performed with T4 ligase (Fermentas) at room temperature for 30 minutes at an ideal molar ratio of plasmid to insert 1:16. The vector was transformed into NEB Turbo *E. coli* cells and isolated as described above. The correct insertion was detected by sequencing (ACTG, Inc.) and through a combination of restriction enzyme digest with *BamHI* and *ScaI*, where fragments were resolved on a 1.5% (w/v) agarose gel.

**Protein Expression and Purification**

pET24bhpnikr plasmid was transformed into BL21 Star *E. coli* cells and a single colony was inoculated in LB media containing 50 µg/ml kanamycin and grown to an optical density at 600 nm (OD$_{600}$) of 0.8-0.9. Protein expression was induced with 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 15°C for 4 hours. The cells were harvested, lysed by sonication in Protein Buffer (20 mM Tris and 100 mM NaCl, pH 7.6) and centrifuged at 18,000 rpm for 1 hour at 4°C using a Sorvall RC6+ SH3000 rotor. The supernatant was loaded onto 2 milliliters of Ni$^{2+}$-nitrilotriacetic acid (NTA) (Qiagen) resin previously equilibrated with 50 volumes of equilibration buffer (100 mM potassium phosphate, 500 mM NaCl and 10 mM imidazole, pH 8.0). Following loading, the resin
was washed again with 25 volumes of equilibration buffer. The protein was eluted with 5 volumes of 100 mM potassium phosphate, 10 mM Tris and 250 mM imidazole, pH 7.6. The purity of each fraction was determined by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) stained with Coomassie Blue dye. To keep HpNikR reduced and metal-free, 2 mM dithiothreitol (DTT) and 10 mM ethylenediaminetetraacetic acid (EDTA) were added respectively to the sample, which was then dialyzed in Protein Buffer containing 2 mM DTT. The dialyzed protein was then loaded onto an UnoQ column (BioRad) for anion exchange chromatography, initially equilibrated in 20 mM Tris pH 7.6 and eluted with a linear NaCl gradient. Fractions containing protein were verified via 12% SDS-PAGE, concentrated, and stored at 4 °C. HpNikR concentration was determined in Protein Buffer by using electronic absorption spectroscopy at 280 nm and using an extinction coefficient of 8480 M⁻¹cm⁻¹ (36, 37). The oxidation state of the protein was detected by performing a 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) assay. A standard curve of 7 to 56 µM β-mercaptoethanol was prepared in 6 M GuHCl, 1 mM EDTA and 400 µM DTNB. The absorbance for the standards and samples prepared in the same conditions were measured at 412 nm. Samples more than 90% reduced were used.
Table 2.1 Primers for plasmid construction and qPCR

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Primer name</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>HpG27_1499</td>
<td>p1499F</td>
<td>CTGGGTCGACTCAGCGAATAGT</td>
</tr>
<tr>
<td>HpG27_1499</td>
<td>p1499R</td>
<td>GATTTCGGCCGAAGCCGATTAG</td>
</tr>
<tr>
<td>HpG27_866</td>
<td>p866F</td>
<td>CACTCGGCCGAACGCTTGTCAATTCATT</td>
</tr>
<tr>
<td>HpG27_866</td>
<td>p866R</td>
<td>CATCGTCGACTGCGTTCAGTTGAAAGGG</td>
</tr>
<tr>
<td>Gene</td>
<td>qPCR</td>
<td></td>
</tr>
<tr>
<td>ureA</td>
<td>qUreAF</td>
<td>GATCTGATGATGCGTGGC</td>
</tr>
<tr>
<td>HpG27_1499</td>
<td>q1499F</td>
<td>CTTGCATTTTGGCCAGTTTT</td>
</tr>
<tr>
<td>HpG27_1499</td>
<td>q1499R</td>
<td>GCTTAGCCCTGATCTTGTGG</td>
</tr>
<tr>
<td>HpG27_866</td>
<td>q866F</td>
<td>CGCGGCTTTAAATGTCTCGCCTTT</td>
</tr>
<tr>
<td>HpG27_866</td>
<td>q866R</td>
<td>ATCCACCCAAACCTCCAGCCATA</td>
</tr>
<tr>
<td>16S rRNA</td>
<td>q16SF</td>
<td>CAGCGTCAGTAAATGCCAG</td>
</tr>
<tr>
<td>16S rRNA</td>
<td>q16SR</td>
<td>TAGAGTGGGGAGGAGTGG</td>
</tr>
</tbody>
</table>

Electrophoretic Mobility Shift Assays

The 194-bp 866 promoter and the 194-bp 1499 promoter were PCR amplified using the same primers used for plasmid construction (Table 2.1). The DNA probes were labeled at both ends with \( \gamma^{32}P \)-ATP (Perkin Elmer) using T4 polynucleotide kinase for 2 hours at 37 °C. Unincorporated nucleotides were removed with a G-25 microspin column (GE Healthcare). The amount of label incorporated was determined using a Packard Tri-Carb 2900TR Liquid Scintillation Counter (LSC). The radiolabeled DNA (10,000 counts per minute (cpm)) was incubated with increasing concentrations of apo- and holo-HpNikR (the latter being protein loaded with stoichiometric nickel) at room temperature for 1 hour in the presence of binding buffer (20 mM Tris, pH 7.5, 100 mM KCl, 3 mM MgCl\(_2\), 0.1% octylphenoxypolyethoxyethanol (IGEPAL), 5% glycerol, 0.1 mg/ml bovine serum albumin and 0.1 mg/ml sonicated herring sperm DNA (Promega)). The reactions were resolved on 6% native Tris-Borate (TB) (300 mM borate and 75 mM Tris-HCl, pH 7.5) polyacrylamide gels containing either 800 µM NiSO\(_4\), 3 mM MgSO\(_4\), or 1 mM
EDTA for 3 hours at 350 V and 4 °C after pre-running the gel for 1 hour in TB running buffer (300 mM borate and 75 mM Tris-HCl, pH 7.5, with either 800 µM NiSO₄, 3 mM MgSO₄, or 1 mM EDTA). The gel was vacuum-dried and exposed overnight to a phosphor screen, scanned with Pharos Fx™ Plus Molecular Imager (BioRad) and analyzed with Quantity One software. The fraction of DNA bound was determined by measuring the radioactive intensity of bound DNA divided by the intensity of total DNA in each lane. Data were fit to a Hill equation: 

\[ r = \frac{[HpNikR]^n}{(K_{d\text{ app}}^n) + [HpNikR]^n} \]

where \( r \) is the fraction of DNA bound to protein and \( K_{d\text{ app}} \) is the protein concentration required for 50% DNA binding.

**DNase I Footprinting Assays**

Oligonucleotide 1499 only was radiolabeled with γ-³²P-ATP using T4 kinase as described above. To achieve single-strand labeling, one of the 5’ labeled ends was cut with SalI, generating a 179 bp probe. Alternatively, for more efficient labeling of the 866 and 1499 promoters, pBS-SK866 and pBS-SK1499 vectors were cut with EagI and the 3’ overhang was filled in with α-³²P-GTP and 10 mM dCTP using Klenow Fragment, generating labeled blunt ends on both sides of the plasmid. Unincorporated nucleotides were removed with a G-25 microspin column. Cutting the linear plasmid with SalI generated the 185 bp and 184 bp 866 and 1499 footprint probes, respectively. At this stage, both digested products (866 and 1499) were resolved on a 7% native polyacrylamide gel and the ~200 bp DNA band was cut and electro-dialyzed at 100 V for 2 hours in 89 mM Tris-HCl, pH 7.5, and 87 mM borate buffer followed by precipitation with 2.5 volumes anhydrous ethyl alcohol and 0.3 M sodium acetate. The amount of labeled probe was measured by scintillation counting. To confirm that the correct DNA
sequence was obtained and to localize the footprint, the Maxam-Gilbert chemical reaction for guanine bases was performed as described (38, 39).

Increasing concentrations of holo-HpNikR were incubated for 1 hour at room temperature with the radiolabeled promoters (~40,000 cpm/sample) in DNase binding buffer (100 mM KCl, 20 mM Tris-HCl, pH 7.6, 1 mM MgCl₂, 1 mM CaCl₂ and 0.5% (v/v) glycerol and 0.01% IGEPAL). To each sample was added 1 µL of 2 µg/ml RNase-free DNaseI enzyme (Fermentas) and the reactions were quenched after 4 min by the addition of 80 µL DNase stop buffer (20 mM Tris, pH 8, 40 mM EDTA, 1% (w/v) SDS, 0.1 µg/µL herring sperm DNA). The enzyme was removed from the reaction with 100 µL of 24:25:1 phenol:chloroform:isoamyl alcohol. The DNA in the top layer was extracted and precipitated with 2.5 volumes of anhydrous ethyl alcohol and 1 µL of 10 mg/mL oyster glycogen at -20 °C overnight. The samples were then spun for 15 min at 12,000 g, the pellets dried under speed-vacuum, resuspended in 4 µL formamide loading dye and denatured at 90 °C for 3 min before loaded on an 8% denaturing polyacrylamide gel. The gel was pre-run to 50 °C at 1500 V. The samples were then run at 1700 V for 2 hours in TBE buffer (89 mM Tris-HCl, pH 7.5, 87 mM borate and 20 mM EDTA). The gel was vacuum-dried and exposed overnight to a phosphor screen, scanned with Pharos Fx™ Plus Molecular Imager (BioRad) and analyzed with Quantity One software.

**H. pylori Growth Conditions and Acid Shock**

The following experiments were performed under the supervision of Michael Jones in our laboratory. WT *H. pylori* and its isogenic ΔnikR strain were plated on Columbia agar plates containing 5% horse blood under a microaerobic environment (5% O₂, 10% CO₂ and 85% N₂) at 37 °C for three days and replated on new Columbia blood
agar plates for a further 22 hours. The colonies were then cultured in 30 mL of Brucella broth with 10% fetal bovine serum (Gibco BRL) while shaking at 100 rpm at 37 °C under the microaerobic environment for 10-15 hours up to an OD₆₀₀ of 0.5 – 0.6. The bacteria were collected and resuspended in 5 mL of fresh Brucella broth, pH 7.0, with or without 200 µM NiCl₂. The cells were incubated for 15 minutes at 37 °C in a microaerobic environment and 10 µL of the bacteria was used to make three ten-fold serial dilutions in Brucella broth and 5 µL of the dilutions were plated on blood agar plate and incubated for 3 days to measure cell viability. The rest of the bacteria were pelleted at 4000 rpm at 4 °C for 10 minutes and the supernatant was removed using a vacuum pump. The pellets were stored immediately at -80 °C to minimize RNA degradation.

**RNA Isolation and Reverse Transcription**

*H. pylori* G27 RNA was isolated by resuspending and lysing the bacterial pellet for 5 min in 1 mL of TRIazol reagent (Invitrogen). Following addition of 200 µL chloroform, all samples were centrifuged at 12,000 g for 15 min at 4 °C. The collected top layer containing the RNA was submitted to a further 200 µL chloroform extraction. The top layer was carefully removed and the RNA precipitated by the addition of 500 µL of isopropanol for at least 30 min at -20 °C. The RNA samples were centrifuged at 12,000 g for 15 min at 4 °C. After the supernatant was discarded, RNA pellets were washed with 70% ethanol and the RNA was air-dried. All RNA was resuspended in 30 µL DEPC-treated water. Traces of genomic DNA were removed with 178 U of RNase-free DNase I (Invitrogen) treatment for 5 min at room temperature. The RNA concentration was determined from the absorbance at 260 nm using an Agilent 8453 spectrophotometer, where an OD₆₀₀ of 1 equals a concentration of 40 ng/µL for single
stranded RNA. The RNA quality was monitored by the absorbance ratio at 260 nm/280 nm, where a ratio of > 1.8 represents good RNA quality, and the RNA integrity was inspected on a 1.5% agarose gel.

To convert RNA to cDNA, Superscript First-Strand synthesis system (Invitrogen) was used. RNA was incubated with 100 ng of random hexamers and 10 mM dNTPs at 65 °C for 5 min. Upon cooling in ice, 0.1 M DTT, 1x first-strand buffer, 20 U of RNase inhibitor RNaseOUT, and 100 U SuperScript II Reverse Transcriptase were added to the mixture and incubated at 50 °C for 1 hour. The reaction was stopped by incubation at 70 °C for 15 min and cDNA was stored at -20 °C.

**Quantitative PCR**

Amplification of target genes, ureA, 1499 and 866 was performed with the primers listed in Table 2.1 using the cDNA template transcribed from total RNA as described above. The internal control used was 16S rRNA. *H. pylori* G27 genomic DNA dilutions of 1 µg to 1 ng were used to make templates for the standard curve. A 20 µL reaction mixture per well for each reaction condition was run in duplicate in a 96-well plate. Each reaction mixture contained 2 µL (~ 60 ng) of cDNA or standard genomic DNA template and 10 µL of 2x Master Mix (Invitrogen), 5.5 µL water, 0.5 µL of 10 mM mixed forward and reverse primers and 2 µL of 1 in 100 diluted ROX reference dye to correct for variation from one well to the next. The two negative controls were DEPC-treated water only and reaction mixture with no DNA or no cDNA template. Samples were run a Roche LightCycler® 480 Real-Time PCR machine (Dr. Brenda Andrews’ lab, CCBR, University of Toronto) for 45 cycles (10 seconds at 95 °C, 35 seconds at 50 °C and 35 seconds at 72 °C). The data collected in real time were recorded as florescence units per
cycle. A melting curve was measured at the end of the reaction, which ensured the specificity of each primer. The relative expression of the target genes was normalized to the 16S rRNA level for each sample and then normalized to its own expression level for wild type *H. pylori* pH 7.0.
3. Results

3.1 HpNikR Purification and Characterization

HpNikR was overexpressed from the pET24 plasmid and was initially purified by a Ni²⁺-NTA column. HpNikR coordinates to the nickel in the column and elutes only with a high concentration of imidazole, which competes out HpNikR for nickel binding. The protein was then buffer exchanged through dialysis in the presence of DTT and EDTA to reduce the cysteines and minimize any metal binding, respectively. The protein was further purified with an UnoQ anion exchange column to remove other proteins that may have eluted from the Ni-NTA column. The collected fractions of each purification step were run on an SDS-PAGE gel to detect and confirm the presence of HpNikR. A single band at approximately 17 kDa was observed following UnoQ purification of the protein, confirming the presence of monomeric HpNikR. Electrospray ionization mass spectrometry revealed the correct molecular weight of the protein (MW_{calc} = 17,147.2 Da compared to MW_{obs} = 17,147 Da). To check the oxidation state of HpNikR, a DTNB assay was performed. There are two cysteine residues per HpNikR monomer and each reacted with 5,5'-Dithio-bis(2-nitrobenzoic acid) producing two TNB (5-thio-2-nitrobenzoic acid) products that were detected at 412 nm (40). Samples reduced more than 90% were kept for further experiments.

To confirm that HpNikR binds to stoichiometric metal as previously reported (35), nickel was titrated into a fixed concentration of HpNikR. Similar to the electronic absorption spectrum of *E. coli* NikR (35), nickel binding correlated with the manifestation of a peak at 302 nm with an extinction coefficient of 5100 M⁻¹ cm⁻¹, which is characteristic of ligand to metal charge transfer (LMCT) between the sulfur group of
cysteine and nickel (Figure 3.1) (41, 42). The maximum absorbance was reached in the presence of 1:1 HpNikR to nickel, thus confirming the nickel binding stoichiometry of HpNikR (Figure 3.1: inset).

![Figure 3.1 Nickel binding to HpNikR at pH 7.6](image)

**Figure 3.1 Nickel binding to HpNikR at pH 7.6**

Difference electronic absorption spectrum of nickel bound to HpNikR was generated by subtracting the spectrum of HpNikR bound to 1 equivalents of nickel from the spectrum of apo-HpNikR. Inset: titration of 20 µM HpNikR with increasing amounts of nickel at pH 7.6 revealed saturation of HpNikR with 1 equivalent of metal. The extinction coefficient at 302 nm was calculated to be $5100 \pm 800 \, \text{M}^{-1} \, \text{cm}^{-1}$ from four independent experiments ± standard deviation.

### 3.2 Identifying and analyzing new HpNikR targets

HpNikR is pleiotropic transcription factor and unlike its *E. coli* homologue, it has a wide repertoire of promoters that it binds to and regulates. Since HpNikR is a nickel-dependent transcription factor important in maintaining nickel and pH homeostasis in *H. pylori*, it is predicted that this repertoire may be larger than currently known.

Chromatin immunoprecipitation followed by sequencing (ChIP-seq) was previously performed in our lab by Xiaojun Yin in collaboration with Yunchen Gong (Department of Cells and Systems Biology at University of Toronto). This is a method
that has been widely used to define the regulatory network of many transcription factors (28). Preliminary results revealed known HpNikR targets such as nikR, ure, fecA3 and frpB4 in addition to annotated genes not known to be regulated by HpNikR. Two novel genes from the list were chosen to study more closely in order to determine if they are direct nickel-dependent HpNikR targets: HpG27_1499 and HpG27_866 (referred to as 1499 and 866). These genes are annotated as iron-regulated periplasmic and outer membrane protein transporters, respectively (30). Previous work has shown that several genes originally believed to be iron-transporters were reassigned as nickel-selective (32, 33). Given the fact that there is a lack of nickel-regulated membrane proteins in H. pylori’s nickel metabolism network, it is reasonable that these genes identified in the ChIP-seq experiment are components of nickel homeostasis.

3.3 DNA-binding activity of HpNikR to 1499 and 866

A simple and straightforward method to detect protein-DNA interactions \textit{in vitro} is to perform an electrophoretic mobility shift assays (EMSA). Generally, TB buffer is the preferred salt used in the gel and running buffer (as described in section 2.2 Methods) to resolve the bound from the unbound DNA. Previous work with nickel-bound HpNikR revealed enhanced detection of DNA-binding activity when excess cations are included in the gel and running buffer (24, 35). In this study, we examined the DNA binding of nickel-activated HpNikR in the presence of either excess Ni(II) or Mg(II) in the gel and running buffer (Section 2.2). Similarly, apo-HpNikR DNA-binding assays were performed in excess Mg(II) and excess EDTA as controls.

The 1499 gene encodes a putative inner membrane Fe(III) transporter of the ABC superfamily and is predicted to be an iron-regulated gene. However, our ChIP-seq results
indicated that it might be regulated by HpNikR in the presence of nickel. To prove that
the promoter for 1499 binds directly to nickel-activated HpNikR, mobility shift assays
were carried out. Figure 3.2a demonstrates that HpNikR (loaded with 1:1 nickel) binds to
1499 in the presence of 800 µM NiSO₄ in the gel and running buffer with a binding
constant of 140 nM (Table 3.2). Similarly, the HpNikR binding affinity for the 866
promoter, a gene encoding a putative iron outer membrane protein (OMP), was calculated
to be approximately 60 nM (Figure 3.2b). The DNA binding activity in excess Ni(II) is
exhibited in a graphic representation in Figures 3.4a and c for the 1499 and 866 DNA
probes, respectively. In both cases, no visible shift was observed with either 35 µM or
75 µM NiSO₄, but with 800 µM NiSO₄. This result is consistent with a previous study in
which Benanti et al. showed that a surplus of Ni(II) cations in the gel and running buffer
were required to detect a shift with HpNikR (24), although why such a high concentration
of nickel is required is not clear.

**Figure 3.2 DNA Binding of HpNikR in excess nickel.**
a) 1499 promoter was incubated with holo-HpNikR (1 nM - 600 nM) and b) 866
promoter was incubated with holo-HpNikR (0.1 nM to 1 µM). The reactions were
analyzed on a 6% native gel with 800 µM NiSO₄ in the gel and running buffer.
F indicates free DNA and B indicates protein-bound DNA. Affinities are calculated as
described in Materials and Methods and are listed in Table 3.1.
Figure 3.3 Magnesium as a cationic requirement of holo-HpNikR for DNA binding. Holo-HpNikR (0.1 nM – 600 nM) was incubated with a) the 1499 DNA probe and b) the 866 DNA probe and analyzed on a 6% native gel with 3 mM MgSO$_4$ in the gel and running buffer. $F$ indicates free DNA and $B$ indicates protein-bound DNA. The DNA-binding affinities are calculated as described in Materials and methods and listed in Table 3.1.

To further demonstrate that promoter binding is activated only by holo-HpNikR and not by weak Ni(II) binding to HpNikR due to presence of excess Ni(II), EMSAs of holo-HpNikR were performed in 3 mM MgSO$_4$ in the gel and running buffer (Figure 3.3). The calculated HpNikR affinities for 1499 and 866 measured in excess Mg(II) were similar to the ones measured in excess Ni(II), as listed in Table 3.1. The high salt concentration was required for a visible shift and revealed that approximately 1 x 10$^{-7}$ M protein was required for half-maximal binding on both novel promoters, as demonstrated by the fits to the Hill equation (Figure 3.4b,d).
Figure 3.4 Binding curves from mobility shift assays of the 866 and 1499 promoters. Representative data from mobility shift assays of holo-HpNikR, such as that shown in Figures 3.2 and 3.3, are fit to a Hill equation as described in Materials and Methods. The DNA-binding affinity of holo-HpNikR is determined for the 1499 DNA probe in a) 800 µM NiSO\(_4\), b) 3 mM MgSO\(_4\) and for the 866 DNA probe in c) 800 µM NiSO\(_4\), d) 3 mM MgSO\(_4\). The concentrations of holo-HpNikR at half-maximal saturation for four independent experiments and their respective Hill coefficients are listed in Table 3.1.

Table 3.1 Apparent calculated DNA-binding affinities (nM)

<table>
<thead>
<tr>
<th></th>
<th>EMSA</th>
<th>Footprinting</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Holo-HpNikR</td>
<td>Apo-HpNikR</td>
<td>Holo-HpNikR</td>
</tr>
<tr>
<td>1499</td>
<td>800 µM NiSO(_4)</td>
<td>3 mM MgSO(_4)</td>
<td>3 mM MgSO(_4)</td>
</tr>
<tr>
<td></td>
<td>140 ± 30</td>
<td>130 ± 80</td>
<td>NA</td>
</tr>
<tr>
<td>Hill coefficient</td>
<td>1.3 ± 0.3</td>
<td>1.9 ± 0.8</td>
<td>NA</td>
</tr>
<tr>
<td>866</td>
<td>60 ± 50</td>
<td>22.0 ± 5.0</td>
<td>NA</td>
</tr>
<tr>
<td>Hill coefficient</td>
<td>1.4 ± 0.2</td>
<td>1.9 ± 0.4</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA- No binding observed/ binding observed at [apo-protein] >> K\(_d\) calculated for holo-protein
Furthermore, apo-HpNikR did not interact with either promoter in the presence of 1 mM EDTA, required to keep HpNikR metal-free (data not shown). Additionally, mobility shift assays of apo-HpNikR in the absence of added nickel, but in the presence of 3 mM MgSO₄ in the gel and running buffer, revealed an observed half-maximal saturation to 866 and 1499 much weaker than the affinity measured for holo-HpNikR in similar conditions (Figure 3.5). Binding was only observed with > 50 µM protein, however the shift observed is smaller than with holo-HpNikR. This difference may indicate that non-specific interactions can occur and the complex may not be functional or relevant to HpNikR nickel regulation. Furthermore, the interaction could also be explained by the presence of excess MgSO₄ playing a role in the conformation of apo-HpNikR, yet nickel contamination may also be a factor for the weaker binding observed.

![Figure 3.5 DNA binding activity of apo-HpNikR.](image)

Reactions with apo-HpNikR (50 nM – 100 µM) incubated with a) 1499 DNA probe and b) 866 DNA probe were resolved on a 6% native gel with 3 mM MgSO₄. Binding is observed only at maximal HpNikR concentrations.
3.4 Localizing HpNikR’s Binding Site

As mentioned before, a consensus sequence for HpNikR DNA binding has not been clearly defined. Therefore, new targets must be identified by means other than sequence identity to allow us to expand the already large HpNikR regulation repertoire and to further understand the role of this transcription factor in nickel homeostasis. After demonstrating nickel-mediated DNA binding of 1499 to HpNikR with EMSAs, DNase I footprinting assays with holo-HpNikR were performed, revealing a 40 bp AT-rich protected region on 1499 (Figure 3.6a), as underlined in Figure 3.6b, with an affinity of 230 nM. Although not a conserved sequence, the binding site follows the pseudo-palindrome TATTAT\textsubscript{11}N\textsubscript{11}AATAATA (Figure 3.7), proposed by Delany et al. and later by Dosanjh et al. (23, 27). Another putative pseudo-palindrome has been suggested by Stoof et al., TRWYAN\textsubscript{15}TRWYA, also resembling the observed protected region of 1499 (not shown) (43). Although both recognition sequences loosely align with the 1499 recognition sequence, Evans et al. concurred with Dosanjh’s identification of DNA bases that are crucial for protein binding such as thymine 10 at the 3’ half-site, important in distinguishing the tight binders from the weak ones (44). This thymine is also present in the 1499 promoter recognition sequence (Figure 3.7).

The same footprint region, shown in Figure 3.8, and binding constant (Table 3.1) was observed in another independent experiment of 1499 incubated with increasing concentration of HpNikR in the presence of excess nickel. This suggests that excess nickel is not required for tight binding of the promoter.
Figure 3.6 DNA-binding activity of HpNikR with the 1499 promoter.

a) DNaseI footprinting assay of apo-HpNikR (50 nM, 100 nM, 500 nM, 5 µM) and holo-HpNikR (50 nM, 100 nM, 200 nM, 300 nM, 500 nM, 1 µM, 5 µM) incubated with $^{32}$P-radiolabeled 179 bp DNA probe for 30 minutes before adding DNase I. The reactions are resolved on an 8% polyacrylamide denaturing gel and the area of protection is indicated. Key: U, undigested 195-bp probe; U2, undigested 179-bp probe; G, Maxam-Gilbert G reaction; G*, same G-reaction exposed for a shorter period of time for clarity; C, control DNase I reaction of 179-bp probe with no HpNikR. The apparent DNA-binding affinity was determined to be 230 ± 80 nM with a Hill coefficient of 2.1 ± 0.3, suggesting a small degree of cooperativity. The number reported is an average of four independent experiments such as that shown ± standard deviation.

b) DNA sequence of 1499 promoter. The binding region is underlined and the guanines within in the footprint are bolded and noted on the footprint gel.
### Figure 3.7 Alignment of the HpNikR binding sites from various promoters (32).

The two parts of the palindrome are bolded and numbered according to Evans et al. (44), and in red for the promoters themselves. The numbers on the right indicate the position of the last protected base on the 3’ end relative to the +1 transcriptional start site. The transcription start site of 1499 is not known. N represents any nucleotide and W represents either an A or T residue.

<table>
<thead>
<tr>
<th>Position</th>
<th>Consensus</th>
<th>1 2 3 4 5 6 7</th>
<th>8 9 10 11 12 13 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>1499</td>
<td>TATWATT----N_{11}----AATWATA</td>
<td>TTCGTATGATTTATGAAGCGATTATAACAATATTCAAG</td>
<td>?</td>
</tr>
<tr>
<td>ureA</td>
<td>CAAAGATAAACACTAATTCTTAAATAATAAT</td>
<td>-56</td>
<td></td>
</tr>
<tr>
<td>nikR</td>
<td>ATCCAGTTTGATTAAATGTTCTATTTTTAAATTAAT</td>
<td>+10</td>
<td></td>
</tr>
<tr>
<td>nixA</td>
<td>AAATATATTACAATACCACAAAAAAGTATTATTTC</td>
<td>+21</td>
<td></td>
</tr>
<tr>
<td>frpB4</td>
<td>AAATTTAAGGTATTATTAATAGAATAATGTAATAA</td>
<td>-8</td>
<td></td>
</tr>
<tr>
<td>fecA3</td>
<td>ATCCGCACTATTAAAGTTTTTTTTGTTTTATTACT</td>
<td>+31</td>
<td></td>
</tr>
<tr>
<td>exbB2</td>
<td>ATGGACTTTGTATTATAAAAAATAAATACAAACAAC</td>
<td>+1</td>
<td></td>
</tr>
<tr>
<td>fur</td>
<td>TCTATGTTTTCATCGCATTATATTGTAATAATAATTC</td>
<td>+1</td>
<td></td>
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</tbody>
</table>
Figure 3.8 DNA-binding activity of HpNikR in excess amounts of nickel with the 1499 promoter.

a) HpNikR (50 nM, 100 nM, 200 nM, 250 nM, 300 nM, 500 nM, 1 µM, 5 µM) was incubated with a \( ^{32} \text{P} \)-labeled 184-bp DNA probe in the presence of 35 µM NiCl\(_2\) for 30 minutes at room temperature prior to the addition of DNase I. The reactions are resolved on an 8% polyacrylamide denaturing gel and the area of protection is indicated. Key: G, Maxam-Gilbert G reaction. The apparent DNA-binding affinity was determined to be 330 ± 40 nM with a Hill coefficient of 2.02 ± 0.04, suggesting a small degree of cooperativity. The number reported is an average of two independent experiments ± standard deviation. 

b) DNA sequence of 1499 promoter (reverse complement of sequence in Figure 3.6b). The binding region is underlined and the guanines within the footprint are bolded and noted on the footprint gel.
3.5 Distortion of the 866 DNA probe in the presence of HpNikR

Although no footprint of HpNikR on 866 promoter sequence is observed, DNase I hypersensitive bands were detected instead, indicated with arrows in Figure 3.9a. Hypersensitive bands suggest a distortion of DNA when complexed with HpNikR, making it more accessible to DNase I cleavage (20). Hypersensitive bands were observed when 866 DNA probe was incubated with increasing holo-HpNikR concentration, however no footprint was detected. Similar work with the nikR promoter revealed strong binding to HpNikR in mobility shift assays, yet a weak binding constant with fluorescence anisotropy (23, 24). The promoter however was not able to display a footprint in DNase I assays, likely due to the protein-DNA complex’s short half-life (24). A similar event may also be occurring with 866 promoter. Although the affinity calculated for the 866 in DNA shift assays is strong (~10^{-8} M), excess metal in the gel and running buffer may have affected the promoter’s true affinity, as observed by West et al. (45). The 866 DNA sequence is very AT rich and thus it can be loosely aligned to the pseudo-palindrome in multiple ways (not shown).
Figure 3.9 DNA-binding activity of HpNikR with the 866 promoter.

a) DNasel footprinting assay of holo-HpNikR (1 nM, 10 nM, 50 nM, 100 nM, 150 nM, 200 nM, 300 nM, 500 nM, 5 µM) incubated with \(^{32}P\) radiolabeled 185 bp DNA probe for 30 minutes before adding DNase I. The reactions are resolved on a 8% polyacrylamide denaturing gel. Key: U, undigested 195-bp probe; U2, undigested 185-bp probe; G, Maxam-Gilbert G reaction; C, control DNase I reaction of 185-bp probe with no HpNikR; A, apo-HpNikR (5 µM) with 185-bp DNA probe. There is no apparent binding affinity, possible due to the short half-life of the DNA-protein complex. Arrows point to hypersensitive bands.

b) DNA sequence of 866 promoter. Guanines are bolded for clarity and indicated on the gel.

5’
TCGACTGCGTGGTTTGAAGGGTTTTTGTGGGATTTTCTAAAATTTATCA
TTGTTTATTAAATGGCTAGCTCCTTTTAAGGTGATTGTAATATTTTTTGG
TTATAATAAACACCTTATTTTTTAAAG2G3AAATTATTATCATATTTTAGG4G5ATT
TTAAGAATGAATGACAAGCGTTCGGCCC – 3’
3.6 In vivo regulation of 1499 and 866

To quantify the influence of nickel on the transcription of 1499 and 866, and the dependence of the response on HpNikR, quantitative PCR (qPCR) was performed. WT *H. pylori* G27 and a ΔnikR strain were grown in Brucella broth supplemented with 50 mM urea at physiological pH in the absence and presence of 200 μM nickel (30). After RNA isolation, qPCR was performed to measure the expression of 1499 and 866 genes relative to *16S* transcript levels, the internal control of the experiment.

As a positive control for our in vivo analysis, the *ureA* gene expression was examined alongside the two genes being studied. Urease is an abundant protein in *H. pylori* and its nickel-metalloenzyme activity is crucial in acid-resistance defense (10, 46, 47). Therefore, upregulation of *ureA* is essential upon metal-shock, and recently it was found to be the first gene to respond to increased nickel concentration (22). In WT *H. pylori*, the data show a moderate upregulation of *ureA* upon addition of nickel, however not statistically significant (*p* > 0.05) (Figure 3.10). It has been established that nickel upregulates *ureA* over three-fold as a primary defense mechanism against metal stress, however our experiments indicated less than a two-fold increase in transcription (48). Possibly, *ureA* expression levels may vary depending on the *H. pylori* strain, since all previous experiments were not performed with *H. pylori* G27 but instead with *H. pylori* 26695 or other *H. pylori* strains (19, 22, 48). It is also possible that the high nickel concentration may have affected *ureA* expression, however cell viability assays, which are described in Methods, revealed that *H. pylori* survived even when supplemented with 200 μM nickel (not shown). More replicates with a different nickel concentration in
growth media or longer incubation times upon nickel-shock are required to check if ureA upregulation becomes significant in WT *H. pylori* G27 strain.

Furthermore, our results show that ureA is significantly downregulated in the ΔnikR strain compared to WT *H. pylori* without nickel (p < 0.05). Regulation of ureA is HpNikR-dependent as expected, based on previous work (22). ureA expression is nickel independent in the ΔnikR mutant since the mRNA levels in our qPCR data remain the same whether ΔnikR *H. pylori* is supplemented with nickel or not (Figure 3.10). Our data revealed abrogation of nickel induction of urease and is corroborated by work from van Vliet *et al.* (6).

In WT *H. pylori*, the in vivo data indicate that the 1499 mRNA levels decrease significantly upon the addition of nickel to the growth media, to almost half relative to the mRNA levels transcribed in the absence of added nickel (p < 0.05) (Figure 3.10). This suggests that nickel-activated DNA binding inhibits the transcription of 1499 when nickel levels rise in the cell. This effect of nickel was not observed in the ΔnikR strain, revealing that nickel does not affect 1499 regulation in the ΔnikR mutant. Similarly, no change of 1499 expression between WT and ΔnikR strain with nickel was observed. This indicated that 1499 regulation is exclusively dependent on nickel-activated HpNikR, similar to fecA3 and frpB4 promoters now established to be nickel transporters (32, 33).

In WT *H. pylori*, the 866 gene expression was not observed to change significantly upon nickel supplementation. Also, no changes in regulation were observed with the ΔnikR strain in the absence or when supplemented with nickel (Figure 3.10). Although it would be expected that a hypothesized nickel transporter be repressed in excess nickel, the lack of an HpNikR footprint and the current in vivo data showing no
regulation suggests that other factors may play a role in the regulation of 866 promoter and HpNikR is not the main transcription factor regulating 866. Perhaps HpNikR may play a role in 866 regulation during the later stages of nickel homeostasis or in other growth phases.

Figure 3.10 *In vivo* regulation of two novel HpNikR-targeted genes

qPCR data from three independent experiments display nickel regulation of two new potentially HpNikR-targeted genes, 1499 and 866 in WT *H. pylori* G27 strain and its isogenic ΔnikR mutant grown to mid-log phase. Transcript levels of 1499 and 866 were measured by qPCR and normalized to relative 16S expression under the same conditions ± standard error. The WT G27, pH 7 data are set at an arbitrary unit of 1, and all other mRNA levels were calculated relative to that. Statistically significant differences of expression levels between WT *H. pylori* without nickel versus other conditions tested are marked with an asterisk (p < 0.05, Student’s *t*-test). *ureA* is the positive control for the qPCR experiments.
4. Discussion

*H. pylori* thrive in the human stomach and gastric mucosa, where the pH drops as low as 2 (8, 9). Two key enzymes, urease and hydrogenase, give the gram-negative bacteria the unique ability to colonize in such unforgiving conditions. Urease converts urea into ammonia, thus buffering cytosolic pH upon acid shock, and hydrogenase catalyzes the reversible oxidation of hydrogen to protons electrons, hence providing energy for the bacteria to colonize (2, 9–13, 25, 46). Both enzymes require nickel in their active site. Consequently, *H. pylori* constantly require a supply of nickel. Nickel uptake and distribution is regulated by the pleiotropic transcriptional regulator HpNikR (6, 15). The high demand for nickel had prompted us to look for new HpNikR targets that are regulated in a nickel-dependent fashion. This will also help us expand the HpNikR recognition motifs and in turn give us better insight into the mechanism of action of HpNikR in nickel and proton homeostasis in *H. pylori*. Until recently, the mechanism by which nickel is taken up into the periplasm was not known, but it was assumed that a high-affinity OMP was required for low bioavailable molecules. These OMPs need the energy derived from the proton motive force (PMF) of the inner membrane TonB/ExbB/ExbD protein complex, much like the transport of iron complexes and cobalamin (49–51). FrpB4 was found to be a nickel-regulated OMP and was recently established to be a nickel importer, thus providing a model to explain extracellular nickel acquisition into the periplasm, where it is then transported into the cytosol by the NixA transporter (32, 33, 52).

A previous ChIP-sequencing experiment revealed an array of known and unknown genes associated with holo-HpNikR. Among them are two genes annotated as
inner and outer membrane iron-transporters, HpG27_1499 and HpG27_866 respectively (30). Since the main disadvantage of ChIP-seq is non-specific binding that leads to high noise background and false positives (53), we set out to determine if HpNikR targets the two new genes in response to nickel. In *vitro* DNA-binding assays were performed with the genes’ respective promoters. Our results reveal nickel-responsive HpNikR binding to both promoters, where ~$10^{-7}$ M HpNikR is required for half-maximal binding in both cases. Although both promoters bind with high affinity, it was noted that excess nickel or magnesium was required for binding in the EMSAs. This supports the work done by Benanti *et al.* showing that some type of excess cation is required in EMSAs with HpNikR, along with the stoichiometric nickel (24). It is presumed that cations prevent repulsive electrostatic interactions between acidic residues of HpNikR and the negatively charged DNA backbone (24).

It is interesting to define what drives HpNikR to recognize multiple promoters with different affinities and why those promoters respond differently to nickel-activated HpNikR. One of these factors is the flexible N-terminal DNA-binding arm on the DBD of HpNikR, which decreases non-specific interactions and prohibits binding to low-affinity DNA sequences (24). Also, HpNikR’s MBD possesses 2 nickel-binding sites with distinct coordination geometries as discovered by recent crystallography studies (17, 54). The MBD asymmetry is proposed to have an allosteric effect on the DNA-binding abilities of HpNikR (17, 26). It has been suggested that HpNikR conformation adapts relative to the sequence that it is recognizing, thus HpNikR’s structural flexibility may also explain its pleiotropic DNA-binding abilities (26). Consequently, DNA sequence is an important factor determining the affinity with which HpNikR binds. Therefore, we set
out to define the DNA sequence of the two promoters targeted by HpNikR through footprinting assays. Holo-HpNikR bound to a 40-bp sequence within the 1499 promoter, encoding for a putative periplasmic Fe(III) ABC membrane transporter, with an affinity of 230 nM, similar to the binding affinity calculated from mobility shift assays. The 1499 recognition sequence is highly conserved on the 5’ end and it has a thymine at position 10 as indicated by the alignment in Figure 3.7, postulated to be a strategic base HpNikR uses to distinguish the strong binders from the weak ones (44). There were no changes observed in the binding affinity when excess nickel was added to the footprint reactions, verifying the lack of any functional additional nickel-binding site.

It is hypothesized that HpNikR exerts a region-specific regulation depending on where on the promoter it binds. Activation is known to occur if binding takes place well upstream of the transcription start site (-50 to -90) while repression occurs when binding takes place near the start site (-10 to +1) (19, 27). At this time, the location of the 1499 transcription start site is not known. Revealing the 1499 transcriptional start site relative to the binding site will complement the in vivo data that displays nickel-dependent repression of 1499 by HpNikR.

In contrast, the 866 promoter, encoding for a predicted iron-regulated OMP, did not display a footprint. Nevertheless, the hypersensitive bands observed with increasing HpNikR concentration clearly show that the DNA is somehow being distorted when protein is present, making it more sensitive to DNase I cleavage. Only one band in the footprint is protected from cleavage. It may be possible that the true complex’s half-life is short or that the affinity is weaker than observed in DNA shift assays due to the presence of excess cations in EMSAs. Previous work done to calculate the binding constant of
HpNikR for its own promoter, nikR, displayed a range of affinities depending on the experiment performed. EMSAs revealed a tighter affinity (120 nM), possibly due to the presence of excess cation, while fluorescence anisotropy displayed a weak binding affinity (5 µM) (23, 24). Perhaps a similar effect is also occurring with the 866 DNA promoter displaying tight binding in mobility shift assays due to the presence of excess cations.

Thus far we have proven that the promoters of 866 and 1499, annotated as iron transporter genes, bind to HpNikR in a nickel-responsive manner. As possible candidates of nickel transporters, we predicted that these genes are repressed in H. pylori grown in the presence of too much nickel, in order to shut down nickel import as a defense mechanism and maintain nickel homeostasis inside the cell. To investigate their in vivo regulation, mRNA transcription levels were measured by performing qPCR. When WT H. pylori were grown to mid-log phase, transcription of 1499 was significantly repressed upon nickel supplementation, suggesting nickel-responsive regulation. No significant changes were observed between WT H. pylori without nickel the ΔnikR strain with or without nickel suggesting that regulation of 1499 is strictly dependent on nickel-loaded HpNikR. HpNikR-dependent repression of 1499 is likely occurring as a defense mechanism against nickel stress, suggesting that 1499 is a plausible inner membrane nickel importer in H. pylori.

The ureA gene however, used as our positive control, did not display a significant upregulation when supplemented with nickel as is expected based on previous work (19, 22, 48). It is probable that ureA expression levels may vary among different H. pylori strains used. Previous experiments were performed with H. pylori 26695 or others instead
of *H. pylori* G27 (19, 22, 48), used in our experiments. Perhaps a different nickel concentration for metal shock is required to maximize *ureA* expression of WT *H. pylori* G27 strain. There is also a chance that the high nickel concentration may have decreased cell survival, however our cell viability assays revealed normal growth of *H. pylori* when supplemented with excess nickel (not shown).

Nonetheless, *ureA* expression was significantly repressed in the *ΔnikR* strain compared to WT *H. pylori* without nickel, which verifies that regulation of *ureA* is HpNikR-dependent. The expression of the *ureA* transcript did not change between WT levels without nickel and *ΔnikR* supplemented with nickel. A possible source of error may be the slow growth of the *ΔnikR* *H. pylori* in the third qPCR replicate, which only reached an OD$_{600}$ ~ 0.3 (early-log phase). The *ΔnikR* strain is grown simultaneously with the WT strain and it might be possible that *ureA* expression in *H. pylori* is log-phase dependent, however, there are no studies to support this hypothesis. It is also possible that *ureA* transcription in the *ΔnikR* G27 strain may not respond to nickel in a similar manner as other knockout strains. Therefore, nickel concentration and incubation time when supplementing *H. pylori* with nickel are two main factors to consider when performing additional qPCR replicates.

In contrast, qPCR data do not show 866 mRNA levels changing in the conditions tested. It is very likely that the 866 gene encodes for a protein that is not regulated by nickel-loaded HpNikR, but by other factors that induce *H. pylori* to respond to stresses, such as acid shock or iron stress (25, 55, 56). Although we hypothesized the 866 promoter to be HpNikR-targeted in a nickel-dependent fashion, it is probable that 866
encodes for a protein that is indeed an iron-importer, thus allotting FrpB4 and FecA3 as the only known OM nickel transporters in *H. pylori*. 
5. Conclusions and Future Work

The DNA–binding activity of HpNikR for promoters of 1499 and 866 was assessed. In vitro DNA mobility shift assays and footprinting assays revealed strong DNA binding affinity for the 1499 promoter and also revealed a binding site within the 1499 promoter that loosely resembles the pseudo-site (TATTATTN_{11}AATAATA). A thymine in position 10 was noted, which is known to play an important role in distinguishing the tight binders from the weak ones. Footprinting assays in excess nickel also supported the absence of low-affinity nickel-binding site on HpNikR when no additive effect was seen in DNA binding. HpNikR did not reveal a binding site on the 866 promoter even though mobility shift assays displayed a strong nickel-dependent DNA binding, suggesting that the excess cations may have played a role in increasing the binding affinity of the 866 promoter for HpNikR.

In vivo quantitative-PCR data of 1499 in WT H. pylori revealed a significant downregulation of the gene in enhanced nickel growth conditions, while transcription remained constant in the ΔnikR strain with or without nickel. The results suggest that nickel-loaded HpNikR regulates 1499 transcription, thus adding another target to the HpNikR regulatory network. The 1499 gene is a promising candidate as an HpNikR-regulated inner membrane nickel transport protein.

To demonstrate that 1499 and 866 are not iron-responsive genes, qPCR experiments with WT and ΔnikR H. pylori grown in Fe(III) supplemented media will be performed. To further elucidate the role that 1499 plays in H. pylori’s nickel homeostasis, a Δ1499 knockout mutant must be generated. If 1499 is a membrane nickel transport protein, then nickel uptake and regulation of nickel-responsive genes should change
accordingly in the isogenic Δ1499 knockout. Future experiments will include examining nickel uptake as well as in vivo transcriptional regulation of nickel-responsive genes such as ureA between WT H. pylori and Δ1499 as a way to assess intracellular nickel availability, consequently validating 1499 to be a inner membrane nickel transporter of the ABC superfamily.
6. References


