The evolution and regulation of DNA-binding by the nickel-dependent transcription factor NikR

Erin Benanti

Follow this and additional works at: http://openscholarship.wustl.edu/etd

Recommended Citation


http://openscholarship.wustl.edu/etd/35

This Dissertation is brought to you for free and open access by Washington University Open Scholarship. It has been accepted for inclusion in All Theses and Dissertations (ETDs) by an authorized administrator of Washington University Open Scholarship. For more information, please contact digital@wumail.wustl.edu.
WASHINGTON UNIVERSITY

Division of Biology and Biomedical Sciences

Program in Molecular Microbiology and Microbial Pathogenesis

Dissertation Examination Committee:
Peter T. Chivers, Chair
Douglas E. Berg
Michael G. Caparon
Eduardo A. Groisman
Himadri B. Pakrasi
Gary D. Stormo

The evolution and regulation of DNA-binding by the Ni^{2+}-dependent transcription factor NikR

by

Erin Lynn Benanti

A dissertation presented to the Graduate School of Arts and Sciences of Washington University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

August 2009

Saint Louis, Missouri
Transition metal homeostasis is critical for all cells to balance cellular metal requirements with metal availability. One common homeostatic mechanism in bacteria is metal-dependent transcriptional regulation. The Ni$^{2+}$-dependent transcription factor NikR is a member of the ribbon-helix-helix (RHH) family of DNA-binding proteins and is widespread among bacteria and archea with vastly different nickel physiologies. The goal of this thesis was to better understand basic aspects of cellular transition metal homeostasis by examining the activity and regulatory properties of NikR family members from different bacterial species. One organism that exhibits a prominent and well-defined nickel physiology is *Helicobacter pylori*, making it an ideal system with which to examine various aspects of metal homeostasis. Genetic studies demonstrated that NikR activation is controlled by a hierarchy of nickel-trafficking in *H. pylori,*
where nickel is preferentially trafficked to the urease assembly pathway. NikR differentially regulates multiple nickel-related genes in response to distinct extracellular nickel concentrations, functioning to coordinate multiple activities important for metal homeostasis. Differential gene regulation resulted from NikR binding to promoters from different genes with a range of affinities and in distinct conformations, due to a flexible N-terminal arm that makes different DNA contacts at two promoters. In addition, the arm expands the specific DNA interactions by NikR as compared to previously characterized RHH transcription factors. Examination of additional previously uncharacterized NikR family members revealed that the N-terminal arm has been adapted differently in some cases but is also critical for DNA-binding affinity and specificity. This structural feature provides a molecular basis for tuning NikR activity to the physiology of the cell. These studies provide insight into how multiple metal-dependent activities in cells are coordinated and controlled in response to fluctuations in environmental metal. Further, they establish a robust experimental system with which to further investigate the molecular details of the evolution of transcriptional regulation, an integral component of metal homeostasis.
Acknowledgements

I would like to extend my utmost gratitude to my thesis advisor, Peter Chivers. He has provided invaluable guidance and support throughout my graduate training, even on the rare occasions when we disagree, and has consistently inspired me to keep pushing for answers. His excitement and enthusiasm for science are infectious, and I know he will continue to inspire me in the future.

I also would like to thank everyone who has worked in the Chivers lab - it has been a truly enjoyable place to work. In particular I have to acknowledge our former technician, Jessica Rowe, who not only is an excellent scientist and made significant contributions to discoveries in our lab, but has also been a never-ending source of support, insightful advice and inspiration for me.

I am forever indebted to my undergraduate advisor, Peggy Cotter, who was the first to share her passion for science with me. Her perspective that science is so incredibly exciting and "we actually get paid to do it!" convinced me that research was worth a try, and I haven't considered changing directions since.

My gratitude also goes to my colleagues at Washington University, including those in the Biochemistry and the Microbiology departments with whom I've interacted with over the years. This has been an exciting place to do science, and someone always has interesting results and discoveries to share.

I also want to acknowledge my committee members, who have not only provided a wealth of knowledge and experience, but who also have patiently guided me throughout my thesis work. I truly appreciate all of their time and insight - my training is very much a reflection of their input. My deepest appreciation also goes to Doug Berg, who has shared his technical expertise, his reagents and his lab members with me, and has also been a constant source of enthusiasm and inspiration.

Finally, I would like to thank my parents who have always supported me and my decisions, and my mother for encouraging me to experience life outside of California. My gratitude also goes to my older sister, who has paved the road to science for me, and who has set the bar incredibly high. She has tirelessly provided me with thoughtful, practical advice, and has always encouraged me to strive for the best. I also want to give my heartfelt thanks to Jeff Iwig and the Iwigs and Iwig-Woods. Despite spending hours at work and home together, Jeff remains extremely supportive and patient. His competitiveness and refusal to accept less than the best continue to make me a better scientist.
# Table of Contents

Abstract ........................................................................................................... ii
Acknowledgements ........................................................................................... iv
Table of Contents ............................................................................................... v
List of Figures ........................................................................................................ viii
List of Tables ........................................................................................................ xi

## Chapter 1  Introduction .................................................................................. 1
Transition metals in biology .................................................................................. 2
Important aspects of metal utilization .................................................................. 7
  Kinetic and thermodynamic properties of metal-binding .................................. 7
  Metal transport ................................................................................................. 8
  Metal chaperones ............................................................................................. 8
  The importance of metal regulation .................................................................. 9
Transition metal homeostasis .............................................................................. 10
  Intracellular metal trafficking ......................................................................... 10
    Metal transfer between proteins .................................................................. 10
    Thermodynamic competition for metals ...................................................... 12
    Kinetic competition for metals .................................................................... 13
  Transcriptional regulation .............................................................................. 14
  Post-transcriptional regulation ........................................................................ 16
  Regulation of transporter localization ........................................................... 17
The importance of nickel in biology ................................................................... 18
  Helicobacter pylori, a model system to study nickel homeostasis ................. 22
    Nickel utilization by Helicobacter pylori ...................................................... 22
    Nickel storage ............................................................................................. 26
    Nickel uptake .............................................................................................. 26
    Nickel-dependent gene regulation ............................................................ 27
The Ni\textsuperscript{2+}-dependent transcription factor NikR ................................................. 28
  Basic properties of NikR ............................................................................... 28
  The Ni\textsuperscript{2+}-binding domain ................................................................ 28
  The DNA-binding domain ............................................................................ 31
  The RHH family of DNA-binding proteins .................................................. 32
  The evolution of DNA-binding in the NikR family ....................................... 34
Summary ............................................................................................................ 35
References .......................................................................................................... 37

## Chapter 2  Differential Ni\textsuperscript{2+}-dependent gene regulation and DNA-binding
by Helicobacter pylori NikR .................................................................................. 62
Overview ............................................................................................................. 63
Overview ............................................................................................................. 264
Abstract ............................................................................................................... 265
Introduction ........................................................................................................ 266
Results ............................................................................................................... 270
Discussion ......................................................................................................... 295
Materials and Methods ................................................................................... 303
References ........................................................................................................ 312

Chapter 7  Conclusions and future directions .................................................. 319
Conclusions ........................................................................................................ 320
Summary ............................................................................................................ 337
References ........................................................................................................ 339

Appendix 1  In vivo analysis of H. pylori NikR gene regulation  ............... 348
Summary ............................................................................................................ 349
Preliminary results and interpretation ............................................................... 351
References ........................................................................................................ 359

Appendix 2  Two separable pathways for nickel transport in H. pylori ...... 361
Summary ............................................................................................................ 362
Preliminary results and interpretation ............................................................... 364
References ........................................................................................................ 372
List of Figures

<table>
<thead>
<tr>
<th>Chapter 1</th>
<th>Introduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1</td>
<td>Summary of known and predicted nickel-binding proteins and nickel pathways of <em>Helicobacter pylori</em></td>
</tr>
<tr>
<td>Figure 1.2</td>
<td>NikR undergoes dramatic conformational changes to bind DNA</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter 2</th>
<th>Differential Ni(^{2+})-dependent gene regulation and DNA-binding by <em>Helicobacter pylori</em> NikR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 2.1</td>
<td>Ni(^{2+}) and NikR dependent regulation in <em>H. pylori</em> strain 26695 at pH 7</td>
</tr>
<tr>
<td>Figure 2.2</td>
<td>Ni(^{2+}) and NikR dependent regulation in <em>H. pylori</em> strain 26695 at pH 5.5</td>
</tr>
<tr>
<td>Figure 2.3</td>
<td>NikR directly binds to multiple promoters with a range of affinities</td>
</tr>
<tr>
<td>Figure 2.4</td>
<td>NikR (\beta)-sheet residues are required for DNA-binding</td>
</tr>
<tr>
<td>Figure 2.5</td>
<td>NikR with stoichiometric or no NiCl(_2) has decreased affinity for the <em>nixA</em> and <em>ureA</em> promoters</td>
</tr>
<tr>
<td>Figure 2.6</td>
<td>Direct binding of Ni(^{2+}) to NikR occurs with high affinity and increases the stability of the protein</td>
</tr>
<tr>
<td>Figure 2.7</td>
<td>NikR recognizes poorly conserved DNA sequences</td>
</tr>
<tr>
<td>Figure 2.8</td>
<td>NikR requires additional cations for DNA-binding in gel mobility shifts</td>
</tr>
<tr>
<td>Figure 2.9</td>
<td>High-affinity binding of NikR to (P_{nixA}) requires Mg(^{2+})</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter 3</th>
<th>An intact urease assembly pathway is required to compete with NikR for nickel ions in <em>Helicobacter pylori</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 3.1</td>
<td>Ni(^{2+}) and NikR-dependent transcriptional regulation in <em>H. pylori</em> strain 26695 measured by S1 nuclease protection</td>
</tr>
<tr>
<td>Figure 3.2</td>
<td>Deletion of <em>ureE</em>, <em>hypB</em>, <em>hypA-slyD</em> or <em>ureAB</em> results in NikR activation under Ni(^{2+})-limiting conditions</td>
</tr>
<tr>
<td>Figure 3.3</td>
<td>Inhibition of urease activity does not activate NikR</td>
</tr>
<tr>
<td>Figure 3.4</td>
<td>(^{63})Ni accumulation in mutant strains inversely correlates with NikR activity</td>
</tr>
<tr>
<td>Figure 3.5</td>
<td>Gene deletions in <em>H. pylori</em> strain G27 show similar effects on nickel physiology</td>
</tr>
</tbody>
</table>

| Chapter 4 | A role for the N-terminal arm of *Helicobacter pylori* NikR in DNA-binding |
Figure 4.1  \textit{H. pylori} NikR contains a unique N-terminal arm .......... 164
Figure 4.2 N-terminal arm mutants are unaffected in stability .......... 168
Figure 4.3 The N-terminal arm does not make specific DNA-contacts ............................................................. 169
Figure 4.4 1:1 Ni\textsuperscript{2+}:nt9-NikR has increased affinity for the \textit{nixA} and \textit{ureA} promoters ................................................................. 171
Figure 4.5 The N-terminal arm inhibits low-affinity and non-specific DNA binding ........................................................................................................ 172
Figure 4.6 nt9-NikR does not bind to the \textit{ureA}, \textit{fur} or \textit{nikR} promoters in the absence of added cations ....................................................... 175
Figure 4.7 The N-terminal arm imposes a \textit{nixA}-specific cation-requirement on \textit{H. pylori} NikR for DNA-binding ...................................................... 176
Figure 4.8 Asp7 and Asp8 are necessary and sufficient for the \textit{H. pylori} NikR-\textit{P}_{\textit{nixA}} cation requirement .............................................................. 179
Figure 4.9 Asp7Ala-Asp8Ala NikR requires added cations to bind \textit{P}_{\textit{ureA}} in the gel shift assay ........................................................................... 183
Figure 4.10 Model of \textit{H. pylori} NikR N-terminal arm-DNA interactions ................................................................................. 185
Figure 4.11 Alignment of NikR N-terminal arms ........................................ 190

Chapter 5 \textit{Helicobacter pylori} NikR adopts different conformations when bound to the \textit{nixA} and \textit{ureA} promoters

Figure 5.1 Mutagenesis of the N-terminal domain of \textit{H. pylori} NikR ..................................................................................................................................... 218
Figure 5.2 Electrophoretic mobility shift experiments for NikR Cys mutants .............................................................................................................................. 219
Figure 5.3 The RHH domain is in different conformations when NikR is bound to the \textit{nixA} and \textit{ureA} promoters ........................................................................ 222
Figure 5.4 The N-terminal arm is in different conformations when NikR is bound to the \textit{nixA} and \textit{ureA} promoters ......................................................... 225
Figure 5.5 Summary of NikR RHH domain and N-terminal arm DNA interactions as determined by Fe-BABE cleavage ......................... 226
Figure 5.6 A salt bridge between Glu47 and Lys48 of the NikR RHH domain is required for high-affinity binding to the \textit{ureA} promoter .......... 231

Chapter 6 Evolution of DNA-binding among NikR family members via the N-terminal arm

Figure 6.1 Summary of \textit{E. coli} NikR-DNA interactions ................................. 268
Figure 6.2 A Clustal alignment of non-redundant NikR family members based on N-terminal β-sheet sequences and any amino acids N-terminal to the β-sheet ................................................................. 271
| Figure 6.3 | Frequency of DNA-contacting β-sheet sequences and N-terminal arm lengths among non-identical NikR homologs .......... 274 |
| Figure 6.4 | ClustalW multiple sequence alignment of NikR proteins previously characterized and those analyzed in the current study .......... 278 |
| Figure 6.5 | DNase I footprinting of NikR orthologs binding to specific promoter fragments .................................................. 282 |
| Figure 6.6 | NikR homologs bind specifically to multiple promoter fragments ................................................................. 287 |
| Figure 6.7 | The N-terminal arms of GbNikR and GuNikR are required for DNA-binding .......................................................... 289 |
| Figure 6.8 | N-terminal arm truncation or mutation does not significantly affect the secondary structure of Geobacter spp. NikRs .......... 292 |
| Figure 6.9 | Residues of the GuNikR N-terminal arm are important for DNA-binding affinity .................................................. 293 |

**Chapter 7  Conclusions and future directions**

| Figure 7.1 | Transition metal homeostasis involves two related yet distinct mechanisms .......................................................... 321 |

**Appendix 1  In vivo analysis of *H. pylori* NikR gene regulation**

| Figure A1.1 | Mutant *H. pylori* NikR gene regulation as measured by S1 protection assays .......................................................... 353 |
| Figure A1.2 | Kinetics of nickel- and NikR-dependent gene regulation in *H. pylori* .................................................................. 356 |

**Appendix 2  Two separable pathways for nickel transport in *H. pylori***

| Figure A2.1 | NixA-dependent nickel transport occurs exclusively at pH 7.0 ........................................................................ 365 |
| Figure A2.2 | NixA-dependent uptake in a minimal uptake assay requires the proton motive force at pH 7 and urea and urease activity at pH 5.5 ......................................................................................... 368 |
List of Tables

Chapter 1 Introduction
Table 1.1 Commonly used transition metals in biology and their functions ................................................................. 4
Table 1.2 Mechanisms important for transition metal homeostasis .................................................................................. 11
Table 1.3 Well-characterized nickel-dependent enzymes and their activities ................................................................. 21

Chapter 2 Differential Ni$^{2+}$-dependent gene regulation and DNA-binding by Helicobacter pylori NikR
Table 2.1 Apparent binding affinities of NikR for different promoters ........................................................................... 78
Table 2.2 Primers used for H. pylori gene and promoter amplification and qRT-PCR ........................................................ 101

Chapter 3 An intact urease assembly pathway is required to compete with NikR for nickel ions in Helicobacter pylori
Table 3.1 Urease activity in different H. pylori strains ................................................................................................. 121
Table 3.2 Oligonucleotide primers ............................................................................................................................ 143
Table 3.3 Bacterial strains ............................................................................................................................................ 146

Chapter 4 A role for the N-terminal arm of Helicobacter pylori NikR in DNA-binding
Table 4.1 Apparent binding affinities of NikR and nt9-NikR for different promoters ......................................................... 173
Table 4.2 Apparent DNA-binding affinities of N-terminal arm mutants under different mobility shift conditions ............... 180
Table 4.3 Primers used for H. pylori gene and promoter amplification .......................................................................... 198

Chapter 5 Helicobacter pylori NikR adopts different conformations when bound to the nixA and ureA promoters
Table 5.1 Apparent DNA-binding affinities relative to wild-type NikR of Cys mutants ......................................................... 220
Table 5.2 Apparent DNA-binding affinities of NikR RHH domain charge mutants ........................................................ 232
Table 5.3  Apparent DNA-binding affinities of wild-type and Lys48Ala NikR for hybrid \textit{nixA-ureA} promoters .................................................. 235
Table 5.4  Primers used for \textit{H. pylori} NikR mutagenesis, hybrid promoter construction and promoter fragment labeling .................................. 251

Chapter 6  Evolution of DNA-binding among NikR family members via the N-terminal arm
Table 6.1  NikR homologs that were over-expressed in \textit{E. coli}, purified and tested for DNA-binding activity \textit{in vitro} ........................................... 279
Table 6.2  Apparent binding affinities of GuNikR N-terminal arm mutants .................................................................................................................. 294
Table 6.3  Primers used for \textit{nikR} amplification ................................................. 308
Table 6.4  Primers used for target promoter amplification .......................... 310
Chapter 1. 

Introduction
I. Transition metals in biology

Life predominantly consists of just eleven elements (40). Carbon, oxygen, hydrogen and nitrogen make up 99.0% of the human body, existing in large part as the major macromolecules in cells. The other seven - sodium, potassium, calcium, magnesium, phosphorus, sulfur and chlorine - make up 0.9% of the total atoms in humans. An additional 17 elements are also important in biology. Among these are the first-row transition metals manganese, iron, cobalt, nickel, copper and zinc. Proteins have evolved to take advantage of the unique properties of these metals, which include positive charge transfer, multiple stable oxidation states, easy ionic bond breaking, significant polarizing power, adjustable bond directions and lengths, and selective interactions with organic ligands. Whereas the major elements of life (carbon, oxygen, hydrogen, nitrogen, phosphate and sulfur) provide the building blocks of macromolecules and non-transition metals (e.g., magnesium and calcium) often act as signaling molecules or messengers, transition metals most distinctive role is to act in enzymes as acid-base and redox catalysts. Additionally, transition metals can play structural or regulatory roles in proteins.

Cells have been known to require transition metals for growth for more than 140 years, based on observations like those of Raulin in 1869 who noted that *Aspergillus niger* required zinc for growth (122). Why metals are required for growth was eventually attributed to their interactions with enzymes in cells, which was initially studied using proteins such as copper-dependent hemocyanin (53),
iron-dependent myoglobin (145), manganese-dependent xanthine oxidase (125) and zinc-dependent carbonic anhydrase (61). Scientists were initially skeptical as to the relevance of the transition metal dependence of these enzymes, considering that *in vitro* activation by a particular metal did not directly demonstrate the structural association of the enzyme with the metal, nor the physiological metal-dependence of the enzyme (149). However, the accumulation of increasing information on metal-protein complexes and their properties eventually led to general acceptance of the idea that cells require transition metals for use in metal-specific proteins.

The focus of this thesis is on the regulation of the transition metal nickel in cells, a metal which has only recently been appreciated for its unique roles in biology (105). However, as these studies are likely applicable to transition metal biology more generally, the following paragraphs describe some key differences in the chemical properties of metals, which are summarized in Table 1.1, and provide context for the broader applicability of this work. Some of these properties undoubtedly influence mechanisms of metal homeostasis.

The most common first row transition metals in biology are Fe, Zn, Cu, Mn, Ni and Co, with Fe, Zn and Cu being required by all organisms [see Table 1.1 for relative environmental abundance; (40)]. Iron is perhaps the most versatile, being capable of redox reactions, bioenergetics and acid-base catalysis [Table 1.1; (40, 103)]. Consistent with this versatility, cellular iron exists in a number of forms including free iron, iron-sulfur clusters and heme iron [iron held in the
Table 1.1. Commonly used transition metals in biology and their functions\textsuperscript{a}.

<table>
<thead>
<tr>
<th>Metal (Abundance\textsuperscript{b})</th>
<th>Porphyrin ring</th>
<th>General function(s)</th>
<th>Examples of specific functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron ((10^{-9};) hydroxy complex, oxycation or oxyanion)</td>
<td>Heme</td>
<td>Acid-base catalysis, redox catalysis</td>
<td>Transport of and reactions with (O_2), electron transfer, magnetic and gravitational sensors</td>
</tr>
<tr>
<td>Zinc ((10^{-5};) cation)</td>
<td>na\textsuperscript{c}</td>
<td>Structural functions, acid-base catalysis</td>
<td>Component of and synthesis of macromolecules, food digestion/extracellular proteolysis, regulation (e.g., zinc fingers)</td>
</tr>
<tr>
<td>Copper ((10^{-6};) carbonate complex)</td>
<td>na</td>
<td>Redox catalysis</td>
<td>Transport of and reactions with (O_2), electron transfer</td>
</tr>
<tr>
<td>Manganese ((10^{-8};) cation)</td>
<td>na</td>
<td>Redox catalysis</td>
<td>Oxygen evolution/photosynthesis</td>
</tr>
<tr>
<td>Nickel ((10^{-5};) cation)</td>
<td>F-430</td>
<td>Acid-base catalysis, redox catalysis</td>
<td>Methanogenesis, energy generation, urea hydrolysis</td>
</tr>
<tr>
<td>Cobalt ((10^{-6};) cation)</td>
<td>Cobalamin/vitamin B12</td>
<td>Redox catalysis</td>
<td>Reduction of nucleotides</td>
</tr>
</tbody>
</table>

\textsuperscript{a}, Compiled from (40).

\textsuperscript{b}, Abundance ratio of each metal in the ocean/earth’s crust (which is a general indication of metal accessibility), followed by the main species formed by that metal.

\textsuperscript{c}, Not applicable.
context of a porphyrin ring (40)]. An important consequence of iron utilization, however, is that Fe\textsuperscript{2+} carries out the Fenton reaction, which converts hydrogen to superoxide radicals that are dangerous to cells (148).

Zinc is also highly prevalent in biological organisms (40). However, the filled $d$ orbital of Zn\textsuperscript{2+} precludes this metal from performing redox functions (150), which also makes it unable to catalyze Fenton-like reactions. Instead, Zn\textsuperscript{2+} serves as an electron pair acceptor (i.e., Lewis acid) in enzymes and is extremely versatile due to a lack of preference for a specific ligand geometry. Notably, this property of Zn\textsuperscript{2+} enables faster exchange rates with different ligands relative to all other transition metals (150). The kinetics of Zn\textsuperscript{2+} binding to proteins, therefore, is important for cellular reactions that require fast metal hand-offs between molecules.

Last of the ubiquitous transition metals, copper is found in biological systems in both the Cu\textsuperscript{1+} and Cu\textsuperscript{2+} oxidation states (65). Cu\textsuperscript{2+} displays the highest electron affinity of all the transition metals, consistent with the Irving-Williams series of general protein metal-binding preferences [$\text{Mg}^{2+}$, $\text{Ca}^{2+}$ (weakest) $< \text{Mn}^{2+} < \text{Fe}^{2+} < \text{Co}^{2+} < \text{Ni}^{2+} < \text{Cu}^{2+} > \text{Zn}^{2+}$]. This makes it the most effective in binding to organic molecules (in both oxidation states) and the best attacking acid at neutral pH (40). In addition, while the appearance of oxygen in the atmosphere resulted in a severe decrease in bioavailable Fe\textsuperscript{2+}, it appears to have led to an increase in bioavailable copper, as a result of the better solubility of cupric salts (96).
In contrast to the three metals described above, manganese, cobalt and nickel are more selectively used throughout biology (40). Manganese is used as Mn\(^{2+}\), Mn\(^{3+}\) and Mn\(^{4+}\) depending on the enzyme, and the chemical properties of Mn\(^{2+}\) are intermediate to those of Mg\(^{2+}\), Ca\(^{2+}\) and Zn\(^{2+}\). The higher oxidation states, particularly Mn\(^{4+}\), provide powerful oxidative activities.

Cobalt is used in biological organisms mostly in the context of vitamin B\(_{12}\), or cobalamin, where the cobalt ion is ligated by a corrin ring. An important distinction of cobalt (and nickel) relative to iron is that these metals are electron rich. Due to the orbital preference of 3d electrons, cobalt and nickel are particularly good electron donors (40). These features likely at least partially account for the greater use of cobalt and nickel early in evolution (i.e., in many archeal and eubacterial species). Consistent with this, most examples of cobalt-dependent enzymes are from prokaryotes, many of which are also capable of vitamin B\(_{12}\) synthesis (164). In addition, a small number of proteins have been suggested to use cobalt in the absence of the corrin ring, however for most cases strict metal specificity has not been demonstrated (69).

Despite many similar properties, nickel is distinguishable from cobalt in that the majority of known nickel-dependent enzymes use the metal in the absence of a ring structure [the one exception being methyl-CoM reductase (40)]. In addition, the electron-rich character of nickel makes it particularly suited for reactions with H\(_2\) (such as that carried out by [Ni-Fe] hydrogenase). Also, nickel is significantly more available in the environment than cobalt. Fewer than ten
nickel-utilizing enzymes are known (see below), however these play prominent roles in microbial physiology. Only a single nickel-dependent enzyme, urease, is produced in eukaryotes, although it is widely expressed in plants and fungi (105). The limited, yet essential, role of nickel in biology makes this metal ideal for examining the details of transition metal homeostasis, and is one reason why this thesis has focused on the development of a tractable experimental system for the detailed investigation of nickel homeostasis.

II. Important aspects of metal utilization

The following sections briefly introduce some general features of cellular metal utilization which are important when considering metal homeostasis in cells and will be discussed as they specifically pertain to nickel later in this chapter.

*Kinetic and thermodynamic properties of metal-binding*

The kinetics, or rates of association and dissociation, and thermodynamics, or affinity, of metal-binding to proteins are properties central to metal utilization in cells. Every specific metal-protein interaction displays characteristic kinetics and thermodynamics which can be altered in response to changes in conditions, including metal, small molecule or protein concentration, or the presence or absence of additional metal-binding proteins. In particular, the rate of metal-association and metal-dissociation are key determinants of proteins that function to transport metals (e.g., transporters, metallochaperones; discussed below), and
the activity of metal sensor proteins strongly depends on the stability, or thermodynamics, of metal-binding. However, these properties of metal interactions are often either completely overlooked or rigidly extrapolated from *in vitro* studies to *in vivo* situations, which contributes to our poor understanding of intracellular metal behavior (discussed in more detail below).

**Metal transport**

A critical component of metal utilization in cells is the acquisition of sufficient metal ions from the environment. Various combinations of metal-specific, energy-dependent transport systems ensure that intracellular concentrations of metals are sufficient to satisfy each cell’s metal requirements (45, 50). Similar transporters function in the reverse direction to export excess metal out of the cytoplasm to maintain homeostasis as metal requirements change, or in the case of multicellular organisms, to facilitate the disbursal of metals throughout other tissues. To prevent unnecessary or deleterious metal acquisition and export both activities must be tightly regulated in the cell, which occurs via a number of distinct mechanisms in various combinations (see below).

**Metal chaperones**

Despite the ability of cells to acquire relatively high concentrations of transition metals compared to the environment, some metal-dependent enzymes require accessory factors termed ‘metallochaperones’ for the successful assembly of
their metal-containing active sites (107). Metallochaperones are typically soluble proteins that function to transport and facilitate the insertion of metals into apoenzymes. As the name implies, metallochaperones prevent the bound metal from making inappropriate interactions with other macromolecules inside the cell. Surprisingly, the discovery of metal chaperones is recent, and they are known only for iron-, copper- and nickel-dependent enzymes (6, 44, 63, 74, 78, 104, 106, 107, 112, 113, 119, 121, 142).

*The importance of metal regulation*

Consistent with the ubiquitous requirements for metals in biology, cells are capable of concentrating metals to high levels (~nM – mM, depending on the metal), making the commonly used term “trace” metals misleading (111, 149). However, high intracellular metal concentrations can be toxic due to non-specific binding to proteins or other molecules, or by catalysis of deleterious reactions, such as the generation of reactive oxygen species. Cells have evolved a number of mechanisms to balance intracellular metal levels to satisfy the cell’s requirements but prevent excess accumulation. Our current understanding of cellular metal homeostasis includes well-studied examples for many of the different transition metals from a variety of cells and organisms. Common themes have emerged from these studies, although differences do exist for distinct metals and cell types. These general mechanisms are discussed in more detail in the following section and summarized in Table 1.2 to put the results of
this thesis into a broader context that takes into consideration our current level of understanding of cellular metal regulation.

III. Transition metal homeostasis

Intracellular metal trafficking

The movement and localization of intracellular metal ions are poorly understood, however metal trafficking is no doubt a critical element of metal homeostasis. Following metal import into the cell, a combination of protein-protein interactions and kinetic and thermodynamic properties of metal-protein interactions dictate the behavior of each metal in the cytoplasm (154). The following sections describe these aspects of intracellular metal behavior in more detail.

Metal transfer between proteins

Obvious examples of direct metal transfer between proteins are those which occurs from metallochaperones to apo-enzymes (119, 142). A second example is metal transfer from a transporter to a chaperone or from a chaperone to a transporter [(51, 140); Table 1.2]. Each of these trafficking steps are critical for the proper targeting of sufficient metal for enzyme assembly, yet very few direct interactions have been demonstrated. One difficulty in detecting metal-transfer events is their inherent instability, and in the absence of more information this remains a poorly understood aspect of metal homeostasis.
Table 1.2. Mechanisms important for transition metal homeostasis*a

<table>
<thead>
<tr>
<th>General mechanism</th>
<th>Prokaryotic examples</th>
<th>Eukaryotic examples</th>
<th>Coupled mechanisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transcriptional regulation</td>
<td>Many (all biological metals)</td>
<td>Aft1,2, GATA-type TFs (Fe(^{2+}), fungi only); (Cu(^{2+}), fungi, <em>Chlamydomonas</em>)</td>
<td>Transporter re-localization</td>
</tr>
<tr>
<td>Post-transcriptional regulation</td>
<td>RyhB (Fe(^{2+}))</td>
<td>IRPs and IREs (Fe(^{2+}))</td>
<td>Transcriptional regulation</td>
</tr>
<tr>
<td>Transporter re-localization</td>
<td>na(^{b})</td>
<td>ATP7A,B, Ctr1 (Cu(^{2+}), humans, yeast, resp.); ZIP4 (Zn(^{2+}), mouse); IRT1 (Fe(^{2+}), <em>Arabidopsis</em>)</td>
<td>Transcriptional regulation (Cu(^{2+}))</td>
</tr>
<tr>
<td>Chaperone-enzyme metal transfer</td>
<td>UreDEFG-urease (*Ni(^{2+}), <em>Klebsiella</em>)</td>
<td>CCS-SOD1 (Cu(^{2+}), <em>Saccharomyces</em>)</td>
<td>Transcriptional regulation, Transporter re-localization (Cu(^{2+}))</td>
</tr>
<tr>
<td>Transporter-chaperone metal transfer</td>
<td>CopA-CopZ, CopZ-CopY (Cu(^{2+}), <em>Enterococcus</em>)</td>
<td>Atx1-Ccc2, Atox1-ATP7A,B (Cu(^{2+}), <em>Saccharomyces</em>, humans, resp.)</td>
<td>Transcriptional regulation, Transporter re-localization</td>
</tr>
</tbody>
</table>

* a, See text for specific references.

* b, Not applicable.
Thermodynamic competition for metals

The identification of chaperones that are required for the assembly of eukaryotic copper-dependent enzymes initially suggested that metal ions might be severely restricted in the intracellular milieu (28, 39). The measurement of extremely high affinities of some metal-dependent transcriptional regulators for their cognate metals further supported the concept that, at least for some transition metals, no “free” metal ions exist in the cell cytoplasm (19, 22, 39, 58, 111, 154). Further, differences in the in vitro measured affinities of various metal-binding proteins suggests a specific order or hierarchy of metal-binding to proteins in vivo (19, 22, 39, 58, 111, 154). However, this hypothesis remains untested due to the technical difficulty of directly measuring free, or labile, metal ions in the cell, as well as distinguishing between free and weakly- or stably-bound intracellular metals. At least for some cellular compartments, indirect experimental evidence suggests that “free” metal ions may in fact refer to metals weakly-complexed to various macromolecules or other abundant ligands (118). Metal-binding affinities of different proteins involved in all aspects of metal homeostasis continue to be determined, however it remains to be seen if affinities determined for multiple proteins from a single system (i.e., a specific metal and cell type) accurately predict the metal trafficking behavior inside the cell. It is clear that, similar to metal-transfer reactions, novel experimental
approaches are required to address how *in vitro* determined affinities relate to the intracellular milieu.

**Kinetic competition for metals**

The kinetics of metal association and dissociation from proteins and other ligands inside the cell is an important, yet commonly overlooked, determinant of intracellular metal trafficking. Fewer studies have examined the metal-association and dissociation rates of isolated metal-binding proteins compared to metal affinity measurements, and are limited to reports describing the metal-binding kinetics of a heavy metal-dependent transcriptional regulator (17), a copper-dependent enzyme (11) and a few nickel chaperones (74). Similar to *in vitro* measured metal affinities, it is not clear how the kinetics of metal-binding to purified proteins will relate to studies examining metal-binding *in vivo*.

Nonetheless, it is apparent that combinations of approaches are required for a complete understanding of intracellular metal dynamics, as well as the changes in metal trafficking in response to variations in extracellular metal concentrations.

A well-characterized system is necessary to begin to understand the various components of intracellular metal trafficking that are described above. This includes access to a variety of experimental readouts assessing parameters such as metal content and specific metal-dependent activities. The development of such a system was one focus of this thesis.
Transcriptional regulation

The most prevalent, or certainly the best studied, mechanism of controlling metal-specific transport and metal homeostasis activities occurs via transcriptional regulation of metal-related genes (Table 1.2). This regulation requires metal-dependent transcription factors, or metalloregulators, that mediate at least two distinct activities: metal- and DNA-binding. Almost all known prokaryotic metalloregulators contain both of these activities within a single protein, and regulators from prokaryotes have been identified that are specific for each of the commonly used transition metals [i.e., iron, zinc, copper, manganese, cobalt and nickel (45)]. Only a small number of eukaryotic metalloregulators have been demonstrated to directly bind metal, recognizing iron, copper or zinc (128), although this observation may reflect the small number of biochemically characterized eukaryotic regulators.

Prokaryotic metalloregulators are grouped into seven classes based on protein structure and are the ArsR, MerR, RcnR/CsoR, CopY, Fur, DtxR and NikR families (45). Of these seven classes, five use a helix-turn-helix motif in the context of a winged helix domain to specifically bind DNA, whereas RcnR/CsoR contain a novel DNA-binding domain and NikR contains a ribbon-helix-helix (RHH) domain (19, 20, 45, 67, 79, 130, 136, 151). Metal binding occurs via distinct domains or at the interface of two domains in all classes (19, 20, 45, 67,
79, 130, 136, 151) with the exception of RcnR/CsoR, which are single-domain proteins (79). The first four regulators repress transcription in their apo, or metal free, forms with metal binding to the protein inducing transcription by either inhibiting DNA-binding (ArsR, MerR and RcnR) or leading to promoter remodeling (MerR). The latter three classes of regulators are activated to bind DNA and repress transcription in response to metal binding. In general, regulators belonging to the first four classes described above induce the expression of genes involved in metal detoxification, storage and/or export in response to increasing metal concentrations, while the latter three classes of regulators repress genes encoding metal uptake systems at higher metal concentrations (45).

Additional detailed studies of prokaryotic metalloregulators have begun to reveal the mechanistic bases for the allosteric regulation of DNA-binding by metal-binding, with metal coordination geometry playing a major role in changes in the oligomeric state and/or dynamics of the protein (13, 16, 22, 24, 33, 38, 76, 137, 151, 158). Additionally, one interesting area of current investigation has followed from the observation that a number of metalloregulators respond specifically to a given metal in vivo, however multiple metals similarly affect the regulator in vitro (45, 147, 154). These studies suggest that a combination of metal-binding features of the protein and the metal physiology of the cell dictate the specificity of metalloregulation.
Much less is known about eukaryotic metalloregulation, and of the
metalloregulators that have been identified none appear to contain domains that
occur in prokaryotic regulators (45, 128). Multiple iron-dependent transcriptional
regulators have been identified exclusively in fungi, where, similar to prokaryotic
gene regulation, decreasing concentrations of iron result in the up-regulation of
iron transporter and siderophore production and transport genes (128). In
addition, copper-dependent metalloregulators have been identified in fungi,
plants, insects (128) and the photosynthetic algae *Chlamydomonas reinhardtii*,
where they up-regulate genes encoding high-affinity copper transporters or
alternative iron-utilization pathways in response to copper limitation or genes
important for copper sequestration in response to excess copper levels (96, 128).
In contrast to most prokaryotic regulators, however, there are multiple
mechanisms for eukaryotic metal-dependent regulator activation, including the
differential localization of regulators in response to variations in metal
concentrations, as well as direct metal-binding by the regulator (128).

*Post-transcriptional regulation*

Control of metal-specific transport can also be mediated via post-
transcriptional mechanisms (Table 1.2). One well-understood example involves
the eukaryotic regulation of iron-dependent enzymes, transporters, storage and
export proteins by the iron-responsive proteins (IRP1 and IRP2). Both proteins
Regulate mRNA levels via iron-responsive elements (IREs) present in untranslated regions (UTRs) of their mRNA targets (103). IRP binding to 5’ UTRs inhibits the translation of iron storage proteins, Fe$^{2+}$-dependent enzymes and an iron exporter, and IRP binding to 3’ IREs stabilizes iron importer transcripts. As a result, iron-deficiency leads to an upregulation of iron-specific transporter genes and repression of iron storage, utilization and export genes.

An example of prokaryotic post-transcriptional regulation occurs in *Escherichia coli* (and a number of additional bacterial species), where a small RNA (RyhB) functions to base-pair with mRNAs encoding iron-dependent enzymes and target them for degradation, preventing their expression (89, 90). *ryhB* is negatively regulated by the iron-dependent transcription factor Fur, which links the repression of iron-specific transporter genes by Fur to the post-transcriptional repression of iron-utilization genes by RyhB under iron-deficient conditions (89, 90).

*Regulation of transporter localization*

The activity of metal-specific transporters can also be controlled by differential transporter localization in response to changes in extracellular metal levels (Table 1.2). The best studied example involves the two human copper-transporters, the Menkes (ATP7A) and Wilson (ATP7B) disease proteins, that are normally localized in the trans-Golgi network (TGN) where they transport copper into the secretory pathway for the assembly of copper-dependent
enzymes (1, 35, 57, 80, 81, 114, 126, 133, 160). As extracellular copper increases, ATP7A relocates from the TGN to the cytoplasmic membrane, presumably to aid in the export of excess intracellular copper (114). In contrast, ATP7B relocates to cytoplasmic vesicles thought to be involved in excess copper sequestration or storage (57). The copper-dependent relocation is reversible, indicating that re-localization of ATP7A and ATP7B is a dynamic mechanism coordinating copper-specific transport activity with extracellular copper levels (57, 114).

Metal transporters specific for copper, zinc and iron have also been shown to be endocytosed from the plasma membrane and subsequently degraded in eukaryotic cells to decrease their activity (2, 64, 66, 110, 115). In each case, evidence suggests that direct metal-binding by each transporter is required to trigger endocytosis and degradation, although the mechanistic details governing relocation are not known. Homologs of some of these metal transporters, as well as many additional members of the larger transporter families represented by the examples described above, exist in eubacteria and archea, although it is not known if transporter re-localization occurs in these organisms.

IV. The importance of nickel in biology

This thesis focuses on mechanisms of microbial nickel regulation to better understand metal homeostasis in general. Nickel is essential for many biological
processes important throughout the domains of life. To provide background on
the importance of this metal in addition to the properties that make it an ideal
model metal, the various functions of nickel are discussed in more detail below.

Nickel is often described as an “early life” catalyst, given its prominent role in
enzymes expressed by archeal and bacterial species that reside in anaerobic
niches (71). The electron-rich environment that gave rise to early life had
significant levels of dihydrogen, carbon dioxide and metal sulfides, all of which
are substrates of nickel-dependent enzymes. The limited distribution of nickel in
higher organisms suggests that the introduction of dioxygen into the atmosphere
limited the evolution of nickel utilization in biological processes, most likely due to
the significant decrease in availability under aerobic conditions. However, nickel
remains an important element critical for life, often specifically in anaerobic
environments, but also directly linked to human health given the anaerobic or
facultatively anaerobic bacterial and archeal species present in the intestinal
‘microbiome’ (29, 30, 48, 93, 131). Additionally, several pathogenic bacteria
require nickel-dependent enzymes for virulence (36, 37, 60, 92, 100, 105, 108),
and these organisms adversely affect human health.

Nine nickel-dependent enzymes have been identified, seven of which are
relatively well-characterized and listed in Table 1.3 (105). These enzymes play
integral roles in microbial metabolism. \( \text{H}_2 \text{ase, CODH, ACDS and MCR} \) (see
Table 1.3 for abbreviations) are central to energy generation in cells. Urease is
important for cell growth by allowing for nitrogen assimilation (105). In at least two cases - *Helicobacter pylori* and *Proteus mirabilis* - the ammonia generated by urease also serves to protect cells from acidic growth conditions (36, 37, 100). NiSOD and Glx I are important in detoxification processes inside cells (25, 161). Interestingly, examples exist of SOD and glyoxylase that use different transition metals in their active sites, such as Cu and Zn, Fe or Mn for SOD (162) and Zn for glyoxylase (52). This contrasts with the first six enzymes discussed above, which are only active with Ni\(^{2+}\) in their active sites.

Much less is known about the last two enzymes recently identified as Ni\(^{2+}\)-dependent. Methylenediurease was isolated from a *Burkholderia* species and determined to be a Ni\(^{2+}\)-dependent enzyme distinct from urease, using methyleneureases as substrates instead of urea (105). The aci-reductone dioxygenase enzyme from *Klebsiella pneumoniae* has been purified in a Ni\(^{2+}\)-bound form that catalyzed a reaction distinct from the normal recycling of methylthioadenosine to methionine, which is catalyzed by a Fe-containing enzyme, so the physiological importance of the nickel-bound enzyme remains unknown.
Table 1.3. Well-characterized nickel-dependent enzymes and their activities\(^a\).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Reaction(s) catalyzed</th>
<th>Function in the cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrogenase (H(_2)ase)</td>
<td>(H_2 \leftrightarrow 2H^+ + 2e^-)</td>
<td>Energy generation</td>
</tr>
<tr>
<td>Carbon monoxide dehydrogenase (CODH)</td>
<td>(CO + H_2O \leftrightarrow CO_2 + 2e^- + 2H^+)</td>
<td>Energy generation</td>
</tr>
</tbody>
</table>
| Acetyl-coenzyme A synthetase (ACDS) | I. \(CO + H_2O \leftrightarrow CO_2 + 2e^- + 2H^+\)  
                          II. \(CH_3C(O)-S-CoA + Co(I)-FeSP^{b} \leftrightarrow CH_3-Co(III)-FeSP + CO + CoA\) | Energy generation or acetate synthesis |
| Methyl-coenzyme M reductase (MCR) | \(CH_3-S-CoM^{c} + CoB-SH^{d} \leftrightarrow CH_4 + CoB-S-S-CoM\) | Energy generation                     |
| Urease                        | \((H_2N)_2CO + H_2O \leftrightarrow NH_3 + CO_2\) | Nitrogen assimilation and/or acid stress response |
| Superoxide dismutase (NiSOD)  | \(2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2\)     | Superoxide detoxification             |
| Glyoxylase I (Gix I)          | \(G-S^{d}-CH_2O-CO-CH_3 \rightarrow G-S-CO-(CH_3)(OH)CH\) | Methylglyoxal detoxification          |

\( ^a\), Compiled from (105) and references therein.

\( ^b\), FeSP, corrinoid-iron-sulfur protein.

\( ^c\), methyl-S-coenzyme M.

\( ^d\), CoB, \(N\)-7-mercaptoheptanoylthreonine phosphate or coenzyme B.
Importantly, each of the nickel-dependent enzymes discussed above play distinct roles in microbial physiology, so the nickel requirements of different organisms vary considerably depending on which enzymes they produce and when they are required. For example, *Escherichia coli* use [Ni-Fe] H$_2$ases only under specific anaerobic conditions (5, 132, 166), however methanogenic archea have a constitutive requirement for MCR (152). Differences in nickel requirements directly impact how nickel is regulated in each organism.

V. *Helicobacter pylori*, a model system to study nickel homeostasis

*Nickel utilization by Helicobacter pylori*

The Gram-negative bacteria *Helicobacter pylori* colonize the acidic environment of the stomach of all primates, forming a stable infection that persists for the lifetime of the host in the absence of treatment (4). The two nickel-dependent enzymes encoded in the *H. pylori* genome, [Ni-Fe] H$_2$ase and urease, are required for gastric colonization as demonstrated using mouse models of infection (36, 37, 108), with urease constituting 6% of the total soluble protein of the cell (55). This enzyme production results in a substantial nickel requirement. Extensive studies have shown that urease is critical for the ability of *H. pylori* to survive under acidic conditions, and the current hypothesis is that the generation of ammonia and bicarbonate from urea by urease buffers acid
surrounding the bacteria (101, 129, 144). *H. pylori* also assimilate ammonia nitrogen via urease activity for the synthesis of macromolecules (32).

The urease active site is well-buried within each UreAB heterodimer and consists of two nickel ions bridged by a carbamylated lysine residue (49). Assembly of the active site requires the chaperones UreH (UreD in other bacteria), UreE, UreF and the GTPase UreG (109, 153), as well as carbon dioxide (for lysine carbamylation), GTP and nickel. Extensive biochemical analyses by Hausinger and co-workers using proteins from *K. aerogenes* suggest an ordered association of chaperones with apo-urease (70, 75, 102, 105, 142, 143), with the initial formation of a UreD-apo-urease complex (75). In subsequent steps UreF and UreG bind sequentially to UreD-apo-urease, forming a quarternary UreDFG-apo-urease complex that is fully competent for assembly *in vitro* in the presence of the three required cofactors (102). Nickel-bound UreE enhances the *in vitro* assembly of urease, and is thought to chaperone and insert nickel into the active site as the final step of assembly *in vivo* (142).

The H$_2$ase active site consists of a dinuclear Ni-Fe center present in the Hyd structural subunits (14, 73, 105). Ni-Fe center assembly has been best studied in *Escherichia coli*, although the high level of conservation of hydrogenase enzyme and chaperone proteins among different bacterial species suggests assembly occurs similarly in organisms such as *H. pylori*. H$_2$ase assembly is a complex process that requires many chaperones (12, 14, 73). Nickel is inserted in the last step of assembly, in a process requiring the chaperones HypA, HypB
and SlyD (56, 59, 74, 82, 86, 163), which are thought to insert the nickel ion together in a GTP-requiring process. Further mechanistic details, including which Ni\(^{2+}\)-binding sites on each chaperone are occupied in each step of the assembly process, as well as which protein inserts Ni\(^{2+}\) into Hyd, remain to be determined.

In *H. pylori*, genetic analyses have demonstrated similar gene requirements for urease and H\(_2\)ase assembly as those described above, with the exception that a role for *slyD* in assembly has not been examined [see Figure 1.1 for a schematic of *H. pylori* nickel utilization; (8, 109, 153)]. A notable distinction in *H. pylori* is that the nickel insertion chaperones HypA and HypB are required for urease and H\(_2\)ase assembly, as demonstrated by a number of approaches (9, 109), although the specific functions they perform in each pathway are not known. Studies suggest that HypA may function to deliver Ni\(^{2+}\) to UreE in *H. pylori*, although experiments attempting to detect metal-transfer between the two proteins have been unsuccessful (9). A key difference between *E. coli* and *H. pylori* HypB is the absence of a high-affinity N-terminal Ni\(^{2+}\)-binding motif in *H. pylori* HypB (72), although both HypB proteins display detectable GTPase activity (87, 94). The HypA proteins bind stoichiometric zinc and nickel with comparable affinities (3, 56, 62, 95). Ample evidence indicates that SlyD from *E. coli* is a significant Ni\(^{2+}\)-binding protein in the cell (15, 54, 97), raising the question of how *H. pylori* SlyD functions in nickel enzyme assembly in this organism. Given the absence of one nickel binding site on *H. pylori* HypB, it is possible SlyD performs
Figure 1.1. Summary of known and predicted nickel-binding proteins and nickel pathways of *Helicobacter pylori* (7, 84, 95, 139).
some functions attributable to HypB in *E. coli*. The differences in HypB proteins from *H. pylori* and *E. coli*, together with the unique combinations of nickel pathways in the two organisms, suggests that distinct nickel-trafficking occurs within the two cell types, however the mechanisms of nickel movement and localization have not been explored in any organism.

**Nickel storage**

In addition to the nickel-dependent enzymes and their cognate chaperones, *H. pylori* also express two small, histidine-rich proteins, Hpn (for *H. pylori* nickel protein) and Hpn-like, that are hypothesized to be nickel storage proteins [Figure 1.1; (43, 46, 99)]. Recent data suggest that these proteins may compete with urease assembly when nickel is limiting (139), although this result is not consistent with previous reports (43, 46). Integration of nickel storage by the Hpn proteins with other nickel-utilization pathways in the cell, as well as Hpn regulation, have not been examined.

**Nickel uptake**

*H. pylori* express a nickel-specific permease, NixA, that is a member of the HoxN family of nickel and cobalt transporters [Figure 1.1; (98)]. NixA is an eight transmembrane-spanning protein located in the inner membrane of *H. pylori*, and residues important for Ni^{2+} transport have been identified through scanning and targeted mutagenesis (41, 42, 159). Recently, the outer membrane protein
FrpB4, a homolog of the ExbBD-TonB-dependent iron-siderophore and cobalamin transporters, was demonstrated to be critical for nickel transport under acidic pH conditions in a manner that required TonB (134). Earlier macroarray studies had predicted a role for the product of the frpB4 gene, as well as the fecA3 gene, in nickel uptake given that each gene was repressed by high concentrations of nickel (26), however a role for FecA3 in nickel uptake has not been examined. The homology of FrpB4 and FecA3 to iron-siderophore and cobalamin transporters strongly suggests that nickel is initially transported into the periplasm of H. pylori as a complex with an unidentified metallophore (134), however there are currently no known biologically used nickel-complexes.

Nickel-dependent gene regulation

H. pylori encode a homolog of the Ni^{2+}-dependent RHH transcriptional regulator NikR [Figure 1.1; (26)], first identified in E. coli (21, 31). E. coli NikR represses transcription of a single operon, nikABCDE, that encodes an ABC-type nickel transporter (21, 23, 31). Interestingly, macroarray studies comparing the gene expression of wild-type and nikR mutant H. pylori strains grown with high concentrations of NiCl₂ suggested that NikR regulates 39 genes in 31 operons in response to increased intracellular nickel (26). The predicted NikR-dependent regulation included the activation of ureAB, nixA, hpn and hpn-like, and the repression of frpB4, fecA3, exbB-exbD-tonB and fur, encoding an iron-dependent transcription factor. These results suggest a much larger repertoire of NikR-
dependent gene regulation in *H. pylori* as compared to *E. coli*, consistent with the fewer number of identifiable transcriptional regulators encoded in the *H. pylori* genome (146), which implies that the regulators that are present more broadly control gene expression in this organism. A major focus of this thesis is the molecular basis for the apparent expansion of nickel-dependent gene regulation in *H. pylori*, as compared to *E. coli*.

VI. The Ni$^{2+}$-dependent transcription factor NikR

*Basic properties of NikR*

NikR consists of an N-terminal RHH domain that mediates DNA-binding (21) and a C-terminal ACT domain that contains a high-affinity Ni$^{2+}$-binding site and is responsible for tetramerization [Figure 1.2; (22)]. Ni$^{2+}$-binding to the C-domain increases the affinity of NikR for specific DNA (21, 23, 137), and the DNA sequence to which NikR binds is a perfect six bp inverted repeat that is separated by a 16 bp spacer [GTATGA -16- TCATAC; (23)]. This DNA recognition sequence occurs only once in the *E. coli* genome (in the promoter region of *nikABCDE*) so NikR is believed to regulate only a single operon in response to increased extracellular nickel.

*The Ni$^{2+}$-binding domain*

X-ray absorption spectroscopy (XAS) first determined that the C-domain Ni$^{2+}$-binding site is square planar (18), and subsequent crystal structures confirmed
Figure 1.2. NikR undergoes dramatic conformational changes to bind DNA. The x-ray crystal structures of *E. coli* NikR (a) in the absence of DNA [PDB ID 2HZA; (137)] and (b) with stoichiometric Ni\(^{2+}\) bound to specific DNA [PDB ID 2HZV; (137)]. The Ni\(^{2+}\) ions are shown as black spheres. Shown below the co-crystal structure is the sequence of the *E. coli* NikR operator present in the *nikABCDE* promoter. The molecular graphics images were produced using the UCSF Chimera package from the Resource of Biocomputing, Visualization, and Informatics at the University of California, San Francisco [supported by NIH P41 RR-01081; (116)].
this result (136). The structures identified the residues His87, His89, Cys95 (all from the same NikR monomer) and His76 (from a second monomer) as Ni\textsuperscript{2+}-binding ligands [Figure 1.2a; (136)]. Several studies using different approaches have also shown that nickel binds in a square planar geometry, which is most preferred by nickel and likely accounts for the nickel specificity of NikR (34, 76, 155). More recently, XAS (76) and crystallography (117) determined that Cu\textsuperscript{2+} also binds to NikR with a square planar geometry, but only Ni\textsuperscript{2+} activates NikR for DNA-binding in vivo (76).

Despite the C-domain being responsible for the tetramerization of NikR, the oligomeric state of the protein is unaffected by Ni\textsuperscript{2+}-binding to this domain (22). Instead, crystal structures of NikR alone [Figure 1.2a; (137)] and bound to its operator site [Figure 1.2b; (137)], as well as a number of other experimental results (16, 22, 34, 76), indicate that Ni\textsuperscript{2+}-binding to the C-domain allosterically controls DNA-binding by allowing NikR to adopt a conformation in which the N- and C-domains are oriented differently relative to one another. The DNA-bound conformation of NikR indicates a dramatic re-organization where the antiparallel β-sheet motifs from the two NikR N-domain dimers face the same direction, allowing for binding of the protein to one face of the DNA helix [Figure 1.2; (137)].
The DNA-binding domain

DNase I footprinting with stoichiometric Ni\textsuperscript{2+} demonstrated that the affinity of NikR for specific DNA increased from undetectable [\(> 1 \ \mu\text{M}\)], the minimum concentration tested; (22)] to 30 nM (13, 22), consistent with NikR directly sensing Ni\textsuperscript{2+} by binding metal to the C-domain, which then activates the protein to bind to the \textit{nikABCDE} promoter and repress transcription (22, 23). Interestingly, the affinity of NikR for specific DNA measured by electrophoretic mobility shift assays in the presence of 50 \(\mu\text{M}\) NiCl\textsubscript{2} (in the gel and running buffer) is 20 pM (23). The two distinct DNA affinities combined with the significantly higher NiCl\textsubscript{2} requirement in the mobility shift assays suggested that NikR contains two types of Ni\textsuperscript{2+}-binding sites, which were named the high-affinity and low-affinity sites (13, 22, 23). However, the importance of each of these in the biological function of NikR remains to be determined (127).

The crystal structure of full-length \textit{E. coli} NikR bound to its operator revealed details of the DNA contacts made by this protein [Figure 1.2b; (137)]. Specific DNA interactions are made by side chains of the \(\beta\)-sheet residues Arg3 and Thr5, with five of the six bases that constitute an operator half-site being contacted by the \(\beta\)-sheet. Roughly 14 nonspecific polar interactions occur between residues of the RHH motif and the phosphate backbone of the DNA, mostly spanning regions of the DNA corresponding to half-sites but also including backbone interactions midway in-between the two half-sites (137). Hydrogen
bonds formed by the N-terminus of helix $\alpha_2$ and the phosphate backbone are included among these interactions, and similar to other RHH protein-DNA complexes (47, 77, 83, 91, 124, 137, 138, 141, 156, 165), function to anchor and orient the domain relative to the DNA. Non-specific interactions between the side chains of residues located throughout the RHH domain and the backbone were also observed, in addition to a small number contributed by side chains and backbones of C-domain residues.

*The RHH family of DNA-binding proteins*

The prototypical RHH protein is the 53 residue bacteriophage P22-encoded transcriptional repressor Arc (123, 135). Through detailed studies of Arc (124), as well as other RHH proteins such as the phage-encoded Mnt repressor (68, 88), the S-adenosylmethionine (AdoMet)-responsive MetJ repressor (141) and the plasmid copy number repressor CopG (47), it is well-understood how these proteins interact with specific DNA sequences. In almost all cases, the RHH motif is located at the N-terminus of the primary amino acid sequence, and the N-terminal $\beta$ ribbon forms an antiparallel $\beta$-sheet with the $\beta$ ribbon of a second polypeptide that inserts into the major groove of DNA, making specific and non-specific contacts with the bases and phosphate backbone (47, 77, 83, 91, 124, 137, 138, 141, 156, 165). A consequence of the RHH fold is that family
members are obligate dimers to allow for antiparallel β-sheet formation, although some family members form higher oligomers (e.g., MetJ, NikR).

Experimentally characterized RHH family members are activated to bind to DNA by a diverse array of signals (135). Examples of control of RHH activity include the oligomerization of the regulator [e.g., monomer-dimer equilibrium and cooperativity between DNA-bound dimers for Arc (10) and CopG (27)], binding of a cofactor [e.g., MetJ activation by AdoMet (120), NikR activation by Ni^{2+} (22, 23)], and hetero-oligomerization with a second protein [e.g., FitA activation via FitB-binding (157)]. Consistent with these varied roles in gene regulation, the RHH fold can exist in isolation, such as is the case for Arc, as well as in the context of additional protein domains, including the examples of NikR, Mnt and MetJ. Differences in the presence or absence of additional domains determines the spacing of regulator DNA binding sites, which always consist of at least two half-sites due to the occurrence of multiple RHH dimers binding to the DNA. For proteins similar to Arc, DNA half-sites are located very close to one another, however for tetrameric proteins like NikR the DNA half-sites are separated by many base-pairs. One consequence of such flexibility of the RHH motif is that even with extensive biochemical, biophysical and structural data for many RHH proteins, a detailed understanding of how these regulators are activated to bind DNA, as well as how they interact with DNA (and therefore the DNA sequences they recognize), are not easily predicted from primary sequence alone.
As mentioned earlier, RHH-containing transcription factors make specific contacts with DNA via the antiparallel β-sheet motif sitting in the major groove of the DNA (123). Base specific contacts are made by the side chains of three of the six residues of one β ribbon, with the first base-contacting position most often being an arginine or a lysine residue, and making the most DNA contacts. Intriguingly, the two RHH proteins *E. coli* NikR and CopG contain identical DNA-contacting β-sheet residues (Arg-Thr-Thr) but recognize different DNA half-sites (GTATGA for NikR and YRACGT for CopG, where Y and R indicate pyrimidines and purines, respectively), indicating that the specificity of DNA-binding is determined both by the β-sheet residues and additional, unknown properties of the proteins (23, 135). This observation further implies that specific DNA binding sites for RHH family members for which little is known are not easily predicted, and by extension, predicting the genes regulated by each RHH protein is not a trivial task.

*The evolution of DNA-binding in the NikR family*

NikR homologs are present in over 100 different microorganisms, including bacterial and archeal species, that encode varying numbers and combinations of nickel-dependent enzymes and nickel-specific transporters in their genomes (164). Clearly, the regulatory capability of different NikR family members must adapt to the distinct physiologies and gene content of these organisms, raising
the question of how NikR activity varies between family members, and if activity does vary, are there amino acid sequence changes that are responsible? The only information available for NikR homologs outside of that described above for *E. coli* and *H. pylori* NikR consists of a few crystal structures of an archeal NikR homolog from *Pyrococcus horikoshii* (24). While this structural characterization provided important details regarding Ni\(^{2+}\)-binding and activation of NikR for DNA-binding, the gene(s) regulated by this protein, as well as its DNA binding site, are not known. Only studies examining the metal- and DNA-binding activities of additional NikR family members will begin to reveal how this family of transcriptional regulators has evolved in response to the rich physiological diversity of microbes.

**VII. Summary**

As described above, much is known about the interactions of RHH family members with DNA, as well as the structural basis for DNA recognition by *E. coli* NikR (137). Macroarray predictions for gene regulation by *H. pylori* NikR suggest that it displays significantly broader regulatory capabilities (26), however comparison of promoter DNA sequences from genes predicted to be regulated by this NikR ortholog do not indicate a conserved binding site. In addition, closer inspection of the target genes potentially controlled by *H. pylori* NikR suggests that many aspects of nickel physiology, including metal uptake, storage and
utilization, are coordinated at least in part by transcriptional regulation. The nickel physiology of *H. pylori* has been well-defined due to its critical role in colonization and infection of the stomach by this notable pathogen (36, 37, 85, 108). Combined with the ability to genetically and environmentally manipulate this organism *in vitro*, this biological system represents a strong candidate with which to examine mechanistic details important for transition metal homeostasis.

This thesis examines several aspects of nickel-dependent gene regulation in *H. pylori* that impact the understanding of mechanisms of nickel homeostasis. Individual components of transition metal homeostasis have been identified for many systems but how the different activities are integrated is unknown. Chapter 2 identifies differential regulation by *H. pylori* NikR of multiple genes which encode proteins with various roles in nickel physiology, demonstrating that NikR coordinates nickel homeostasis in response to variations in extracellular nickel. Chapter 3 demonstrates that nickel is preferentially trafficked to the urease assembly pathway in *H. pylori* cells, which serves to prevent NikR activation under nickel-limiting conditions. The molecular basis for differential gene regulation and coordination of nickel homeostasis by *H. pylori* NikR is further explored in Chapters 4 and 5, where protein structure and DNA sequence are both shown to influence DNA-binding affinity and specificity. Finally, Chapter 6 demonstrates the generality of these observations with additional NikR homologs.
References


2001. Supramolecular assembly and acid resistance of Helicobacter pylori 


Intracellular copper routing: the role of copper chaperones. Trends 

Determination of the structure of Escherichia coli glyoxalase I suggests a 

verschiedener Hamocyanine. Hoppe-Seyler's Z Physiol Chem 216:110- 
119.

Rahfeld. 1997. The Escherichia coli SlyD is a metal ion-regulated 


maturation in Escherichia coli: role of accessory proteins HypA and HybF. 


166. **Zinoni, F., A. Beier, A. Pecher, R. Wirth, and A. Bock.** 1984. Regulation of the synthesis of hydrogenase (formate hydrogen-lyase linked) of E. coli. Arch Microbiol **139:**299-304.
Chapter 2

Differential Ni\textsuperscript{2+}-dependent gene regulation and DNA-binding by *Helicobacter pylori* NikR

Parts of this chapter were published previously and are reprinted here with the permission of the American Society of Biochemistry and Molecular Biology.

Overview

To develop *H. pylori* as a model system for studying metal homeostasis, my initial studies focused on determining the gene target(s) of the Ni\(^{2+}\)-dependent transcription factor NikR in the cell, as well as biochemically characterizing the DNA-binding activity of this protein. Interestingly, *H. pylori* NikR differentially regulates multiple facets of Ni\(^{2+}\) physiology (i.e., transport, utilization and storage) in response to variations in extracellular Ni\(^{2+}\) concentrations. This regulation can at least in part be explained by differences in the DNA-binding affinity of NikR for different gene promoters, likely resulting from changes in promoter recognition sequences. Given that transcriptional regulation is a common mechanism contributing to transition metal homeostasis for a variety of metals and cell types, these results strengthen the utility of *H. pylori* nickel physiology as a model system for investigating the molecular details of cellular metal responses. Accordingly, results from this chapter have prompted studies focusing on the regulation of NikR activity in *H. pylori* cells as another level of metal-dependent regulation, as well as the ability of NikR to discriminate between promoters containing distinct recognition sequences.
Abstract

*H. pylori* expresses the Ni\(^{2+}\)-dependent transcription factor NikR, which is a likely candidate for controlling gene expression critical for the prominent Ni\(^{2+}\) physiology of this organism. A previous macroarray study predicted that high concentrations of extracellular NiCl\(_2\) and NikR regulate multiple genes encoding proteins with different nickel-related activities, such as nickel transport, storage and utilization. The predicted regulation was not validated in that study, so it is not known whether NikR regulates these genes directly. Additional studies have indicated that NikR may function as an indirect acid sensor, possibly due to the increase in Ni\(^{2+}\) solubility at acidic pH and data suggesting that regulation of some NikR-controlled genes occurs at pH 5.5.

To determine if NikR regulates nickel-related target genes I have used quantitative RT-PCR to measure transcript levels in wild-type and nikR mutant cells over a range of NiCl\(_2\) concentrations at pH 7. To compare NikR-dependent regulation at neutral and acidic pH I also measured transcripts in cells exposed to a range of NiCl\(_2\) concentrations at pH 5.5. Consistent with the array study, NikR up-regulated ureA transcription in response to increased NiCl\(_2\) at pH 7. In contrast to array predictions, NikR repressed nixA levels in response to increasing NiCl\(_2\), repressed nikR only under low NiCl\(_2\) conditions, and repressed fur only at high NiCl\(_2\) concentrations. Although NikR-dependent regulation was observed for some genes in response to individual NiCl\(_2\) concentrations at pH 5.5, the overall trends in gene expression do not support a model where NikR
functions as an indirect acid sensor. In addition, I demonstrated that purified NikR binds directly to multiple promoter fragments in vitro with a range of affinities that correlate with the Ni$^{2+}$-dependent gene regulation observed in vivo. In contrast to the well-characterized NikR protein from *Escherichia coli*, which recognizes a single perfect inverted repeat in one promoter, *H. pylori* NikR recognizes two poorly conserved sequences present in the *nixA* and *ureA* promoters, and requires high-affinity Ni$^{2+}$-binding and Mg$^{2+}$ to bind DNA, as well as added cations in gel mobility shift assays.
Introduction

*H. pylori* encodes one identifiable Ni$^{2+}$-dependent transcriptional regulator, NikR, that is a member of the ribbon-helix-helix (RHH) family of transcription factors. A previous study examined NikR-dependent regulation in *H. pylori* under high extracellular NiCl$_2$ conditions and predicted 39 genes in 31 operons to be either repressed or activated by NikR (9). This regulation included the activation of *ureAB*, encoding the urease enzyme, *nixA*, encoding a nickel transporter and *hpn* and *hpn-like*, encoding nickel storage proteins. NikR was predicted to repress *fur*, encoding an iron-dependent transcription factor, *frpB4* and *fecA3*, encoding predicted TonB-dependent iron siderophore transporters, and the divergently transcribed *exbBDtonB* and *nikR* genes. The predicted regulation of Fur and iron-related genes suggests that some regulation attributed to NikR may be indirect. Experiments designed to validate the array data were unconvincing. In addition, observation of the repression of some (*frpB4, fecA3*), but not all (*nixA*) nickel transporter genes is puzzling, especially given the function of *E. coli* NikR in repressing nickel transport. A subsequent report (14) demonstrated that *nixA* is repressed by Ni$^{2+}$ and NikR, rather than activated as the macroarray study suggested, highlighting the need for further testing of the array predictions.

In addition to its role as a Ni$^{2+}$-dependent regulator, *H. pylori* NikR has also been proposed to function as an indirect acid sensor as a result of the increased solubility of Ni$^{2+}$ at acidic pH (3, 21, 22). Increased urease activity in cells exposed to mildly acidic conditions (pH 5.5) was NikR-dependent and increased
amidase and formamidase activities were NikR- and Fur-dependent (3, 22), suggesting that NikR directly regulates ureAB and fur in response to acid, and the repression of fur is necessary to relieve the repression of amidase and formamidase genes. Microarray studies also indicated that a number of NikR- and Fur-regulated genes were repressed or activated in response to acidic conditions, and a nikR-fur double mutant in H. pylori strain SS1 was significantly reduced in its ability to colonize a mouse model of infection (3). These studies suggest that NikR and Fur are activated by acidic conditions in the stomach and their activity is important for infection. However, direct examination of NikR-dependent gene regulation under acidic conditions has not been carried out.

To determine if NikR regulates the genes predicted by the array studies, and to compare NikR-dependent regulation at pH 7 with regulation at pH 5.5, I have used quantitative RT-PCR (qRT-PCR) to measure transcript levels of a subset of predicted NikR target genes that are important for nickel physiology. In addition, I tested the ability of purified NikR to bind to these gene promoters and determined the metal requirements for DNA-binding using multiple in vitro assays. My results demonstrate that NikR upregulates and represses multiple genes in response to increasing concentrations of NiCl₂ at pH 7. However, acidic pH did not result in the regulation of all of the target genes controlled by NikR at pH 7, indicating that NikR does not function as an indirect acid sensor. Purified NikR bound to multiple promoters with a range of affinities, and recognized a poorly conserved inverted repeat present in the nixA and ureA promoters. Ni²⁺
and Mg\textsuperscript{2+} were both necessary for DNA-binding, and electrophoretic mobility shift assays required additional cations in the gel and running buffer. These results demonstrate that \textit{H. pylori} NikR regulates multiple genes in response to changes in intracellular Ni\textsuperscript{2+} concentrations, and suggest that differential regulation by NikR is a result of differences in DNA-binding affinity for distinct DNA recognition sites.
Results

**NikR-dependent gene regulation at neutral and acidic pH**

To determine if NikR regulates nickel-related genes predicted by a previous array study (9), I measured transcript levels in *H. pylori* strain 26695 and an isogenic nikR mutant exposed to increasing concentrations of NiCl$_2$ during overnight growth in Brucella Broth, pH 7.0 buffered with 100 mM MOPS using qRT-PCR. There was no significant difference in transcript levels in the parent and nikR mutant strains grown in the absence of added NiCl$_2$ (Figure 2.1a). In the parent strain, nixA transcript levels were repressed ~10-fold in response to greater than 100 nM added NiCl$_2$, ureA transcript levels were upregulated a maximum of 10-fold in response to greater than 1 µM added NiCl$_2$, and fur transcription was repressed 20-fold in response to 500 µM added NiCl$_2$ (Figure 2.1b-d). nikR transcript levels oscillated over the range of NiCl$_2$ tested, with transcripts increasing ~3-fold in response to 1-100 nM added NiCl$_2$, decreasing 2-fold with 1 µM NiCl$_2$ and increasing ~10-fold with 500 µM NiCl$_2$. Deletion of nikR resulted in a loss of nixA repression at high NiCl$_2$ concentrations, as well as ureA upregulation and fur repression at 500 µM NiCl$_2$, demonstrating that NikR represses nixA and fur and upregulates ureA in response to increasing concentrations of NiCl$_2$. nikR promoter activity was measured in the nikR::cat mutant strain using primers recognizing the start of the cat gene and part of the upstream untranslated region of the nikR promoter (Table 2.2). Quantitation of this hybrid transcript demonstrated that nikR::cat levels decreased at 100 pM
Figure 2.1. Ni\textsuperscript{2+}- and NikR dependent regulation in *H. pylori* strain 26695 at pH 7. qRT-PCR was used to determine the total level of (a,b) *nixA*, (a,c) *ureA*, (a,d) *fur*, (a,e) *nikR*, and (a,f) *hpn* transcripts present in the parent (solid circles, lines) and *nikR* mutant (open circles, dashed lines) strains grown overnight with no added NiCl\textsubscript{2} (a) or increasing concentrations of NiCl\textsubscript{2} (b-f). Total transcript levels, including the control to which each transcript was normalized (*recA*), were determined by extrapolation from standard curves of known concentrations of each DNA template run in parallel with experimental samples. Each sample was normalized to the parent strain grown at pH 7 without added NiCl\textsubscript{2} and averaged from three RT-PCR replicates from at least two biological samples. Y-axis scales of (b) through (f) are plotted logarithmically.
NiCl$_2$, and remained at ~10-fold lower levels compared to nikR in the parent strain, suggesting that NikR regulates its own expression in a biphasic manner. This result is consistent with a study demonstrating the presence of multiple NikR binding sites in the exbBDtonB – nikR intergenic region (12). No significant changes in hpn transcript levels were detected under any of the conditions tested (Figure 2.1a, f).

Previous studies using microarrays (3), Northern blotting (22) and enzymatic assays (3, 22) have suggested that NikR is activated by a shift from neutral to acidic pH, however NikR-dependent regulation has not been directly measured under steady-state acidic growth conditions. To determine if NikR is activated by a decrease in pH, and to see how Ni$^{2+}$- and NikR-dependent regulation is affected by acidic pH, transcript levels were measured in the parent and nikR mutant strains grown overnight in Brucella broth, pH 5.5 buffered with 100 mM MES and increasing concentrations of NiCl$_2$. All five transcripts (nixA, ureA, fur, nikR and hpn) increased ~5- to 10-fold at pH 5.5 relative to pH 7.0 in the absence of NiCl$_2$ (Figure 2.2a). Deletion of nikR abrogated the increase in nixA transcripts and significantly increased the level of nikR transcript under this condition, indicating that NikR upregulates nixA and represses nikR in response to a decrease in pH. In contrast, no significant changes were detected in ureA, fur or hpn transcripts in the nikR mutant, suggesting that additional factors are responsible for the increase in expression of these genes at pH 5.5.
Changes in *nixA*, *ureA* and *fur* levels at pH 5.5 as a function of added NiCl$_2$ were smaller than those observed at pH 7 (Figure 2.2b, c vs 2.1b, c), although both *nixA* and *ureA* decreased and *fur* increased gradually with increasing NiCl$_2$. *nikR* and *hpn* transcript levels increased ~10-fold and ~30-fold, respectively, in response to 10 nM NiCl$_2$ (Figure 2e, f). The Ni$^{2+}$-dependent changes in *nixA*, *ureA* and *hpn* transcripts were absent in the *nikR* mutant strain, and interestingly, *fur* levels increased ~10-fold in response to 10 nM NiCl$_2$ and the hybrid *nikR* transcript showed an overall increase of ~10-100 fold at all added NiCl$_2$ concentrations (Figure 2.2e, f). Together these data indicate that NikR regulates a subset of genes in response to acidic pH, the most significant being regulation of its own gene. In addition, Ni$^{2+}$- and NikR-dependent regulation at pH 5.5 is significantly altered from that which occurs at pH 7, suggesting that additional cellular factors modulate NikR activity and/or NikR regulates these genes indirectly.

*NikR directly binds to multiple promoters with a range of affinities*

To determine if NikR directly binds to the promoters from the *nixA*, *ureA*, *fur*, *nikR* and *hpn* genes, the protein was over-expressed in *E. coli*, purified and assayed for DNA-binding using electrophoretic mobility shift assays. *H. pylori* NikR displayed a hierarchy of DNA-binding affinities to these promoter fragments, exhibiting the highest and equal affinity of 4 nM for $P_{nixA}$ and $P_{ureA}$ and progressively weaker affinities for $P_{fur}$, $P_{nikR}$ and $P_{hpn}$ (Figure 2.3, Table 2.1). No
Figure 2.2. \( \text{Ni}^{2+} \)- and NikR dependent regulation in \textit{H. pylori} strain 26695 at pH 5.5. qRT-PCR was used to determine the total level of (a,b) \textit{nixA}, (a,c) \textit{ureA}, (a,d) \textit{fur}, (a,e) \textit{nikR}, and (a,f) \textit{hpn} transcripts present in the parent (solid circles, lines) and \textit{nikR} mutant (open circles, dashed lines) strains grown overnight in BBF5 with no added NiCl\(_2\) (a) or increasing concentrations of NiCl\(_2\) (b-f). Total transcript levels, including the control to which each transcript was normalized (\textit{recA}), were determined by extrapolation from standard curves of known concentrations of each DNA template run in parallel with experimental samples. Each sample was normalized to the parent strain grown at pH 7 without added NiCl\(_2\) and averaged from three RT-PCR replicates from at least two biological samples. Y-axis scales of (a) and (e) are plotted logarithmically.
binding was detected to a 143-bp internal fragment of *H. pylori rpoD* that was used as a negative control. Consistent with the mode of DNA-binding displayed by the RHH protein family, mutation of β-sheet residues Arg 12 to Ala or Met, Ser 14 to Ala or Ser 16 to Ala resulted in a protein unable to bind to the *nixA* or *ureA* promoter fragments (Figure 2.4).

*High affinity Ni\(^{2+}\)-binding by NikR is required for high-affinity DNA-binding*

Gel mobility shift assays with *E. coli* NikR require excess concentrations of NiCl\(_2\) (> 50 µM) in the gel and running buffer for DNA-binding (7), and the affinity of *E. coli* NikR for DNA is increased in this assay ~1000-fold relative to the affinity observed with stoichiometric NiCl\(_2\) in DNase I footprinting assays (6). To determine if high-affinity Ni\(^{2+}\)-binding was required for DNA-binding, and if *H. pylori* NikR displayed a similar change in affinity for the *nixA* and *ureA* promoters in the presence of excess and stoichiometric Ni\(^{2+}\), DNase I footprinting assays were used to measure NikR binding to the *nixA* and *ureA* promoters in the presence or absence of stoichiometric NiCl\(_2\). NikR displayed 6- and 9-fold increase in affinity for *nixA* and *ureA* in the presence of stoichiometric NiCl\(_2\), with affinities of 49 and 45 nM, respectively (Figure 2.5, Table 2.1). In addition, the affinity differences for both promoters under excess and stoichiometric Ni\(^{2+}\) conditions (mobility shifts vs DNase I) were only ~12-fold, indicating that *H. pylori* NikR does not behave similarly to *E. coli* NikR in the presence of 50 µM Ni\(^{2+}\), although stoichiometric Ni\(^{2+}\) is still required for the highest DNA affinities.
Figure 2.3. NikR directly binds to multiple promoters with a range of affinities. Gel mobility shifts with 50 µM NiCl$_2$ in the gel and running buffer. Full-length *H. pylori* NikR was 1.7-fold serially diluted from concentrations of 1.0 µM to 349 pM protein. F, free DNA; B, protein-bound DNA; and SS, a super-shifted protein-DNA complex. Each full titration was run on two separate gels in parallel and a vertical line separates each pair of gel images. Each titration represents one of at least two replicates. Affinities were calculated as described in the Materials and Methods and are listed in Table 2.1. Highest to lowest protein concentrations are indicated by the black arrowhead above each gel.
Figure 2.4. NikR β-sheet residues are required for DNA-binding. Gel shift assays with 100 nM (nixA) or 200 nM (ureA) protein with nixA or ureA promoter fragments. Reactions were run on gels with 50 µM NiCl₂ in the gel and running buffer. F, free DNA; and B, bound DNA. The vertical lines represent where the same image from one gel has been cut and pasted together.
Figure 2.5. NikR with stoichiometric or no NiCl$_2$ has decreased affinity for the *nixA* and *ureA* promoters. DNase I footprinting of *H. pylori* NikR with stoichiometric Ni$^{2+}$ (a, b) serially diluted 1.5-fold from 1.0 µM to 677 pM or in the absence of Ni$^{2+}$ (c, d) serially diluted 1.3-fold from 5.0 µM to 75 nM titrated against $P_{nixA}$ (a, c) and $P_{ureA}$ (b, d). Highest to lowest protein concentrations are indicated by the black arrowhead above each gel.
Table 2.1 Apparent binding affinities of NikR for different promoters

<table>
<thead>
<tr>
<th></th>
<th>nixA</th>
<th>ureA</th>
<th>fur</th>
<th>nikR</th>
<th>hpn</th>
<th>rpoD</th>
</tr>
</thead>
</table>
| 50 µM NiCl₂      | 3.8(±0.8) | 3.8(±0.3) | 29(±6) | 120(±50) | 180(±70) | nb  
| (mobility-shift) |      |      |     |       |       |      |
| 1:1 Ni²⁺         | 49 (±7)  | 45 (±10) | nd  | nd    | nd    | nd   |
| (DNase I footprint) |      |      |     |       |       |      |
| No Ni²⁺          | 311 (±154) | 417 (±236) | nd  | nd    | nd    | nd   |
| (DNase I footprint) |      |      |     |       |       |      |

*a* Affinities are reported in nM.

*b* No detectable binding.

*c* No data collected.
**H. pylori NikR binds Ni\(^{2+}\) with high affinity**

Similar to *E. coli* NikR, a peak at 302 nm was present in the difference spectrum of holo- vs apo-*H. pylori* NikR samples. Titrations of Ni\(^{2+}\) against *H. pylori* NikR in the presence of the competitor EGTA indicated that the affinity for Ni\(^{2+}\) is 2 pM (Figure 2.6a), similar to that measured for *E. coli* NikR [2-6 pM; (6, 25)]. Consistent with these results, stability measurements monitoring NikR folding by circular dichroism in the presence or absence of Ni\(^{2+}\) demonstrated that Ni\(^{2+}\)-binding to the protein increases the concentration of urea required to unfold NikR (Figure 2.6b). The presence of two distinct transitions in the holo-NikR denaturation curve relative to one transition observed for apo-NikR together with the Ni\(^{2+}\)-dependent shift in the second half of the holo-NikR curve suggest that the N- and C-domains of NikR unfold separately, and that the second half of the curve represents unfolding of the Ni\(^{2+}\)-binding C-domain.

**NikR recognizes poorly conserved binding sites at the nixA and ureA promoters**

DNase I footprinting indicated that NikR protected a 36 bp region of \(P_{\text{nixA}}\) [–14 to +22 relative to the start of transcription; (14)] and a 38 bp region of \(P_{\text{ureA}}\) [–91 to –54 relative to the start of transcription, (2); Figure 2.7]. Hydroxyl radical footprinting revealed that NikR protects four regions of four bases each that are separated by four to six bases at each promoter, demonstrating that NikR binds to one face of \(P_{\text{nixA}}\) and \(P_{\text{ureA}}\) that spans approximately two turns of the double helix. DMS protection at positions –4 of the template strand and +11 of the non-
Figure 2.6. Direct binding of Ni$^{2+}$ to NikR occurs with high affinity and increases the stability of the protein. (a) Ni$^{2+}$-EGTA was serially diluted 1.6-fold from a concentration of 950 µM to 322 nM against 50.0 µM NikR and Ni$^{2+}$-binding was monitored by absorbance at 302 nM. (b) NikR in the absence (filled circles) or presence (open circles) of Ni$^{2+}$ was unfolded with increasing concentrations of urea and folding of the protein was monitored by ellipticity at 230, as measured by circular dichroism.
Figure 2.7. NikR recognizes poorly conserved DNA sequences. Fe-EDTA and DNase I footprinting with 1.0 µM NikR and 50 µM Ni$^{2+}$ and the top strands of the \textit{nixA} (a) or \textit{ureA} (b) promoters. Black vertical lines next to gels indicate regions of protection. (c) Schematic of NikR protection at \textit{P}_{nixA} and \textit{P}_{ureA}. Brackets, DNase I protection; grey shaded boxes, Fe-EDTA protection; and black triangles, protected guanine bases (DMS data not shown). Base positions indicated are relative to the start of transcription (2, 14) and the predicted half-sites are boxed.
template strand of $P_{nixA}$ and positions –81 and –79 of the template strand of $P_{ureA}$ demonstrates that base-specific contacts made by NikR include these guanines (see Figure 4.3). Furthermore, the DMS protection helped to define the likely half-sites recognized by NikR at $P_{nixA}$ and $P_{ureA}$. Based on these results I predict the NikR recognition site at $nixA$ to be TATTAC - 13 - GTATTA and that at $ureA$ to be TAACAC - 13 - ATAATA (boxed sequences in Figure 2.7c).

**NikR requires additional cations for DNA-binding in gel mobility shift assays**

The initial experiments examining *H. pylori* NikR DNA-binding using electrophoretic mobility shift assays were performed under conditions used for studies with *E. coli* NikR, which has two different affinities for the $nikA$ promoter depending on the amount of NiCl$_2$ present (6). To determine if *H. pylori* NikR displays a similar requirement for excess NiCl$_2$ in the gel and running buffer of gel mobility shift assays, a titration of NikR with the $nixA$ promoter was performed in the absence of NiCl$_2$ in the gel and running buffer, but with stoichiometric NiCl$_2$ in the binding reaction. No DNA-binding was detected under these conditions, suggesting the presence of a second Ni$^{2+}$-binding site that may not increase DNA affinity to the extent observed for *E. coli* NikR (Figure 2.8).

To explore the specificity of this metal requirement in the mobility shift assay, NikR binding to $P_{nixA}$ was also measured in the presence of 1 mM MgCl$_2$ and KCl in the gel and running buffer (Figure 2.8c, f). Interestingly, each of these salts resulted in a mobility shift, indicating that NikR does not contain a
Figure 2.8. NikR requires additional cations for DNA-binding in gel mobility shifts. Titrations of *H. pylori* NikR serially diluted 3-fold from concentrations of 500 nM to 229 pM against *P*<sub>nixA</sub> with (a) 50 μM NiCl<sub>2</sub>, (b) no added cations, (c) 1 mM MgCl<sub>2</sub>, (d) 50 μM NiSO<sub>4</sub> or (e) 1 mM MgSO<sub>4</sub> in the gel and running buffer. (f) NikR was serially diluted 1.7-fold from 500 nM to 2 pM against *P*<sub>nixA</sub> with 1 mM KCl in the gel and running buffer. (g, h) E39A or D43A NikR were 3-fold serially diluted from concentrations of 500 nM to 229 pM protein against *P*<sub>nixA</sub> with either (g) no added cations or (h) 50 μM NiCl<sub>2</sub>. (i) NikR was serially diluted 1.7-fold from 500 nM to 2 pM against *P*<sub>nixA</sub> with 50 μM NiCl<sub>2</sub> and 1 mM MgCl<sub>2</sub> in the gel and running buffer. The vertical lines indicates where two gel images were pasted together. Highest to lowest protein concentrations are indicated by the black arrowhead of decreasing height above the gel.
second Ni$^{2+}$-binding site, but rather displays a non-specific cation requirement, at least in this assay. DNA-binding by NikR also occurred when 50 μM NiSO$_4$ or 1 mM MgSO$_4$ was added to the gel and running buffer (Figure 2.8g, h), demonstrating that NikR required added cations and not Cl$^-$ ions for activity. Furthermore, mutation of Glu39 or Asp43 to Ala, residues implicated in low-affinity cation-binding by *E. coli* NikR (8), had no measurable effect on the DNA-binding activity of *H. pylori* NikR in the presence or absence of added cations in the mobility-shift assay (Figure 8f, g). Also, the addition of NiCl$_2$ and MgCl$_2$ did not produce an additive effect on DNA-binding affinity (Figure 8i).

Mg$^{2+}$ and K$^+$ are normally present at 10 and 100 mM, respectively, in *E. coli* (17), while Ni$^{2+}$ is maintained at significantly lower levels [< 10 μM; (17)]. To begin to address which cation is physiologically relevant for *H. pylori* NikR activity, an apparent affinity of NikR for Mg$^{2+}$ was estimated. DNase I footprinting of stoichiometric Ni$^{2+}$:NikR-$P_{nixA}$ titrations was performed at different constant Mg$^{2+}$ concentrations, because it was not possible to titrate MgCl$_2$ with a constant NikR concentration due to the Mg$^{2+}$-dependence of DNase I which prohibited quantitation. Without MgCl$_2$ the 1:1 Ni:NikR-$P_{nixA}$ affinity was ~400 nM in a buffer containing 100 mM KCl (Figure 2.9). The highest NikR-DNA binding affinity (10 nM) was observed at MgCl$_2$ concentrations ≥ 3 mM and this value was constant up to the highest concentration tested (50 mM). An estimate from a plot of the calculated $K_d$ values of NikR for $P_{nixA}$ as a function of MgCl$_2$ concentration indicates an affinity of the NikR-DNA complex for Mg$^{2+}$ in the μM to mM range.
(Figure 2.9b). The estimated affinity of NikR for Mg$^{2+}$ suggests that it may act as the cation required for NikR activity in vivo, assuming H. pylori maintains intracellular metal concentrations at levels similar to E. coli. Together these data demonstrate that the second Ni$^{2+}$-binding site is not conserved between H. pylori and E. coli NikR but do not reveal a structural basis for the altered NikR cation requirement.
Figure 2.9. High-affinity binding of NikR to $P_{\text{nixA}}$ requires Mg$^{2+}$. (a) DNase I footprinting of NikR that was 5-fold serially diluted from concentrations of 1.0 µM to 1.6 nM [corresponding to Lanes 1-5; (-) lane contains no protein] and incubated with $P_{\text{nixA}}$ in the presence of increasing concentrations of MgCl$_2$. (b) NikR affinity for $P_{\text{nixA}}$, as determined by DNase I footprinting titrations of NikR serially diluted 2-fold from concentrations of 1.0 µM to 2 nM and incubated with $P_{\text{nixA}}$, as a function of MgCl$_2$ (gels not shown). The dashed horizontal line indicates the affinity of NikR for $P_{\text{nixA}}$ in the absence of MgCl$_2$. Data from two independent titrations is plotted with the calculated standard error.
Discussion

*H. pylori* NikR has been predicted to regulate 39 genes arranged in 31 operons, including both activation and repression of gene expression, in response to increased Ni$^{2+}$ (9), and has also been suggested to function as an indirect acid sensor (3, 22). In this work I attempted to validate the Ni$^{2+}$- and NikR-dependent regulation of five target genes involved in nickel physiology and compared changes in transcript levels of these genes at neutral and acidic pH. NikR repressed and activated genes in response to increasing NiCl$_2$ (*nixA, ureA*, respectively), and repressed genes at discrete NiCl$_2$ concentrations (*nikR, fur*) at neutral pH. Purified NikR directly bound to the promoters of each gene *in vitro* with a range of affinities that correlated with the lowest concentration of added NiCl$_2$ required to trigger regulation of *nixA, ureA, fur* and *nikR in vivo*. Together these results demonstrate that NikR differentially regulates genes in response to changing environmental Ni$^{2+}$ concentrations.

NikR also regulated *nixA, ureA, fur* and *nikR* at pH 5.5, however a decrease in pH in the absence of added NiCl$_2$ did not trigger regulation of all of the genes, nor did it uniformly shift the Ni$^{2+}$-responsive regulation of each gene. These results argue against the idea that the increased solubility of Ni$^{2+}$ at acidic pH serves as an indirect signal to the cell via NikR. The most prominent NikR-dependent regulation observed at pH 5.5 is the ~10-100 fold upregulation of *nikR*. Urease activity also increases in response to a decrease in pH, suggesting that increased NikR levels in the cell may be necessary to respond to larger
overall concentrations of intracellular Ni$^{2+}$. Together with the NikR-dependent regulation of hpn transcripts at pH 5.5, a gene whose promoter NikR displayed the lowest affinity for in vitro, the data suggest that greater levels of NikR protein at acidic pH may allow for the regulation of additional target genes not regulated by NikR at neutral pH.

Recent studies, together with the results presented here, indicate that the iron-responsive transcription factor Fur is capable of repressing nikR expression (12). The apparent reciprocal repression of nikR by Fur and of fur by NikR in addition to the auto-repression by each regulator makes it difficult to interpret data for either transcript. One possibility is that the repression observed for each transcript by both regulators increases the sensitivity of the negative auto-regulatory loops. Dual regulation of each transcript by NikR and Fur also links the expression of both regulators to extracellular Ni$^{2+}$ and Fe$^{2+}$ levels. One obvious reason for the cell to link its Ni$^{2+}$ and Fe$^{2+}$ responses is that hydrogenase requires both metals for activity. Hydrogenase generates electrons for the electron transport chain, which consists of many iron-dependent proteins, so in this way Ni$^{2+}$ and Fe$^{2+}$ availability in the environment may control the overall metabolism of H. pylori via NikR and Fur. It is less obvious why urease may be linked to Fe$^{2+}$ physiology, however it is not unreasonable to speculate that Fe$^{2+}$-independent Ni$^{2+}$ responses (and Ni$^{2+}$-independent Fe$^{2+}$ responses) are also important for the cell, given that two separate metal-dependent transcription factors (NikR and Fur) mediate responses to the different metals.
NikR protected positions –14 to +22 relative to the start of transcription of $P_{nixA}$ (14), consistent with its role in the repression of $nixA$. Because the NikR binding site overlaps the transcription start site, it is likely that repression occurs by RNA polymerase occlusion from the $nixA$ promoter. NikR protected positions –91 to –54 of $P_{ureA}$, consistent with a previous report where the region from –67 to –49 was demonstrated to be necessary for the Ni$^{2+}$-dependent increase in $ureA$ transcription in vivo (23). The location of the binding site suggests that NikR functions as a class I transcriptional activator (18), however, more detailed studies with a reconstituted $H. pylori$ RNA polymerase will be required to determine the precise mechanism of $ureA$ induction.

DMS protection assays allowed me to define the half-sites NikR recognizes at the $nixA$ and $ureA$ promoters, which consist of two imperfect inverted repeats (TATTAC -13- GTATTA at $nixA$ and TAACAC -13- ATAATA at $ureA$). Mutagenesis of individual bases within each binding site will be required to determine the relative contributions of specific operator positions to NikR binding, and it will be interesting to compare the affects of base substitutions at analogous positions in either promoter. One prediction is that the ability of NikR to function as a repressor or activator of transcription is governed solely by the location of its binding site relative to the start of transcription. The differences in the NikR binding site sequences suggest an alternative, not mutually exclusive, possibility where different NikR binding sites result in unique conformations of DNA-bound NikR, leading to distinct activities. Further experiments examining the
conformation of NikR bound to different promoters are required to distinguish between these possibilities (Chapter 5).

High-affinity DNA-binding by NikR required Ni\(^{2+}\)-binding, as apo-NikR displayed affinities of ~300 and ~400 nM for the nixA and ureA promoters, respectively, and holo-NikR had affinities of 49 and 45 nM (Table 2.1, Figure 2.5). Although the >30-fold increase in affinity of \textit{E. coli} NikR for DNA in response to Ni\(^{2+}\)-binding is a lower limit estimate because DNA-binding by the apo form of this protein is undetectable (6), it is notable that \textit{H. pylori} NikR displays only a ~7-fold increase in affinity for these promoters in response to high-affinity Ni\(^{2+}\)-binding. The diminished effect of Ni\(^{2+}\)-binding on \textit{H. pylori} NikR suggests that the apo-protein exists in a conformation(s) more competent for DNA-binding compared to \textit{E. coli} NikR. Another distinction from \textit{E. coli} NikR is the requirement for Mg\(^{2+}\) for high-affinity DNA-binding by \textit{H. pylori} NikR. An intracellular Mg\(^{2+}\) concentration in \textit{H. pylori} similar to the 10 mM measured for \textit{E. coli} (17), together with the estimated affinity of <3 mM of \textit{H. pylori} NikR for Mg\(^{2+}\), suggest that this site probably exists in a Mg\(^{2+}\)-bound state in the cell. The lowered specificity and affinity of apo- and holo-\textit{H. pylori} NikR for DNA suggests that additional stabilization is required for the formation of a protein-DNA complex, perhaps provided by protein-cation and cation-DNA interactions not important in \textit{E. coli} NikR DNA-binding.

Mobility-shift assays with different cations showed that NikR does not contain a low-affinity Ni\(^{2+}\)-binding site that significantly increases DNA-binding affinity at
$P_{r{x}A} \text{ and } P_{u{r}A}$. These results are consistent with a previous study that showed only a five-fold increase in DNA-binding affinity to $P_{u{r}A}$ when excess NiCl$_2$ was present in a mobility shift assay (1). My data is partially consistent with a recent report suggesting that NikR contains a low-affinity metal-binding site that is specific for Mg$^{2+}$, Ca$^{2+}$ or Mn$^{2+}$ (13). In that study, fluorescence anisotropy was used to detect changes in NikR-$P_{u{r}A}$-binding. In striking contrast to the gel-based assays [this study and (1)], the researchers were unable to detect DNA-binding in the presence of 3 mM NiCl$_2$. Importantly, the anisotropy experiments contained no added cations while the footprinting experiments contained significant concentrations of cations [100 mM KCl, 1 mM MgCl$_2$, 1 mM CaCl$_2$ in (1) and 100 mM KCl, 3 mM MgCl$_2$ in our study]. This difference does not explain the cation dependence of the mobility-shift assays, which are very sensitive to added cations, while the footprinting reaction was sensitive to MgCl$_2$ even in the presence of 100 mM KCl. Each DNA-binding assay (footprinting, mobility-shift, and fluorescence anisotropy) likely provides different information regarding NikR-DNA interactions that will require further detailed studies to reconcile.

Interestingly, mutation of Glu39 or Asp43 to Ala [corresponding to Glu30 and Asp34 in *E. coli* NikR, which are implicated in low-affinity Ni$^{2+}$-binding (8, 19)] had no significant effect on DNA-binding in mobility-shift assays. This result supports the hypothesis that *H. pylori* NikR does not contain the low-affinity Ni$^{2+}$-binding site present on *E. coli* NikR. Furthermore, the lack of observable DNA-binding effect from mutation of Glu39 or Asp43 suggests that *H. pylori* NikR exists in a
significantly different conformation on DNA, as compared with *E. coli* NikR. The half-site spacing of 13 base pairs observed in *H. pylori* NikR-regulated promoters, relative to the 16 base pair spacer of *E. coli* NikR (7), likely places different constraints on the NikR DNA-binding domains relative to the C-terminal Ni\textsuperscript{2+}-binding domains.

Biologically, the presence or absence of a low-affinity Ni\textsuperscript{2+} site could reflect the different regulatory functions and physiological contexts of the *H. pylori* and *E. coli* NikR proteins. *H. pylori* expresses hydrogenase and urease, several Ni\textsuperscript{2+}-binding chaperones important in enzyme assembly, and Ni\textsuperscript{2+} storage proteins, whereas *E. coli* expresses four hydrogenase isoenzymes under specific anaerobic conditions. The increased complexity of Ni\textsuperscript{2+} physiology in *H. pylori* may have imposed selective pressure on *H. pylori* NikR, resulting in the expansion of Ni\textsuperscript{2+}-dependent regulation and the observed biochemical differences in *H. pylori* NikR-DNA interactions compared to *E. coli* NikR.

The discovery that *H. pylori* NikR binds DNA with less specificity and affinity than *E. coli* NikR is consistent with the expanded regulation initially predicted (9) and partially validated [this study, (11, 14, 15)] for this NikR homolog, however the apparent discrepancies in NikR-dependent regulation observed in this study and earlier work indicate that further experiments are required before the complete NikR regulon can be unequivocally defined. What is clear from these studies is that this second experimentally-characterized NikR family member has evolved to regulate multiple aspects of nickel physiology in *H. pylori* through
alterations in metal- and DNA-binding activities of the protein. Elucidating the molecular bases for the alterations in NikR-DNA interactions will provide further insight into how this family of transcription factors has evolved in response to the physiologies of different microorganisms.
Materials and Methods

Bacterial strains and culture conditions

*H. pylori* strain 26695 (from Helene Kling-Bäckhed and Jeffrey Gordon; Washington University School of Medicine) and derivatives (see below) were maintained as glycerol stocks at -80° C and passaged no more than five times after recovery from stock. Cells were grown at 37° C under microaerobic conditions using a BD BBL GasPak Jar (Fisher, Pittsburgh, PA) with microaerobic gas packs (Mitsubishi Gas Chemical Co, VWR, Westchester, PA). Growth on solid medium used Brucella blood agar plates (BD Difco, Fisher) containing 10% horse blood (Colorado Serum Company, Denver, CO), 3 μg/ml vancomycin (Sigma, Saint Louis, MO), 1 μg/ml trimethoprim (Sigma) and 10 μg/ml amphotericin B (Sigma). Chloramphenicol was used at 20 μg/ml (Fisher). Liquid growth used Brucella broth (BD Difco, Fisher) containing 5% fetal bovine serum (Sigma) and 100 mM MOPS buffer (initial pH of 7.0) or 100 mM MES (initial pH of 5.5). For liquid growth, cells were incubated as described above, except 25 ml flasks containing 10 ml cultures with shaking (120 rpm). Liquid cultures were inoculated with cells grown for 24 h on Brucella blood agar plates after at least one passage from frozen stock, resuspended in liquid media and added to a final starting OD$_{600}$ of 0.05.

The *nikR* isogenic mutant of *H. pylori* 26695 was constructed using double homologous recombination with overlapping PCR products (4). Briefly, three separate PCR reactions amplified ~500 bp regions upstream or downstream of
* nikR* from 26695 genomic DNA, and an antibiotic resistance gene from plasmid pHel2 (*cat*) (a gift from Rainer Haas; Max von Pettenkofer Institut für Hygiene und Medizinische Mikrobiologie). Primer sequences are listed in Table 2.2. To ensure a non-polar ORF deletion, the upstream region reverse primer contained three stop codons in each reading frame followed by a ribosome binding site (RBS). The downstream region forward primer contained a RBS followed by a start codon. PCR products were purified using a Qiagen PCR Purification Kit (Qiagen, Valencia, CA), mixed and used as the template for a second round of PCR. Purified PCR product was used to naturally transform 24 h plate-grown *H. pylori* (10). Cells were grown on solid media for an additional 20 h, then plated onto chloramphenical-containing media. The mutant strain was checked for the expected deletion by PCR.

**RNA isolation, cDNA synthesis and qRT-PCR**

Overnight-grown cells were harvested by centrifugation at 4°C (2600 x g, 5 min), resuspended in 1 ml Trizol (Invitrogen) and RNA was isolated according to the manufacturer’s instructions. RNA was resuspended in 32 µl DEPC-H₂O and treated with 4 U DNase I (Invitrogen) for 30 min at 22°C, ethanol precipitated, quantified and stored at -80°C. cDNA was generated from 10 µg total RNA using Random Primers and Superscript II RNase- Reverse Transcriptase as per the manufacturer’s instructions (Invitrogen).
qRT-PCR was performed in triplicate in 96-well plates with SYBR Green qRT-PCR reagent (Invitrogen) and the BioRad iCycler iQ Real-Time PCR Detection System (Biorad, Hercules, CA) using 2 µl of the 40 µl cDNA synthesis reaction as template. Standard curves of each transcript were generated by titrating known concentrations of DNA template (10³ to 10⁸ copies/well) in duplicate on the same plate as matched experimental samples, and experimental sample copy number was determined by extrapolation from the known samples. Primers used for qRT-PCR are listed in Table 2.2. Reactions using primers recognizing the constitutively-expressed transcript to recA and standard curves of recA-containing DNA were run in parallel with experimental transcripts. After calculating the total copy number of transcript in each well, the experimental samples were normalized to recA and further normalized to the parental strain, *H. pylori* 26695, grown at pH 7 in the absence of added NiCl₂. The average of at least two independent biological replicates for which three qRT-PCR replicates were performed is reported with the calculated standard deviation. Significant variation was often observed for individual transcripts in single wells during the qRT-PCR assays, likely as a result of technical aspects of the assay. These samples, which represented replicates greater than the two reported, were excluded from the data presented here.
Protein overexpression and purification

*H. pylori* strain 26695 nikR (HP1338) was PCR amplified from genomic DNA (a generous gift from Doug Berg, Washington University School of Medicine) using the primers PC121 and PC122 (Table 2.2; Integrated DNA Technologies, Coralville, IA) and cloned into pET22-b using the *Nde*I and *Xho*I restriction sites (Novagen, Madison, WI) to create pEB116. The DNA sequence was verified by sequencing (SeqWright, Houston, TX).

Native *H. pylori* NikR and variants were expressed and purified as described previously for *E. coli* NikR (6, 7) except that gel-filtration was used as a second purification step instead of ion exchange. Protein concentration was determined in 6 M guanidine hydrochloride (GuHCl) using $\varepsilon_{276} = 9895$ M$^{-1}$ cm$^{-1}$, as predicted by primary sequence analysis (16). To remove Ni$^{2+}$ from purified protein, the Ni-NTA eluate was incubated with 50 mM EDTA for 48 h at 4°C, followed by gel-filtration (the second purification step). The removal of Ni$^{2+}$ ions was confirmed using UV-visible spectroscopy at 302 nm.

Promoter fragments - cloning and labeling

DNA fragments for promoter regions were amplified by PCR using the oligonucleotide pairs described in Table 2.2. A subset of fragments (Table 2.2) were cloned into pBluescriptII SK (Stratagene, La Jolla, CA) using standard molecular biology techniques. The cloned promoter sequences were confirmed by DNA sequencing.
Promoter fragments for DNA-binding assays were generated by PCR as follows: 0.5 µM forward (5’) primers (listed first in Table 2.2) for the nixA, ureA, fur, nikR, hpn and rpoD fragments were 5’-end labeled with [γ-32P]-ATP [GE Biosciences, Piscataway, NJ] and T4 polynucleotide kinase (New England Biosciences, Beverly, MA) in a total volume of 40 µl. Excess [γ-32P]-ATP was removed by desalting and the purified primers were used in a PCR reaction with the corresponding reverse primers (listed second in Table 2.2) using plasmids pEB106, pEB131 or pEB104 as templates for the nixA, ureA and nikR promoters or H. pylori 26695 genomic DNA for the fur, hpn and rpoD fragments. The 143-bp fragment of the H. pylori rpoD gene had comparable length and GC content to the other fragments and was used as a negative control. The resulting labeled fragments were purified using a Qiagen PCR Purification Kit (Qiagen).

**Electrophoretic mobility shift assays**

Mobility shift assays were performed using 7% polyacrylamide gels and electrophoresis buffer containing 50 mM Tris (pH 8.8), 25 mM boric acid and with NiCl₂, MgCl₂, NaCl, KCl, NiSO₄ or MgSO₄ as described in the text and figure legends. The binding buffer was identical to that used for DNase I footprinting. The same end-labeled DNA fragments as those used for footprinting were incubated with NikR or mutant proteins at 22°C for 30 min and 20 µl of the 50 µl total volume was loaded directly onto a running gel (120 V).
Footprinting assays

DNase I footprinting was performed as described previously (5, 6) in a binding buffer containing 10 mM Tris-Cl (pH 8.0), 100 mM KCl, 3 mM MgCl₂, 10 µg/ml oxidized E. coli thioredoxin [used to prevent non-specific NikR accumulation in the gel wells], and 4 ng/µl salmon sperm DNA (Fisher Scientific). Labeled DNA fragments were incubated with protein at 22°C for 1 hour prior to DNase I (Sigma) addition (final concentration 300 ng/mL). NiCl₂ was added to the binding reactions as described in the text and figure legends.

Hydroxyl radical footprinting was performed as described previously (20) with the following modifications: binding reactions were performed in 50 µl of DNase I footprinting buffer with 10 mM MgCl₂. Fe(II)-EDTA (2 mM stock) was added to a final concentration of 167 µM, followed by the addition of 833 µM sodium ascorbate (20 mM stock) and 0.05% H₂O₂ (30% v/v stock). The reaction was carried out for 1 min at 22°C and was quenched with 10 µl of 0.1 M thiourea and 1 µl of 0.5 M EDTA (pH 8.0). Dimethyl sulfate (DMS) protection experiments were carried out as previously described (24) except binding reactions were in a total volume of 100 µl and the buffer contained 10 mM sodium cacodylate (pH 8.0) instead of Tris-Cl.

Apparent affinities measured by mobility shift assays were calculated from binding curves determined by the ratio of bound (all shifted species) vs. free counts as quantified using a Molecular Dynamics Storm 840 Phosphoimager and
IMAGEQUANT Version 5.2 software. Apparent affinities measured by DNase I footprinting were calculated from binding curves determined by the ratio of the protected DNA region normalized to a region of DNA not protected from the same lane vs the same ratio from identical regions of a protein-free lane on the same gel. The data were fit using MICROMATH SCIENTIST Version 2.01 and the following equation:

\[ y = \frac{1}{1 + \left( \frac{K_d}{x} \right)^n} \]

where: \( y \), fraction DNA bound (ratios described above); \( K_d \), protein concentration required for half-maximal DNA-binding; \( x \), protein concentration; and \( n \), Hill coefficient. All reported affinities are the average of at least two independent experiments using a dilution series of at least 15 protein concentrations. The reported error is the standard deviation between the calculated affinities of at least two independent experiments.

**UV-visible and CD spectroscopy**

UV-visible spectra were collected on a Shimadzu UV-2401PC spectrophotometer using a 100 \( \mu \)l sample volume. CD spectra were collected on a Jasco J-715 spectropolarimeter using a 900 \( \mu \)l sample volume in a cylindrical cuvette with a 1 cm pathlength. All spectra were collected at 22°C in a buffer containing 20 mM Tris (pH 7.5), 100 mM NaCl.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Name</th>
<th>Pylori Gene Coordinates (5'→3')</th>
<th>Sequence (5'→3')&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Product use</th>
</tr>
</thead>
<tbody>
<tr>
<td>nikR 5'</td>
<td>EB085</td>
<td>1399283-1399263</td>
<td>gttagattcttggttaaatcc</td>
<td>Gene deletion</td>
</tr>
<tr>
<td>nikR 3'</td>
<td>EB090</td>
<td>1397817-1397837</td>
<td>gcctaaaccctaaaaactagcc</td>
<td>Gene deletion</td>
</tr>
<tr>
<td>cat</td>
<td>EB223</td>
<td>na&lt;sup&gt;b&lt;/sup&gt;</td>
<td>TGACTAACTAGGAGGAATAatgcaattcacaagattgatata</td>
<td>Gene deletion</td>
</tr>
<tr>
<td>cat</td>
<td>EB224</td>
<td>na</td>
<td>CATTATCCCTCCAGGTAatttatcagcaagtctgtaa</td>
<td>Gene deletion</td>
</tr>
<tr>
<td>nikR</td>
<td>PC121</td>
<td>1398772-1398755</td>
<td>att ttc tca tAT GGA TAC ACC CAA TAA AG</td>
<td>Overexpression (pEB116)</td>
</tr>
<tr>
<td>nikR</td>
<td>PC122</td>
<td>1398327-1398341</td>
<td>ttt gta tct cga gCT ATT CAT TGT ATT C</td>
<td>Overexpression (pEB116)</td>
</tr>
<tr>
<td>nixA</td>
<td>EB006</td>
<td>1137016-1136993</td>
<td>gga tta gaa ttc AAA ATT TTT TAG GGC AAT TTG CAG</td>
<td>DNA-binding (pEB106)</td>
</tr>
<tr>
<td>nixA</td>
<td>EB007</td>
<td>1136850-1136873</td>
<td>cca tta ccc ggg CAA TGC ATG CAA GAA CAC AAT CGC</td>
<td>DNA-binding (pEB106)</td>
</tr>
<tr>
<td>ureA</td>
<td>EB047</td>
<td>78187-78163</td>
<td>gtc tca cgg cgg TTC TCA TTT TGG TGC GAG TTT TTG</td>
<td>DNA-binding (pEB131)</td>
</tr>
<tr>
<td>ureA</td>
<td>EB036</td>
<td>77927-77950</td>
<td>gta tca gtc gac CTT GTC TAA CTC TTT TGG GGT GAG</td>
<td>DNA-binding (pEB131)</td>
</tr>
<tr>
<td>fur</td>
<td>EB031</td>
<td>1090060-1090083</td>
<td>gta ctc cgg cgg AGT TAC ATT AAA ATG CGA CAA TGG</td>
<td>DNA-binding</td>
</tr>
<tr>
<td>fur</td>
<td>EB032</td>
<td>1090221-1090199</td>
<td>gca ttc gtc gac ATC TTT TCA TGC TGA TAT CTT CC</td>
<td>DNA-binding</td>
</tr>
<tr>
<td>nikR</td>
<td>EB004</td>
<td>1398872-1398847</td>
<td>ccc atc gaa ttc AAA TCC ATG TTG TAT TAT AAT TGT TC</td>
<td>DNA-binding (pEB104)</td>
</tr>
<tr>
<td>nikR</td>
<td>EB005</td>
<td>1398747-1398771</td>
<td>cca tat ccc ggg TGA ATC GTC TTT ATT GGG TGT ATC C</td>
<td>DNA-binding (pEB104)</td>
</tr>
<tr>
<td>hpn</td>
<td>EB033</td>
<td>1497308-1497284</td>
<td>gtc tat cgg cgg ATA ATT CAA AAT TTA GGG AAT ATG G</td>
<td>DNA-binding</td>
</tr>
<tr>
<td>Gene</td>
<td>Accession</td>
<td>Start-End</td>
<td>Sequence</td>
<td>Technique</td>
</tr>
<tr>
<td>--------</td>
<td>-----------</td>
<td>-----------</td>
<td>---------------------------</td>
<td>-----------------------</td>
</tr>
<tr>
<td><em>hpn</em></td>
<td>EB034</td>
<td>1497146-1497169</td>
<td><code>cga tta gtc gac</code> CCG TGT TGT TCT TCA TGG TGT GCC</td>
<td>DNA-binding</td>
</tr>
<tr>
<td><em>rpoD</em></td>
<td>PC687</td>
<td>93095-93078</td>
<td><code>ttc ggt atc gat</code> GAT GAA AGC GAT CGA ACT</td>
<td>DNA-binding</td>
</tr>
<tr>
<td><em>rpoD</em></td>
<td>PC688</td>
<td>92952-92972</td>
<td><code>gag aaa ccc tgg act</code> CAA ATG CGC AAA TAG TTT CTC</td>
<td>DNA-binding</td>
</tr>
<tr>
<td><em>nixA</em></td>
<td>EB099</td>
<td>1136058-1136078</td>
<td><code>CTT TTT CAA GTC GTT AGC GAG</code></td>
<td>qRT-PCR</td>
</tr>
<tr>
<td><em>nixA</em></td>
<td>EB100</td>
<td>1135941-1135961</td>
<td><code>AAA GAA CGA TCC TAG AAA CGC</code></td>
<td>qRT-PCR</td>
</tr>
<tr>
<td><em>ureA</em></td>
<td>EB118</td>
<td>77345-77365</td>
<td><code>TTT AAC GCG TTG GTT GAT AGG</code></td>
<td>qRT-PCR</td>
</tr>
<tr>
<td><em>ureA</em></td>
<td>EB119</td>
<td>77262-77282</td>
<td><code>TAG TTG TCA TCG TTT TTA GCG</code></td>
<td>qRT-PCR</td>
</tr>
<tr>
<td><em>fur</em></td>
<td>EB122</td>
<td>1090515-1090535</td>
<td><code>TGT TTG CAT TGC GGT AAG ATC</code></td>
<td>na qRT-PCR</td>
</tr>
<tr>
<td><em>fur</em></td>
<td>EB123</td>
<td>1090629-1090649</td>
<td><code>TTG GCA TTC TTT ACA CCA CAC</code></td>
<td>na qRT-PCR</td>
</tr>
<tr>
<td><em>nikR</em></td>
<td>EB120</td>
<td>1398443-1398463</td>
<td><code>AGC ATA ATT GCT TGG AGA CGA</code></td>
<td>qRT-PCR</td>
</tr>
<tr>
<td><em>nikR</em></td>
<td>EB121</td>
<td>1398348-1398368</td>
<td><code>AGA CGC CTT AGT CAA TTT AGC</code></td>
<td>qRT-PCR</td>
</tr>
<tr>
<td><em>nikRcat</em></td>
<td>EB239</td>
<td>1398792-1398812</td>
<td><code>GCT TTT AAC GAA CTC ATG CCA</code></td>
<td>qRT-PCR</td>
</tr>
<tr>
<td><em>nikRcat</em></td>
<td>EB240</td>
<td>na</td>
<td><code>acc gtc ata cta tat gtg cag</code></td>
<td>qRT-PCR</td>
</tr>
<tr>
<td><em>hpn</em></td>
<td>EB124</td>
<td>1497139-1497156</td>
<td><code>GAA CAA CAC GGC GGG CAC</code></td>
<td>qRT-PCR</td>
</tr>
<tr>
<td><em>hpn</em></td>
<td>EB125</td>
<td>1497004-1497023</td>
<td><code>GTG GCA GCA ACC TTC TTC TT</code></td>
<td>qRT-PCR</td>
</tr>
<tr>
<td><em>recA</em></td>
<td>EB101</td>
<td>163038-163058</td>
<td><code>GAT TGA CTC TAT TTC TAC AGG</code></td>
<td>qRT-PCR</td>
</tr>
<tr>
<td><em>recA</em></td>
<td>EB102</td>
<td>163147-163167</td>
<td><code>AAT ATG CAA GCT TAG AGT GGT</code></td>
<td>qRT-PCR</td>
</tr>
</tbody>
</table>
a Underlined bases correspond to restriction sites and capitalized bases correspond to regions complementary to the PyloriGene coordinates listed.

b Not applicable.
References


104


Interaction: Metal Ion Requirements and Sequence Specificity.


Chapter 3

An intact urease assembly pathway is required to compete with NikR for nickel ions in *Helicobacter pylori*

This chapter was published previously and is reprinted here with the permission of the American Society of Microbiology.

Overview

A commonly described mechanism central to transition metal homeostasis is metal-dependent transcriptional regulation. However, how transcription factor activity is coordinated with additional metal-dependent pathways in the cell is an often overlooked aspect of metal homeostasis. In addition, intracellular trafficking of transition metals, particularly metals such as copper and nickel for which metal-specific chaperone proteins have been identified, is also poorly understood. This problem is particularly relevant for *Helicobacter pylori* nickel homeostasis, where the Ni\(^{2+}\)-dependent transcription factor NikR differentially regulates the expression of genes encoding proteins that participate in various nickel-requiring pathways. In the following study, I took a genetic approach to identify nickel-requiring pathways in *H. pylori* that influence Ni\(^{2+}\)-dependent regulation by NikR. The results indicate that a hierarchy of nickel trafficking exists in *H. pylori*, which represents an underappreciated aspect of cellular transition metal homeostasis. Although the effects of disrupting one metal-dependent pathway on other metal-dependent pathways in a cell are not known for other systems, it is likely that preferential metal trafficking exists for additional cell types and metals, given that transcriptional regulation is a common feature of metal homeostasis and many pathways requiring a single metal are present in all cells.
Abstract

The toxicity of excess intracellular transition metals demands that cells tightly control metal trafficking in the cytoplasm. However, how newly imported or recycled cytoplasmic metal ions are allocated to different destinations in the cell is not well understood. The Gram-negative bacterium *Helicobacter pylori* possesses two nickel-dependent enzymes, urease and hydrogenase, and the Ni$^{2+}$-dependent transcription factor NikR. We examined the effects of deleting genes required for urease and hydrogenase assembly on NikR activation in *H. pylori* strains 26695 and G27. Disruption of the urease assembly pathway, but not loss of urease activity, increased NikR activity under Ni$^{2+}$-limiting conditions as measured by increased NikR-dependent repression of *nixA* and *frpB4* transcripts as well as decreased $^{63}$Ni accumulation in cells. In addition, the hydrogenase assembly chaperone SlyD partially compensated for the absence of HypA in urease assembly in *H. pylori* strain 26695, but not G27. These results demonstrate the presence of competition for nickel ions between a Ni$^{2+}$-dependent enzyme assembly pathway and NikR. Additionally, this work establishes a new system for investigating the mechanisms of intracellular metal trafficking and homeostasis.
Introduction

Transition metals are used by an estimated one-third of all proteins (25). They act as cofactors to catalyze distinct biochemical reactions, stabilize structure, or act as modulators of protein activity. However, high intracellular metal levels can be toxic due to non-specific binding to proteins or other molecules, or by catalysis of deleterious reactions, such as the generation of reactive oxygen species. A diverse complement of proteins is dedicated to the acquisition, trafficking, and regulation of intracellular metal ions. The mechanisms by which each cell integrates these activities to allocate the appropriate proportion of metal to different metal-binding proteins to ensure optimum activity but prevent toxicity are not well understood.

Studies of the equilibrium metal-binding properties of proteins important for metal homeostasis have indicated that the metal chelation capacity of cells exceeds total intracellular metal concentrations under metal-limiting conditions (25, 47). For example, Cu$^{1+}$- and Zn$^{2+}$-responsive transcriptional regulators from *E. coli* avidly bind their cognate metals, $K_d \approx 10^{-21} – 10^{-15}$ M (13, 47). Because a single free metal ion in an *E. coli* cell has a concentration of $10^{-9}$ M (1 nM) under equilibrium conditions, the metalloregulators would be expected to bind any available metal ion. These observations have lead to the proposal that there are no “free” transition metal ions inside the cell (11, 13, 25, 47, 50). Instead, a combination of protein-protein interactions and metal transfer reactions are proposed to ensure the fidelity of metal trafficking to metal-dependent enzymes.
Detailed investigation of this hypothesis is encumbered by the complexity of metal homeostasis networks that often contain numerous essential metalloenzymes. It is thus difficult to perturb metal ion homeostasis and maintain cell viability. Microbial nickel physiology provides an ideal system for studying metal homeostasis due to the small number of enzymes that require nickel ions (43). Importantly, in several bacteria the loss of Ni$^{2+}$-dependent enzyme activity is not lethal under laboratory growth conditions, facilitating the study of the effects of eliminating these proteins on other nickel-dependent activities in the cell.

*Helicobacter pylori* is a Gram-negative bacterium that colonizes the stomach of primates (3). Nickel metabolism is critical to the survival of *H. pylori* in the acidic environment of the stomach because the two Ni$^{2+}$-dependent enzymes urease and hydrogenase are required for efficient colonization of animal models of infection (21, 22, 45), but are non-essential under laboratory growth conditions. Urease hydrolyzes urea to buffer cells from acid (38). The [Ni-Fe] hydrogenase oxidizes H$_2$, using the available reducing equivalents to generate energy by respiration (36). Additionally, urease is produced at extremely high levels in the cell [~6% cytoplasmic protein; (29)], resulting in a substantial intracellular nickel binding capacity.

Urease and hydrogenase both require conserved, well-orchestrated pathways for metallocofactor assembly, including an absolute requirement for nickel-insertion chaperones under metal-limiting conditions. Studies of urease
assembly in *Klebsiella aerogenes* by Hausinger and co-workers identified key aspects of this process [reviewed in (33, 43)], including a role for UreE in binding and inserting nickel into the UreAB catalytic subunits (14, 56). Similarly, Böck and co-workers have identified many proteins essential for hydrogenase assembly in *E. coli* [reviewed in (8)], including the two chaperones, HypA and HypB, required for the nickel insertion step (30, 35). In addition, the *E. coli* chaperone SlyD has been shown to participate in hydrogenase assembly in association with HypB (34, 71). Both enzyme assembly pathways are present in *H. pylori* and, interestingly, the hydrogenase chaperones HypA and HypB are required for urease assembly in *H. pylori* (46). SlyD function in *H. pylori* has not been examined, although a potential SlyD-HypB interaction has been suggested from a proteomics experiment that pulled down a large complex of hydrogenase assembly proteins (60).

*H. pylori* nickel homeostasis is maintained in part by the Ni$^{2+}$-responsive transcription factor NikR. Several genes are activated or repressed by NikR in response to increased extracellular nickel, including up-regulation of *ureAB* (23) (encoding the urease structural subunits) and *hpn* (15) (encoding a Ni$^{2+}$ storage protein). NikR also represses genes associated with nickel import, including *nixA* (23, 68), *fecA3* (24), *frpB4* (18, 24) and *exbBDtonB* (24), as well as *fur* (19, 65) (encoding an Fe(II)-dependent transcription factor), and *nikR* itself (15). Direct interactions between NikR and each of these promoters have been shown biochemically (1, 5, 15, 18-20, 23, 24). NikR has also been linked to the growth
and adaptation of *H. pylori* to acidic conditions (10, 65), an environment in which urease activity is critical. NikR requires Ni\(^{2+}\) to bind specific DNA sequences (1, 5, 19, 20, 70), which results in the repression of all currently known nickel import genes (*nixA*, *fecA3*, *frpB4* and *exbBDtonB*) (18, 23, 24, 68). It is important, therefore, that Ni\(^{2+}\)-dependent enzyme biosynthesis pathways acquire Ni\(^{2+}\) before NikR is activated and repression of nickel-uptake genes occurs.

The purpose of this study was to determine whether nickel-dependent enzyme biosynthesis pathways compete with NikR for Ni\(^{2+}\) in the cell. We find that disruption of genes required for urease assembly, but not inhibition of urease activity itself, results in increased NikR-dependent repression of nickel-import genes under conditions of limiting extracellular nickel, when compared to a parent strain. The demonstration of a nickel trafficking network in *H. pylori* establishes a simple, yet robust, model system to investigate mechanisms required for the maintenance and regulation of intracellular metal homeostasis.
Results

Conditions that alter NikR activity are easily detected by monitoring levels of transcripts directly regulated by NikR (15, 18, 19, 23, 24, 65, 68). To control for variation in nickel concentration between different batches of Brucella broth and fetal bovine serum, both components of the growth medium used here, cells (OD$_{600}$ = 1.0) were exposed to either 100 $\mu$M of dimethylglyoxime (DMG), a Ni$^{2+}$-selective chelator, or 100 $\mu$M NiCl$_2$ for 40 min to generate Ni$^{2+}$-restricted and Ni$^{2+}$-replete conditions. An S1 nuclease protection assay was used to determine the levels of $nixA$, $frpB4$ and $ureA$ transcript levels (see Methods). Consistent with previous studies (18, 23, 24, 68), $nixA$ and $frpB4$ were repressed five- and six-fold, respectively, (Figure 3.1a, b), and $ureA$ levels increased 1.2-fold in a Ni$^{2+}$- and NikR-dependent manner (Figure 3.1c). The timescale for the changes in transcript levels observed here is similar to that seen in a previous study of $nixA$ regulation (68). Additionally, under these conditions regulation of all three transcripts was not affected by deletion of $fur$ (Figure 3.1), which has been shown to affect $nikR$ expression and shares a subset of target genes with NikR (19). Based on these data, the $nixA$ and $frpB4$ transcripts were chosen as reporters of NikR activity for subsequent experiments because of their greater response to changes in Ni$^{2+}$ levels.

*Gene deletions in the urease assembly pathway increase NikR activity under Ni$^{2+}$-limiting conditions.*
Figure 3.1. Ni\textsuperscript{2+} - and NikR-dependent transcriptional regulation in *H. pylori* 26695 measured by S1 nuclease protection. Cells were exposed to DMG (D) or NiCl\textsubscript{2} (N) for 40 min and levels of nixA (a), frpB4 (b) or ureA (c) transcript were measured using 10 µg input RNA. Data in each panel was normalized to the transcript level of 26695 cells exposed to DMG. Plotted data are the average of three independent cultures and error bars represent the calculated standard error. One representative gel for each probe is shown and the arrowhead indicates bands corresponding to the probes for nixA, frpB4 or ureA. Upper bands in all gels represent undigested probe. P-values are indicated (*, <0.1; **, <0.05).
Individual deletions of \textit{ureE}, \textit{hypA}, \textit{hypB} and \textit{slyD} were constructed in strain 26695 (see Methods) to test for effects on NikR-dependent transcriptional repression. Strains lacking \textit{ureE} or \textit{hypB} expressed significantly less \textit{nixA} and \textit{frpB4} after treatment with DMG (Figure 3.2a, b) compared to the parent strain, suggesting that NikR activity was increased in these mutants. Deletion of \textit{hypA} or \textit{slyD} showed no difference in \textit{nixA} or \textit{frpB4} levels. Because the hydrogenase chaperones HypA and HypB are required for urease assembly in \textit{H. pylori} \cite{46}, but only deletion of \textit{hypB} affected NikR-regulated transcripts, we explored whether SlyD and HypA have redundant roles in \textit{H. pylori} nickel physiology that mask effects on NikR. A \textit{hypA-slyD} double mutant displayed significantly less \textit{nixA} and \textit{frpB4} transcript relative to the parent strain or the single mutants in response to DMG treatment (Figure 3.2a, b).

In all cases the decrease in \textit{nixA} and \textit{frpB4} transcripts in DMG-exposed cells was NikR-dependent as deletion of \textit{nikR} in the \textit{ureE}, \textit{hypB} and \textit{hypAslyD} backgrounds resulted in constitutively high \textit{nixA} and \textit{frpB4} levels (Figure 3.2c, d). Inhibition of translation by treating cells with erythromycin or chloramphenicol had no effect on NikR activity in the different mutant strains, ruling out the possibility that de novo synthesis of NikR is responsible for the increase in activity (data not shown). These data demonstrate that cells lacking UreE, H ypB, or HypA and SlyD have increased levels of active NikR under Ni$^{2+}$-limiting conditions, and suggest that competition for nickel ions exists between urease assembly and NikR.
Figure 3.2. Deletion of *ureE*, *hypB*, *hypA-slyD* or *ureAB* results in NikR activation under Ni$^{2+}$-limiting conditions. Cells were exposed to DMG (D) or NiCl$_2$ (N) for 40 min and levels of *nixA* (a and c) and *frpB4* (b and d) transcript were measured using 10 µg input RNA. Data in each panel was normalized to the transcript level of 26695 cells exposed to DMG. Plotted data are the average of three independent cultures and error bars represent the calculated standard error. One representative gel for each probe is shown and the arrowhead indicates bands corresponding to the probes for *nixA* or *frpB4*. Upper bands in all gels represent undigested probe. P-values are indicated (*, <0.1; **, <0.05).
Urease (UreAB) and hydrogenase (HydABC) are the terminal destinations of nickel handled by the chaperones and also represent significant Ni\(^{2+}\)-binding capacity within the cell. A *ureAB* mutant strain displayed significantly less *nixA* and *frpB4* relative to the parent strain in the presence of DMG (Figure 3.2a, b) and the decrease in both transcripts was NikR-dependent (Figure 3.2c, d). In contrast, deletion of *hydABC* had no effect on *nixA* and *frpB4* compared to the parent strain.

Genes involved in other aspects of nickel homeostasis had no effect on NikR function. Single or double mutant strains deleted for genes involved in Ni\(^{2+}\) transport [*nixA* (39) and *exbBD*tonB (53)], Ni\(^{2+}\) storage [*hpn* and *hpn-like*; (6, 26, 40, 55)] or Ni\(^{2+}\) efflux [*cznABC*; (58)] displayed NikR-dependent regulation similar to the parent strain (data not shown). These results are consistent with multiple pathways for Ni\(^{2+}\) import, as has been established (53), and also suggest that processes likely acting downstream of Ni\(^{2+}\)-dependent enzyme assembly (ie, Ni\(^{2+}\) storage, efflux under excess Ni\(^{2+}\) levels) are not in competition with NikR.

Together, these results support the idea that disruption of the urease assembly pathway increases NikR activation under Ni\(^{2+}\)-limiting conditions, when competition for Ni\(^{2+}\) is likely at its highest levels because urease active sites are not saturated (15, 54, 65).

*Urease activity is not required for increased NikR activity.*
One consequence of the deletion of nickel insertion chaperones is a loss of Ni\textsuperscript{2+}-dependent enzyme activity, i.e. urease, which may affect NikR activity. To test this possibility urease activity was measured in soluble extracts (55) from cells exposed to DMG or NiCl\textsubscript{2} for 40 min. Strain 26695 exhibited urease activities of 13.55 and 20.71 µmol NH\textsubscript{3}/min*mg protein in response to DMG or NiCl\textsubscript{2}, respectively (Table 3.1). Urease activity was increased 1.3-fold in a nikR mutant strain under both nickel-limiting and nickel-replete conditions. As expected, the ureE and hypB mutants displayed significantly decreased urease activity (≥100-fold; Table 3.1). The hypA mutant exhibited a partial decrease in urease activity (2- to 3-fold) compared to the parent strain, while the slyD mutant was unaffected. However, the hypA-slyD double mutant exhibited significantly reduced urease activity (≥100-fold) under both conditions. Deletion of hydABC had no effect on urease activity. The loss of urease activity in the hypB and hypA-slyD mutant strains was not dependent on NikR because deletion of nikR in either strain did not restore activity (data not shown). Together these data suggest that HypA and SlyD function redundantly in urease assembly in strain 26695 and provide evidence that SlyD plays a role in nickel physiology in H. pylori, as has been suggested by a recent proteomic study (60).

To determine if decreased urease activity is directly responsible for increased NikR activation, urease activity was inhibited in 26695 cells by treatment with flurofamide, a direct competitive inhibitor of urease activity that requires Ni\textsuperscript{2+} insertion into the enzyme (2, 48). Flurofamide addition (100 µM) decreased
### TABLE 3.1. Urease activity in different *H. pylori* strains

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Urease activity&lt;sup&gt;a&lt;/sup&gt; (µM NH₃ min⁻¹ mg protein⁻¹)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>26695</td>
<td>G27</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DMG</td>
<td>NiCl₂</td>
<td>DMG</td>
</tr>
<tr>
<td>wild-type</td>
<td>13.55 (±0.52)</td>
<td>20.71 (±1.88)</td>
<td>25.76 (±1.98)</td>
</tr>
<tr>
<td><em>nikR</em></td>
<td>17.64 (±1.43)*</td>
<td>26.80 (±0.89)*</td>
<td>29.56 (±0.95)*</td>
</tr>
<tr>
<td><em>ureE</em></td>
<td>0.11 (±0.03)**</td>
<td>0.24 (±0.07)**</td>
<td>0.13 (±0.02)**</td>
</tr>
<tr>
<td><em>hypA</em></td>
<td>4.76 (±0.31)**</td>
<td>10.52 (±0.53)*</td>
<td>0.05 (±0.02)**</td>
</tr>
<tr>
<td><em>hypB</em></td>
<td>0.09 (±0.00&lt;sup&gt;b&lt;/sup&gt;)**</td>
<td>0.23 (±0.02)**</td>
<td>0.12 (±0.00)**</td>
</tr>
<tr>
<td><em>slyD</em></td>
<td>13.96 (±0.56)</td>
<td>25.80 (±0.58)</td>
<td>32.92 (±2.53)*</td>
</tr>
<tr>
<td><em>hypAslyD</em></td>
<td>0.07 (±0.01)**</td>
<td>0.23 (±0.04)**</td>
<td>0.01 (±0.00)**</td>
</tr>
<tr>
<td><em>ureAB</em></td>
<td>0.04 (±0.04)**</td>
<td>0.01 (±0.00)**</td>
<td>0.01 (±0.00)**</td>
</tr>
<tr>
<td><em>hydABC</em></td>
<td>15.21 (±5.05)</td>
<td>25.26 (±3.15)</td>
<td>nd&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>wild-type</td>
<td>14.38 (±1.59)</td>
<td>0.13 (±0.01)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Reported values are the average of three independent cultures with the standard error for each strain and condition.

<sup>b</sup> Error is <0.01. The lowest value measurable by this method is A₆₂₅ = 0.001; ~0.5-1 nM NH₃.

<sup>c</sup> Not determined.

* P < 0.1, mutant vs parent strain; ** P < 0.01, mutant vs parent strain.
urease activity >100-fold, but had no affect on \textit{nixA} and \textit{frpB4} levels (Table 3.1, Figure 3.3). A DMSO control had no effect in either assay. These data suggest that disruption of the urease assembly pathway in the chaperone mutants, rather than loss of urease activity, is responsible for NikR activation under Ni\textsuperscript{2+}-limiting conditions, i.e., Ni\textsuperscript{2+} flux through the assembly pathway is necessary for successful competition with NikR for Ni\textsuperscript{2+}.

We note that the urease activity in the \textit{hypB} mutant in the presence of added NiCl\textsubscript{2} are much lower than that reported for the \textit{hypB} mutant in a previous study (46). In that report, a different \textit{H. pylori} parent strain was used and cells were grown on solid media for a longer time prior to measurement of urease activity. The latter may diminish the decrease in activity observed in cells with a less efficient assembly pathway.

\textit{NikR activity inversely correlates with} \textit{\textsuperscript{63}Ni accumulation}.

A likely consequence of the premature activation of NikR under Ni\textsuperscript{2+}-limiting conditions is decreased Ni\textsuperscript{2+} accumulation due to reduced nickel transporter levels, because of reduced \textit{frpB4} and \textit{nixA} transcript levels (Figure 3.2). \textit{\textsuperscript{63}Ni} accumulation was measured in cells exposed to either low (10 nM \textit{\textsuperscript{63}NiCl\textsubscript{2}}) or high (50 nM \textit{\textsuperscript{63}NiCl\textsubscript{2}}/100 \mu M NiCl\textsubscript{2}) Ni\textsuperscript{2+} concentrations (see Methods). The 26695 parent strain accumulated \textasciitilde6000-fold higher \textit{\textsuperscript{63}Ni} in high vs low NiCl\textsubscript{2} conditions (Figure 3.4). \textit{\textsuperscript{63}Ni} accumulation in a \textit{nikR} mutant was increased 1.2-fold relative to the parent strain under nickel-limiting conditions, consistent with a
Figure 3.3. Inhibition of urease activity does not activate NikR. Cells were exposed to DMSO (D) or flurofamide (F) for 40 min and levels of \textit{nixA} (a) and \textit{frpB4} (b) transcript were measured using 10 μg input RNA. Data in each panel was normalized to the transcript level of 26695 cells exposed to DMSO. Plotted data are the average of three independent cultures and the error bars represent the calculated standard error. One representative gel for each probe is shown and the arrowhead indicates bands corresponding to the probes for \textit{nixA} or \textit{frpB4}. Upper bands in both gels represent undigested probe. P-values are indicated (*, <0.1; **, <0.05).
Figure 3.4. $^{63}$Ni accumulation in mutant strains inversely correlates with NikR activity. Cells were incubated with 10 nM $^{63}$NiCl$_2$ (a and c) or 50 nM $^{63}$NiCl$_2$ and 100 µM NiCl$_2$ (b and d) for 40 min. $^{63}$Ni levels were measured as described in Methods. Reported values are the average of three independent cultures with the standard error for each strain. Total $^{63}$Ni values varied between experiments, but relative accumulation between different strains was reproducible. P-values are indicated (*, <0.1; **, <0.05).
low level of NikR-dependent repression of \textit{nixA} and \textit{frpB4} under this condition (Figure 3.2c, d). The \textit{ureE}, \textit{hypB}, \textit{hypAslyD} and \textit{ureAB} strains accumulated less $^{63}\text{Ni}$ relative to the parent strain under both Ni$^{2+}$-limiting and Ni$^{2+}$-replete conditions, although the chaperone mutants showed a greater difference relative to the parent strain than the \textit{ureAB} strain (Figure 3.4a, b). The decrease in $^{63}\text{Ni}$ accumulation in the urease pathway mutants reflected increased NikR activity, since deletion of \textit{nikR} in each mutant background restored $^{63}\text{Ni}$ accumulation to levels similar to the \textit{nikR} mutant strain (Figure 3.4c, d). In contrast, the \textit{hypA}, \textit{slyD} and \textit{hydABC} strains took up levels of $^{63}\text{Ni}$ similar to the parent strain under both conditions. These data further support an increase in NikR activity in the chaperone mutants under Ni$^{2+}$-limiting conditions and indicate that the decrease in \textit{nixA} and \textit{frpB4} levels results in decreased NixA and FrpB4 protein levels during the course of this experiment (40 min), consistent with a previous report of the rate of NixA turnover under similar conditions (68).

\textit{The effect of a hypA deletion on NikR activity shows strain variability.}

\textit{H. pylori} is well known for inter-strain variability in gene content and physiology (32, 52). To determine if urease assembly proteins affect NikR activation similarly in a second strain background, individual deletions of \textit{ureE}, \textit{hypA}, \textit{hypB}, \textit{slyD} and \textit{ureAB} as well as a double \textit{hypA-slyD} mutant were constructed in \textit{H. pylori} strain G27, a clinical isolate commonly used in laboratory studies (16). NikR activity in each strain was assayed by measuring \textit{frpB4} levels.
Similar to strain 26695, the *ureE*, *hypB* and *ureAB* deletions decreased *frpB4* levels under Ni\(^{2+}\)-limiting conditions (Figure 3.5a). However, the G27 *hypA* mutant displayed decreased *frpB4* levels under this condition, while the G27 *slyD* mutant had *frpB4* levels similar to the parent strain. These data indicate that HypA and SlyD function differently in urease assembly and Ni\(^{2+}\) homeostasis in G27 and 26695. A hydrogenase (*hydABC*) mutant in strain G27 was not examined given the absence of any affect on NikR activity in strain 26695.

Urease activity in the G27 *ureE*, *hypA*, *hypB*, *hypA-slyD* and *ureAB* mutants displayed the same trends as for strain 26695 mutants (Table 3.3), although the basal levels of the G27 parent strain were higher (25.5 and 36.6 \(\mu\)mol NH\(_3\)/min*mg protein under Ni\(^{2+}\)-limiting and Ni\(^{2+}\)-replete conditions). Differences in basal urease activities in different strain backgrounds have been observed before (9). Similar to all strains examined previously (46, 66), deletion of *ureE*, *hypB* and *ureAB* in the G27 background resulted in significantly reduced urease activity (Table 3.3). Interestingly, the G27 *hypA* mutant displayed a >100-fold decrease in urease activity, which is in contrast to a *hypA* mutant in strain 26695 (this study) but similar to a *hypA* mutant in strain 43504 (46). These data suggest that there are mechanistic differences in urease assembly amongst *H. pylori* strains, but whether this variability arises from differences in efficiency of Ni\(^{2+}\)-insertion due to mutations in the chaperones or results from other differences in physiology is unclear.
Figure 3.5. Gene deletions in *H. pylori* strain G27 show similar effects on nickel physiology. (a) S1 nuclease protection measuring *frpB4* levels using 10 µg input RNA from DMG (D) or NiCl$_2$ (N) exposed cells. The transcript level of each strain was normalized to the transcript level of G27 cells exposed to DMG. Plotted data are the average of three independent cultures and error bars represent the standard error. One representative gel is shown and the arrowhead indicates bands corresponding to *frpB4*. Other bands are likely cross-reacting mRNA.
Ni accumulation in cells exposed to (b) 10 nM $^{63}\text{NiCl}_2$ or (c) a mixture of 50 nM $^{63}\text{NiCl}_2$ and 100 µM NiCl$_2$ for 40 min. The average of three independent cultures with the standard error is plotted. P-values are indicated (*, <0.1; **, <0.05).
The G27 parent strain accumulated ~1100-fold more $^{63}$Ni in high vs low Ni$^{2+}$ conditions (Figure 3.5b). A nikR mutant strain accumulated 1.6- and 1.1-fold more $^{63}$Ni relative to the parent strain under Ni$^{2+}$-limiting and Ni$^{2+}$-replete conditions, respectively. $^{63}$Ni import in the urease pathway mutant strains (ureE, hypA, hypB, hypAslyD and ureAB) was decreased relative to the parent strain and inversely correlated with NikR activity under Ni$^{2+}$-limiting conditions (Figure 3.5a, b). Under Ni$^{2+}$-replete conditions the ureE and hypB mutants also accumulated significantly less $^{63}$Ni than the parent strain (Figure 3.5c). Conversely, the hypA mutant (and the hypAslyD double mutant) showed significantly increased $^{63}$Ni accumulation relative to the parent strain, which contrasts with the data for these deletions in strain 26695 and further supports a distinction between HypA activity in the two strains. A mechanistic basis for this difference is not apparent and was not pursued further in this work.
Discussion

The difficulty of directly monitoring small pools of transition metal ions both spatially and temporally in a bacterial cell demands a combination of experimental approaches to dissect metal trafficking pathways. The known nickel-dependent enzyme assembly pathways of *H. pylori* and the defined readout of NikR activity provide a relatively simple system with which to elucidate mechanisms of intracellular metal trafficking. We find that an intact urease assembly pathway preferentially secures nickel ions under non-saturating concentrations, as disruption of this pathway increases NikR activity at the same ambient extracellular nickel concentration. Increased NikR activity was observed as a decrease in NikR-regulated transcript levels, which was additionally manifested as a decrease in $^{63}$Ni accumulation. These effects were observed in short time frames (< 40 min) and did not require new protein synthesis, suggesting that relatively rapid responses to changes in Ni$^{2+}$ availability do not depend upon turnover of NikR protein.

NikR activation in *H. pylori* could be accomplished in one of two ways with respect to external nickel levels. If competition for nickel occurs, NikR activity would be predicted to change in response to altered availability of metal in the cytoplasm, and independently of changes in extracellular nickel. In the absence of competition, NikR activity would be expected to correlate with a fixed, i.e. threshold, intracellular nickel concentration that is independent of Ni$^{2+}$-dependent enzyme expression or Ni$^{2+}$-dependent enzyme biosynthesis, but dependent on
the total metal concentration of the system. The observation that NikR activity increases in urease assembly mutants at the same total nickel concentration relative to the parent strain (Figure 3.2) indicates that intracellular nickel competition exists and that the urease assembly pathway preferentially acquires nickel ions.

Competition between NikR and other nickel-trafficking pathways has also been observed in *E. coli*, where the Ni\(^{2+}\) efflux protein RcnA acts to limit premature activation of NikR activity under nickel-limiting conditions (31). Deletion of *rcnA* results in increased NikR-dependent repression of nickel uptake genes, a decrease in total intracellular Ni\(^{2+}\), and a diminution of Ni\(^{2+}\)-dependent enzyme activity compared to a wild-type strain under the same conditions. In *H. pylori*, deletion of genes encoding the nickel efflux proteins CznABC did not affect NikR activity, suggesting that differences in nickel trafficking may exist among disparate bacteria. Given the different nickel physiologies of each organism, including the high urease expression in *H. pylori*, some variation in nickel partitioning is not unexpected.

Interestingly, additional genes linked to nickel physiology in *H. pylori* did not compete with NikR for nickel ions. Deletion of the genes encoding the [Ni-Fe] hydrogenase structural subunits had no effect on NikR activity (Figure 3.2a, b). Similarly, deletion of genes encoding the Ni\(^{2+}\) storage proteins Hpn and Hpn-like did not affect NikR or urease activity under the Ni\(^{2+}\)-limiting or Ni\(^{2+}\)-replete conditions used in this study (data not shown). These results suggest a lack of
competition between these Ni\(^{2+}\)-binding proteins and NikR. Hydrogenase in \(H. pylori\) strain 43504 is expressed and active under growth conditions similar to those used in this study (6, 7, 45, 55) so this nickel pool is either small relative to that for urease or hydrogenase acquires nickel ions in a manner that does not compete with NikR.

The loss of the Ni\(^{2+}\) storage proteins Hpn and Hpn-like probably has little effect on NikR function because these proteins likely acquire nickel ions under Ni\(^{2+}\)-replete conditions, when NikR is already fully active. A previous study observed an increase in urease activity under low Ni\(^{2+}\) conditions when \(hpn, hpn\)-like or both genes were deleted (55), suggesting that the storage proteins can, in fact, compete for nickel ions. The discrepancy between that study and the observations reported here is likely due to the significantly different growth conditions used, late-log liquid cultures in the present work vs 2 d on solid media or 24 h in BHI broth in (55). Differences in the nickel physiologies of the parent strains used in this and the previous study could also account for this apparently contradictory result.

The identification of a hierarchy for nickel ion trafficking in \(H. pylori\) that favors urease assembly over NikR defines a system with which to establish the mechanistic basis for preferential metal targeting in cells. Because the deletion of several genes linked to the same pathway produce the same effect on NikR function, it seems likely that the encoded proteins form a tightly coupled pathway. The presence of an oligomeric complex seems most plausible based on studies.
of urease assembly (14, 42, 56, 57, 60). Previous biochemical studies of urease assembly suggest a possible mechanistic explanation for the competition we observed. Notably, NikR displays an affinity for Ni\(^{2+}\) [2 pM (1, 5); 12 nM (69)] that is several orders of magnitude tighter than those reported for UreE or HypA [1 \(\mu\)M (6, 37)] under similar in vitro conditions. However, Hausinger and co-workers have shown that *K. aerogenes* UreE inserts Ni\(^{2+}\) into apo-urease in the presence of strong Ni\(^{2+}\) chelators (iminodiacetic or nitrilotriacetic acid), indicating that UreE, in conjunction with the urease assembly complex, shields nickel ions from chelation (56). There are other cases for the role of chaperone dependent metal-transfer reactions in metalloenzyme assembly in the face of competition. For example, the *Saccharomyces cerevisiae* yeast superoxide dismutase chaperone (yCCS) inserts Cu\(^{1+}\) into apo-SOD in the presence of the copper chelators BCS and EDTA (50). Full or partial reconstitution of *H. pylori* urease assembly in vitro will be necessary to analyze Ni\(^{2+}\) competition biochemically.

Urease assembly in *H. pylori* appears more complex than for *K. aerogenes* because of the involvement of HypA and HypB (46). Whether these proteins play structural roles in urease assembly or bind and transfer nickel ions requires additional biochemical and genetic studies. Nonetheless, a plausible model for the competition that we have observed entails a set of Ni\(^{2+}\)-transfer reactions that are refractory to competition from Ni\(^{2+}\)-chelators, such as NikR. Intracellular nickel is available either from importers, or released upon degradation of Ni\(^{2+}\)-dependent enzymes or Ni\(^{2+}\)-binding proteins. Multiple examples of direct
chaperone-transporter interactions have been identified, including interactions between the *Synechocystis* Cu\(^{1+}\) chaperone Atx1 and the two Cu\(^{1+}\) transporters CtaA and PacS (63), *S. cerevisiae* Atx1 and the Cu\(^{1+}\) transporter Ccc2 (49), as well as the Cu\(^{1+}\) chaperone CopZ and the copper importer CopA in *Enterococcus hirae* (44). In the absence of a direct protein-protein interaction, other mechanisms may contribute to the preferential targeting of nickel ions to enzyme assembly. The intracellular localization of urease assembly near the inner membrane (66) could provide spatial separation of NikR and urease so that each would sense different local concentrations of nickel ions within the cell. A defective urease assembly pathway would allow newly transported nickel to disperse more widely throughout the cell, resulting in increased NikR activity.

These studies have provided some additional insights into *H. pylori* urease assembly independent of the competition with NikR, but also point to difficulties in defining protein function in different *H. pylori* strains. In particular, the different effects of the hypA and slyD deletions in strains 26695 and G27 suggest differences in nickel physiology and the efficiency of urease assembly between strains. In strain 26695, there appear to be partially redundant functions between HypA and SlyD with respect to urease activity, but this redundancy was not observed in strain G27. Additionally, deletion of hypA resulted in increased Ni\(^{2+}\) accumulation in strain G27, although an explanation for this observation is not apparent at this time.
SlyD participates in hydrogenase assembly in *E. coli* via a direct interaction with HypB (34, 71). A recent proteomics study with strain 26695 identified SlyD in a complex with other urease chaperones including HypB (60), so a role for *H. pylori* SlyD in urease assembly is not unexpected. Whether *H. pylori* SlyD participates in hydrogenase assembly has not been determined. One possible explanation for the disparate results in the two strains lies in protein sequence differences. HypA is completely conserved between *H. pylori* strains 26695 (61) and G27 (4), suggesting its intrinsic metal-binding properties are not altered. In contrast, SlyD contains four amino acids that differ between strains 26695 and G27: G23 to D, I26 to E, T43 to A, and I113 to T. The first three amino acid changes are located in the peptidyl-prolyl isomerase domain of SlyD, the activity of which is not required for hydrogenase assembly in *E. coli* (72). Notably, position 113 is located in the “flap” region of SlyD that, in *E. coli* SlyD, is required for interacting with HypB and for hydrogenase assembly (34). HypB contains three conservative amino acid differences between strains 26695 and G27: Q6 to K, V187 to I and K236 to R, with residue 6 located in a region of the protein analogous to the SlyD-interacting domain of *E. coli* HypB (34). The effects of the amino acid substitutions in different SlyD and HypB alleles can be examined using both genetic and biochemical approaches. It is also possible that the differences observed between strains are dependent on changes in expression levels of these chaperones or other proteins not examined in this study.
The major function attributed to *H. pylori* urease is neutralization of acid in the stomach (41, 51, 59), and urease activity significantly increases in response to a decrease in pH as a result of post-translational activation (10, 54, 65) and transcriptional induction (10, 65). An increase in the bioavailability of Ni\(^{2+}\) at acidic pH has been suggested to be one explanation for the increase in urease activity (64), raising the question of how NikR activity is modulated by pH. One prediction from our results is that an increasingly active urease assembly pathway, such as that which occurs in acid-exposed cells, will require an increase in the extracellular nickel concentration required to trigger NikR activation. Examining nickel competition under different growth conditions will test this hypothesis.

The dynamics of intracellular metal trafficking are poorly understood. We have taken advantage of the prominent nickel physiology of *H. pylori* to establish a robust system to study metal trafficking in cells. Our results indicate that the urease assembly pathway successfully out-competes the transcription factor NikR for Ni\(^{2+}\), despite the urease chaperones having significantly lower Ni\(^{2+}\) affinities compared to NikR. These results provide further insight into the nickel physiology of *H. pylori* and establish a framework to investigate the detailed mechanisms of intracellular Ni\(^{2+}\) competition, as well as more general features of cytoplasmic metal dynamics. Different transition metals are found sparingly within the cell (i.e., copper), used for one or two specific functions (i.e., nickel), or widely required (i.e., iron and zinc), making it likely that differences in
homeostatic mechanisms exist. Nonetheless, common features are also likely to be shared among homeostasis systems for different metals both within the same cell and between different cell types.
Materials and Methods

**Bacterial culture conditions and strain construction.**

*H. pylori* strains 26695 (from Helene Kling-Bäckhed and Jeffrey Gordon; Washington University School of Medicine) and G27 (from Daiva Dailidiene and Douglas Berg; Washington University School of Medicine) and their derivatives (see below) were maintained as glycerol stocks at -80°C and passaged no more than five times after recovery from stock. Cells were grown at 37°C under microaerobic conditions using a BD BBL GasPak Jar (Fisher, Pittsburgh, PA) with microaerobic gas packs (Mitsubishi Gas Chemical Co, VWR, Westchester, PA). Growth on solid medium used Brucella blood agar plates (BD Difco, Fisher) containing 10% horse blood (Colorado Serum Company, Denver, CO), 3 µg/ml vancomycin (Sigma, Saint Louis, MO), 1 µg/ml trimethoprim (Sigma) and 10 µg/ml amphotericin B (Sigma). Antibiotics were used at the following concentrations: 20 µg/ml chloramphenicol (Fisher), 15 µg/ml kanamycin (Fisher) and 10 µg/ml erythromycin (Fisher). Liquid growth used Brucella broth (BD Difco, Fisher) containing 5% fetal bovine serum (Sigma; initial pH of 7.0, adjusted using NaOH). For liquid growth, cells were incubated as described above, except 25 ml flasks containing 10 ml cultures with shaking (120 rpm). Liquid cultures were inoculated with cells grown for 24 h on Brucella blood agar plates after at least one passage from frozen stock, resuspended in liquid media and added to a final starting OD_{600} of 0.05. 40 min exposure to dimethylglyoxime
(DMG; Fisher), NiCl$_2$ (Fisher), DMSO (Fisher) and flurofamide (Tocris, Ellisville, MO) was carried out aerobically with shaking.

*H. pylori* 26695 and G27 were used as the parental strains from which all mutants were constructed. Mutant strains were constructed using double homologous recombination with overlapping PCR products (12). Briefly, three separate PCR reactions amplified ~500 bp regions upstream or downstream of the target open reading frame (ORF) from 26695 genomic DNA, and an antibiotic resistant gene from either plasmid pHel2 (*cat*), pHel3 (*aphA-3*) (28) (gifts from Rainer Haas; Max von Pettenkofer Institut für Hygiene und Medizinische Mikrobiologie) or genomic DNA from an *erm*-containing *H. pylori* strain [a gift from Daiva Dailidiene and Douglas Berg; Washington University School of Medicine; (17)]. Primer sequences are listed in Table 3.2. To ensure non-polar ORF deletions, the upstream region reverse primer contained three stop codons in each reading frame followed by a ribosome binding site (RBS). The downstream region forward primer contained a RBS followed by a start codon. PCR products were purified using a Qiagen PCR Purification Kit (Qiagen, Valencia, CA), mixed and used as the template for a second round of PCR. Purified PCR products were used to naturally transform 24 h plate-grown *H. pylori* (17). Cells were grown on solid media for an additional 20 h, then plated onto selective media. All gene deletions were initially constructed in *H. pylori* 26695. *H. pylori* strain G27 mutants were constructed by natural transformation
of genomic DNA from the corresponding 26695 mutant strain. All strains were checked for the expected deletions by PCR (Table 3.2).

RNA isolation and S1 nuclease protection assay.

Cells (OD$_{600}$ 1.0) were exposed to various compounds (see Results) for 40 min, immediately put on ice, and harvested by centrifugation at 4°C (2600 x g, 5 min). Cells were resuspended in 1 ml Trizol (Invitrogen) and RNA was isolated according to the manufacturer’s instructions. RNA was resuspended in 32 µl DEPC-H$_2$O and treated with 4 U DNase I (Invitrogen) for 30 min at 22°C, ethanol precipitated, quantified and stored at -80°C.

Nuclease protection assays were performed as previously described (27). Single-stranded DNA oligonucleotide probes (Invitrogen, Carlsbad, CA) complementary to specific transcripts (EB360 – nixA, EB357 – frpB4, EB346 – ureA; see Table 3.2) were end-labeled with $^{32}$P and purified using standard methods. Labeled probe (2 x 10$^4$ cpm) was added to 10 µg total RNA. Hybridized (55°C, overnight) and digested samples were ethanol precipitated and analyzed by denaturing gel electrophoresis (10% acrylamide). Gels were analyzed using a GE Healthcare Typhoon Trio Variable Mode Imager and ImageQuant Version 5.1 software. Bands representing specific transcripts were quantitated, normalized to the DMG-treated parent strain and averaged. RNA from three independent cultures of each strain and growth condition was
analyzed in parallel with the parent and nikR mutant strains used as controls. Each experiment was repeated at least twice.

$^{63}$Ni uptake assay

OD$_{600}$ values of three independent cultures of each strain were measured and either 10 nM $^{63}$NiCl$_2$ (specific activity 9.87 mCi mg$^{-1}$; Perkin-Elmer, Boston, MA) or 100 µM NiCl$_2$ + 50 nM $^{63}$NiCl$_2$ was added directly to each culture, followed by incubation at 37°C (40 min at 120 rpm). Two 1 ml aliquots of each culture were collected in duplicate and cells were harvested by centrifugation (16,000 x g, 1 min), rinsed with 900 µl 50 mM HEPES (pH 7.0), 50 mM EDTA and resuspended in 200 µl 10 µM acetic acid, then mixed with 1 ml of scintillation fluid (ScintiSafe; Fisher). $^{63}$Ni content was measured by scintillation counting on a Beckman LS7000 using a pre-programmed 10 min acquisition window (0 to 1.31 MeV). $^{63}$Ni counts per min (cpm) were converted to atoms of $^{63}$Ni per cfu and the average of each strain grown in triplicate was reported. OD$_{600}$ values were converted to colony-forming units (cfu) using a standard curve determined for cells grown under identical conditions. The reported $^{63}$Ni atoms/cell represent a lower limit on Ni$^{2+}$ content because of cold nickel present in the growth medium.

Urease assay.

Cells were grown and treated with DMG or NiCl$_2$ as described for RNA isolation. Soluble cell extracts were prepared and urease activity was measured
using a previously published protocol and with technical advice from Stéphane Benoit and Rob Maier (6, 55) with minor modifications. Cells were immediately put on ice, harvested by centrifugation at 4° C (2600 x g, 5 min), rinsed three times with 900 µl 50 mM HEPES (pH 7.0), resuspended in 750 µl of the same buffer and lysed by sonication (2 x 30 s pulses). Soluble extracts were collected after centrifugation of the resulting lysate (16,000 x g, 5 min). The total protein concentration of each sample was determined using the BioRad Protein Assay (BioRad, Hercules, CA). Samples were either used directly (low urease activity) or diluted 20-fold (high urease activity) in 50 mM HEPES (pH 7.0) or left undiluted before adding 5 µl to 245 µl 50 mM HEPES (pH 7.0) containing 25 mM urea (1 M freshly prepared stock in dd H₂O). The samples were mixed and incubated at 37° C for either 10 min (high activity) or 30 min (low activity). The NH₃ present in each sample was measured using the phenol-hypochlorite (Bertholot) reaction as previously described (67).
<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (PyloriGene sequences in italics)</th>
<th>PyloriGene Coordinates (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>EB085</td>
<td>gttagattcttggttaatcc</td>
<td>1399283 - 1399263</td>
</tr>
<tr>
<td>EB090</td>
<td>gcctaaaccctaaactagcc</td>
<td>1397817 - 1397837</td>
</tr>
<tr>
<td>EB153</td>
<td>ggagtttttgatgggctaacg</td>
<td></td>
</tr>
<tr>
<td>EB154</td>
<td>cccagtttgctgactgataattcatgcataactaaccctt</td>
<td>920350 - 920368</td>
</tr>
<tr>
<td>EB155</td>
<td>atccacttttcaatctatcgccggaataaccgatgcaaga</td>
<td>920013 - 919993</td>
</tr>
<tr>
<td>EB156</td>
<td>ctaagcgctcactaaatcg</td>
<td></td>
</tr>
<tr>
<td>EB157</td>
<td>gatattgctcaagtatcgac</td>
<td>75145 - 75125</td>
</tr>
<tr>
<td>EB160</td>
<td>tgattggctaatcgaattcc</td>
<td>73837 - 73857</td>
</tr>
<tr>
<td>EB164</td>
<td>aaaaagctgaaacagccgctaa</td>
<td>1189667 - 1189647</td>
</tr>
<tr>
<td>EB165</td>
<td>ttactggatgaatttttagtttgcattgggttcatctct</td>
<td>1189159 - 1189179</td>
</tr>
<tr>
<td>EB166</td>
<td>atggttcgtggttttatccatggtagtaaggtatagga</td>
<td>1188617 - 1188598</td>
</tr>
<tr>
<td>EB167</td>
<td>ttatccccataatcctttg</td>
<td></td>
</tr>
<tr>
<td>EB221</td>
<td>tgactaactagaggaataatacttaaaatgagaatatcacc*</td>
<td>*Complementary to pHel3</td>
</tr>
<tr>
<td>EB222</td>
<td>cattatccctccaggtactaaacaattcactcagaatctaa*</td>
<td>*Complementary to pHel3</td>
</tr>
</tbody>
</table>
EB223  tgactaactggaggaataatgcaattcacaagaattgatata**  **Complementary to pHel2
EB224  cattatcctccaggtatatattcagcaagtcttgtaa**  **Complementary to pHel2
EB225  ttattcctctagttagtcattgagagaatccctttttg
EB226  tagtacctggaggaataatgaatatcaatgaatagcgtctta
EB227  1398774 - 1398794
EB228  cattattccctccaggtatatattcagcaagtcttgtaa
EB229  ttattcctctagttagtcattgagagaatccctttttg
EB230  74747 - 74768
EB231  tagtacctggaggaataatgaatatcaatgaatagcgtctta
EB232  74232 - 74210
EB233  tttagaccggttttgtgcagttgggataaag
EB234  78467 - 78447
EB235  ttattcctctagttagtcattgagagaatccctttttg
EB236  77957 - 77977
EB237  taatacctggaggaataatgaatatcaatgaatagcgtctta
EB238  75526 - 75506
EB239  atttttgccttgcatcaagc
EB240  677444 - 677464
EB241  tttagaccggttttgtgcagttgggataaag
EB242  677957 - 677937
EB243  ttattcctctagttagtcattgagagaatccctttttg
EB244  681509 - 681529
EB245  tagtacctggaggaataatgaatatcaatgaatagcgtctta
EB246  682056 – 682036
EB247  tcagcgatacatctcaagcgc
EB248  682056 – 682036
EB249  accaaagtgtgtgtgatagc
EB250  75041 - 75061
EB251  agtttttctctgaagacataacaagatttggatgtaatgtagcaattttgttg
EB252  77976 - 78035
EB253  cctttttatctttttctaaaaatttagtcaagtttttaacctgaggt†
EB254  1584437 - 1584392
EB255  †Non-complementary bases

144
<table>
<thead>
<tr>
<th></th>
<th>Sequence</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>EB360</td>
<td>tcacagcgcaccccttttaagaaaaataaatcttttggtaattgt</td>
<td>1136897 - 1136941</td>
</tr>
<tr>
<td>EB390</td>
<td>cagtcacccaaatcggctacc</td>
<td>1089745 - 1089765</td>
</tr>
<tr>
<td>EB391</td>
<td>ttattcctcctagttagtcagctgatcttttccctttatccgt</td>
<td>1090211 - 1090191</td>
</tr>
<tr>
<td>EB392</td>
<td>taatacctggaggaataatgtgcacagagagatgaatgttaa</td>
<td>1090644 - 1090664</td>
</tr>
<tr>
<td>EB393</td>
<td>taagggctgtgagttgcttg</td>
<td>1091141 - 1091120</td>
</tr>
<tr>
<td>EB394</td>
<td>tgactaactaggaggaataatgaacgagaaaaatataaacac</td>
<td>Complementary to erm</td>
</tr>
<tr>
<td>EB400</td>
<td>catttacctcctcagtgattctattaataatttagct</td>
<td>Complementary to erm</td>
</tr>
<tr>
<td>EB401</td>
<td>catttacctcctcagtgattctattaataatttagct</td>
<td>Complementary to erm</td>
</tr>
<tr>
<td>EB461</td>
<td>gtgctgaatgaaaaattttcc</td>
<td>953309 - 953289</td>
</tr>
<tr>
<td>EB462</td>
<td>ttattcctcctagttagtcagttcttttctttttaaattttcg</td>
<td>952820 - 952845</td>
</tr>
<tr>
<td>EB463</td>
<td>tagtacacctggaggaataatggaaaaaacgtgaaattacc</td>
<td>952130 - 952111</td>
</tr>
<tr>
<td>EB464</td>
<td>tgatgtagcgcagttcataagc</td>
<td>951591 - 951612</td>
</tr>
</tbody>
</table>
Table 3.3. Bacterial strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>26695 gene number</th>
<th>Genotype</th>
<th>Primers(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>26695</td>
<td>—</td>
<td>Parent strain (61)</td>
<td>na</td>
</tr>
<tr>
<td>G27</td>
<td>—</td>
<td>Parent strain (16)</td>
<td>na</td>
</tr>
<tr>
<td>nikR(^b)</td>
<td>HP1338</td>
<td>nikR::aphA-3</td>
<td>EB085, EB225, EB226, EB090</td>
</tr>
<tr>
<td>nikRerm</td>
<td>HP1338</td>
<td>nikR::erm</td>
<td>EB085, EB225, EB226, EB090</td>
</tr>
<tr>
<td>fur</td>
<td>HP1027</td>
<td>fur::cat</td>
<td>EB390, EB391, EB392, EB393</td>
</tr>
<tr>
<td>nikRfur</td>
<td>HP1338, HP1027</td>
<td>nikR::aphA-3, fur::cat</td>
<td>same as single deletions</td>
</tr>
<tr>
<td>ureE</td>
<td>HP0075</td>
<td>ureE::aphA-3</td>
<td>EB157, EB318, EB319, EB160</td>
</tr>
<tr>
<td>hypA</td>
<td>HP0869</td>
<td>hypA::cat</td>
<td>EB153, EB154, EB155, EB156</td>
</tr>
<tr>
<td>hypB</td>
<td>HP0900</td>
<td>hypB::aphA-3</td>
<td>EB461, EB462, EB463, EB464</td>
</tr>
<tr>
<td>slyD</td>
<td>HP1123</td>
<td>slyD::aphA-3</td>
<td>EB164, EB165, EB166, EB167</td>
</tr>
<tr>
<td>hypAslyD</td>
<td>HP0869, HP1123</td>
<td>hypA::cat, slyD::aphA-3</td>
<td>same as for single deletions</td>
</tr>
<tr>
<td>ureAB</td>
<td>HP0072-73</td>
<td>ureAB::cat</td>
<td>EB320, EB321, EB322, EB344</td>
</tr>
<tr>
<td>hydABC</td>
<td>HP0619-621</td>
<td>hydABC::aphA-3</td>
<td>EB324, EB325, EB326, EB327</td>
</tr>
<tr>
<td>ureEnikR</td>
<td>HP0075, HP1338</td>
<td>ureE::aphA-3, nikR::erm</td>
<td>same as single deletions</td>
</tr>
<tr>
<td>hypBnikR</td>
<td>HP0900, HP1338</td>
<td>hypB::aphA-3, nikR::erm</td>
<td>same as single deletions</td>
</tr>
<tr>
<td>hypAslyDnikR</td>
<td>HP0869, HP1123, HP1338</td>
<td>hypA::cat, slyD::aphA-3, nikR::erm</td>
<td>same as single deletions</td>
</tr>
<tr>
<td>ureABnikR</td>
<td>HP0072-73, HP1338</td>
<td>ureAB::cat, nikR::erm</td>
<td>same as single deletions</td>
</tr>
</tbody>
</table>

\(^a\) See Table 3.2 for primer sequences.

\(^b\) The 26695 nikR::aphA-3 strain was used for the experiments shown in Figure 3.2. The nikR::erm strain was used for all additional experiments with both parental strains 26695 and G27.
References


70. Zambelli, B., A. Danielli, S. Romagnoli, P. Neyroz, S. Ciurli, and V. Scarlato. 2008. High-affinity Ni\(^{2+}\) binding selectively promotes binding of


Chapter 4

A role for the N-terminal arm of *Helicobacter pylori* NikR in DNA-binding

Parts of this chapter were published previously and are reprinted here with the permission of the American Society of Biochemistry and Molecular Biology.

Overview

Outside of the important role of transcriptional regulation in transition metal homeostasis, transcription factors are critical for the ability of a cell to sense and respond to a wide array of environmental changes. While the multitude of environmental signals remains relatively constant for any environmental niche, the physiology of individual organisms varies significantly. This raises the intriguing question of how transcriptional regulation, and in particular transcription factor function, evolves in response to variations in cellular physiology. The work presented in the following chapter addresses this problem by investigating the molecular basis for the unique aspects of DNA-binding activity displayed by *Helicobacter pylori* NikR compared to its previously characterized homolog from *Escherichia coli*. The results indicate that an additional structural motif at the N-terminus of *H. pylori* NikR (the N-terminal arm) is a major determinant responsible for the ability of this homolog to bind to different DNA sequences, a property essential for the expanded regulatory capabilities of this protein. Comparison of different NikR family members indicates that significant variation exists in the length and sequence of the N-terminal arm, suggesting that the evolution of DNA-binding activity by different family members is due in part to changes in the arm. These results identify one mechanism by which transcriptional regulation can evolve in response to different cellular physiologies: through transcription factor amino acid changes affecting DNA-binding activity.
Abstract

The Ni\textsuperscript{2+}-dependent transcription factor NikR is widespread amongst microbes. The two experimentally characterized NikR orthologs from \textit{Helicobacter pylori} and \textit{Escherichia coli} directly regulate the expression of five and one operon(s), respectively, in response to increased intracellular Ni\textsuperscript{2+}. Here, we demonstrate that the nine residue N-terminal arm present in \textit{H. pylori} NikR plays a critical role in the expanded regulatory capabilities of this NikR family member. Specifically, the N-terminal arm is required to inhibit NikR binding to low-affinity and non-specific DNA sequences and is also linked to a cation requirement for NikR binding to the \textit{nixA} promoter. Site-directed mutagenesis and arm truncation variants of NikR indicate that two residues, Asp7 and Asp8, are linked to the cation requirement for binding. Pro4 and Lys6 are required for maximal DNA-binding affinity of the full-length protein to both the \textit{nixA} and \textit{ureA} promoters. The N-terminal arm is highly variable amongst NikR family members and these results suggest that it is an adaptable structural feature that can tune the regulatory capabilities of NikR to the nickel physiology of the microbe in which it is found.
Introduction

Nickel is an essential cofactor in several metalloenzymes (34, 36), which are expressed primarily in microorganisms. Many microbes are capable of expressing at least one Ni$^{2+}$-enzyme, however no microbial genome encodes more than four known Ni$^{2+}$-enzymes. The cellular Ni$^{2+}$ content is often directly proportional to Ni$^{2+}$-enzyme expression levels. Facultative anaerobes, such as *E. coli*, require Ni-Fe hydrogenases for growth under specific anaerobic conditions (4, 48, 65). The induction of hydrogenase expression results in a concomitant increase in Ni$^{2+}$ transporter expression and intracellular Ni$^{2+}$ levels (31, 47). In some cases, microbes maintain high Ni$^{2+}$-enzyme levels in the apo-form and only increase intracellular Ni$^{2+}$ under conditions in which enzyme activity is required. For example, *H. pylori* urease can comprise up to 10% of the total protein of cells grown at neutral pH (5), where the acid-buffering activity of urease is not necessary for cell viability (23, 54), and only a small proportion of Ni$^{2+}$-bound urease exists (38, 56). When *H. pylori* is grown in acidic pH conditions urease activity is required (5, 35, 52, 53), and the levels of intracellular Ni$^{2+}$ (49) and active Ni$^{2+}$-bound urease (11, 52, 59) significantly increase. In both examples, the coordinated regulation of Ni$^{2+}$ import with the nickel requirements of each organism is critical for optimizing Ni$^{2+}$-enzyme activity to specific growth conditions while also preventing the accumulation of excess nickel ions, which are potentially toxic.
The microbes described above, in addition to many others, possess a gene that encodes the Ni\(^{2+}\)-dependent transcriptional regulator NikR. *E. coli* NikR was the first identified family member (13, 19) and is the best characterized ortholog. *E. coli* NikR represses the transcription of a Ni\(^{2+}\)-specific transporter (NikABCDE) (15, 19, 47), which is required for hydrogenase activity under anaerobic growth conditions (63, 64). In contrast, *H. pylori* NikR regulates many genes in response to elevated Ni\(^{2+}\) concentrations, including the repression of Ni\(^{2+}\) transport (1, 16, 18, 20, 24, 25, 60). Direct regulation by *H. pylori* NikR has been previously demonstrated, including the activation of *ureA* (1, 20, 22, 24) and the repression of *nixA* (24), *fur* (20), *nikR* (1, 16, 20), *exbB* (20), *fecA3* (25) and *frpB4* (18, 25). *H. pylori* NikR also plays a role in the acid response (11, 59) and acts in a transcriptional hierarchy with Fur (11, 20, 59), the well-known iron-dependent transcriptional regulator. The *E. coli* NikR DNA-binding site is a perfect inverted repeat present in the promoter region of the nikABCDE operon (15). In contrast, the binding sites that have been identified in the *H. pylori* NikR-regulated promoter regions described above (1, 20, 24, 25) do not share a well-conserved binding site.

NikR is a member of the ribbon-helix-helix (RHH) family of DNA-binding proteins (13, 43, 50). DNA-recognition by this family of proteins occurs through specific contacts with DNA bases via the surface residues of the antiparallel \(\beta\)-sheet (ribbon) at the N-terminus of this domain [Figure 4.1a; (43)]. Important DNA contacts that affect DNA-binding affinity and specificity have also been
Figure 4.1. *H. pylori* NikR contains a unique N-terminal arm. (a) Structure of the *E. coli* NikR-DNA complex showing the N- and C-terminal domain arrangement of the NikR tetramer (50). The locations of the nt9 arms are indicated by arrows. (b) N-terminal amino acid sequence alignment of *H. pylori* and *E. coli* NikR. The additional nine residues of *H. pylori* NikR are referred to as the N-terminal arm (nt9). The grey shaded residues are located in the β-sheet motif and are responsible for making specific DNA contacts in other RHH proteins. The relative location of the high-affinity Ni$^{2+}$-binding sites in each sequence are indicated as dark grey boxes. The molecular graphics image was produced using the UCSF Chimera package from the Resource of Biocomputing, Visualization, and Informatics at the University of California, San Francisco [supported by NIH P41 RR-01081; (40)].
identified in residues that precede the β-sheet (33, 43, 45, 55), although the sequence and length of these N-terminal extensions are variable. More specifically, the well-characterized RHH proteins Arc, Mnt and MetJ have N-terminal extensions immediately adjacent to the RHH motif that play important roles in DNA-recognition, including the formation of critical hydrophobic, phosphate and electrostatic interactions with DNA operator sites (33, 45, 55).

The NikR RHH domain is linked to a C-terminal domain that is homologous to an ACT ligand-binding domain and contains a high-affinity Ni$^{2+}$-binding site (29, 50). This domain is tetrameric (14) and results in a dimer of dimers quaternary structure for NikR. These domains and their topological arrangement are unique compared to other known metal-responsive transcriptional regulators (39).

Here, we show that the nine-residue N-terminal arm of *H. pylori* NikR (Figure 4.1), not visible in the recent crystal structures of *H. pylori* NikR (21), plays a critical role in NikR DNA-binding. The arm inhibited low-affinity and non-specific NikR-DNA interactions. Additionally, removal of the arm relieved a cation requirement for DNA-binding specifically at the *nixA* promoter, providing evidence that the arm plays distinct structural roles at different promoters. Mutagenesis of individual arm residues identified amino acids responsible for the *P$_{nixA}$* cation requirement as well as those necessary for high-affinity DNA-binding. These results suggest that the N-terminal arm of *H. pylori* NikR is critical for the ability of this transcription factor to recognize degenerate DNA-binding sites and thus regulate many genes to integrate the complex Ni$^{2+}$ physiology of this
organism in response to changes in intracellular Ni$^{2+}$ levels. The N-terminal arms of different NikR family members vary widely in sequence and length suggesting that this structural feature may be important for evolving regulatory specificity in accordance with cell physiology.
Results

The N-terminal arm of *H. pylori* NikR is not required for binding to *P*\textsubscript{nixA} or *P*\textsubscript{ureA}.

*H. pylori* NikR has an extension of nine amino acids at its N-terminus, (Figure 4.1). To examine the role of this arm in DNA binding, a truncation mutant of *H. pylori* NikR (nt9-NikR) lacking the first nine residues was constructed. This mutant also contained an Ile to Met substitution at the new N-terminus to ensure protein translation. UV-visible spectroscopy indicated that, similar to full-length *H. pylori* NikR, nt9-NikR bound Ni\textsuperscript{2+} upon elution from a Ni-NTA column (data not shown). Nickel ions were removed from the protein as described in the Materials and Methods. The stability of nt9-NikR in the presence or absence of stoichiometric Ni\textsuperscript{2+} was similar to full-length NikR (Figure 4.2), suggesting that loss of the arm does not affect the overall secondary structure of the protein or high-affinity Ni\textsuperscript{2+}-binding.

DNA-binding to the *nixA* and *ureA* promoters (*P*\textsubscript{nixA} and *P*\textsubscript{ureA}) was measured for nt9-NikR (at 1:1 Ni\textsuperscript{2+}:protein) using different footprinting techniques in the presence or absence of excess NiCl\textsubscript{2} (50 \textmu M). DNase I, hydroxyl radical (Fe-EDTA) and dimethyl sulfate (DMS) protection of *P*\textsubscript{nixA} and *P*\textsubscript{ureA} by nt9-NikR was identical to that observed for full-length NikR (Figure 4.3; data not shown for *P*\textsubscript{nixA} bottom strand or for *P*\textsubscript{ureA}), suggesting that the N-terminal arm does not directly interact with either DNA sequence.

The contribution of the N-terminal arm to DNA-binding affinity was tested using DNase I footprinting titrations (1:1 Ni\textsuperscript{2+}:protein). The affinities of full-length NikR
Figure 4.2. N-terminal arm mutants are unaffected in stability. Apo-protein (a) or holo-protein (1:1 Ni\textsuperscript{2+}:protein) (b) stability was measured by monitoring the circular dichroism signal of 50.0 µM protein at 230 nm. The fraction unfolded represents the difference between signal at 0.5 M urea (completely folded protein in this assay) and each increasing urea concentration. ●, full-length, native \textit{H. pylori} NikR; ●, nt9-NikR; ■, K6M; ▲, D7A; ♦, D8A; △, D7AD8A; □, nt5-NikR.
Figure 4.3. The N-terminal arm does not make specific DNA-contacts. Different footprinting methods of 1.0 µM full-length or nt9-NikR binding to the top strand of the *nixA* promoter. (a) DNase I, Fe-EDTA and DMS footprinting in the presence or absence of full-length NikR with 50 µM NiCl₂. (b) DNase I and DMS footprinting of nt9-NikR with 50 µM NiCl₂. This image consists of two pairs of lanes from the same gel image pasted together. (c) Fe-EDTA footprinting of full-length and nt9-NikR with stoichiometric or 50 µM NiCl₂ and DNase I footprinting with 50 µM NiCl₂.
for $P_{\text{nixA}}$ and $P_{\text{ureA}}$ are 49 and 45 nM (Table 4.1, Figure 2.5, Table 2.2), respectively. nt9-NikR bound to both $P_{\text{nixA}}$ and $P_{\text{ureA}}$ with affinities of ~5 nM (Figure 4.4, Table 4.1), which are 10-fold higher than full-length NikR, suggesting that the N-terminal arm may actually decrease DNA-binding affinity under these assay conditions.

**The N-terminal arm inhibits non-specific DNA-binding**

The importance of the N-terminal arm in DNA-binding was further tested using mobility shift assays containing 50 $\mu$M NiCl$_2$ (Figure 4.5) with promoters that have been shown to be directly bound by *H. pylori* NikR [$nixA$, $ureA$, $fur$, $nikR$ and $hpn$; Chapter 2; (1, 16, 20, 24)]. Full-length NikR displays a hierarchy of DNA-binding affinities to these fragments, exhibiting the highest affinity for $P_{\text{nixA}}$ and $P_{\text{ureA}}$ and progressively weaker affinities for $P_{\text{fur}}$, $P_{\text{nikR}}$ and $P_{\text{hpn}}$, and does not detectably bind to a 143-bp internal fragment of *H. pylori* rpoD (Table 4.1, Figure 2.3, Table 2.2). nt9-NikR bound $P_{\text{nixA}}$, $P_{\text{ureA}}$ and $P_{\text{fur}}$ with affinities similar to full-length NikR (Figure 4.5, Table 4.1). Interestingly, nt9-NikR displayed significantly increased affinity for $P_{\text{nikR}}$ and $P_{\text{hpn}}$ and formed distinct shifted complexes with each fragment, in contrast to the more diffuse shifts displayed by full-length NikR. In addition, nt9-NikR was able to bind to the rpoD fragment (Figure 4.5), although the lack of any detectable binding by full-length NikR to rpoD prohibited a quantitative comparison between the calculated affinities of each protein. These
Figure 4.4. 1:1 Ni$^{2+}$:nt9-NikR has increased affinity for the *nixA* and *ureA* promoters. DNase I footprinting of nt9-NikR with stoichiometric Ni$^{2+}$ serially diluted 1.5-fold from 100 nM to 228 pM titrated against $P_{nixA}$ (a) and $P_{ureA}$ (b). Highest to lowest protein concentrations are indicated by the black arrowhead above each gel.
Figure 4.5. The N-terminal arm inhibits low-affinity and non-specific DNA binding. Gel shifts with 50 µM NiCl₂ in the gel and running buffer. nt9-NikR was 1.7-fold serially diluted from concentrations of 1.0 µM to 349 pM protein. F, free DNA; B, protein-bound DNA; and SS, a super-shifted protein-DNA complex. Each full titration was run on two separate gels in parallel and a vertical line separates each pair of gel images. Each titration represents one of at least two replicates. Affinities were calculated as described in the Materials and Methods and are listed in Table 4.2. Highest to lowest protein concentrations are indicated by the black arrowhead above each gel.
Table 4.1 Apparent binding affinities\textsuperscript{a} of NikR and nt9-NikR for different promoters

<table>
<thead>
<tr>
<th></th>
<th>nixA</th>
<th>ureA</th>
<th>fur</th>
<th>nixR</th>
<th>hpn</th>
<th>rpoD</th>
</tr>
</thead>
<tbody>
<tr>
<td>full-length NikR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 µM NiCl\textsubscript{2} (mobility-shift)</td>
<td>3.8(±0.8)</td>
<td>3.8(±0.3)</td>
<td>29(±6)</td>
<td>120(±50)</td>
<td>180(±70)</td>
<td>nb\textsuperscript{b}</td>
</tr>
<tr>
<td>1:1 Ni\textsuperscript{2+} (DNase I footprint)</td>
<td>49(±7)</td>
<td>45(±10)</td>
<td>nd\textsuperscript{c}</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>nt9-NikR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 µM NiCl\textsubscript{2} (mobility-shift)</td>
<td>3.5(±0.9)</td>
<td>2.9(±0.8)</td>
<td>24(±6)</td>
<td>67(±20)</td>
<td>88(±50)</td>
<td>230(±90)</td>
</tr>
<tr>
<td>1:1 Ni\textsuperscript{2+} (DNase I footprint)</td>
<td>4.7(±0.2)</td>
<td>5.2(±4.5)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

\textsuperscript{a}, Apparent affinities of full-length NikR or nt9-NikR measured by mobility-shift or DNase I footprinting assays were calculated as described in the Materials and Methods. Affinities are reported in nM and the averages of two independent experiments are shown.

\textsuperscript{b}, No detectable binding.

\textsuperscript{c}, No data collected.
data demonstrate that the N-terminal arm functions to inhibit non-specific DNA-binding by *H. pylori* NikR.

**The N-terminal arm plays a distinct role in *H. pylori* NikR binding to *P*$_{nixa}$**

Similar to *E. coli* NikR, full-length *H. pylori* NikR requires high-affinity Ni$^{2+}$-binding to the C-domain to activate the protein for DNA-binding (Chapter 2). In contrast to *E. coli* NikR, *H. pylori* NikR does not contain a second Ni$^{2+}$-binding site, but instead requires Mg$^{2+}$ for high-affinity DNA-binding. Evidence for the Mg$^{2+}$ requirement is manifested by an increase in affinity for $P_{nixa}$ with increasing concentrations of MgCl$_2$, as measured by DNase I footprinting titrations (Figure 2.9), and as a requirement for added MgCl$_2$ in the gel and running buffer of gel mobility shift assays (Figure 2.8). nt9-NikR (1:1 Ni:protein) was able to bind to $P_{nixa}$ with a $K_d$ of 3.3 nM in the mobility shift assay in the absence of additional cations in the gel and running buffer (Figure 4.7, Figure 4.8, Table 4.2), indicating that the N-terminal arm is at least partly responsible for the cation requirement of full-length NikR for binding to $P_{nixa}$. nt9-NikR still required stoichiometric Ni$^{2+}$ to bind to DNA, indicating that the protein is activated by high-affinity Ni$^{2+}$-binding similar to full-length NikR (data not shown). nt9-NikR was also tested for binding to $P_{ureA}$, $P_{fur}$ and $P_{nikR}$ in the absence of added cations in the gel shift assay, however no binding to these promoters was observed except at the highest protein concentrations tested (> 300 nM for $P_{fur}$ and 1.0 µM for $P_{ureA}$ and $P_{nikR}$).
Figure 4.6. nt9-NikR does not bind to the *ureA*, *fur* or *nikR* promoters in the absence of added cations. *P*_{ureA} (a) and *P*_{fur} (b) binding was tested with nt9-NikR that was 1.7-fold serially diluted from concentrations of 1.0 µM to 349 pM. (c) *P*_{nikR} binding was tested with full-length or nt9-NikR that were 2.5-fold serially diluted from concentrations of 1.0 µM to 1.6 nM protein. Reactions were run on gels and in running buffer with no added cations. F, free DNA and B, bound DNA. The vertical lines indicate where two gel images were pasted together.
Figure 4.6). These data suggest that the N-terminal arm is playing a distinct structural role at $P_{nixA}$ as compared with $P_{ureA}$, $P_{fur}$ and $P_{nikR}$.

*Individual residues of the N-terminal arm play specific roles in DNA-binding by H. pylori NikR*

To determine the contribution of individual N-terminal arm residues to specific DNA-binding and the $P_{nixA}$ cation requirement, amino acids were individually mutated to either alanine (residues 1-5; 7-9), valine (Asp2) or methionine (Lys6; Figure 4.1b). Each mutant described below showed no change in stability relative to native *H. pylori* NikR in the presence or absence of Ni$^{2+}$ (Figure 4.2). To qualitatively test for contributions to cation binding in the NikR-$P_{nixA}$ complex, the mutant proteins (200 nM; 1:1 Ni$^{2+}$:protein) were examined in mobility shift assays with or without 50 µM NiCl$_2$ (Figure 4.7a). $P_{nixA}$-binding of the Asp7Ala and Asp8Ala mutants was detected in the absence of 50 µM NiCl$_2$, suggesting that Asp7 and Asp8 are linked to the cation requirement of full-length NikR. All of the mutants showed at least some binding to $P_{nixA}$ in the presence of 50 µM NiCl$_2$, demonstrating that none of the proteins were completely non-functional in this assay. However, the Pro4Ala and Lys6Met mutants did not quantitatively shift the $P_{nixA}$ fragment, suggesting that Pro4 and Lys6 are required for maximal DNA-binding affinity.

A similar qualitative screen of each site mutant for the ability to bind to $P_{ureA}$ indicated that no mutant gained the ability to bind to this promoter in the absence
Figure 4.7. The N-terminal arm imposes a *nixA*-specific cation-requirement on *H. pylori* NikR for DNA-binding. Gel shifts of 1:1 Ni$^{2+}$:protein (200 nM) of full-length NikR, nt9-NikR or each N-terminal arm mutant with *P*$_{nixA}$ (a) or *P*$_{ureA}$ (b) in the presence or absence of 50 µM NiCl$_2$. Portions of 3 gels are shown including all 10 site mutants and full-length and/or nt9-NikR on each gel as controls. Lanes denoted with an asterisk indicate mutants capable of binding in the absence of added cations and lanes denoted with a diamond indicate mutants displaying decreased affinity.
of added cations, however the Pro4 and Lys6 mutants showed similar apparent
decreases in binding affinity for \( P_{\text{ureA}} \) (Figure 4.7b). The mutants were also
screened for the ability to bind to a non-promoter fragment (\( rpoD \)) under
conditions in which nt9-NikR is able to bind to non-specific DNA (250 nM protein,
50 \( \mu \)M NiCl\(_2\)), however no mutants were capable of shifting this fragment under
the condition tested (data not shown), suggesting that removal of the entire N-
terminal arm is necessary for increased non-specific DNA-binding by \( H. \text{pylori} \)
NikR.

DNA-binding of individual mutant proteins to \( P_{\text{nixA}} \) and \( P_{\text{ureA}} \) was examined in
further detail because of the differential effect of the N-terminal arm truncation on
binding to each promoter. Protein titrations of Pro4Ala, Lys6Met, Asp7Ala, or
Asp8Ala NikR (1:1 Ni\(^{2+}\):protein) with \( P_{\text{nixA}} \) and \( P_{\text{ureA}} \) were performed in the
presence or absence of 50 \( \mu \)M NiCl\(_2\) or 1 mM MgCl\(_2\) in a mobility shift assay
(Figure 4.8, Table 4.2; data not shown for Pro4Ala, Lys6Met). The Pro4Ala and
Lys6Met mutants had decreased affinity with added NiCl\(_2\) in the gel and running
buffer for both \( P_{\text{nixA}} \) (Pro4Ala, 38-fold; Lys6Met, 2-fold) and \( P_{\text{ureA}} \) (Pro4Ala, 259-
fold; Lys6Met, 8-fold; Table 4.2). Lys6Met NikR also had a 2.7- and 2.2-fold
decrease in affinity for \( P_{\text{nixA}} \) and \( P_{\text{ureA}} \), respectively, with added MgCl\(_2\). Pro4Ala
NikR DNA-binding could not be measured with 1 mM MgCl\(_2\) due to protein
aggregation. The cause of this aggregation was not explored further. Both
Pro4Ala and Lys6Met were unable to bind to either DNA fragment in the absence
of additional NiCl\(_2\) or MgCl\(_2\), similar to native NikR (data not shown).
Figure 4.8. Asp7 and Asp8 are necessary and sufficient for the *H. pylori* NikR-\(P_{\text{luxA}}\) cation requirement. Titrations of NikR mutants serially diluted 1.7-fold from concentrations of 500 nM to 1.7 pM with no added cations, 50 µM NiCl\(_2\) or 1 mM MgCl\(_2\). Each full titration was run on two separate gels in parallel and a vertical line separates each pair of gel images. Each titration represents one of at least two replicates. Affinities were calculated as described in the Materials and Methods and are listed in Table 4.2. Highest to lowest protein concentrations are indicated by the black arrowhead above each gel.
Table 4.2 Apparent DNA-binding affinities\(^a\) of N-terminal arm mutants under different mobility-shift conditions

<table>
<thead>
<tr>
<th>Protein</th>
<th>wt</th>
<th>nt9</th>
<th>P4A</th>
<th>K6M</th>
<th>D7A</th>
<th>D8A</th>
<th>D7AD8A</th>
<th>nt5</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{nixA})</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>no added cations</td>
<td>nb(^b)</td>
<td>3.3 (±2.2)</td>
<td>nb</td>
<td>nb</td>
<td>1.0</td>
<td>3.4 (±2.3)</td>
<td>6.5 (±4.5)</td>
<td>nb</td>
</tr>
<tr>
<td>50 µM NiCl(_2)</td>
<td>3.8(^c) (±0.8)</td>
<td>3.5(^d) (±0.9)</td>
<td>145</td>
<td>27.2 (±15.8)</td>
<td>1.0 (±0.2)</td>
<td>1.8 (±0.1)</td>
<td>2.4 (±1.0)</td>
<td>3.4 (±0.5)</td>
</tr>
<tr>
<td>1 mM MgCl(_2)</td>
<td>7.4 (±1.2)</td>
<td>4.8 (±1.7)</td>
<td>nd(^d)</td>
<td>19.7 (±10.3)</td>
<td>3.8 (±1.7)</td>
<td>5.3 (±3.7)</td>
<td>2.8 (±0.5)</td>
<td>3.7 (±3.5)</td>
</tr>
<tr>
<td>(\text{ureA})</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 µM NiCl(_2)</td>
<td>3.8(^d) (±0.3)</td>
<td>2.9(^d) (±0.8)</td>
<td>985</td>
<td>29.4 (±1.2)</td>
<td>agg(^e)</td>
<td>1.4 (±0.1)</td>
<td>1.8 (±0.7)</td>
<td>6.7 (±0.2)</td>
</tr>
<tr>
<td>1 mM MgCl(_2)</td>
<td>12.1 (±6.4)</td>
<td>6.5 (±1.2)</td>
<td>nd</td>
<td>26.8 (±2.2)</td>
<td>6.1 (±1.3)</td>
<td>5.6 (±4.4)</td>
<td>2.4 (±0.1)</td>
<td>6.0 (±2.0)</td>
</tr>
</tbody>
</table>

\(^a\) Apparent affinities of mutant NikR proteins measured by mobility-shift assays were calculated as described in the Materials and Methods. Affinities are reported in nM and the averages of two independent experiments are shown.

\(^b\) No detectable binding.

\(^c\) From Table 4.1.

\(^d\) No data was collected.

\(^e\) No affinity measured due to protein aggregation.
The Asp7Ala and Asp8Ala mutants bound to $P_{\text{nixA}}$ in the presence or absence of excess cations and to $P_{\text{ureA}}$ in the presence of excess cations with similar affinities of $\sim$1-6 nM, although the affinity of Asp7Ala for $P_{\text{ureA}}$ with 1 mM MgCl$_2$ was not measured due to protein aggregation under this condition (Figure 4.8, Table 4.2). The Asp7Ala and Asp8Ala affinities for these promoters are similar to those measured for full-length NikR and nt9-NikR under all the conditions tested, including a modest decrease in affinity for all proteins binding to either promoter in the presence of 1 mM MgCl$_2$.

**Asp7 and Asp8 are necessary and sufficient for the cation-dependence of *H. pylori* NikR-$P_{\text{nixA}}$ binding**

The effects of the Asp7Ala or Asp8Ala mutations on *H. pylori* NikR $P_{\text{nixA}}$ binding suggested that these residues are responsible for the cation requirement for binding to this promoter, but neither mutant yielded a reproducible discrete mobility-shifted species, in contrast to that observed for nt9-NikR (Figure 4.8). A Asp7Ala-Asp8Ala double mutant was constructed to determine if the absence of both aspartic acid residues better mimics nt9-NikR binding to $P_{\text{nixA}}$. The presence of a discrete shifted species in the mobility shift assay with Asp7Ala-Asp8Ala recapitulated the nt9-NikR binding result for $P_{\text{nixA}}$ (Figure 4.8) in the absence of additional cations, indicating that the cation-dependence of DNA-binding to the nixA promoter by full-length NikR is mediated by both Asp7 and
Asp8. Similar to nt9-NikR, Asp7Ala-Asp8Ala NikR required added cations in the gel and running buffer to bind to \( P_{\text{ureA}} \) (Figure 4.9). Protein titrations of Asp7Ala-Asp8Ala NikR to \( P_{\text{ntixA}} \) or \( P_{\text{ureA}} \) in the presence (or absence for \( P_{\text{ntixA}} \)) of additional \( \text{NiCl}_2 \) or \( \text{MgCl}_2 \) indicated this mutant was unaltered in binding affinity to both promoters, as compared with full-length NikR (Figure 4.8, Table 4.2).

A truncation mutant lacking the first five amino acids of \( H. \text{pylori} \) NikR (nt5-NikR) was constructed to test if Asp7 and Asp8 are sufficient to impose a cation requirement on NikR for binding to \( P_{\text{ntixA}} \) or if the presence of other N-terminal arm residues is required. The resulting protein also contained a Lys6Met mutation necessary for protein expression. Purified nt5-NikR was unable to bind to \( P_{\text{ntixA}} \) in the absence of excess cations, consistent with the cation requirement imposed by Asp7 and Asp8 (Figure 4.8). The Lys6Met mutation in the nt5-NikR construct did not influence DNA-binding affinity in the presence of excess cations (Table 4.2), suggesting that Lys6 is required for maximum DNA-binding affinity specifically in the context of the full-length N-terminal arm.
Figure 4.9. Asp7Ala-Asp8Ala NikR requires added cations to bind $P_{\text{ureA}}$ in the gel shift assay. Asp7Ala-Asp8Ala NikR was 1.7-fold serially diluted from concentrations of 500 nM to 1.7 pM protein. Reactions were run on gels with either no added cations or 50 µM NiCl$_2$ in the gel and running buffer. F, free DNA and B, bound DNA. The vertical lines indicate where two gel images were pasted together.
Discussion

Site-directed mutation or truncation of residues in the N-terminal arm revealed important features of *H. pylori* NikR-DNA interactions. These experiments demonstrated differential contributions from several residues and are consistent with previous results for other RHH proteins, such as Arc and Mnt, that are discussed in more detail below (8, 32, 41, 42, 55). The inherently asymmetric nature of the tetrameric NikR-DNA complex (51) precludes a detailed molecular interpretation of the data. For example, of the four N-terminal arms present in the *H. pylori* NikR-DNA complex, there will be at least two distinct conformations (Figure 4.1a). Discerning the individual contributions of these different conformations to DNA-binding interactions is complex, as demonstrated by detailed experiments with the Mnt repressor (7), and will require further experiments either selectively monitoring individual arms [as in (7) with Mnt], or using an assay that simultaneously measures single arm-DNA interactions (Chapter 5).

Our results show a cation requirement for *H. pylori* NikR binding to *P*$_{nixA}$ that is linked to Asp7 and Asp8 of the N-terminal arm (Figure 4.8). This observation, combined with knowledge of several RHH-DNA complexes, suggests that the cation may be necessary to prevent repulsive electrostatic interactions between the negatively charged Asp residues and the negatively charged phosphodiester backbone (Figure 4.10). Adjacent Asp residues that coordinate Mg$^{2+}$ in the presence of nucleic acids have been observed both functionally and structurally
Figure 4.10. Model of *H. pylori* NikR N-terminal arm-DNA interactions. The positively charged Asn and Lys residues may make stabilizing electrostatic interactions with the DNA phosphate backbone, and/or the negatively charged Asp7 and Asp8 residues may require a cation(s) to prevent repulsive interactions with the phosphate backbone. The molecular graphics image was produced using the UCSF Chimera package from the Resource of Biocomputing, Visualization, and Informatics at the University of California, San Francisco [supported by NIH P41 RR-01081; (40)].
(3, 17, 30). Notably, a structure of HIV reverse transcriptase reveals a Mg$^{2+}$
coordinated in part by vicinal Asp residues and an $\alpha$-phosphate oxygen of bound
dTTP (30). Alternatively, cations may be required to prevent repulsive
interactions between Asp7 and Asp8 and other negatively charged amino acids,
such as Glu39 and Asp43, that may be in close proximity when the protein is
bound to DNA.

DNA-binding by nt9-NikR to five promoters of NikR-regulated genes indicated
that the arm is necessary for maintaining a hierarchy of binding affinities and
decreases DNA-binding to non-specific sites (Figure 4.5, Table 4.1). Several
RHH family members, including Arc, Mnt, and MetJ (33, 43, 45, 55), contain N-
terminal arms that play important roles in DNA recognition. Arc repressor
contains seven amino acids N-terminal to the $\beta$-sheet motif that are disordered in
the absence of DNA but assume fixed conformations in the presence of operator
sites (42) with individual residues making hydrophobic, phosphate (43) or
electrostatic (42) contacts with the DNA. N-terminal arm mutants of Arc exhibit
significant DNA-binding defects, although they still display wild-type hydroxyl
radical footprints (43), similar to the nt9-NikR behavior observed in this study
(Figure 4.3). The tetrameric Mnt repressor depends on Arg2 of its four amino
acid arm for DNA-binding (33) and analysis of hybrid Arc and Mnt proteins
identified two residues (one arm and one $\beta$-sheet position) that determine DNA-
binding specificity (44). One hybrid protein, containing the Mnt arm residue and
the Arc $\beta$-sheet residue, bound both Mnt and Arc operators as well as non-
specific DNA (44), an effect similar to that observed for nt9-NikR (Figure 4.5). It is clear that *H. pylori* NikR belongs in the subset of RHH proteins that utilize N-terminal amino acids to modulate their DNA-binding activity.

Asp7Ala-Asp8Ala NikR was unaffected in cation-dependent DNA-binding to $P_{\text{ureA}}$, $P_{\text{fur}}$ or $P_{\text{nikR}}$ (Figure 4.9, data not shown), indicating that the nt9 arm is conformationally distinct when NikR is bound to $P_{\text{nixA}}$ compared to the other three promoters. The differential roles of the N-terminal arm in NikR binding to $P_{\text{nixA}}$, a NikR-repressed gene, and $P_{\text{ureA}}$, an NikR-activated gene, might also suggest that the arm is important for NikR-DNA interactions at one promoter and NikR-protein interactions at the other. For example, the arm might be involved in NikR-RNA polymerase interactions specifically at $P_{\text{ureA}}$, where NikR binds upstream of the -10 and –35 sequences (Figure 4.3, Figure 2.7) (1, 20, 24), allowing for the up-regulation of *ureAB* expression.

Mutation of Pro4 and Lys6 resulted in proteins with decreased affinities for both $P_{\text{nixA}}$ and $P_{\text{ureA}}$. Interestingly, the loss of the arm and specific changes in amino acid composition of the arm had opposing effects on DNA-binding, since nt9-NikR has higher affinity for DNA and Pro4Ala and Lys6Met NikR have decreased affinity for DNA (Table 4.2). The decrease in affinity displayed by Pro4Ala NikR most likely reflects the propensity of proline residues to display unusual peptide bond angles. Our results suggest that Ala substitution for Pro4 may result in greater conformational flexibility in the first five residues of the arm that may adversely impact DNA-binding affinity.
The decrease in DNA-binding affinity exhibited by Lys6Met NikR, but not nt5-NikR, suggests that Lys6 may interact with one or more of the N-terminal five amino acids present in Lys6Met NikR. Replacement of Lys with Met in this case might destabilize the protein-DNA complex by imposing non-favorable interactions between other arm residues and Met6, whereas the N-terminal Met, in the case of nt5-HpNikR, would be relieved from these constraints. It is also possible that the positively charged N-terminus of nt5-NikR is able to substitute for Lys6, making additional electrostatic interactions with the DNA or other arm residues.

Previous studies examining *H. pylori* NikR DNA-binding *in vitro* have used versions of purified NikR with significantly altered N-termini, including a protein containing a 15 amino acid N-terminal Strep-tag [MASWSHPQFEKIEGR; (24, 25)], a protein with three extra N-terminal amino acids following cleavage of a His-tag [GSH; (20)] and a protein with a single added alanine remaining after cleavage of N-terminal His- and S-tags (18). Strep-NikR and GSH-NikR protected a similar region of \( P_{nixA} \) [Strep-NikR (24)] and \( P_{ureA} \) [Strep-NikR and GSH-NikR (20, 24)] as compared with native NikR [Figure 4.3; Figure 2.7; (1)], a result not surprising since nt9-NikR displayed identical protection of \( P_{nixA} \) and \( P_{ureA} \) as compared with full-length NikR (Figure 4.3). The dramatic changes in DNA-binding affinity and specificity that result from loss of the NikR N-terminal arm (Figure 4.4, Figure 4.5, Table 4.1) suggest that Strep-NikR and GSH-NikR might exhibit altered affinity and specificity for DNA, however the limited
experimental data available for each protein [a single, high protein concentration with a high Ni\(^{2+}\) concentration for Strep-NikR binding to \(P_{\text{nixA}}\) (24), \(P_{\text{ureA}}\) (24), \(P_{\text{fecA3}}\) (25) and \(P_{\text{rpB4}}\) (25) and four- or five-point protein titrations with a high Ni\(^{2+}\) concentration that are not quantitated for GSH-NikR binding to the \(exbB\)-\(nikR\) intergenic region, \(P_{\text{ureA}}\) and \(P_{\text{fur}}\) (20)] precludes a more quantitative assessment of the effects of each non-native N-terminal extension. The importance of the N-terminus of \(H.\ pylori\) NikR in DNA-binding, as shown by the current study, further indicates that experiments using NikR variants with altered N-termini should be interpreted with caution.

A comparison of NikR family members predicted from genome sequence annotations indicates arm lengths up to 32 amino acids N-terminal to the \(\beta\)-sheet (Figure 4.11). Significant variability exists in arm sequences of NikR from different \(H.\ pylori\) strains as well as different Helicobacter species. Notably, \(H.\ pylori\) isolate HPAG1 NikR contains a Pro4His change and \(H.\ acinonychis\) str. Sheeba NikR contains a Pro4Asn change. \(H.\ mustelae\) NikR contains a completely unique 10 amino-acid N-terminal arm: MRTMEKEKNS (http://www.sanger.ac.uk/Projects-/H_mustelae/). Interestingly, the \(H.\ mustelae\) arm lacks Asp residues and instead contains two alternating Glu and Lys residues at positions 5-8 as well as an Arg at position 2. The rodent pathogen \(H.\ hepaticus\) contains a NikR with five N-terminal amino acids, only one of which is charged (Lys2). Detailed biological data characterizing the Ni\(^{2+}\) physiologies of these bacteria is currently lacking, although one study has begun to address the
Figure 4.11. Alignment of NikR N-terminal arms. Representative N-terminal NikR protein sequences retrieved from an *H. pylori* 26695 NikR BLASTP search (2).

All retrieved sequences were initially aligned using ClustalW (57). Sequences N-terminal to and including the β-sheet residues were included if the entire sequence contained all four high-affinity Ni²⁺ site ligands and more than one additional amino acid N-terminal to the antiparallel β-sheet. If multiple identical
sequences were present, only one was included, except that all available *H. pylori* and closely related bacterial NikR sequences were included. Sequence order was altered to list *E. coli* and *H. pylori* NikRs at the top for reference, so order is not significant. \(\beta\)-sheet residues responsible for making DNA-contacts are shown in blue.
role of the Ni\textsuperscript{2+}-enzyme urease in \textit{H. hepaticus} metabolism (6). It is likely that significant differences exist, particularly between \textit{H. pylori}, \textit{H. mustelae} and \textit{H. hepaticus}, given that they colonize the gastric mucosa of humans (9), ferrets (26) and the rodent liver and intestine (27, 62), respectively. The structural consequences of these arm sequence changes are difficult to predict since the target genes of each NikR ortholog are unknown, however the sequence disparity suggests that the amino acid changes may result in altered NikR function in these different bacteria.

\textit{H. pylori} NikR has likely adopted its N-terminal arm to respond differently than \textit{E. coli} NikR to increased intracellular Ni\textsuperscript{2+}. High-affinity Ni\textsuperscript{2+}-binding is structurally conserved between the two NikR orthologs (21, 50), as is its effect on DNA-binding affinity. Additional metal binding by each protein and the related DNA-binding responses are distinct. It is likely that additional structural differences in \textit{H. pylori} NikR, relative to \textit{E. coli} NikR, are necessary to fully modulate the activity of the former. Nevertheless, the unique properties of \textit{H. pylori} NikR attributable to the N-terminal arm indicate that regulatory function can be tuned through localized changes in protein structure. Alignments of predicted bacterial transcription factors that have distinct DNA-binding motifs (10, 37, 46) indicate that the addition of extra amino acids adjacent to DNA-binding domains is a common occurrence, suggesting that this may represent a widespread mechanism of regulator evolution.
Materials and Methods

H. pylori NikR – Cloning and mutagenesis

H. pylori strain 26695 nikR (HP1338) was PCR amplified from genomic DNA (a generous gift from Doug Berg, Washington University School of Medicine) using the primers PC121 and PC122 (Table 4.3; Integrated DNA Technologies, Coralville, IA) and cloned into pET22-b using the NdeI and XhoI restriction sites (Novagen, Madison, WI) to create pEB116. nt9- and nt5-NikR were created using primers EB058 or EB190, respectively, and PC122 to amplify a 5’ truncated nikR from pEB116. The resulting products were digested with NdeI and XhoI and ligated into pET22-b digested with the same enzymes to create pEB149 (nt9-NikR) and pEB202 (nt5-NikR). Site-directed mutagenesis of individual NikR residues (described in the Results) was carried out using the Quik Change Site-directed Mutagenesis protocol (Stratenege, La Jolla, CA) using complementary oligonucleotides with the mutated codon and Pfu DNA polymerase. The DNA sequence of each construct was verified by sequencing (SeqWright, Houston, TX).

Protein purification and expression

Native H. pylori NikR and variants were expressed and purified as described previously for E. coli NikR (14, 15) except that gel-filtration was used as a second purification step instead of ion exchange. Protein concentration was determined in 6 M guanidine hydrochloride (GuHCl) using $\varepsilon_{276} = 9895 \text{ M}^{-1} \text{ cm}^{-1}$, as predicted.
by primary sequence analysis (28). To remove Ni\(^{2+}\) from purified protein, the Ni-NTA eluate was incubated with 50 mM EDTA for 48 h at 4°C, followed by gel-filtration (the second purification step). The removal of Ni\(^{2+}\) ions was confirmed using UV-visible spectroscopy at 302 nm.

**Promoter fragments - cloning and labeling**

DNA fragments for promoter regions were amplified by PCR using the oligonucleotide pairs described in Table 4.3. A subset of fragments (Table 4.3) were cloned into pBluescriptII SK (Stratagene, La Jolla, CA) using standard molecular biology techniques. The cloned promoter sequences were confirmed by DNA sequencing.

Promoter fragments for DNA-binding assays were generated by PCR as follows: 0.5 µM forward (5') primers (listed first in Table 4.3) for the nixA, ureA, fur, nikR, hpn and rpoD fragments were 5'-end labeled with \([\gamma-^{32}P]\)-ATP [GE Biosciences (formerly Amersham), Piscataway, NJ] and T4 polynucleotide kinase (NEB, Beverly, MA) in a total volume of 40 µl. Excess \([\gamma-^{32}P]\)-ATP was removed by desalting and the purified primers were used in a PCR reaction with the corresponding reverse primers (listed second in Table 4.3) using plasmids pEB106, pEB131 or pEB104 as templates for the nixA, ureA and nikR promoters or *H. pylori* 26695 genomic DNA for the fur, hpn and rpoD fragments. The 143-bp fragment of the *H. pylori* rpoD gene had comparable length and GC content to the other fragments and was used as a negative control. The resulting labeled
fragments were purified using a Qiagen PCR Purification Kit (Qiagen, Valencia, CA).

**DNA-binding assays**

DNase I footprinting was performed as described previously (13, 14) in a binding buffer containing 10 mM Tris-Cl (pH 8.0), 100 mM KCl, 3 mM MgCl$_2$, 10 µg/ml *E. coli* thioredoxin [used to prevent non-specific NikR accumulation in the gel wells] (12), and 4 ng/µl salmon sperm DNA (Fisher Scientific, Pittsburgh, PA). Labeled DNA fragments were incubated with protein at 22°C for 1 hour prior to DNase I (Sigma, St. Louis, MO) addition (final concentration 300 ng/mL). NiCl$_2$ was added to the binding reactions as described in the text and figure legends.

Hydroxyl radical footprinting was performed as described previously (58) with the following modifications: binding reactions were performed in 50 µl of DNase I footprinting buffer with 10 mM MgCl$_2$. Fe$^{2+}$-EDTA (2 mM stock) was added to a final concentration of 167 µM, followed by the addition of 833 µM sodium ascorbate (20 mM stock) and 0.05% H$_2$O$_2$ (30% v/v stock). The reaction was carried out for 1 min at 22°C and was quenched with 10 µl of 0.1 M thiourea and 1 µl of 0.5 M EDTA (pH 8.0). Dimethyl sulfate (DMS) protection experiments were carried out as previously described (61) except binding reactions were in a total volume of 100 µl and the buffer contained 10 mM sodium cacodylate (pH 8.0) instead of Tris-Cl.
Electrophoretic mobility shift assays were performed using 7% polyacrylamide gels and electrophoresis buffer containing 50 mM Tris (pH 8.8), 25 mM boric acid and with NiCl₂, MgCl₂, KCl, NiSO₄ or MgSO₄ as described in the text and figure legends. The binding buffer was identical to that used for DNase I footprinting. The same end-labeled DNA fragments as those used for footprinting were incubated with NikR or mutant proteins at 22°C for 30 min and 20 µl of the 50 µl total volume was loaded directly onto a running gel (120 V).

Apparent affinities measured by mobility shift assays were calculated from binding curves determined by the ratio of bound (all shifted species) vs free counts as quantified using a Molecular Dynamics Storm 840 Phosphoimager and IMAGEQUANT Version 5.2 software. Apparent affinities measured by DNase I footprinting were calculated from binding curves determined by the ratio of the protected DNA region normalized to a region of DNA not protected from the same lane vs the same ratio from identical regions of a protein-free lane on the same gel. The data were fit using MICROMATH SCIENTIST Version 2.01 and the following equation:

\[ y = \frac{1}{1 + \left(\frac{K_d}{x}\right)^n} \]

where: \( y \), fraction DNA bound (ratios described above); \( K_d \), protein concentration required for half-maximal DNA-binding; \( x \), protein concentration; and \( n \), Hill coefficient. All reported affinities are the average of at least two independent experiments using a dilution series of at least 15 protein concentrations. The
reported error is the standard deviation between the calculated affinities of at least two independent experiments.

**UV-visible and CD spectroscopy**

UV-visible spectra were collected on a Shimadzu UV-2401PC spectrophotometer using a 100 µl sample volume. CD spectra were collected on a Jasco J-715 spectropolarimeter using a 900 µl sample volume in a cylindrical cuvette with a 1 cm pathlength. All spectra were collected at 22°C in a buffer containing 20 mM Tris (pH 7.5), 100 mM NaCl.
Table 4.3 Primers used for *H. pylori* gene and promoter amplification

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Name</th>
<th>Pylori Gene Coordinates (5'→3')</th>
<th>Sequence (5'→3')&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Plasmid Name</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>nikR</em></td>
<td>PC121</td>
<td>1398772-1398755</td>
<td>att ttc taC TAT GGA TAC ACC CAA TAA AG</td>
<td>pEB116</td>
</tr>
<tr>
<td><em>nikR</em></td>
<td>PC122</td>
<td>1398327-1398341</td>
<td>ttt gta tCt cga gag gCT ATT CAT TGT ATT C</td>
<td>pEB116</td>
</tr>
<tr>
<td><em>nt5</em></td>
<td>EB058</td>
<td>1398742-1398726</td>
<td>gtc act acA tat gAT CCG CTT TTC GGT TTC TCT T</td>
<td>pEB149</td>
</tr>
<tr>
<td><em>nt5</em></td>
<td>EB190</td>
<td>1398755-1398732</td>
<td>gta cat caT atg GAC GAT TCA ATC ATC CGC TTT TCG</td>
<td>pEB202</td>
</tr>
<tr>
<td><em>nixA</em></td>
<td>EB006</td>
<td>1137016-1136993</td>
<td>gga tta gaA tcC AAA ATT TTT TAG GGC AAT TTG CAG</td>
<td>pEB106</td>
</tr>
<tr>
<td><em>nixA</em></td>
<td>EB007</td>
<td>1136850-1136873</td>
<td>cca tta ccc ggg CAA TGC ATG CAA GAA CAC AAT CGC</td>
<td>pEB106</td>
</tr>
<tr>
<td><em>ureA</em></td>
<td>EB047</td>
<td>78187-78163</td>
<td>gtc tca ccc ccc TTC TCA TTT TTT GTC GAG TTT TTG</td>
<td>pEB131</td>
</tr>
<tr>
<td><em>fur</em></td>
<td>EB036</td>
<td>77927-77950</td>
<td>gta tca gtc gag CTT GTC TAA CTC TTT TGG GGT GAG</td>
<td>pEB131</td>
</tr>
<tr>
<td><em>fur</em></td>
<td>EB031</td>
<td>1090060-1090083</td>
<td>gta cta ccc ccc AGT TAC ATT AAA ATG CGA CAA TGG</td>
<td>na&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>fur</em></td>
<td>EB032</td>
<td>1090221-1090199</td>
<td>gca tct gtc gag ATC TTT TCA TGC TGA TAT CTT CC</td>
<td>na</td>
</tr>
<tr>
<td><em>nikR</em></td>
<td>EB004</td>
<td>1398872-1398847</td>
<td>ccc atc gaa tcc AAA TCC AGT TTG TAT TAT AAT TGT TC</td>
<td>pEB104</td>
</tr>
<tr>
<td><em>nikR</em></td>
<td>EB005</td>
<td>1398747-1398771</td>
<td>cca tat ggc ggg TGA ATC GTC TTT ATT TGG GTG TGT ATC C</td>
<td>pEB104</td>
</tr>
<tr>
<td><em>hpn</em></td>
<td>EB033</td>
<td>1497308-1497284</td>
<td>gtc tct ccc ccc ATA ATT CAA AAT TTA GGG AAT ATG G</td>
<td>na</td>
</tr>
<tr>
<td><em>hpn</em></td>
<td>EB034</td>
<td>1497146-1497169</td>
<td>cga tta gtc gag CCG TGT TGT TCT TCA TGG TGT GCC</td>
<td>na</td>
</tr>
<tr>
<td><em>rpoD</em></td>
<td>PC687</td>
<td>93095-93078</td>
<td>ttc ggt atc gag GAT GAA AGC GAT CGA ACT</td>
<td>na</td>
</tr>
<tr>
<td><em>rpoD</em></td>
<td>PC688</td>
<td>92952-92972</td>
<td>gaa aac ccc tgc act CAA ATG CGC AAA TAG TTT CTC</td>
<td>na</td>
</tr>
</tbody>
</table>

<sup>a</sup>, Underlined bases correspond to restriction sites and capitalized bases correspond to regions complementary to the PyloriGene coordinates listed.

<sup>b</sup>, Not applicable; fragment was not cloned.
References


Helicobacter pylori NikR adopts different conformations when bound to the nixA and ureA promoters.
Overview

One of the major questions regarding DNA-binding proteins is how they recognize specific DNA sequences, particularly considering the vast amount of DNA present on even a single prokaryotic chromosome in a cell. In addition, transcriptional regulators often control the expression of entire regulons consisting of many genes that contain 'consensus' sites within their promoters, which can represent highly conserved binding motifs or largely degenerate sequences. In an earlier chapter of this thesis the ribbon-helix-helix (RHH) protein *H. pylori* NikR was shown to bind to multiple promoters with different DNA sequences. Here, the NikR RHH domain interactions with two promoter DNA sequences are examined in further detail. In contrast to the general belief that transcriptional regulator DNA-binding specificity results from rigid protein-DNA interactions, I show that NikR exists in two different conformations when bound to distinct DNA sequences. The results presented here suggest that despite powerful tools such as structural biology, a combination of approaches will be required to fully elucidate the rules dictating sequence specific DNA-binding.
Abstract

_H. pylori_ NikR binds to promoters from genes within its regulon with a hierarchy of affinities, which is likely a consequence of sequence differences in the DNA binding sites. The ability of NikR to discriminate specific binding sites resides partly in its nine amino acid N-terminal arm, the deletion of which collapses the hierarchy of binding affinities NikR displays for different promoters and increases non-specific DNA binding. Additionally, indirect evidence indicates that the N-terminal arm exists in different conformations when the protein is bound to the _nixA_ and _ureA_ promoters. I have directly examined the NikR conformation when the protein is bound to _nixA_ and _ureA_ by tethering the chemical nuclease Fe-BABE to different individual positions in the RHH domain. The presence of different cleavage patterns directly demonstrates that both the RHH domain and the N-terminal arm adopt different conformations on the _nixA_ and _ureA_ promoters. Additionally, the two RHH domain dimers of the NikR tetramer are in distinct conformations at the _ureA_ promoter. In a complementary approach, site directed mutagenesis studies identified a salt bridge that is remote from the DNA-binding interface and was required for high-affinity binding to _ureA_, but not _nixA_. Finally, DNA affinity measurements of wild-type NikR and a _nixA_-selective salt bridge mutant (Lys48Ala) to hybrid _nixA-ureA_ promoters demonstrated that a combination of imperfect inverted repeat half sites, spacers and flanking DNA is required for sequence-specific DNA-binding by NikR, with the largest contribution to sequence specificity made by the repeat half sites.
These results provide further insight into RHH domain-DNA interactions, and suggest that understanding DNA-binding specificity in this family requires consideration of protein-DNA interactions outside of the $\beta$-sheet-major groove contacts.
Introduction

Consistent with the many diverse functions of RHH proteins in cells, which include the regulation of bacteriophage genes (50), plasmid maintenance and segregation (18, 30), plasmid encoded antitoxin and repressor functions (31) and metabolite- (40) and metal-dependent (10, 17) gene regulation, family members contain a wide variety of additional domains that control the DNA-binding activity of the RHH domain (46). A consequence of these various activities is that the DNA sequence motifs, as well as the sequence motif number and arrangement, that are recognized by each RHH protein vary significantly (5, 6, 12, 16, 27, 35-37, 45, 48, 51, 52, 54, 56). Despite structural characterization of several RHH protein-DNA complexes (28, 35, 37, 38, 44, 47-49, 53, 56), accurate predictions of the DNA binding sites of uncharacterized RHH proteins remain difficult (43, 46), as well as identifying additional potential binding sites for proteins with at least one known binding site.

The Ni$^{2+}$-dependent RHH protein NikR from *Helicobacter pylori* regulates the expression of multiple genes in response to increasing extracellular nickel by directly binding to these gene promoters [Chapter 2; (1, 7, 14, 15, 19, 23, 24)]. The DNA sequences of the recognition sites in each promoter are defined by a series of poorly conserved six bp imperfect inverted repeat half sites [Chapter 2; (7, 19, 22)], which results in NikR binding to these promoter DNA fragments with a range of affinities [Chapter 2; (7, 22)]. In contrast, *Escherichia coli* NikR binds
to a DNA recognition site consisting of a six bp perfect inverted repeat separated by 16 bp (10, 12).

Extensive structural studies of other RHH proteins, including the Arc and MetJ repressors over 15 years ago, demonstrated that the β-sheet motif of the RHH domain sits in the major groove of DNA, with three solvent exposed residues making base-specific hydrogen bonds via their side chains (43, 44, 49). Additional non-specific DNA phosphate contacts are made, some by tandem turn regions N-terminal to the β-sheets and others by the N-terminus of helix α2 of the RHH domain (43, 44, 49). These protein-phosphate interactions are hypothesized to attach the N-terminus and helix α2 to the DNA backbone, as well as link these structural elements to the β-sheet (43). Additionally, Arc and MetJ contain residues preceding the β-sheet (7 and 22 residues, respectively), similar to the N-terminal arm of *H. pylori* NikR, that are disordered in the absence of DNA and make four phosphate interactions with the DNA half-sites (44, 49). The N-terminal arm of Arc also makes hydrophobic interactions with the β-sheet via three Met residues (44). Several possibilities have been suggested for the role of the flexible N-terminal arms in DNA-binding by Arc and MetJ, including adapting to different DNA structures or coupling the arm-phosphate contacts with specific β-sheet-base contacts to reduce non-specific DNA-binding (43). Furthermore, detailed studies of the related Mnt repressor have shown that the N-terminal arm of this RHH protein makes two base specific contacts with the center of the DNA
recognition site (33), although so far this situation appears to be unique within the RHH family.

Eight more recently solved RHH protein-DNA co-crystal structures have included proteins that bind to a larger number of binding sites aligned along one stretch of DNA, such as CopG (28), Omega (53) and ParR (48), as well as antitoxin repressors [FitA (38) and RelB (35)] that, similar to Arc and MetJ, bind to two inverted repeats. Common to all of these structures, including those of Arc and MetJ, is a set of non-specific interactions made by helix α2 of the RHH domain to the phosphate backbone (28, 37, 44, 46, 47, 49, 53). However, these proteins contain no or only a few residues preceding the β-sheet motif (CopG, ParR, FitA and RelB), or the structure was solved in the absence of the N-terminal arm (Omega), so additional information regarding specific DNA interactions is limited to variations in the hydrogen bonds formed between the β-sheet side chains and the DNA bases. The one exception is the case of CopG, which contains two Lys residues between the N-terminal Met and the first β-sheet residue. Lys2 made a single phosphate interaction at each CopG half-site (28).

The *E. coli* NikR-operator DNA co-crystal structure revealed protein-DNA interactions that included five base specific hydrogen bonds made by Arg3 and Thr5 of the RHH β-sheet (47). Additionally, residues from both the RHH domain and the C-terminal domain of NikR interact with the DNA phosphate backbone flanking the inverted repeat half-sites, including two contacts between C-domain residues and the center of the inverted repeat binding site (47). No protein-DNA
or protein-protein interactions were observed for *E. coli* NikR residues preceding the β-sheet, which contrasts with results showing that the nine amino acids at the N-terminus of *H. pylori* NikR are important for DNA-binding (Chapter 4). There is, however, no structure available for *H. pylori* NikR bound to any DNA sequence, so any differences that exist between the protein-DNA interface for the two NikR proteins are not known. Additionally, the conformation(s) of the N-terminal arm in the absence of DNA is unclear (20).

In previous work, indirect evidence demonstrated that the N-terminal arm of *H. pylori* NikR exhibits conformational differences when NikR is bound to the *nixA* and *ureA* promoters, as distinguished by alanine scanning mutagenesis (7). In this chapter I further examine this conformational difference using chemically modified and site-directed mutants of NikR in combination with engineered promoter sequences to demonstrate that NikR exists in two different conformations when the protein is bound to *nixA* and *ureA*. These data firmly establish novel features of RHH domain-DNA interactions, including changes in protein conformation in response to DNA sequence differences and the likely involvement of DNA structure in specific DNA-binding by *H. pylori* NikR.
Results

The chemical modification of introduced Cys residues (Figure 5.1) required mutation of one native Cys residue (Cys96), which does not affect DNA-binding activity (Figure 5.2, Table 5.1), and protection of the high-affinity Ni$^{2+}$-binding site Cys by carrying out modifications in the presence of Ni$^{2+}$. Thus, the wild-type protein used in these experiments is Cys96Ala and is referred to as NikR$^*$. 

*Individual Cys mutations impair NikR binding to nixA and ureA to varying degrees*

To directly test if *H. pylori* NikR adopts distinct conformations when NikR is bound to the *nixA* and *ureA* promoters, I used the chemical nuclease Fe-BABE to covalently modify NikR mutants containing single Cys residues substituted throughout the N-terminal arm and helix α1 of the RHH domain (Figure 5.1), similar to a previous study with the Mnt repressor (8). Fe-BABE modified Cys residues located within ~22 Å of DNA can cleave the phosphodiester backbone of radiolabeled DNA under appropriate conditions due to hydroxyl radical production by the chelated Fe$^{2+}$ (29).

The effects of individual Cys mutations on NikR binding (without the Cys96Ala mutation) to the *nixA* and *ureA* promoters were assessed using electrophoretic mobility shift assays (Figure 5.2, Table 5.1). All mutants had decreased affinities (Table 5.1) for the *nixA* and *ureA* promoters that ranged from modest (e.g., Asn20Cys, 0.7- and 8.0-fold for *nixA* and *ureA*, respectively) to severe (e.g.,
Figure 5.1. Mutagenesis of the N-terminal domain of *H. pylori* NikR. (a) *H. pylori* NikR N-domain primary sequence with the secondary structure elements noted above and Cys mutants denoted by arrows. (b) The position of residues mutated in this study are shown on the structure of the N-domain of *H. pylori* NikR [only one Glu47-Lys48 pair is shown for clarity; (20)], which is shown superimposed on B-form DNA. The structure and DNA was modified using the Chimera package from the Resource of Biocomputing, Visualization, and Informatics at the University of California, San Francisco [supported by National Institute of Health Grant P41 RR-01081; (42)].
Figure 5.2. Electrophoretic mobility shift experiments for NikR Cys mutants.

Mobility shifts of (a) NikR* (Cys96Ala), (b) Met1Cys, (c) Thr3Cys, (d) Asn5Cys, (e) Asp7Cys, (f) Ser9Cys, (g) Asn20Cys, (h) Asp23Cys, (i) Asn27Cys and (j) Ile30Cys NikR mutants serially diluted 3-fold from 500nM to 228 pM with nixA (left-side panels) or ureA (right-side panels) promoter fragments. The left-most lane in each titration is DNA alone. Differences in the mobility of different protein-DNA complexes are not significant due to minor variations in gel run times and the different sizes of the nixA and ureA DNA fragments.
Table 5.1. Apparent DNA-binding affinities relative to wild-type NikR of Cys mutants.

<table>
<thead>
<tr>
<th>Protein</th>
<th>nixA</th>
<th>ureA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cys96Ala</td>
<td>1.0(^a)</td>
<td>1.6</td>
</tr>
<tr>
<td>Met1Cys</td>
<td>5.8</td>
<td>27.5</td>
</tr>
<tr>
<td>Thr3Cys</td>
<td>20.2</td>
<td>nb(^b)</td>
</tr>
<tr>
<td>Asn5Cys</td>
<td>14.7</td>
<td>43.4</td>
</tr>
<tr>
<td>Asp7Cys</td>
<td>1.9</td>
<td>16.8</td>
</tr>
<tr>
<td>Ser9Cys</td>
<td>4.3</td>
<td>16.2</td>
</tr>
<tr>
<td>Asn20Cys</td>
<td>0.7</td>
<td>8.0</td>
</tr>
<tr>
<td>Asp23Cys</td>
<td>2.6</td>
<td>17.8</td>
</tr>
<tr>
<td>Asn27Cys</td>
<td>6.5</td>
<td>14.1</td>
</tr>
<tr>
<td>Ile30Cys</td>
<td>5.8</td>
<td>20.7</td>
</tr>
</tbody>
</table>

\(^a\) Affinities were calculated using best fits to Eq. 5-1 (see Materials and Methods) and divided by the affinity measured for wild-type NikR tested in parallel with the mutants.

\(^b\) No detectable binding.
Thr3Cys, 20- and >100-fold for nixA and ureA). However, a number of these mutants did cleave DNA, allowing for the qualitative analysis of protein-DNA proximity and protein conformation (discussed in detail below). One possible reason why the Cys mutants showed decreased affinities may be that the Cys substitutions result in alterations in protein stability, conformation or disrupt protein-protein contacts important for DNA-binding. Alternatively, the formation of disulfide bonds between the newly-introduced Cys side chains under mobility shift conditions could impair the formation of stable protein-DNA complexes.

The NikR DNA-binding domains are in different conformations at the nixA and ureA promoters

Proteins were purified under reducing conditions, modified with Fe-BABE according to published protocols (39) and analyzed for Cys side chain proximity to DNA by Fe-BABE cleavage reactions. NikR* did not produce any detectable cleavage at either the nixA or ureA promoter (Figures 5.3 and 5.4).

nixA

Of the four helix α1 Fe-BABE modified mutants, Asn20Cys generated reproducible cleavage of the nixA promoter (Figure 5.3). Cleavage by Asn20Cys spanned the DNase I footprint of nixA, and extended slightly past the outer boundaries of the footprint [3 and 2 bases at the upper and lower boundaries, respectively; Figure 5.5a -- mapping presented here was based on previous
Figure 5.3. The RHH domain is in different conformations when NikR is bound to the nixA and ureA promoters. DNase I protection and Fe-BABE cleavage footprinting reactions of NikR* and Asn20Cys Fe-BABE modified mutants with (a) the nixA promoter, and (b) the ureA promoter. DNase I protection is indicated at the left of each panel with a solid black bar, and Fe-BABE cleavage by Asn20Cys is indicated at the right of each panel with multiple black (stronger cleavage) and grey (weaker cleavage) bars.
DNase I footprinting using a similar DNA fragment that was labeled differently, which produced a distinct DNase I cleavage pattern and slightly altered footprint; Chapter 2; (7)]. For nixA there were seven distinct symmetrically arranged cleavage regions that were grouped as two pairs of outer cleavage regions (outside the inverted repeat half-sites) and one set of three inner regions (Figure 5.3a and 5.5a). The three inner regions were centered around position +3, one base upstream of the midpoint of the DNase I footprint (black oval in Figure 5.5a). The inner cleavage region of each outer pair together with the outermost region of the inner set of three straddle the half sites (the grey boxes in Figure 5.5a), consistent with the location of Asn20 at the end of helix α1 and near the two edges of the antiparallel β-sheet (Figure 5.1b), which sits in the major groove of DNA (47). H. pylori NikR binds to DNA as a tetramer, so the increased number of cleavage regions suggests that each Fe-BABE modified Cys exists in more than one conformation, resulting in more than four cleavage regions.

ureA

Asn20Cys produced eight distinct cleavage regions that can be grouped into two pairs of strong cleavage regions outside of the inverted repeat half-sites and four weaker cleavage regions inside the half-sites (Figure 5.3b). The four inner regions are centered 2 bases upstream of the midpoint of the ureA DNase I footprint (position -72; Figure 5.5b), with the two upstream and two downstream pairs separated by 1 base each. The inner four regions are separated by the two
outer pairs of cleavage regions by 4 and 1 bp (upstream and downstream, respectively). While the two outer paired regions are each separated by 6 bp, the differences in outer pair vs inner pair spacing and the slightly offset center of Fe-BABE cleavage result in an asymmetry of cleavage of the outermost regions, with the upstream region extending 7 bases up from the DNase I footprint and the downstream region extending only 1 base down from the footprint. Similar to the results for modified Asn20Cys cleavage of \textit{nixA}, the eight cleavage regions at \textit{ureA} indicate that each modified Cys is present in two different conformations. Together with the asymmetrical cleavage observed at \textit{ureA}, these data suggest that the two DNA-binding domains of the NikR tetramer are in distinct conformations at this promoter.

\textit{The NikR N-terminal arms are in different conformations at the \textit{nixA} and \textit{ureA} promoters}

\textit{nixA}

Of the five N-terminal arm Cys mutants, Thr3Cys and Asn5Cys gave reproducible cleavage of \textit{nixA} (Figure 5.4). Both modified proteins exhibited identical cleavage patterns at the two promoters, which is not surprising given their proximity in primary sequence. Cleavage at \textit{nixA} consisted of eight distinct regions that spanned the DNase I footprint, extending 5 and 4 bases upstream and downstream, respectively, and including both stronger and weaker cleavage
Figure 5.4. The N-terminal arm is in different conformations when NikR is bound to the \( \text{nixA} \) and \( \text{ureA} \) promoters. DNase I protection and Fe-BABE cleavage footprinting reactions of NikR\(^*\), Thr3Cys and Asn5Cys Fe-BABE modified mutants with (a) the \( \text{nixA} \) promoter, and (b) the \( \text{ureA} \) promoter. DNase I protection is indicated at the left of each panel with a solid black bar, and Fe-BABE cleavage by Thr3Cys and Asn5Cys is indicated at the right of each panel with multiple black bars.
Figure 5.5. Summary of NikR RHH domain and N-terminal arm DNA interactions as determined by Fe-BABE cleavage. The Asn20Cys-cleavage (i) and Thr3Cys- and Asn5Cys-cleavage (ii) of nixA (a) and ureA (b) are mapped onto B-form DNA (in black). (iii) The promoter sequences are shown with the previously identified half-sites highlighted in grey boxes [Chapter 2; (7)]. The numbering refers to the sequence positions relative to the start of transcription determined for each promoter (2, 23). Top strand sequences cleaved by Asn20Cys-modified NikR are underlined and sequences cleaved by Thr3Cys- and Asn5Cys-modified NikRs are indicated by lines above. B-form DNA was generated using the
‘make-na’ website [http://structure.usc.edu/make-na/; (3, 4, 25, 34)] and modified using the Chimera package (42).
regions (Figure 5.4a). The eight regions were centered 2 bases downstream from the midpoint of the DNase I footprint (position +7; Figure 5.5a) and were similarly spaced, with all but one pair of regions separated by 2 bases. The eight total regions indicate that, as was the case for modified Asn20Cys, the 4 modified Cys at residues 3 or 5 of the NikR tetramer exist in two conformations each, with no overlapping regions of cleavage. The cleavage observed for Thr3Cys and Asn5Cys overlapped with that seen for Asn20Cys (compare Figure 5.3a and 5.4a), although the arm and helix $\alpha_1$ cleavage was offset by 1-2 bases per region. This suggests that Thr3 and Asn5 may be located in a similar, although not identical, position as Asn20 relative to the DNA, which would in part be dictated by the angle of helix $\alpha_1$ with the DNA.

*ureA*

Modified Thr3Cys and Asn5Cys cleaved *ureA* at six regions that can be grouped as two outer pairs and one inner pair of cleavage regions (Figure 5.4b). The inner pair was centered 2 bases upstream from the midpoint of the DNase I footprint (position -74; Figure 5.5b) and, in contrast to all other cleavage, consisted of 6 bases per region spanning much of the DNA spacer between the two half-sites. The inner pair was separated from the upstream outer pair by 5 bases and from the downstream outer pair by 2 bases. In addition, the upstream outer pair was separated by 9 bases, while the downstream outer pair was separated by only 6 bases. Together, this resulted in significant cleavage
asymmetry, with the upstream Thr3Cys and Asn5Cys cleavage extending 11 bases outside of the DNase I footprint while the downstream cleavage extended only 2 bases. This cleavage asymmetry was similar to that observed for Asn20Cys cleavage of *ureA*, and is consistent with the two arms and one RHH domain dimer from each half of the NikR tetramer being in two different conformations at *ureA*. The results for *ureA* contrast with those for *nixA*, where both the helix $\alpha_1$ and the two arm modified proteins mediated symmetrical cleavage patterns.

The distinct cleavage patterns observed for both the helix $\alpha_1$ and arm mutants at the two promoters clearly demonstrate that the NikR tetramer is in different conformations when the protein is bound to *nixA* and *ureA*.

*Mutation of a single RHH residue that does not contact DNA selectively impairs ureA-binding*

The existence of two DNA-bound conformations of NikR suggests that different amino acid interactions, likely within the RHH domain and/or N-terminal arm, occur when NikR is bound to one promoter but not the other. To identify potential residues that might contribute to the altered DNA-binding properties of the *H. pylori* NikR RHH domain, an alignment of the *H. pylori* and *E. coli* NikR RHH domains was examined for non-conserved amino acids. Lys48 of *H. pylori* NikR was an obvious candidate because the equivalent position in *E. coli* NikR is
an Ala residue (Ala39) and this position was poorly conserved among the entire NikR family. A RHH domain mutant of helix α2, Lys48Ala (Figure 5.1), was identified that bound to nixA with affinity similar to wild-type NikR but bound to ureA with significantly reduced affinity (62-fold decrease; Figure 5.6; Table 5.2). The loss of a positively charged Lys residue suggested that a salt bridge between Lys48 and a nearby negatively charged residue is critical for high-affinity ureA-binding by NikR.

Lys48 participates in a salt bridge that is required for high-affinity ureA-binding

Examination of the H. pylori NikR crystal structures (20) indicated that two negatively charged residues, Glu47 from the opposite polypeptide chain and Asp52 from the same chain, are both close to Lys48 (~3.5 Å). To determine whether these residues were linked to Lys48, individual Ala mutants were constructed and tested for nixA- and ureA-binding by mobility shift assays. Both mutants bound to nixA and ureA with only slightly weakened affinity (2-7 fold; Figure 5.6; Table 5.2). The absence of a significant effect for either mutation suggests that these residues are unimportant for DNA-binding, or that only one residue is important for interacting with Lys48 and one can substitute for the other. However, the Glu47Ala-Asp52Ala double mutant had lower affinity for the nixA and ureA promoters relative to the single mutants (12- and 37-fold, respectively; Table 5.2), with a more significant reduction in affinity for ureA.
Figure 5.6. A salt bridge between Glu47 and Lys48 of the NikR RHH domain is required for high-affinity binding to the *ureA* promoter. Electrophoretic mobility shifts of NikR N-domain mutants serially diluted 1.7-fold from 500 nM to 175 pM with *nixA* (left-side panels) or *ureA* (right-side panels) promoter fragments. (a) Lys48Ala, (b) Glu47Ala, (c) Asp52Ala, (d) Glu47Ala-Asp52Ala, (e) Glu47Lys-Lys48Glu. The left-most lane in each titration is DNA alone. F, free DNA. B, bound DNA. Each titration was run on two gels in parallel which are denoted by vertical black lines.
Table 5.2. Apparent DNA-binding affinities of NikR RHH domain charge mutants.

<table>
<thead>
<tr>
<th></th>
<th>wild-type</th>
<th>K48A</th>
<th>E47A</th>
<th>D52A</th>
<th>E47A-D52A</th>
<th>E47K-K48E</th>
<th>K48D-D52K</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>nixA</em></td>
<td>3.8 (±0.8)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.7 (±0.2)</td>
<td>7.3 (±0.6)</td>
<td>16.6 (±1.8)</td>
<td>46.8 (±2.8)</td>
<td>6.3 (±2.4)</td>
<td>621.2 (±514.5)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>ureA</em></td>
<td>3.8 (±0.3)</td>
<td>237.0 (±75.0)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23.8 (±13.2)</td>
<td>27.7 (±13.5)</td>
<td>140.4 (±29.6)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.0 (±0.1)</td>
<td>nb</td>
</tr>
</tbody>
</table>

<sup>a</sup> Affinities were calculated from best fits using Eq. 5-1 and are reported in nM and the average of at least two experiments is reported with the standard deviation. Wild-type values are from Chapter 2 (7).

<sup>b</sup> A lower limit estimate.
compared to \textit{nixA}. These data support the idea that one or both negatively charged residues interacts with Lys48 when NikR is bound to \textit{ureA}, although the double mutant is still not as impaired as Lys48Ala, possibly due to charge-charge repulsion of the remaining Lys.

\textit{Inverting the candidate salt bridges identifies a critical Glu47-Lys48 interaction}

The results described above do not conclusively identify the residue(s) that interact with Lys48. As an alternative mutagenesis approach residue pairs were swapped (i.e., Glu47Lys-Lys48Glu and Lys48Asp-Asp52Lys). The Glu47Lys-Lys48Glu mutant displayed only minor decreases in affinity for \textit{nixA} and \textit{ureA} of 1.5- and 2-fold, respectively, whereas the Lys48Asp-Asp52Lys mutant had 164-fold lower affinity for \textit{nixA} and an even greater reduction in affinity for \textit{ureA} (no binding was detected up to 5 \text{µM} protein; Figure 5.6; Table 5.2). The similar behavior of the Lys48Ala and Lys48Asp-Asp52Lys mutants suggests neither contains the protein-protein interaction required for \textit{ureA}-binding. Further, the restoration of DNA-binding in the Glu47Lys-Lys48Glu mutant strongly suggests a salt bridge between these two residues is necessary for \textit{ureA}-binding.

\textit{Analysis of promoter sequence contributions to NikR high-affinity binding to nixA and ureA}
The inverted repeat sequences present in RHH protein recognition sites are generally believed to contain all of the information necessary for specific protein-DNA complex formation (43, 46). Comparison of the inverted repeats in the nixA and ureA promoters, which are based on DNase I, Fe-EDTA, DMS and KMnO₄ footprinting [Chapter 2 and (7)], indicates that 4 of the 12 bps of the nixA and ureA half-sites are different (Figure 5.5, Table 5.3). Because almost all of the base-specific contacts made by RHH proteins are to repeat half sites in the various DNA recognition sites (28, 37, 44, 46, 47, 49, 53), changes in half-site sequences represent likely candidates for influencing the conformation of NikR when bound to nixA and ureA. However, the Fe-BABE cleavage results indicate that the N-terminal arm and helix α1 of the RHH domain are also in close proximity to the spacer region between the half-sites, as well as DNA outside of the half-sites and outside of the DNase I footprint. To determine the specific DNA sequences necessary to promote a ‘nixA-like’ conformation of NikR, several nixA-ureA hybrid promoters were constructed in which the two half sites and the intervening spacer regions of the two promoters were arranged in different combinations (Table 5.3).

Wild-type NikR had reduced affinities for all four hybrid promoters, with relatively small effects with only the half sites swapped (~4-fold; Table 5.3). Replacing both the spacer and the half site sequences caused an additional, modest (1.5 to 2-fold) decrease in affinity. The limited overall decreases in
Table 5.3. Apparent DNA-binding affinities of wild-type and Lys48Ala NikR for hybrid *nixA-ureA* promoters.

<table>
<thead>
<tr>
<th>Promoter</th>
<th>HS</th>
<th>SP</th>
<th>HS</th>
<th>wild-type</th>
<th>K48A</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>nixA</em></td>
<td>T A T T A C</td>
<td>A A T T A C</td>
<td>A A A A A A</td>
<td>3.8 (±0.8)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.7 (±0.2)</td>
</tr>
<tr>
<td><em>ureA</em></td>
<td>T A A C A C</td>
<td>A A T T A C</td>
<td>C A A A A A</td>
<td>16.8 (±13.2)</td>
<td>62.2 (±33.7)</td>
</tr>
<tr>
<td><em>HSSPnixA</em></td>
<td>T A A C A C</td>
<td>T A T T C A</td>
<td>T T T T A A</td>
<td>26.0 (±11.7)</td>
<td>423.7 (±459.3)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>ureA</em></td>
<td>T A A C A C</td>
<td>T A A T T C</td>
<td>T T T T T A</td>
<td>3.8 (±0.3)</td>
<td>237.0 (±75.0)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>HSSSureA</em></td>
<td>T A T T A C</td>
<td>T A A T T C</td>
<td>T T T T T A</td>
<td>13.8 (±7.1)</td>
<td>258.1 (±288.5)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>HSSPureA</em></td>
<td>T A T T A C</td>
<td>A T T A C</td>
<td>C A A A A A</td>
<td>24.2 (±8.6)</td>
<td>67.2 (±66.2)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Affinities were calculated from best fits using Eq. 5-1 and are reported in nM and the average of at least two experiments are reported with the standard deviation. Wild-type values are from Chapter 2 and (7) and Lys48Ala values for the wild-type *nixA* and *ureA* promoters are from Table 5.2.

<sup>b</sup> HSSP, half site and spacer; HS, half site (e.g., *ureA*HSSP*nixA*, *ureA* half-sites and spacer in the *nixA* promoter). Half site sequences are highlighted in grey and bases that differ from the wild-type promoter are boxed.

<sup>c</sup> A lower limit estimate.
affinity (<10-fold) indicate that the sequence substitutions in the hybrid promoters did not remove any key protein-DNA contacts, and further demonstrate that both half site and spacer DNA sequences are necessary for high-affinity DNA-binding by NikR, with the half-sites making larger contributions relative to the spacer sequences.

In contrast, Lys48Ala NikR was very sensitive to substitutions that increase the *ureA* sequence content of the promoter (Table 5.3), showing a 9-fold decrease in affinity when the *ureA* half sites were inserted into the *nixA* promoter and an additional 7-fold decrease in affinity when both the *ureA* half sites and spacer were inserted into *nixA*. However, these effects were context dependent, as the introduction of the *nixA* half sites into the *ureA* promoter did not improve Lys48Ala NikR binding, whereas introduction of the *nixA* half sites and spacer into the *ureA* promoter increased the affinity only 3.5-fold. These data argue that sequences outside of the half sites and spacer of *ureA* destabilize the Lys48Ala NikR-DNA interaction.

The affinity changes observed for the two proteins support two key aspects of NikR DNA recognition: sequence elements throughout individual promoter sequences are important for DNA-binding and protein conformational flexibility is required for NikR to recognize the two promoters. This flexibility likely accounts for the modest changes in affinity of wild-type NikR for the *nixA* and *ureA* hybrid
promoters, while Lys48Ala NikR cannot stably adopt the conformation required to interact with the ureA promoter with high affinity.
Discussion

Comparison of multiple RHH protein crystal structures in the presence and absence of DNA indicates that the RHH domain undergoes little or no large scale structural changes in response to DNA-binding (28, 37, 44, 46, 47, 49, 53). Instead, the side chain orientations of the DNA-contacting β-sheet residues are thought to change to mediate optimal protein-DNA interactions (46). This idea is consistent with the differences in the number, location and symmetry of hydrogen bonds made between β-sheet residues of different RHH proteins and their DNA recognition sites (43, 46). The experimental data presented here builds upon that in previous chapters and clearly shows that *H. pylori* NikR exists in two distinct conformations when the protein is bound to the *nixA* and *ureA* promoters (Figures 5.3 and 5.4; Chapter 4). One possibility is that structural alterations in the NikR C-terminal domain change the spacing of the two N-terminal RHH domains relative to one another, which would result in differences in the alignment of the domains with the DNA helix. A second, not mutually exclusive, possibility is that conformational changes are localized to the RHH domain and either or both of the α1 helix and/or N-terminal arm structure is different when NikR is bound to the two promoters.

Because the majority of RHH proteins [e.g., Arc, (52); Mnt, (51); FitA, (54)] are known to bind to only one recognition site in their respective genomes, little is known about the potential for degenerate DNA recognition as observed for *H.*
*pylori* NikR. MetJ is the only RHH protein other than *H. pylori* NikR that has been shown to directly bind to multiple promoters from genes it regulates *in vivo* (40). Promoters recognized by MetJ contain varying numbers of heptad binding sites with different sequences, ranging from 50 to 100% conservation per heptad relative to the consensus sequence with a minimum of two heptads required for binding (40). However, only one co-crystal has been solved for MetJ bound to a single DNA recognition site (49), so it is not possible to compare the conformations of the MetJ RHH domains when bound to different DNA sequences.

Multiple DNA-bound protein conformations may be a consequence of the Arg-Ser-Ser β-sheet sequence of *H. pylori* NikR. Co-crystal structures have been solved for three RHH proteins that contain individual Ser residues in their β-sheets: MetJ, Lys-Thr-Ser, (49); FitA, Ser-Val-Arg, (38); and RelB, Ser-Asn-Arg, (35). The latter two are slightly unusual in that the basic residues usually present at the N-terminal position of the β-sheet that make the largest number of base contacts are instead located at the last position, although additional examples of this order exist (e.g., Arc and Mnt). In the MetJ-DNA structure the Ser makes no DNA contacts, while the Lys and Thr residues make fewer total base contacts relative to other RHH proteins (28, 35, 37, 38, 44, 47-49, 53, 56). The shared ability of MetJ and *H. pylori* NikR to bind multiple promoters with different recognition sequences suggests that the R-S-S β-sheet of NikR may also make
fewer base-specific contacts, and possibly fewer specific contacts overall, than those observed for other RHH proteins, allowing for greater flexibility in sequence recognition. Although this somewhat contradicts what is observed for R-T-T-T-containing *E. coli* NikR (47), Ser-containing β-sheets (albeit in different primary sequence contexts) represent a large proportion of currently sequenced NikR family members (Figure 6.2), indicating that greater flexibility of DNA sequence recognition may be a common feature of the NikR subgroup of RHH proteins.

One way in which *H. pylori* NikR may compensate for the loss of β-sheet DNA contacts in some or all contexts is via the N-terminal arm, which was shown to be important for maintaining a hierarchy of binding affinities to promoters from multiple NikR-regulated genes, as well as inhibiting non-specific DNA-binding [Figure 4.5, Table 4.2; (7)]. These previous observations support a role for the arm in DNA-binding specificity, although not necessarily by making direct contacts. The Fe-BABE cleavage data (Figure 5.4) indicate that the N-terminal arm is in close proximity to the DNA of both promoters, suggesting that the arm may interact with the DNA phosphate backbone as observed for the N-terminal arms of other RHH proteins (32, 43, 44, 49). Together with the evidence that the arm impacts DNA-binding specificity, this suggests that the structure of the DNA recognition sites outside of the NikR half-sites may be critical for specific DNA-binding, which is discussed further below. Alternatively, the arm of NikR may make base contacts outside of the half-sites, similar to the base specific contacts...
made by the Mnt repressor N-terminal arm, which occur exclusively via the arms internal to the Mnt tetramer at the center of the DNA recognition site (8, 33).

The Fe-BABE cleavage data of the arm modified proteins for \textit{ureA} in the half site and spacer region (Figure 5.4) can be interpreted to indicate greater internal arm flexibility, or that the relative locations of multiple Cys side chain orientations (particularly those of the two internal arms) are different relative to \textit{nixA}. The greater intensity of cleavage of \textit{ureA} promoter DNA suggests that the two internal N-terminal arms of NikR are closer to the DNA than for the \textit{nixA} promoter, clearly indicating a difference in overall arm conformation(s) at the two promoters. This is consistent with previous findings that showed a cation requirement for the arm truncation mutant nt9-NikR only for the \textit{ureA} promoter in mobility shift assays [Chapter 4; (7)], although more detailed studies are still required to determine the role of the arm in the cation requirement.

Fe-BABE cleavage patterns produced by both helix $\alpha$1 and the two arm modified proteins were asymmetric at \textit{ureA} but not \textit{nixA} (Figures 5.3 and 5.4). Similar to the differences in overall cleavage patterns of Asn20Cys at \textit{nixA} and \textit{ureA}, the asymmetry of cleavage at \textit{ureA} could be explained by differences in the C-terminal domain of NikR or the RHH domain that would orient the four modified side chains of each mutant differently relative to the DNA helix.

The identification of a potential Glu47-Lys48 salt bridge between the two $\alpha$2 helices of the RHH domain as a requirement for high-affinity \textit{ureA}-binding, but
not nixA-binding, supports the conclusion that differences in the RHH domain conformation exist between NikR bound to the two promoters. In every RHH protein-DNA co-crystal structure there are interactions between the N-terminus of helix α2 and the phosphate backbone flanking each end of the repeat sequences (32, 43, 44, 49). These protein-DNA contacts are thought to rigidly anchor the β-sheet motif to DNA (43). The location of the Glu47-Lys48 salt bridge in the middle of the α2 helices suggests that this interaction may position the NikR β-sheet motif differently at nixA and ureA, which implies that the RHH domain undergoes a small conformational change that is likely a result of recognition sequence differences in the two promoters. This conformational change could alter the NikR-DNA backbone contacts, perhaps by one or more phosphate groups, which would re-orient the β-sheet side chains.

Despite the RHH domains of apo-H. pylori NikR and holo-Pyroccoccus horikoshii NikR having similar overall structures, the H. pylori NikR structure (20) showed differences in the conformation of helix α2 relative to the structure of P. horikoshii NikR (13), including significantly different angles between the α2 helices and the plane of the C-domain (16 v 7.5 degrees, respectively), an absence of contacts between the RHH- and C-domains in H. pylori NikR, and different interactions between the helix α1-helix α2 loop and helix α4 (20). In addition, three extra amino acids are present in the loop between helix α2 and sheet β2 of H. pylori NikR compared to P. horikoshii NikR, which could increase
the flexibility of the RHH and C-domains relative to one another. Glu47 and Lys48 are not conserved within the NikR family and are not present in *P. horikoshii* NikR. The differences in structures might suggest that this region of the *H. pylori* NikR is more flexible as a result of a decreased number of C-terminal domain interactions, which could be essential for the ability of *H. pylori* NikR to recognize different DNA sequences. It is important to note that the DNA recognition site(s) and target gene(s) regulated by *P. horikoshii* are not known, so it is unclear if this NikR structure represents a family member that binds to multiple gene promoters or just a single DNA site.

Swapping the *nixA* and *ureA* half-sites and spacers within otherwise wild-type promoters further supported this idea because hybrid promoters with the non-cognate half-site and spacer were bound with reduced affinity by wild-type NikR relative to the wild-type promoters, and the introduction of the *nixA* half-sites into the *ureA* promoter was unable to restore Lys48Ala NikR high-affinity DNA-binding (Table 5.3). Additionally, despite the *ureA* half sites and spacer being sufficient to decrease Lys48Ala NikR affinity for an otherwise wild-type *nixA* promoter to a low affinity similar to the wild-type *ureA* promoter, the *nixA* half sites and spacer were not capable of restoring a high affinity of Lys48Ala NikR for an otherwise wild-type *ureA* promoter. These data show that a combination of half-site, spacer and flanking sequences are required for high-affinity DNA-binding.
This idea is consistent with recent studies examining DNA interactions made by the two RHH proteins ParG (55) and PutA (56). The recognition motifs of NikR, ParG and PutA are significantly different from each other [NikR - one 6 bp inverted repeat, (12); ParG - eight 4 bp direct repeats, (5); PutA - five 6 bp direct repeats, (56)]. However, similar to NikR, ParG and PutA DNA sequences flanking the repeats are important for specific, high-affinity DNA-binding (55, 56).

The small amount of evidence, limited to Mnt repressor (33), for base specific contacts by RHH domains outside of the β-sheet interactions suggests that this family uses both specific, or ‘direct read-out’, interactions as well as the conformation of flanking DNA sequences, or ‘indirect read-out’, interactions for specific DNA-binding (41). The fact that the DNA duplexes of many of the RHH-DNA co-crystal structures are bent to varying degrees [Arc - 50°, (44); MetJ - 50°, (49); CopG - 60°, (28); FitA - 44°, (38); NikR - 22°, (47); ParR - 46°, (48)] further supports the idea that RHH proteins with different β-sheet motifs require variations in flanking DNA structure for optimal DNA-binding, and also implies that different protein-DNA contacts can occur. These results suggest that the current thinking of RHH protein DNA recognition needs to be expanded to include the analysis of DNA structural specificity outside of the minimal binding sites often predicted from lower-resolution experiments such as DNase I footprinting.
This work provides additional insight into the mechanistic basis for *H. pylori* NikR recognition promoters with different DNA sequences, and further illuminates features of RHH domain-DNA interactions that cannot be studied using family members that recognize only a single DNA binding site. *H. pylori* NikR directly binds to at least eight different promoters (1, 7, 14, 15, 19, 21-24), although detailed mapping of NikR-DNA interactions has only been performed for two of these [Chapters 2; (7)]. Detailed analyses of NikR-promoter interactions using approaches similar to those described here will determine if NikR interacts with these promoters similarly to *nixA*, *ureA* or adopts additional conformations when bound to each DNA sequence. Future studies that take a comprehensive approach to defining the molecular details of the NikR-*nix*A and -*ure*A complexes, as well as interactions with promoters from other genes of the NikR regulon, will help to better understand how flexibility and specificity in RHH domain-DNA interactions are achieved.
Materials and Methods

Mutagenesis of NikR

Site-directed mutagenesis of individual NikR residues was carried out using the Quik Change Site-directed Mutagenesis protocol (Strategene, La Jolla, CA) using complementary oligonucleotides with the mutated codon (Table 5.4), plasmid pEB116 [Chapter 2; (7)] as template and Pfu DNA polymerase. The Met1Cys mutant was created by amplifying the nikR gene from H. pylori strain 26695 genomic DNA using primers EB580 (Table 5.4) and PC122 [Chapter 2; (7)], followed by digestion with NdeI and XhoI and cloning into pET22b. The DNA sequences of all mutants were verified by sequencing (SeqWright, Houston, TX).

Protein expression and purification

All NikR proteins and mutant variants were expressed and purified as described previously (11, 12) except that an additional ion exchange step was performed following elution off of the Ni-NTA column. Additionally, all Cys mutant proteins were purified in the presence of 1 mM β-mercaptoethanol throughout the purification protocol. Protein concentration was determined in 6 M guanidine hydrochloride (GuHCl) using $\varepsilon_{276} = 9895\, \text{M}^{-1}\, \text{cm}^{-1}$, as predicted by primary sequence analysis (26).

Promoter fragments - cloning and labeling
DNA fragments for promoter regions were amplified by PCR using the oligonucleotide pairs described in Table 5.4. Promoter fragments for the Cys mutant mobility shift, DNase I footprinting and Fe-BABE cleavage assays were generated by end-filling \textit{EagI}-digested, gel-purified PCR products. End-fill reactions used 0.2 \( \mu \text{M} \) PCR product, 1.0 \( \mu \text{l} \) 3'-5' exo' Klenow Fragment (NEB, Beverly, MA) and [\( \alpha^{32}\text{P} \)]-dGTP (Perkin Elmer, Waltham, MA) in a total volume of 40 \( \mu \text{l} \). Excess [\( \alpha^{32}\text{P} \)]-dGTP was removed using the Nucleotide Exchange Kit (Qiagen, Valencia, CA). Fragments used in the RHH domain mutant mobility shift assays with wild-type and hybrid promoters were generated as follows: 0.5 \( \mu \text{M} \) forward (5') primers (listed first in Table 5.4) for each promoter fragment was 5'-end labeled with [\( \gamma^{32}\text{P} \)]-ATP (Perkin Elmer) and 1.0 \( \mu \text{l} \) T4 polynucleotide kinase (NEB) in a total volume of 40 \( \mu \text{l} \). Excess [\( \gamma^{32}\text{P} \)]-ATP was removed by desalting and the purified primers were used in a PCR reaction with the corresponding reverse primers (listed second in Table 5.4) using plasmid DNA as the template. The resulting labeled fragments were purified using a Qiagen PCR Purification Kit (Qiagen).

\textit{Fe-BABE modification and DNA cleavage of modified Cys mutants}

Purified NikR Cys mutants were desalted into 20 mM Tris (pH 8.0), 300 mM NaCl to remove the \( \beta \)-mercaptoethanol and NiCl\(_2\) was added back to apo-protein at a stoichiometry of 2 Ni\(^{2+} \):1 NikR to ensure saturation of the high-affinity binding site. 40 \( \mu \text{M} \) protein was incubated with 15 mM Fe-BABE [(S)-
1\{[Bis\(\text{carboxymethyl}\)amino]methyl\}-2-\{4-\([2\text{-bromoacetyl}]\)amino\}phenylethyl\}(\text{carboxymethyl})amino\}acetic acid, iron\(\text{III}\); Dojindo Laboratories, Kumamoto, Japan] in a final reaction volume of 37 \(\mu l\) and incubated at 37\(^{\circ}\) C for 1 h according to established protocols (39). The reaction was quenched with an equal volume of 1 M Tris (pH 8.0) and desalted two times into 20 mM Tris (pH 8.0) 300 mM NaCl to eliminate unreacted Fe-BABE.

Fe-BABE cleavage reactions were performed in the same buffer used for DNase I footprinting, and used a variation of a previously published protocol (39). The reactions were started with 5 \(\mu l\) of a 10X stock of freshly mixed sodium ascorbate (100 mM; stored in aliquots at -20\(^{\circ}\) C) and H\(_2\)O\(_2\) (250 mM), incubated for 2 min at 22\(^{\circ}\) C and quenched with 36 \(\mu l\) of a 2.5X stock solution of thiourea (100 mM) and EDTA (75 mM).

\textbf{Dnase I footprinting}

DNase I footprinting was performed as described previously (10, 11) in a binding buffer containing 10 mM Tris-Cl (pH 8.0), 100 mM KCl, 3 mM MgCl\(_2\) and 50 \(\mu M\) NiCl\(_2\). Labeled DNA fragments were incubated with protein at 22\(^{\circ}\)C for 30 min prior to DNase I (Sigma, Saint Louis, MO) addition (final concentration 300 ng/mL). Formic acid cleavage of labeled DNA was performed using the standard protocol for Maxam-Gilbert sequencing.
Electrophoretic mobility shift assays

Mobility shift assays were performed using 7% polyacrylamide gels and electrophoresis buffer containing 50 mM Tris (pH 8.8), 25 mM boric acid and with 50 $\mu$M NiCl$_2$. The binding buffer was identical to that used for DNase I footprinting except that 10 $\mu$g/ml E. coli thioredoxin (9) and 4 ng/µl salmon sperm DNA (Fisher Scientific, Pittsburgh, PA) were added to prevent non-specific NikR accumulation in the gel wells. Labeled DNA fragments were incubated with NikR or mutant proteins at 22°C for 30 min and 20 µl of the 25 µl total volume was loaded directly onto a running gel (120 V).

Apparent affinities measured by mobility shift assays were calculated from binding curves determined by the ratio of bound (all shifted species) vs free counts as quantified using a GE Healthcare Typhoon Trio Variable Mode Imager and ImageQuant Version 5.1 software. Apparent affinities measured by DNase I footprinting were calculated from binding curves determined by the ratio of the protected DNA region normalized to a region of DNA not protected from the same lane vs the same ratio from identical regions of a protein-free lane on the same gel. The data were fit using MICROMATH SCIENTIST Version 2.01 and the following equation:

$$y = \frac{1}{1+(K_d/x)^n}$$

Eq. (5-1)

where: $y$, fraction DNA bound (ratios described above); $K_d$, protein concentration required for half-maximal DNA-binding; $x$, protein concentration; and $n$, Hill coefficient. All reported affinities are the average of at least two independent
experiments using a dilution series of at least 8 protein concentrations with at least one 16-point titration, and the standard deviation is also reported.
Table 5.4. Primers used for *H. pylori* NikR mutagenesis, hybrid promoter construction and promoter fragment labeling.

<table>
<thead>
<tr>
<th>Protein/gene</th>
<th>Primer Name</th>
<th>Sequence (5′→3′)</th>
<th>Plasmid Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>C96A</td>
<td>EB059</td>
<td>AGCGGGACGCATGTTTTAgcACCACGCACATTCCACATG</td>
<td>pEB173</td>
</tr>
<tr>
<td></td>
<td>EB060</td>
<td>CATGTGAATGTGCGTGTgcTAAACACTGCGTCGCCGCT</td>
<td></td>
</tr>
<tr>
<td>M1C</td>
<td>EB580</td>
<td>gatcatacatgtgcGATAACCGAACAAGATGAT</td>
<td>pEB294&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>T3C</td>
<td>EB562</td>
<td>GATTTCCTCTCAGATGATgcCCCAATAAGACGATTCA</td>
<td>pEB278</td>
</tr>
<tr>
<td></td>
<td>EB563</td>
<td>TGAATCGTGCTTTATGGGgcaATCCATTGAGAxAATC</td>
<td>pEB286&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>N5C</td>
<td>EB564</td>
<td>TCTCAATGGATACAACCgcaAAAGACGATTTCAATCATC</td>
<td>pEB279</td>
</tr>
<tr>
<td></td>
<td>EB565</td>
<td>GATGATTGACTGCTCTTtgcaGGGTGTATCCATTGAGA</td>
<td>pEB287&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>D7C</td>
<td>EB566</td>
<td>ATGGATACCACCAATAAtgcGATCCATCACTCGCTTTT</td>
<td>pEB280</td>
</tr>
<tr>
<td></td>
<td>EB567</td>
<td>AAAGCGGATGATTTGATGcaTTTATTGGGTGTATCCAT</td>
<td>pEB288&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>S9C</td>
<td>EB568</td>
<td>ACACCCAAAAGACGATgcatGCTCATCCGGCTTTTTCGTT</td>
<td>pEB281</td>
</tr>
<tr>
<td></td>
<td>EB569</td>
<td>AACCCGAAACCGGATGACAACGATTTTATTGAGGTTGCTT</td>
<td>pEB289&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>N20C</td>
<td>EB570</td>
<td>TTGCGTTTCTCTTACAAAtgcTTTATTAGACGAATTAGAC</td>
<td>pEB282</td>
</tr>
<tr>
<td></td>
<td>EB571</td>
<td>GTCTAATTTGCTCTATAAGaTTGTTGAAAGAAACGCA</td>
<td>pEB290&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>D23C</td>
<td>EB572</td>
<td>TTAACAAAAATTTTATTgtcGAATTAGAACGATCTAC</td>
<td>pEB283</td>
</tr>
<tr>
<td></td>
<td>EB573</td>
<td>GATGCGGTGCTCTAATTGcTaTTTTTTTTTGTGTAAT</td>
<td>pEB291&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>N27C</td>
<td>EB574</td>
<td>TTATTAGACGAATTAGACTCggCAACCCATTTAATACGCGC</td>
<td>pEB284</td>
</tr>
<tr>
<td></td>
<td>EB575</td>
<td>GCCGTCTTTATGTGCGcGcatGCTCATATTGCTTAATATA</td>
<td>pEB292&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>I30C</td>
<td>EB576</td>
<td>GAATTAGACAAACCgCAtgcTAAACACGCTATTCTCTTCT</td>
<td>pEB285</td>
</tr>
<tr>
<td></td>
<td>EB577</td>
<td>AGAAGAATAGCGGTTTgcaGATGCGGGTTGTCTAATTC</td>
<td>pEB293&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>K48A</td>
<td>EB268</td>
<td>CGCGACATGATCAGAGAgcaTTAGTAGAGAAGACATTGG</td>
<td>pEB241</td>
</tr>
<tr>
<td></td>
<td>EB269</td>
<td>CCAATTGTCTCTACTAAAttgcTCTTGATCATGTCGCGG</td>
<td>pEB241</td>
</tr>
<tr>
<td>E47A</td>
<td>EB636</td>
<td>GTGCCGGACATGATCAGAgcaAAAATTATGAGAAGACAT</td>
<td>pEB300</td>
</tr>
<tr>
<td></td>
<td>EB637</td>
<td>ATTTGCTTCTACTAATTtgctTCTGATCATGTCGCGG</td>
<td>pEB300</td>
</tr>
<tr>
<td>D52A</td>
<td>EB634</td>
<td>AGAAGAAATTAAGTAGAGAAgcaAAATTTGGCGAGAGACACAA</td>
<td>pEB299</td>
</tr>
<tr>
<td></td>
<td>EB635</td>
<td>GTGTCTTCTCTGCGCAATTtgctTCTACTAAATTTTCTTCT</td>
<td>pEB299</td>
</tr>
<tr>
<td>Genotype</td>
<td>Accession</td>
<td>Sequence</td>
<td>Location</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
<td>--------------------------------------------------------------------------</td>
<td>----------</td>
</tr>
<tr>
<td>E47A-D52A</td>
<td>EB658</td>
<td>GTGCAGCGACATGATGACgcaAAATTAGTAGAAGcaAAT</td>
<td>pEB308</td>
</tr>
<tr>
<td></td>
<td>EB659</td>
<td>ATTgcTTCTACTAATTtgcTCTGATCATGTCGCCAC</td>
<td>pEB308</td>
</tr>
<tr>
<td>E47K-K48E</td>
<td>EB640</td>
<td>GTGCAGCGACATGATGACgcaAAATTAGTAGAAGcaAAT</td>
<td>pEB302</td>
</tr>
<tr>
<td></td>
<td>EB641</td>
<td>CCAATTGCTTCTCTACTAAttcttTCTGATCATGTCGCCAC</td>
<td>pEB302</td>
</tr>
<tr>
<td>K48D-D52K</td>
<td>EB638</td>
<td>CGGCAGACATGATGACgcaAAATTGGGAGAGACAAC</td>
<td>pEB301</td>
</tr>
<tr>
<td></td>
<td>EB639</td>
<td>GTTGTCTTCTTCTTCATTTCATTTGAAGGGGAGGACAC</td>
<td>pEB301</td>
</tr>
<tr>
<td>nixA</td>
<td>EB629</td>
<td>AAAATTTTATGGGACTTTTGCAAGAAA</td>
<td>pEB106'</td>
</tr>
<tr>
<td></td>
<td>EB618</td>
<td>CAATGCATGCAAGAACACAATCGCTAA</td>
<td>pEB106</td>
</tr>
<tr>
<td>ureA</td>
<td>EB632</td>
<td>GTTTTTGAATTTATTATAATTTTTTTAAAGGGGAGG</td>
<td>pEB131'</td>
</tr>
<tr>
<td></td>
<td>EB620</td>
<td>AATCAAGGTGGATGTTATCTGCAAC</td>
<td>pEB131</td>
</tr>
<tr>
<td>nixA flanking</td>
<td>EB662</td>
<td>ctagatggtaccATTTTTCCAATCACATGGGACAC</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td>EB663</td>
<td>glacatagttaccGATCGCAACAGCGCTGATGATGGT</td>
<td>none</td>
</tr>
<tr>
<td>ureA flanking</td>
<td>EB660</td>
<td>ctagatggtaccAACAAAATAATGGGCTAATCCTC</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td>EB661</td>
<td>glacatagttaccCGCTTCATACCCACTTCTGATGAT</td>
<td>none</td>
</tr>
<tr>
<td>ureAHSSPinixA'</td>
<td>EB666</td>
<td>TTCTAAATTAAAAATA[TAACACTAATTCTATTAAAAATAAT][TTTTTTTAAAAGGGTGCC</td>
<td>pEB309</td>
</tr>
<tr>
<td></td>
<td>EB667</td>
<td>CGGCACTTTTTAGAAGAAA[TTTATTTAAAAATGAAATGTGTTA][TTTTTTGTAATTAGGAAGAAG</td>
<td>pEB309</td>
</tr>
<tr>
<td>nixAHSSPuraA</td>
<td>EB664</td>
<td>TATAGCGCTTCAAGATA[TTTACAAATTCACAATGGGACAC</td>
<td>pEB310</td>
</tr>
<tr>
<td></td>
<td>EB665</td>
<td>ATGACGAGACTA[TTATACCTTTTTGTAATTGTAAT][TTTCTTTGAAGCCTAAT</td>
<td>pEB310</td>
</tr>
<tr>
<td>ureAHSnixA</td>
<td>EB652</td>
<td>TTCTAAATTAAAAAATA[TAACAC]ATTACCAAAAAAAATA[ATAATA][TTTTTTTTAAAAAGGTCG</td>
<td>pEB311</td>
</tr>
<tr>
<td></td>
<td>EB653</td>
<td>CGGCACTTTTTTTAAAGAAA[TTTAA][TTTTTTGTATGTAAT][TTTTTTGTGAATGAAGAAGAAGCCA</td>
<td>pEB311</td>
</tr>
<tr>
<td>nixAHSureA</td>
<td>EB650</td>
<td>TATAGCGCTTCAAGATA[TTTAC]TTTCAATTTTAA[GTATTA]ATTATGTATGAAGCCT</td>
<td>pEB312</td>
</tr>
<tr>
<td></td>
<td>EB651</td>
<td>ATGACGAGACTA[TTATACCTTTTTGTAATTGTAAT][TTTCTTTGAAGCCTAAT</td>
<td>pEB312</td>
</tr>
<tr>
<td>wild-type nixA</td>
<td>EB628</td>
<td>ctagatggtaccAAAATTTTTTAGGCAATTTTGCAAAA</td>
<td>pEB106'</td>
</tr>
<tr>
<td></td>
<td>EB618</td>
<td>CAATGCATGCAAGAACACAATCGCTAA</td>
<td>pEB106'</td>
</tr>
<tr>
<td>wild-type ureA</td>
<td>EB631</td>
<td>glacatagttaccGTCCCCATTTTAAATTTCTAAGG</td>
<td>pEB131'</td>
</tr>
<tr>
<td></td>
<td>EB620</td>
<td>AATCAAGGTGGATGTAATTGTAGCAA</td>
<td>pEB131'</td>
</tr>
<tr>
<td>Hybrid</td>
<td>Primer</td>
<td>Sequence</td>
<td>Plasmid</td>
</tr>
<tr>
<td>------------</td>
<td>--------</td>
<td>---------------------------</td>
<td>---------</td>
</tr>
<tr>
<td>nixAs</td>
<td>EB629a</td>
<td>AAAATTTTTTAGGGCAATTTCAGAAA</td>
<td>pEB309/311</td>
</tr>
<tr>
<td>ureA</td>
<td>EB632a</td>
<td>GTTTTGATTTTATAAATTCTAAAGG</td>
<td>pEB310/312</td>
</tr>
</tbody>
</table>

a, Underlined bases correspond to restriction sites, capitalized bases correspond to regions complementary to genomic DNA sequence and bracketed sequences correspond to hybrid promoter sequences introduced into an otherwise wild-type promoter.

b, Met1Cys in Cys96Ala background is pEB295.

c, Second plasmid listed for each primer pair is in Cys96Ala background.

d, See Chapter 2 (7).

e, HSSP, half-site and spacer; HS, half-site.

f, Plasmid used as the template in a labeling PCR reaction.

g, Used with EB618 (nixA) or EB620 (ureA) in a labeling PCR reaction.
References


originate from a common ancestor and possess a similar regulatory region. Proc Natl Acad Sci U S A 83:867-71.


34. **Lakshimanarayanan, A. V. a. S.-r., V.** Stereochemistry of nucleic acids and polynucleotides. Biochimica et Biophysica Acta **204:**49-53.


Chapter 6.

Evolution of DNA-binding among NikR family members via the N-terminal arm
Overview

Transcriptional regulation allows cells to alter their gene expression in response to changes in the environment. Cell types that express homologous transcriptional regulators are often quite diverse as a result of their genotype (e.g., different single-cell organisms) or phenotype (e.g., developmental stage, tissue type). These differences demand that the activities of homologous transcription factors are flexible regardless of their specific activating signal. Much discussion exists in the literature concerning the main factors contributing to the plasticity of transcriptional regulation, which includes changes in DNA recognition site sequences or mutation of the primary sequence of transcriptional regulators, as well as alterations in the connectivity of different transcriptional networks. However, less common is experimental evidence demonstrating the consequences of such changes throughout evolution. The previous chapters of this thesis have identified multiple unique aspects of the DNA-binding and regulatory capabilities of one member of the NikR family of transcription factors. In this last experimental chapter I explore the idea that the general strategy used by one NikR family member has been similarly exploited by additional NikR proteins to change their activity.
Abstract

To explore the versatility of DNA sequence recognition by the NikR family, seven NikR homologs with different N-terminal sequences and lengths were purified and measured for DNA-binding to promoters from predicted target genes. Two NikR proteins bound to two different promoter fragments from their respective genomes, one pair from genes encoding a newly identified family of ABC-type nickel transporters and the second pair from one operon encoding a similar nickel transporter and from an operon encoding an Fe-only hydrogenase. DNase I mapping of NikR binding sites identified novel recognition site architecture, notably NikR from Geobacter uraniireducens protected a ~70 bp region of DNA that spanned two sets of inverted repeats. Mutagenesis of the N-termini of NikRs from two Geobacter spp. demonstrated new roles for the N-terminal arm in NikR DNA-binding, with arm site mutants of G. uraniireducens NikR being affected in both DNA affinity and specificity. These results demonstrate that the N-terminal arm is an adaptable structural feature that has evolved in multiple NikR family members to modulate DNA-binding activity in distinct ways.

265
Introduction

A recent bioinformatics analysis (42) of greater than 700 sequenced genomes from all three domains of life revealed that despite the common notion that the transition metal nickel is rare in biology (17), approximately 60% of the 319 bacterial genomes analyzed, 85% of the 45 archeal genomes analyzed and 30% of the eukaryotic genomes analyzed encode known nickel-dependent enzymes. Eukaryotic nickel utilization is limited to a single nickel-dependent enzyme, urease - that is found in plants and fungi (25), so the smaller proportion of eukaryotes that use nickel is not unexpected. In contrast, prokaryotes encode at least nine nickel-dependent enzymes that are integral to energy generation, nitrogen assimilation and de-toxification, including the [Ni-Fe] hydrogenase, methylCoM-reductase, urease and Ni-superoxide dismutase (Ni-SOD) (25). Notably, the numbers and combinations of nickel-dependent enzymes in bacteria and archea vary significantly, which likely reflects the disparate growth environments and lifestyles of microorganisms.

Similar analysis of the prevalence of known nickel-specific transporters in completed genomes indicated that most nickel-utilizing organisms also encode identifiable nickel transporters, with the most prevalent transporters belonging to the NikMNQO ABC-type family, although the UreE/HupJ and nickel-cobalt permeases (NiCoTs) were also frequent among bacteria (42). Together with the mosaic distribution of nickel-dependent enzymes, these observations indicate
that the content of nickel-related genes among organisms that use nickel for
growth is highly variable, which is likely a reflection of different physiology.

As is the case for other transition metals, the different aspects of nickel
utilization (acquisition, intracellular trafficking, enzyme assembly, storage and
export) must be tightly controlled by cells to prevent the toxicity of excess
intracellular nickel. One mechanism by which prokaryotes mediate nickel
homeostasis is transcriptional regulation by the Ni\(^{2+}\)-dependent transcription
factor NikR (6, 9, 12, 40). Bioinformatics analyses similar to those described
above indicated that many, although not all, nickel-utilizing microorganisms
encode NikR homologs (42), raising the question of how NikR DNA-binding is
modulated in response to different microbial nickel physiologies.

*Escherichia coli* NikR was the first identified member of this large family of
homologous transcription factors (6, 12), and its function is relatively well
understood [see Figure 6.1; (6, 8, 12, 23, 31, 40)]. Previous chapters of this
thesis examined the biological and biochemical functions of a second NikR family
member from *Helicobacter pylori*, and identified aspects of *H. pylori* NikR activity
that are distinct from *E. coli* NikR. However, it remains to be determined if *H.
pylori* and *E. coli* NikR are representative of two different subsets of the NikR
family with very distinct DNA recognition properties, with *E. coli* NikR binding a
single sequence with high specificity (8) and *H. pylori* binding many sequences
(4), or if a spectrum of DNA-binding affinity and specificity occurs throughout the
family.
Figure 6.1. Summary of *E. coli* NikR-DNA interactions. (a) Crystal structure of *E. coli* NikR bound to its operator (34). The DNA-contacting \( \beta \)-sheet residue side chains are shown in black. (b) Schematic of residue-DNA contacts with base contacts in black and phosphate contacts in grey [modified from (34)].
Significant variation in DNA-contacting β-sheet residue identity and N-terminal arm length exists within the NikR family [Chapter 4; (4)], suggesting that differences in regulator amino acid sequence are one way that nickel-dependent regulation may evolve in response to distinct nickel physiology. To better understand how DNA-binding activity varies across the NikR family, this chapter describes the initial characterization of DNA-binding by four additional NikR proteins that contain different N-terminal arm and β-sheet sequences. Several new aspects of NikR-DNA interactions were revealed, including new DNA recognition sequences, the identification of multiple NikR tetramer binding sites within a single promoter, and the first example of NikR binding to a promoter linked to an enzyme requiring a different metal. Additionally, deletion and site-directed mutagenesis of the N-terminal arm of one NikR protein identified an important role for this arm in NikR DNA-binding. These results provide experimental evidence that NikR DNA-binding is modulated in response to different nickel physiology through changes in amino acid sequence at the N-terminus of the protein, and further indicate that information from only two NikR homologs is insufficient for making accurate, complete predictions about the DNA-binding activities of related transcriptional regulators.
Results

To explore differences in DNA-binding activity of NikR proteins from organisms with varying physiologies I took a combined bioinformatic and biochemical approach. Sequence alignments identified NikR proteins containing significant differences relative to the first two biochemically characterized NikRs from *E. coli* and *H. pylori*, as well as homologs from organisms encoding different combinations of known nickel-dependent enzymes in their genomes. A second criterion for pursuing particular homologs was the presence of identifiable candidate target genes likely to be regulated by NikR. Selected NikR family members were subsequently analyzed for their over-expression in *E. coli*, purification using Ni-NTA agarose chromatography and detectable DNA-binding activity.

*Sequence variation at the N-terminus of NikR family members*

Alignment of 74 non-identical N-terminal amino acid sequences from different NikR homologs that contained all four high-affinity Ni\(^{2+}\)-binding site ligands in the NCBI database (first performed, 06-2007; updated data presented here, 04-2009) based on any residues N-terminal to the \(\beta\)-sheet and the \(\beta\)-sheet residues showed a high degree of variability within the NikR subgroup of RHH family members (Figure 6.2).
<table>
<thead>
<tr>
<th>Organism</th>
<th>Accession</th>
<th>Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Campylobacter_hominis</td>
<td>MKELARFSVT</td>
<td>M(X\textsubscript{2,22})RS</td>
</tr>
<tr>
<td>Collinsella_stercoris</td>
<td>MDETHATSHVAPGDVPVHTPPAEARFSVT</td>
<td></td>
</tr>
<tr>
<td>Gemmata_observiglobus</td>
<td>MSELTFRFSVS</td>
<td>M(X\textsubscript{2,3})RS</td>
</tr>
<tr>
<td>delta_proteobacterium_MLMS-1</td>
<td>MLKRFFSVS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Desulfitalea_psychrophila</td>
<td>MLKRFFSIS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Pyrococcus_abyssii</td>
<td>MOLVRFSSIS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Sulfini.hydrogenibium_azerense</td>
<td>MEGLVRSIS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Pyrococcus_horkoehii</td>
<td>MELRFSIS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Methylobacter_petroleumiu</td>
<td>MERTFIS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Deltia_acidovorans</td>
<td>MPPPVSPLLMERFITS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Rhodopseudomonas_palustris</td>
<td>MERVTS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Plesiostocysti_pacifica</td>
<td>MDROFDRVS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Helicobacter_pylori_26695</td>
<td>MDTNPKKDSSIRFVS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Helicobacter_acinonychis</td>
<td>MDTNPKKDSSIRFVS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Helicobacter_pylori_B128</td>
<td>MDTNPKKDSSIRFVS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Helicobacter_pylori_HPAG1</td>
<td>MDTNPKKDSSIRFVS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Campylobacter_conulus</td>
<td>MDENVFVS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Campylobacter_rectus</td>
<td>MENVRFSS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Campylobacter_curvus</td>
<td>MENVRFSS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Helicobacter_hepaticus</td>
<td>MKSSRFSS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Helicobacter_cinaed</td>
<td>MKQQRFSS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Campylobacter_fetus</td>
<td>MEGEDKIRFSS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Sherwannella_pealeana</td>
<td>MSNDTIRTFVS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Sherwannella_sediminis</td>
<td>MSNDTIRTFVS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Persephena_marina</td>
<td>MGKTFIRFSS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Thermococcus_kadakarensis</td>
<td>MKIIRFGVS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Thermoaerobacter_tengongensis</td>
<td>MLLFISKIAQHKGIGCLEGIVRFGV</td>
<td></td>
</tr>
<tr>
<td>Pyrococcus_furfosus</td>
<td>MGVIFRGVS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Thermococcus_sp</td>
<td>MAIVRFGS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Dictyogomus_turgidum</td>
<td>MKAVRFGS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Dictyogomus_thermovilum</td>
<td>MTKIVRFGS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Thermococcus_barophilus</td>
<td>MKIVRFGS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Thermoaerobacter_pseudethano</td>
<td>MGVISLIEIRFVGVS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Aciduliproducens_boonei</td>
<td>MQQRFSS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Prosthecocitrus_austriari</td>
<td>MKQRFSS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Syntrophus_acidifluoridificus</td>
<td>MSDIFVRFSS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Kuenenia_stuttgartensis</td>
<td>MSKQSVTHNLIRMFSSFLVRFVGVS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Chlorobium_phaeobacteroides</td>
<td>MSEYRFGS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Geobacter_unariedouensis</td>
<td>MGGETIRFSGS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Geobacter_bermsiens</td>
<td>MGGETIRFSGS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Lawsonia_intracellularis</td>
<td>MGEXITRFGS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Desulfovibrio_vulgaris</td>
<td>MGTRIRFGS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Desulfovibrio_desulfuricans_G20</td>
<td>MGQTRFVGVS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Desulfovibrio_salexigens</td>
<td>MGQTRFVGVS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Desulfovibrio_piger</td>
<td>MGQTRFVGVS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Desulfovibrio_desulfuricans</td>
<td>MGQARFGVS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Chlorobium_terpum</td>
<td>MSQLYRFGS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Chlorobium_ferrooxidans</td>
<td>MSQLYRFGS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Dethiobacter_alpalkilphus</td>
<td>MSQKYRFGS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Staphylococcus_marinus</td>
<td>MKPVPKFYG</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Geobacter_loveyi</td>
<td>MGDIRFSGS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Pseudomonas_putida</td>
<td>MQRITIT</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>MQRITIT</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Hydrogenobaculum_sp</td>
<td>MRKMDAKVIRCIT</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Methanoperas_kandieri</td>
<td>MNGKGEONLVRTSST</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Haloarchula_mariamortui</td>
<td>MSDKDIRISIT</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Methanothermobacter_thermotrophicus</td>
<td>MMIRSMIS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Methanobrevibacter_smithii</td>
<td>MECFSMMRISMS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Methanosphaera_stadtmaniana</td>
<td>MRIRSMIS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Methanocaldoceoccus_jannaschii</td>
<td>MTMIRSMIS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Methanococcus_maripaludis</td>
<td>MVDMDRISI</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Anaeromyoxobacter_sp</td>
<td>MRAMLIRGSS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Anaeromyoxobacter_dehalogenans</td>
<td>MLIRGSS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Methanoculleus_marinigri</td>
<td>MPMGAEMLRSI</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Methanocorpusculum_labreanum</td>
<td>MVELSIRGSS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Methanoregula_boonei</td>
<td>MTLENSIRGSS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Methanosarcina_mazei</td>
<td>MKKTYVHLHTISLNCIIIRINYKLGDTMETELMRIVGS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Methanosarcina_barkeri</td>
<td>METELMRIVGS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Methanoaseta_thermodicha</td>
<td>MEQELMRIVGS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>uncultured_methanogen</td>
<td>MDQELMRIVGS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Methanosaerica_acetivorans</td>
<td>METELMRIVGS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Methanosaerica_acetivorans_2</td>
<td>METELMRIVGS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Solibacter_usitatus</td>
<td>MSLIRGVS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
<tr>
<td>Archaeoglobus_fulgidis</td>
<td>MEEGTRIGVS</td>
<td>M(X\textsubscript{2,9})RS</td>
</tr>
</tbody>
</table>

271
Figure 6.2. A ClustalW (37) alignment of non-redundant NikR family members based on N-terminal β-sheet sequences and any amino acids N-terminal to the β-sheet. Listed on the far right are the number of amino acids ($X_\beta$) occurring between the N-terminal Met and the first β-sheet residue, with the DNA-contacting β-sheet residues of each NikR subgroup.
**β-sheet variation**

Nine different combinations of DNA-contacting β-sheet residues occur with the largest number of proteins containing Arg-Gly-Ser and Arg-Ser-Ser sequences (37 and 22 of the 74 sequences, respectively; Figure 6.3a). The first Arg residue is almost completely conserved (Arg, 73/74), with the exception of one Lys-containing NikR from *Staphylothermus marinus*. In contrast, five different amino acids occur at the second (Gly - 39/74, Ser - 26/74, Thr - 7/74, Cys - 1/74 or Val - 1/74; Figure 6.3b) and third (Ser - 64/74, Thr - 7/74, Asn - 1/74, Ala - 1/74 or Tyr - 1/74) positions.

Considering that Arg3 of *E. coli* NikR makes four of the five specific base interactions observed in the co-crystal structure [see Figure 6.1; (34)], the high degree of conservation at the first position is not surprising. However, the other base-specific contact observed in the co-crystal structure occurs via the second DNA-contacting sheet position (34), a Thr in *E. coli* NikR. Approximately half of the sequences contain a Gly at this position, an unlikely residue to make specific base contacts, suggesting that additional specific DNA contacts may be present to compensate for the loss of a DNA interaction at this position. Alternatively, DNA-binding may be less specific as a result of fewer specific protein-DNA contacts.
Figure 6.3. Frequency of DNA-contacting β-sheet sequences and N-terminal arm lengths among non-identical NikR homologs. (a) The number of NikR proteins containing each of nine combinations of DNA-contacting β-sheet residues. (b) The relative percentages of each residue occurring at each β-sheet position. (c) The number of NikR proteins containing increasing numbers of residues between the N-terminal (Nt) Met and the first DNA-contacting β-sheet residue.
N-terminal arm length variation

Comparison of the number of residues occurring between the N-terminal Met and the Arg of the β-sheet of each NikR protein indicated that N-terminal arm lengths range from 0 to 32 amino acids, with the largest number of homologs containing four or five amino acids between the Met and Arg (43 of 79 sequences; Figure 6.3c). There was no obvious correlation between N-terminal arm length and β-sheet sequence. One important consideration is that the NikR sequences analyzed here are based on genome annotations, so it is possible that long NikR N-terminal arms containing an internal Met, Val or Leu may represent an erroneous annotation of the nikR gene or a sequencing error.

There are only two (3%) of the non-identical N-terminal NikR sequences that contain Arg-Thr-Thr β-sheets with one amino acid arms and 22 (30%) Arg-Ser-Ser β-sheets with variable length arms (Figure 6.2), indicating that *E. coli* NikR represents one extreme of the NikR family, with only one amino acid between the N-terminal Met and the first β-sheet residue and the last two DNA-contacting β-sheet residues being Thr. *H. pylori* NikR is more representative of the general properties found at the N-terminus of the family, with an Arg-Ser-Ser β-sheet and a longer than average arm length of nine amino acids.

DNA binding site identification

An expected feature of all NikR homologs is that they repress the expression of genes encoding nickel transporters (6, 10, 12, 15, 16), so to identify potential
target genes regulated by each NikR homolog the genomes of each organism were scanned for genes encoding members of the five known nickel transporter families. This approach was also taken in an earlier study (30), where the identification of predicted NikR recognition sites allowed for the assignment and characterization of a new family of nickel-specific ABC-type transporters. NikR binding to the predicted sites was not tested and predictions of \textit{H. pylori} NikR recognition sites from that study were subsequently demonstrated to be incorrect (1, 4, 14, 15), indicating that experimental knowledge of only the \textit{E. coli} NikR recognition site is not sufficient to accurately predict binding motifs for other NikR proteins. I also examined the gene neighborhoods of \textit{nikR} for likely target genes. In cases where \textit{nikR} was present as an isolated gene, binding to the \textit{nikR} promoter was also tested because transcription factor autoregulation is a common occurrence.

\textit{Functional characterization of NikR homologs}

To better understand the consequences that changes in $\beta$-sheet and N-terminal arm sequences have on DNA-binding by different NikR homologs, I selected an array of NikR proteins with $\beta$-sheets and arms significantly different from \textit{E. coli} and \textit{H. pylori} NikR to study further, with a particular focus on Arg-Gly-Ser containing proteins because they represent an unexplored subgroup of the NikR family and make up the largest proportion of the NikRs currently identified in microbial genomes. One constraining factor of this approach was the
willingness of other researchers to provide genomic DNA from little-studied microbial species.

**NikR homolog cloning**

The nine organisms for which I obtained the corresponding genomic DNA represented Arg-Gly-Ser, Arg-Cys-Thr and Lys-Gly-Tyr β-sheet NikR proteins with N-terminal arm lengths of 4, 7, 9 or 18 amino acids. One genomic sample, from *Staphylothermus marinus* strain F1 encodes two putative nikR paralogs, of which only one was present in the original alignment because the second paralog (NikR2) lacks one of the four high-affinity Ni$^{2+}$-binding site ligands (Asp in place of His76 of *E. coli* NikR). NikR2 contains similar patterns of polar and non-polar residues in the β-sheet sequence and the other three Ni$^{2+}$-binding site ligands, so likely represents a related transcriptional regulator that may have evolved a novel metal-binding site specificity, although the studies described here focused only on the DNA-binding properties of NikR proteins, not metal specificity. Because of these properties the *S. marinus* nikR2 gene was also cloned for further studies. Out of ten total cloned nikR genes, seven resulted in significant over-expression of a protein of the appropriate size (~15 kDa) under standard induction conditions. The resulting proteins bound to Ni-NTA agarose resin in the absence of any additional protein tags (Figure 6.4, Table 6.1), which has been observed for both *E. coli* and *H. pylori* NikR (1, 4, 6). Clones of the *Methanospirillum hungatei*, *Hydrogenobaculum* strain Y04ANC1 and *Campylobacter fetus* nikR genes showed no detectable expression of a protein of
Figure 6.4. ClustalW multiple sequence alignment (37) of NikR proteins previously characterized and those analyzed in the current study. Residues that are conserved in 7 or more of the 9 homologs are shaded gray and DNA-contacting β-sheet residues and high-affinity Ni\textsuperscript{2+} binding site ligands are boxed.
Table 6.1. NikR homologs that were over-expressed in *E. coli*, purified and tested for DNA-binding activity *in vitro*.

<table>
<thead>
<tr>
<th>Organism</th>
<th>NikR ORF(^a)</th>
<th>Nt sequence</th>
<th>Potential target genes</th>
<th>Recognition motifs</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. desulfuricans</em></td>
<td>Dde_2956 (DdNikR)</td>
<td>MGQTIRFGVS(^c,d)</td>
<td>Dde_1189 (<em>nikK</em>)&lt;br&gt;Dde_1190 (<em>nikM</em>)&lt;br&gt;Dde_2281 (<em>Fe-hyd</em>)</td>
<td>GTGTTA-15-TAACAC</td>
</tr>
<tr>
<td><em>D. vulgaris</em></td>
<td>Dvul_0474</td>
<td>MGRTIRFGVS(^d)</td>
<td>D_vul0540 (<em>nikK</em>)&lt;br&gt;D_vul1936 (<em>nikM</em>)</td>
<td>na(^e)</td>
</tr>
<tr>
<td><em>G. bemidjiensis</em></td>
<td>Gbem_3562 (GbNikR)</td>
<td>MGETVRFGIS(^d)</td>
<td>Gbem_2644 (<em>nikA</em>)&lt;br&gt;Gbe_2225 (<em>nikM</em>)&lt;br&gt;Gbe_3813 (<em>nikM</em>)&lt;br&gt;Gbe_3814 (<em>nikM</em>)&lt;br&gt;Gbe_3562 (<em>nikR</em>)</td>
<td>GTGTTAC-13-GTGCTAC</td>
</tr>
<tr>
<td><em>G. uraniireducens</em></td>
<td>Gura_0772 (GuNikR)</td>
<td>MGETIRFGIS(^d)</td>
<td>Gura_0780 (<em>nikM1</em>)&lt;br&gt;Gura_0772 (<em>nikR</em>)&lt;br&gt;Gura_2762 (<em>nikM2</em>)&lt;br&gt;Gura_3001 (<em>fur</em>)&lt;br&gt;Gura_1953 (<em>hypE</em>)</td>
<td>GACATAC-13-GTATTCA;&lt;br&gt;GTGCTAC-13-GTGGTAC&lt;br&gt;GTGACA-13-CTTTATA;&lt;br&gt;GTGTTAC-13-GTGCTAC</td>
</tr>
<tr>
<td><em>M. boonei</em></td>
<td>Mboo_1643</td>
<td>MTLENDLSRIGIS</td>
<td>Mboo_1643 (<em>nikR</em>)&lt;br&gt;Mboo_1640 (<em>nikQ</em>)&lt;br&gt;Mboo_2377 (<em>nikA</em>)&lt;br&gt;Mboo_1293 (<em>nikM</em>)</td>
<td>na</td>
</tr>
<tr>
<td><em>S. marinus</em> NikR1</td>
<td>Smar_0006</td>
<td>LKKPVFKGIY</td>
<td>Smar_0001 (<em>nikM</em>)</td>
<td>na</td>
</tr>
<tr>
<td><em>S. marinus</em> NikR2</td>
<td>Smar_0366 (SmNikR2)</td>
<td>MSGKRRFGVS(^d)</td>
<td>Smar_0363/0364 (<em>sbp</em>)</td>
<td>GCACAG-23-CTGTGC</td>
</tr>
</tbody>
</table>
a, ORFs are listed according to genome annotation from the Joint Genome Institute (http://genome.jgi-psf.org/).

b, DNA-binding was detected for those organisms and genes highlighted in bold.

c, DNA-contacting β-sheet residues are underlined.

d, Expressed protein lacks the N-terminal methionine (20).

e, Reported only if DNA-binding was observed
the expected size under standard induction conditions and were not pursued further.

Using the DNA binding site predictions described above, DNA-binding assays were performed for the seven NikR homologs that could be expressed and purified. DNA-binding activity was detected for four of the seven proteins corresponding to NikR homologs from *D. desulfuricans*, *G. bemidjiensis*, *G. uraniireducens* and *S. marinus* NikR2 (Figure 6.5, Table 6.1). No DNA-binding was detected for NikR proteins from *D. vulgaris*, *M. Boonei* and *S. marinus* NikR1. The lack of observable activity may be due to proteins purified in an inactive state, reaction conditions that are incompatible with DNA-binding, or incorrect target promoter predictions. DNA-binding by these homologs was not pursued further.

*NikR homologs bind specifically to promoters from genes encoding NikKMNQO-family nickel transporters and a second ABC-type transporter*

*Desulfovibrio desulfuricans* NikR (DdNikR)

DdNikR DNA-binding was tested initially to a fragment constituting the likely promoter region for an operon encoding a nickel-specific ABC-type transporter belonging to the recently identified NikMNQO family, which was also predicted to contain a NikR recognition motif (30). A second, downstream fragment spanning the 108 bp intergenic region between *nikK* and *nikM* represented another potential promoter and was also tested. DdNikR specifically bound the
Figure 6.5. DNase I footprinting of NikR orthologs binding to specific promoter fragments. The protected regions in each panel are indicated by solid black bars, with the location of each footprint relative to the start codon of each gene listed to the left for panels (b) to (d). The far left lane in panels (b) to (d) is a G+A sequencing cleavage ladder. (a) *D. desulfuricans* NikR binding to $P_{\text{nikK}}$ (1.0 $\mu$M, 200 and 40 nM). (b) *G. bemidjiensis* NikR binding to $P_{\text{nikM}}$ (2-fold serial dilution from 2.0 $\mu$M to 125 nM). (c) *G. uranireducens* NikR binding to $P_{\text{nikM1}}$ (serially diluted 2-fold from 200 nM to 781 pM). (d) *S. marinus* NikR2 binding to $P_{\text{sbp}}$ (2.0 $\mu$M, 1.5 $\mu$M and 1.2 $\mu$M).
nikK promoter (Dde1189) at the highest protein concentration tested (1.0 µM), as detected by DNase I footprinting (Figure 6.5a). While the region of DNA protected by *D. desulfuricans* NikR was not mapped, it is likely that the six base pair perfect inverted repeat (GTGTTA - TAACAC) that is separated by 15 base pairs is the recognition site for this NikR homolog (30).

**Geobacter bemidjiensis** NikR (GbNikR)

GbNikR DNA-binding was tested to a promoter from a *nikA*-containing operon (*nikA* corresponds to Gbem_2644), three candidate promoters from two *nikM*-containing operons (*nikM* homologs correspond to Gbem_2225 and Gbem_3808), as well as its own promoter (Gbem_3562; Table 6.1). GbNikR bound to a promoter fragment from one *nikMQO* operon (Gbem_2225) as detected by DNase I footprinting (Figure 6.5b). Mapping of the DNase I footprint of GbNikR at the *nikM* promoter indicated that a 30 bp region of DNA was protected, spanning an imperfect seven bp inverted repeat (GTGTTAC – GTGCTAC) that is separated by 13 bp, which was predicted previously (30). Binding to this promoter was also detected by gel mobility shift assays (Figure 6.7b), and quantitation of GbNikR titrations in both assays yielded apparent affinities of ~50 nM.

**Geobacter uraniireducens** NikR (GuNikR)

Two candidate promoter fragments were tested for GuNikR DNA-binding, including the likely promoter from a large operon that begins with a gene encoding a NikM homolog (Gura_0780) and also contains various predicted
TonB-related membrane proteins. The last gene in this predicted operon encodes NikR and, because it is located ~260 bp downstream from the closest upstream gene, GuNikR DNA-binding to a fragment spanning this potential nikR promoter was also tested. GuNikR bound specifically to the nikM promoter fragment (Gura_0780; Figure 6.5c), and mapping of the DNase I footprint indicated that this protein protected ~70 bp of DNA spanning two imperfect seven bp inverted repeats (GACATAC - GTATTCA; GTGCTAC-GTGTTAC), each separated by 13 bp and one of which was predicted earlier (30). The size of this DNase I footprint and the presence of two pairs of repeats strongly suggests that two GuNikR tetramers bind to the nikM promoter. Binding to \( P_{\text{nikM}} \) was also detected by gel mobility shift assay (Figure 6.6c and Figure 6.9a), and titration of GuNikR with \( P_{\text{nikM}} \) indicated an apparent affinity of 7.6 nM (Table 6.2).

**Staphylothermus marinus** NikR2 (SmNikR2)

The gene encoding SmNikR2 (Smar_0366) is located immediately downstream of two genes encoding likely ABC-transporter components, substrate binding protein (sbp) Smar_0364 and inner membrane protein Smar_0365, that are annotated as part of a Mn\(^{2+}\) transport system. A single gene oriented in the opposite direction, Smar_0363, and likely divergently transcribed from Smar_0364, is annotated to encode the third ATPase component of this transport system, so SmNikR2 was tested for the ability to bind to a fragment spanning the intergenic region between Smar_0363 and Smar_0364. Combined with a change in one of the four high-affinity site Ni\(^{2+}\)}
ligands in SmNikR2, it is possible that this homolog responds to a metal other than nickel, however experiments measuring SmNikR2-dependent regulation in vivo are required to identify the relevant physiological signal (23, 38).

DNase I footprinting revealed that SmNikR2 protected 21 bp of DNA, although the cleavage pattern of DNase I in this region prohibited unequivocal identification of the footprint (Figure 6.5d). The footprint overlaps with one half site of a six bp perfect inverted repeat (GCACAG - CTGTGC), however these half sites are separated by 23 bp that is a significantly larger spacing than has been observed for any other NikR recognition sequence here or elsewhere (1, 4, 6-8, 14-16).

Two NikR family members bind to multiple promoter fragments

To determine if NikR homologs with variable length N-terminal sequences behave like *H. pylori* NikR and bind to multiple promoters, the DNA recognition sequences identified by DNase I footprinting were used to search the promoter regions of each genome for additional candidate promoters (13). Inverted repeats of the size and sequence identified above were identified that contained one or two mismatches, and then screened for their location relative to the closest annotated open reading frame. Results for the candidate promoters identified by this approach are described below.
DdNikR

A DdNikR recognition sequence containing one mismatch relative to the repeats upstream of the nikK promoter (GTATTA - TAACAC vs GTGTTA - TAACAC for nikK; Table 6.1) was identified upstream of genes encoding an Fe-only hydrogenase enzyme (Dde_2281/2280). Interestingly, this imperfect inverted repeat is located an almost identical distance from the start codon of Dde_2281 as that observed for the inverted repeat located in the nikK promoter (-103 to -77 vs -105 to -79 for nikK), suggesting that DdNikR might regulate the expression of nickel transporter and Fe-dependent hydrogenase genes similarly. DdNikR binding to the promoter from Dde_2281 was detected by electrophoretic mobility shift assays at relatively high protein concentrations (> 500 nM; Figure 6.6a, b). Binding reactions run in parallel with the D. desulfuricans nikR promoter demonstrated that this interaction was specific for the Dde_2281 promoter, albeit of low affinity.

GuNikR

The GuNikR recognition sequence (one pair of inverted repeat half sites) containing two mismatches was identified in promoters from a second nikM gene (nikM2; Gura_2762), fur (Gura_3002) and hypE (Gura_1953; Table 6.1). fur encodes an iron-dependent transcription factor that regulates nikR expression and is regulated by NikR in H. pylori [Chapter 2; (14, 39)], and hypE encodes a chaperone required for [Ni-Fe] hydrogenase assembly (21). GuNikR bound to the nikM2 promoter (Figure 6.6d and 6.9a) as manifested by two distinct shifted
Figure 6.6. NikR homologs bind specifically to multiple promoter fragments. (a) 
*D. desulfovibrio* NikR binding to *P*<sub>Fe-hyd</sub> (3-fold serially dilution from 5.0 µM to 2 nM) and (b) serially diluted 3-fold from 2.0 µM to 55 nM with *P*<sub>nikR</sub> (left four lanes) or *P*<sub>Fe-hyd</sub> (right four lanes). *G. uraniireducens* NikR serially diluted from 5.0 µM to 2 nM with (c) *P*<sub>nikM1</sub> or (d) *P*<sub>nikM2</sub>. Mobility shifts were performed with 5 µM NiCl<sub>2</sub> in the gel and running buffer. F, free DNA. B, protein-bound DNA.
complexes, although with an overall 25-fold lower affinity compared to the *nikM1* promoter (Table 6.2).

*The N-terminal arms of GbNikR and GuNikR are required for DNA-binding*

To determine if residues N-terminal to a Arg-Gly-Ser β-sheet are important for DNA-binding, truncation mutants of GbNikR and GuNikR were constructed. In each case, residues 2-4 (GlyGluThr in both proteins) were deleted and the mutants (Δnt3NikRs) were tested for their ability to bind to DNA. Both Δnt3GbNikR and Δnt3GuNikR were unable to bind to their corresponding promoters, even at high protein concentrations (5 μM; Figure 6.7b-d). These results demonstrate that the arm truncation mutants behave differently than *H. pylori* nt9-NikR [Chapter 4; (4)] and that one or more residues of the N-terminal arm is essential for high-affinity DNA-binding by GbNikR and GuNikR.

*Deletion or mutation of the arm does not affect Geobacter NikR stability*

To determine if the lack of DNA-binding by Δnt3GbNikR and Δnt3GuNikR is a result of altered protein folding, full-length and Δnt3 NikR proteins were examined in the presence or absence of stoichiometric NiCl₂ using circular dichroism (CD). There was no significant difference between the full-length and Δnt3 proteins demonstrating that the absence of detectable DNA-binding by the
Figure 6.7. The N-terminal arms of GbNikR and GuNikR are required for DNA-binding. GbNikR (a) or Δnt3-GbNikR (b) serially diluted 3-fold from 500 nM to 200 pM with $P_{\text{nikM}}$. (c, d) Δnt3-GuNikR serially diluted 3-fold from 5.0 µM to 2 nM with (c) $P_{\text{nikM1}}$ or (d) $P_{\text{nikM2}}$. Mobility shifts performed with 5 µM NiCl$_2$ in the gel and running buffer. F, free DNA. B, protein-bound DNA.
Δnt3 NikRs is due to a role for the arms in protein-DNA interactions and not protein folding (Figure 6.8).

*Identification of GuNikR residues important for DNA-binding affinity*

To more specifically identify contributions of the GuNikR N-terminal arm to DNA-binding affinity arm residues were individually mutated to Ala (Gly2Ala, Glu3Ala, Thr4Ala). Also, an additional Ala was inserted immediately following the N-terminal Met to create Ala2ins (arm sequence - AGETI). Mobility shift assays of each GuNikR mutant with the *nikM1* and *nikM2* promoters demonstrated that differential contributions are made from each residue to DNA-binding affinity and specificity. The Ala2ins, Gly2Ala, Glu3Ala and Thr4Ala mutants displayed decreases in affinity of approximately 11-, 7-, 10- and 35-fold, respectively, for *nikM1* (Figure 6.9, Table 6.2). Ala2ins, Gly2Ala and Thr4Ala also had significant decreases in affinity for the *nikM2* promoter (11-, 6- and 6-fold, respectively). Interestingly, Glu3Ala displayed a modest increase in affinity of 2-fold for *nikM2*.

CD experiments revealed small differences in protein folding between the arm mutant proteins and wild-type GuNikR (Figure 6.8c), however because of where the mutations occur in the NikR protein, it is most likely these differences are the result of small amounts of contaminants in each protein preparation that affect the protein concentration determination by UV-spectroscopy. These data indicate that each residue of the GuNikR arm is required for high-affinity DNA-
binding, although their importance varies for binding to the two \textit{nikM} promoters. The differential affects of each mutation on \textit{nikM1} and \textit{nikM2}, particularly for Glu3Ala GuNikR, suggests a similar role of the GuNikR arm in DNA-binding as that determined for the \textit{H. pylori} NikR arm, which is required for maintaining a hierarchy of binding affinities to different promoters.
Figure 6.8. N-terminal arm truncation or mutation does not significantly affect the secondary structure of *Geobacter* spp. NikRs. CD scans at 22° C of 2.0 µM (a, b) apo-full length (thick black line), holo-full length (thin black line), apo-Δnt3 (thick gray line) and holo-Δnt3 (thin gray line) of (a) GbNikR and (b) GuNikR. (c) GuNikR apo- (thick lines) and holo-(thin lines) site mutants Ala2ins (black), Gly2Ala (medium gray), Glu3Ala (light gray) and Thr4Ala (darkest gray).
Figure 6.9. Residues of the GuNikR N-terminal arm are important for DNA-binding affinity. Wild-type GuNikR (a) or N-terminal site mutants A2ins (b), G2A (c), E3A (d) or T4A (e) were serially diluted 1.7-fold from 5.0 µM to 17.5 nM with $P_{\text{nikM1}}$ (left panels) and $P_{\text{nikM2}}$ (right panels). Each mutant arm sequence is indicated to the upper right of each gel. Mobility shifts performed with 50 µM NiCl$_2$ in the gel and running buffer. F, free DNA. B, protein-bound DNA. The asterisk indicates DNA in wells.
Table 6.2. Apparent binding affinities of GuNikR N-terminal arm mutants.

<table>
<thead>
<tr>
<th></th>
<th>wild-type</th>
<th>A2ins</th>
<th>G2A</th>
<th>E3A</th>
<th>T4A</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>nikM1</em></td>
<td>7.6 (±0.7)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>86.1 (±1.8)</td>
<td>53.7 (±8.6)</td>
<td>76.7 (±14.9)</td>
<td>270.2 (±8.8)</td>
</tr>
<tr>
<td><em>nikM2</em></td>
<td>197.0 (±238.0)</td>
<td>2,088.1 (±858.6)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1,205.0 (±1,549.3)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>99.8 (±47.0)</td>
<td>1,228.3 (±282.5)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>, Affinities were calculated from best fits using Eq. 6.1 and are reported in nM. The average of at least two experiments are reported with the standard deviation.

<sup>b</sup>, A lower limit estimate.
Discussion

The results presented in this chapter extend our understanding of NikR DNA recognition, demonstrate the functional consequences of amino acid changes that occur at the N-terminus of NikR family members, and have implications for nickel-dependent gene regulation in a number of microorganisms with different nickel physiologies. Through analysis of the DNA-binding properties of different NikR proteins and identification of the role of their N-terminal arms in DNA-binding, I have shown that other NikRs in addition to *H. pylori* NikR utilize amino acids N-terminal to the β-sheet motif for their DNA recognition. Specifically, NikRs from *G. bemidjiensis* and *G. uraniireducens* require their N-terminal arms for high-affinity DNA-binding, a function similar to the *H. pylori* NikR arm, although the effects of arm truncation are different for the *Geobacter* spp. and *H. pylori*. These results indicate that NikR DNA-binding activity may adapt by N-terminal sequence changes, perhaps as a result of the varying physiologies of different microorganisms.

**Novel DNA recognition site architecture**

DNase I mapping of GuNikR binding to the *nikM1* promoter and of SmNikR2 binding to the *sbp* promoter indicate significant differences in the DNA interactions of these NikR homologs compared to what is known for *E. coli* and *H. pylori* NikR. GuNikR protected a much larger ~70 bp region of DNA that spanned two sets of imperfect inverted repeats, compared to 40 bp for *E. coli*
NikR (6) and 36 bp for *H. pylori* NikR that each contain one set of repeats (1, 4, 14, 15). Although footprinting was not performed for GuNikR binding to nikM2, two sets of highly similar inverted repeats are present at identical spacing as those in the nikM1 promoter, suggesting that GuNikR may protect a similar region of DNA at nikM2. Two pairs of inverted repeat half sites combined with the larger footprint region of GuNikR strongly suggest that two tetramers of GuNikR may bind to a single promoter, which would represent a novel mode of DNA-binding by a NikR protein.

In the case of SmNikR2 binding to the sbp promoter, the footprinted region of DNA overlaps with one half site of a perfect six bp inverted repeat, however the spacing between the repeat half sites is 23 bp. The DNase I cleavage pattern in this region of the DNA precluded unequivocal interpretation of the data, so there is a possibility that SmNikR2 recognizes this inverted repeat, however this would be in stark contrast to data for *E. coli* and *H. pylori* NikR (with spacers of 16 and 13 bp, respectively), as well as results presented in this chapter for GbNikR and GuNikR (with 7 bp half sites and 13 bp spacers). Notably, the spacing between the center of the two perfect repeat half sites with the 23 bp spacer would be 29 bp, or approximately 2.5 turns of B-form DNA, which would require a dramatically different conformation of a SmNikR2 tetramer on DNA relative to that observed for *E. coli* NikR bound to DNA (34). A spacer of this size would require either significant bending of the DNA upon interaction with SmNikR2, or that SmNikR2 tetramers display significantly altered conformations relative to *E. coli* NikR.
Unique features of GuNikR DNA-binding

GuNikR bound to two nikM promoters, which is not surprising given that two paralogous nikMQO operons exist in the G. uraniireducens genome, and the promoter regions are highly similar. The downstream inverted repeats of the two NikR recognition sequences in nikM1 and nikM2 differ only at two bp (one in each half site) and six bp are different between the two sets of upstream inverted repeats (Table 6.1). The significant difference in affinity of GuNikR for the two promoters indicates that some or all of the variable recognition site positions are critical for high-affinity DNA-binding. Examination of NikR-dependent gene regulation in vivo will be required to determine if NikR regulates both nikMQO operons in G. uraniireducens, which would also clarify whether both operons encode proteins that transport nickel into the cell. It is not clear why G. uraniireducens would encode two highly similar nickel-specific import systems, but one possibility is that the NikMQO family functions to transport nickel-chelate complexes into cells, and the two NikR-regulated NikMQO transporters might recognize different nickel complexes. Evidence exists for prokaryotic utilization of nickel-chelates, although the identity of the complex(es) is not known (32). An alternative possibility is that the two transporters exhibit different kinetic properties or are expressed in response to distinct environmental conditions.

Mutagenesis of the GuNikR arm showed that each residue, as well as the overall length of the arm, is critical for DNA-binding. Thr4Ala had the most
severe decrease in affinity for the \textit{nikM1} promoter (35-fold; Table 6.2), and together with the polar nature of the Thr side chain this may indicate that Thr4 in wild-type GuNikR directly interacts with DNA and/or helps to orient the $\beta$-sheet. Considering the Arg-Ser-Ser $\beta$-sheet sequence of GuNikR relative to Arg-Thr-Thr of \textit{E. coli} NikR, it is possible that Thr4 DNA contacts compensate for the absence of the \textit{E. coli} NikR Thr5 and/or Thr7 contacts which were seen in the co-crystal structure [Figure 6.1; (34)].

Mutation of Glu3 or Thr4 affects GuNikR \textit{nikM2}-binding differently than \textit{nikM1}-binding, while A2ins and Gly2Ala GuNikRs displayed similar decreases in affinity for both promoters (Table 6.2). Thr4Ala GuNikR binding to \textit{nikM2} was affected to a much lower degree than \textit{nikM1}, suggesting that Thr4 makes DNA contacts that are critical for the highest affinity GuNikR-DNA complex in the context of both promoters. The small increase in Glu3Ala GuNikR affinity for \textit{nikM2}, while the same mutant had decreased affinity for \textit{nikM1}, suggests that Glu3 may make different contributions to GuNikR binding to the two promoters. This result is even more interesting considering that \textit{H. pylori} NikR adopts different conformations when bound to promoters with different DNA sequences (Chapter 5), and suggests that GuNikR may similarly bind to DNA with distinct conformations.
**DdNikR binding to multiple promoters**

Binding of DdNikR to promoter fragments from a *nikKLMQO* operon and from an operon encoding a Fe-only hydrogenase enzyme provide the first data suggesting that a NikR family member directly regulates the expression of proteins that require metals other than nickel in the cell. If NikR-dependent regulation of nickel transport in *D. desulfuricans* occurs similar to that in *E. coli* and *H. pylori* (i.e., nickel transport is repressed in response to high nickel concentrations), then DdNikR might also repress the expression of the Fe-only hydrogenase under a condition where Ni-Fe hydrogenase expression is preferred. This regulation is similar to Ni$^{2+}$-dependent regulation of a Fe-SOD in *Streptomyces coelicolor* that is mediated by a Fur homolog, Nur, which also represses *nikABCDE* expression in response to high extracellular nickel concentrations (2). Similar to the situation for *D. desulfuricans* Fe- and [Ni-Fe]-dependent hydrogenases, *S. coelicolor* encodes both Fe- and Ni-dependent SODs, and the Ni-dependent SOD is induced in response to increased concentrations of NiCl$_2$ (2). The resulting expression of only one metal-dependent enzyme (SOD in *S. coelicolor* and potentially [Ni-Fe] hydrogenase in *D. desulfuricans*) ensures that only enzymes for which the appropriate metal cofactor is present are expressed and the unnecessary synthesis of enzymes unable to be assembled is prevented. This regulation also conserves metal ions that are present in limited quantities for the most important metal-dependent proteins. A similar scenario has been demonstrated for two paralogous L31
ribosomal proteins from *Bacillus subtilis*, only one of which is zinc-dependent (26). Zinc-independent L31 is repressed in the presence of high zinc concentrations by the zinc-dependent transcription factor Zur to ensure that the zinc-dependent L31 is synthesized when zinc is available (26). Additionally, the DdNikR recognition sequence is present at conserved locations in the promoters of *nikK* and Fe-hyd genes in other *Desulfovibrio* species such as *D. vulgaris*, suggesting that the possible NikR-dependent regulation of these genes is conserved among the *Desulfovibrio* genus.

*Differences in NikR-DNA interactions*

A common feature of all NikR homologs studied to date is the presence of Arg at the N-terminal position of the β-sheet. In the *E. coli* NikR structure this residue dominates the direct contacts between the protein and DNA [Figure 6.1; (34)]. The sequences of the likely inverted repeats recognized by DdNikR, GbNikR and GuNikR characterized here suggest that the Arg-DNA contacts are not completely conserved throughout the NikR family. Although these repeats begin and end with G-T bps that are contacted directly by Arg3 of *E. coli* NikR (34), the *Geobacter* recognition sites and one of the *D. desulfuricans* promoters are not palindromes (Table 6.1). This alone would demand alterations in NikR-DNA interactions relative to *E. coli* NikR, which makes symmetrical contacts with its operator (8, 34). However, this observation is not entirely surprising given the
asymmetric sites recognized by *H. pylori* NikR [Chapter 2; (4)] as well as additional RHH family members (19, 24, 29, 35, 36, 41, 43).

*NikR variation relative to the RHH family*

The NikR subgroup of the RHH family displays a large amount of sequence variability in and adjacent to the $\beta$-sheet, and results presented here and in earlier chapters indicate that many of the differences observed for different RHH proteins also exist within the NikR subgroup. For example, multiple roles for NikR N-terminal arms in DNA-binding (Figure 6.7-6.9), novel combinations of promoters recognized by individual NikR homologs (Figure 6.6), and possibly unique stoichiometry of DNA-binding and unique architecture of NikR recognition sites (Figure 6.5c). Similar to results for *H. pylori* NikR (Chapters 4 and 5) and GbNikR and GuNikR in this chapter, the N-terminal arms of the RHH proteins Arc, Mnt and MetJ make important contributions to DNA interactions, although in each of these cases the arm has been shown to make contacts with the DNA phosphate backbone (22, 28, 29, 36).

The *E. coli* methionine repressor MetJ regulates the expression of at least six genes under the control of five promoters that contain 2-5 tandem copies of an 8 bp ‘Met-box’ recognition site (27), a promoter architecture that is somewhat reminiscent of the multiple pairs of GuNikR half-sites present at the *nikM1* and *nikM2* promoters. Depending on the number of Met-boxes, an increasing number of MetJ dimers bind to the tandem recognition sequences (27). A similar
response was observed for the RHH omega repressor from Gram-positive bacterial plasmids (11), suggesting that multiple tetramers of GuNikR may bind to the two \textit{nikM} promoters. Consistent with this idea is the presence of multiple shifted complexes in gel mobility shift assays of GuNikR with \( P_{\text{nikM1}} \) (Figure 6.6c) and \( P_{\text{nikM2}} \) (Figure 6.9a).

\textit{Implications for NikR biological activity and nickel physiology}

There are currently more than 250 unique NikR homologs from 194 bacterial and archeal species with genomes in the NCBI database, with more than 70 distinct N-terminal sequences (Figure 6.2). In addition, it was recently predicted that at least 360 prokaryotic species require nickel (42). NikR is present in many of these microbes which contain diverse combinations of nickel-dependent enzymes, as well as various numbers of nickel-specific transporters. Differences in nickel transporter and nickel- and other metal-dependent enzymes encoded in each genome, as well as distinct metabolic capabilities of an organism requiring the expression of nickel-related genes, are a few examples of variations in gene content that could influence NikR DNA-binding activity and gene regulation. It will be interesting to see how changes in protein sequence at the DNA-binding interface of NikR allow for global modulation of activity without requiring DNA sequence changes in each NikR-regulated promoter. Sequence changes altering other properties of NikR, such as \( \text{Ni}^{2+} \)-binding, may also modulate NikR gene regulation in different microorganisms.
Materials and Methods

Bioinformatics

The *H. pylori* 26695 NikR protein sequence (HP1338) was used in a protein BLAST search of the NCBI non-redundant protein sequence database [first performed 06-2007, most recently 04-2009 (3)] and the resulting sequences of significant similarity (minimum score of 100) were filtered for redundant sequences, then screened for the presence of the four high-affinity Ni²⁺-binding site ligands [in *E. coli* NikR these correspond to His76, His87, His89 and Cys95; (33)]. β-sheet sequences plus any amino acids N-terminal to the β-sheet were then filtered for redundant sequences (100% identity) and aligned using ClustalW (37).

Cloning and mutagenesis of NikR homologs

*nikR* genes were PCR amplified using the genomic DNA and primers listed in Table 6.3 (Integrated DNA Technologies, Coralville, IA). Digested PCR products were cloned into pET22-b using the *Nde*I and *Xho*I restriction sites (Novagen, Madison, WI; see Table 6.3 for plasmid names). Δnt3-GbNikR was constructed using primers EB621 and EB422 and Δnt3-GuNikR was constructed using primers EB499 and or EB470, both primer pairs amplifying 5' truncated *nikR* genes from the corresponding full-length NikR-containing plasmids. The resulting products were digested with *Nde*I and *Xho*I and ligated into pET22-b.
digested with the same enzymes to create pEB298 (Δnt3-GbNikR) and pEB296 (Δnt3-GuNikR). Site-directed mutagenesis of individual GuNikR residues was carried out using the Quik Change Site-directed Mutagenesis protocol (Strategene, La Jolla, CA) using complementary oligonucleotides with the mutated codon (Table 6.3) and Pfu DNA polymerase. The DNA sequence of each construct was verified by sequencing (SeqWright, Houston, TX).

Protein purification and expression

All wild-type and mutant NikR proteins and mutant variants were expressed and purified as described previously for *E. coli* NikR (7, 8) except that sequential steps of Q-sepharose ion exchange [20 mM Tris (pH 8.0) and 50 mM or 1 M NaCl] and gel filtration [20 mM Tris (pH 8.0) and 300 mM NaCl] were performed following elution off of a Ni-NTA column. Protein concentration was determined in 6 M guanidine hydrochloride (GuHCl) using ε_{276} predictions from primary sequence analysis (18). To remove Ni^{2+} from purified protein, the Ni-NTA eluate was incubated with either 50 mM EDTA or 20 mM L-histidine for 48 h or 24 h, respectively, at 4°C, followed by ion exchange and gel-filtration (the second and third purification steps). The removal of Ni^{2+} ions was confirmed using UV-visible spectroscopy at 302 nm.

Promoter fragments - cloning and labeling
DNA fragments for promoter regions were amplified by PCR using the oligonucleotide pairs described in Table 6.4. Promoter fragments for DNA-binding assays were generated by PCR as follows: 0.5 µM forward (5') primers (listed first in Table 6.4) for each promoter fragment was 5'-end labeled with [γ-^{32}P]-ATP [GE Biosciences, Piscataway, NJ] and T4 polynucleotide kinase (NEB, Beverly, MA) in a total volume of 40 µl. Excess [γ-^{32}P]-ATP was removed by desalting and the purified primers were used in a PCR reaction with the corresponding reverse primers (listed second in Table 6.4) using genomic DNA as the template. The resulting labeled fragments were purified using a Qiagen PCR Purification Kit (Qiagen, Valencia, CA). Some labeled fragments were generated by end-filling a gel-purified PCR product that was digested with EagI, or that contained a string of four C's at the 5' end of the forward primer (see Table 6.4). End-fill reactions used 0.2 µM PCR product, Klenow Fragment (3'-5' exo⁻; NEB) and [α-^{32}P]-dGTP (GE Biosciences) in a total volume of 40 µl. Excess [α-^{32}P]-dGTP was removed using the Nucleotide Exchange Kit (Qiagen).

**DNA-binding assays**

DNase I footprinting was performed as described previously (6, 8) in a binding buffer containing 10 mM Tris-Cl (pH 8.0), 100 mM KCl, 3 mM MgCl₂ and 50 µM NiCl₂. Labeled DNA fragments were incubated with protein at 22°C for 1 hour prior to DNase I (Sigma, St. Louis, MO) addition (final concentration 300 ng/mL).
Formic acid cleavage of labeled DNA was performed using the standard protocol for Maxam-Gilbert sequencing.

Electrophoretic mobility shift assays were performed using 7% polyacrylamide gels and electrophoresis buffer containing 50 mM Tris (pH 8.8), 25 mM boric acid and with 50 µM NiCl₂. The binding buffer was identical to that used for DNase I footprinting except that 10 µg/ml *E. coli* thioredoxin (5) and 4 ng/µl salmon sperm DNA (Fisher Scientific) were added to prevent non-specific NikR accumulation in the gel wells. Labeled DNA fragments were incubated with NikR or mutant proteins at 22°C for 30 min and 20 µl of the 25 µl total volume was loaded directly onto a running gel (120 V).

Apparent affinities measured by mobility shift assays were calculated from binding curves determined by the ratio of bound (all shifted species) vs free counts as quantified using a GE Healthcare Typhoon Trio Variable Mode Imager and ImageQuant Version 5.1 software. Apparent affinities measured by DNase I footprinting were calculated from binding curves determined by the ratio of the protected DNA region normalized to a region of DNA not protected from the same lane vs the same ratio from identical regions of a protein-free lane on the same gel. The data were fit using MICROMATH SCIENTIST Version 2.01 and the following equation:

\[ y = \frac{1}{1+(K_d/x)^n} \]  
\[ \text{Eq. (6-1)} \]

where: y, fraction DNA bound (ratios described above); Kₐ, protein concentration required for half-maximal DNA-binding; x, protein concentration; and n, Hill
coefficient. All reported affinities are the average of at least two independent experiments using a dilution series of at least 15 protein concentrations. The reported error is the standard deviation between the calculated affinities of at least two independent experiments.

**UV-visible and CD spectroscopy**

UV-visible spectra were collected on a Shimadzu UV-2401PC spectrophotometer using a 100 µl sample volume. CD spectra were collected on a Jasco J-715 spectropolarimeter using a 900 µl sample volume in a cylindrical cuvette with a 1 cm pathlength. All spectra were collected at 22°C in a buffer containing 20 mM Tris (pH 7.5), 100 mM NaCl.
Table 6.3. Primers used for nikR amplification.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Primer Name</th>
<th>Sequence (5'→3')&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Plasmid Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>DdNikR&lt;sup&gt;b&lt;/sup&gt;</td>
<td>EB419</td>
<td>gttacatctatatgGGCCAGACCATCCGATTTC</td>
<td>pEB257</td>
</tr>
<tr>
<td></td>
<td>EB420</td>
<td>gatgagtatTTAGTCTCCTATTTTCCTATC</td>
<td>pEB257</td>
</tr>
<tr>
<td>D. vulgaris NikR&lt;sup&gt;c&lt;/sup&gt;</td>
<td>EB417</td>
<td>cgtatacatatatgGGACGCACCATCCGCTTC</td>
<td>pEB258</td>
</tr>
<tr>
<td></td>
<td>EB418</td>
<td>gttacatctatatgGTAGTGAGGTCCTGTCCGGT</td>
<td>pEB258</td>
</tr>
<tr>
<td>GbNikR&lt;sup&gt;d&lt;/sup&gt;</td>
<td>EB421</td>
<td>gtaactctatatgGGACGCACCATCCGCTTC</td>
<td>pEB259</td>
</tr>
<tr>
<td></td>
<td>EB422</td>
<td>ctgatctcatatgGGACGCACCATCCGCTTC</td>
<td>pEB259</td>
</tr>
<tr>
<td>GuNikR&lt;sup&gt;e&lt;/sup&gt;</td>
<td>EB469</td>
<td>gtacatctatatgGGACGCACCATCCGCTTC</td>
<td>pEB270</td>
</tr>
<tr>
<td></td>
<td>EB470</td>
<td>catgtactcatatgGGACGCACCATCCGCTTC</td>
<td>pEB270</td>
</tr>
<tr>
<td>M. boonei NikR&lt;sup&gt;f&lt;/sup&gt;</td>
<td>EB455</td>
<td>gtagcatatATTTTCCTATATCTTTCCTTC</td>
<td>pEB269</td>
</tr>
<tr>
<td></td>
<td>EB456</td>
<td>gtagcatatATTTTCCTATATCTTTCCTTC</td>
<td>pEB269</td>
</tr>
<tr>
<td>SmNikR1&lt;sup&gt;g&lt;/sup&gt;</td>
<td>EB439</td>
<td>gtagcatatATTTTCCTATATCTTTCCTTC</td>
<td>pEB260</td>
</tr>
<tr>
<td></td>
<td>EB440</td>
<td>gtagcatatATTTTCCTATATCTTTCCTTC</td>
<td>pEB260</td>
</tr>
<tr>
<td>SmNikR2</td>
<td>EB441</td>
<td>gtagcatatATTTTCCTATATCTTTCCTTC</td>
<td>pEB261</td>
</tr>
<tr>
<td></td>
<td>EB442</td>
<td>gtagcatatATTTTCCTATATCTTTCCTTC</td>
<td>pEB261</td>
</tr>
<tr>
<td>Δnt3GbNikR</td>
<td>EB621</td>
<td>ctgatctatatgGTAAGGTTTGTATATCAATGGAC</td>
<td>pEB298</td>
</tr>
<tr>
<td>Δnt3GuNikR</td>
<td>EB499</td>
<td>ctgatctatatgGTAAGGTTTGTATATCAATGGAC</td>
<td>pEB296</td>
</tr>
<tr>
<td>A2ins-GuNikR</td>
<td>EB642</td>
<td>GAAGGAGATATACATATGgcaGGAGAGACCATATTAGTATTGCCGC</td>
<td>pEB303</td>
</tr>
<tr>
<td></td>
<td>EB643</td>
<td>GAATCTAATGTTCTGCTCCTGACTATGTATATGTCCTTC</td>
<td>pEB303</td>
</tr>
<tr>
<td>G2A-GuNikR</td>
<td>EB644</td>
<td>GAAGGAGATATACATATGgcaGGAGAGACCATATTAGTATTGCCGC</td>
<td>pEB304</td>
</tr>
<tr>
<td></td>
<td>EB645</td>
<td>GCCGAATCTAATG TTCTGCTCCTGACTATGTATATGTCCTTC</td>
<td>pEB304</td>
</tr>
<tr>
<td>E3A-GuNikR</td>
<td>EB646</td>
<td>GAAGGAGATATACATATGgcaGGAGAGACCATATTAGTATTGCCGC</td>
<td>pEB305</td>
</tr>
<tr>
<td></td>
<td>EB647</td>
<td>GAATCTAATGTTCTGCTCCTGACTATGTATATGTCCTTC</td>
<td>pEB305</td>
</tr>
<tr>
<td>T4A-GuNikR</td>
<td>EB648</td>
<td>GAAGGAGATATACATATGgcaGGAGAGACCATATTAGTATTGCCGC</td>
<td>pEB306</td>
</tr>
<tr>
<td></td>
<td>EB649</td>
<td>GCCGAATCTAATGTTCTGCTCCTGACTATGTATATGTCCTTC</td>
<td>pEB306</td>
</tr>
</tbody>
</table>

<sup>a</sup>, Underlined bases correspond to restriction sites and capitalized bases correspond to regions complementary to genomic DNA sequence.

<sup>b</sup>, <i>D. desulfuricans</i> genomic DNA was a generous gift from Dr. Judy Wall, University of Missouri, Columbia.

<sup>c</sup>, <i>D. vulgaris</i> genomic DNA was a generous gift from Chris Walker, University of Washington.

<sup>d</sup>, <i>G. bemidjiensis</i> genomic DNA was a generous gift from Dr. Derek Lovley, Unveristy of Massachusetts, Amherst.

<sup>e</sup>, <i>G. uraniireducens</i> genomic DNA was a generous gift from Evgenya Shelobolina, University of Wisconsin, Madison.
<sup>1</sup>, *M. boonei* strain 6A8 genomic DNA was a generous gift from Dr. Suzanna Brauer, Oregon Health and Sciences University.

<sup>9</sup>, *Staphylothermus marinus* strain F1 genomic DNA was a generous gift from Dr. Harold Huber, Universitaet Regensburg.
Table 6.4. Primers used for target promoter amplification.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Gene promoter</th>
<th>Primer name</th>
<th>Sequence (5’ to 3’)a</th>
</tr>
</thead>
<tbody>
<tr>
<td>D. desulfuricans</td>
<td>Dde_1189</td>
<td>EB425</td>
<td>ctagatcgccgTCCGACATGATGCAGCTGTCC</td>
</tr>
<tr>
<td></td>
<td>(nikK)</td>
<td>EB426</td>
<td>ctagatgtagacACCCGTTACCTCCGGTTGTGC</td>
</tr>
<tr>
<td></td>
<td>Dde_1190</td>
<td>EB427</td>
<td>gatctagccgcCTATGCACCTGACGAGTGC</td>
</tr>
<tr>
<td></td>
<td>(nikM)</td>
<td>EB428</td>
<td>ctagatgtagacCCTCCGCATAGTGCAATGC</td>
</tr>
<tr>
<td></td>
<td>Dde_2281</td>
<td>EB622</td>
<td>cccccGTGTTGAGCCATATCGACAAGAGAG</td>
</tr>
<tr>
<td></td>
<td>(Fe-hyd)</td>
<td>EB623</td>
<td>CTGTGCTTTGACGATTCCAGCATAAC</td>
</tr>
<tr>
<td>D. vulgaris</td>
<td>Dvul_0540</td>
<td>EB431</td>
<td>gtatactaggccgCGAGGTCGACGTGGAGGAG</td>
</tr>
<tr>
<td></td>
<td>(nikK)</td>
<td>EB432</td>
<td>gtatactaggccgATGATGACTCCTTCTTGGG</td>
</tr>
<tr>
<td></td>
<td>Dvul_1936</td>
<td>EB429</td>
<td>ctagatcgccgTTAGATGACGAGGCGACTC</td>
</tr>
<tr>
<td></td>
<td>(nikM)</td>
<td>EB430</td>
<td>ctagatgtagacATGTCGTAGGCTCCTCAGG</td>
</tr>
<tr>
<td>G. bemidjensis</td>
<td>Gbem_2644</td>
<td>EB601</td>
<td>cccccAAATCTACGACGACTCTTACAGG</td>
</tr>
<tr>
<td></td>
<td>(nikA)</td>
<td>EB602</td>
<td>GCTAGACACTTCTTTGCTCATGAGG</td>
</tr>
<tr>
<td></td>
<td>Gbem_2225</td>
<td>EB603</td>
<td>cccccGAAGACGTACATACCTTACTACTGCC</td>
</tr>
<tr>
<td></td>
<td>(nikM1)</td>
<td>EB604</td>
<td>GCCTCCGCCATATGCTAGTGGTCTT</td>
</tr>
<tr>
<td></td>
<td>Gbem_3813</td>
<td>EB605</td>
<td>cccccGAGCTAGAAACCATTAAACAGG</td>
</tr>
<tr>
<td></td>
<td>(nikM)</td>
<td>EB606</td>
<td>AGCGCGATTCCAGAAATTAACCTT</td>
</tr>
<tr>
<td></td>
<td>Gbem_3814</td>
<td>EB607</td>
<td>cccccGCAACTACGCCACTTAGAAGATG</td>
</tr>
<tr>
<td></td>
<td>(nikM)</td>
<td>EB608</td>
<td>CAGAATACGCTGGACATACATAAA</td>
</tr>
<tr>
<td></td>
<td>Gbem_3562</td>
<td>EB435</td>
<td>gtatactaggccgAGGAATCAAAACTTGTG</td>
</tr>
<tr>
<td></td>
<td>(nikR)</td>
<td>EB436</td>
<td>gtatactaggccACCACGTGACGTACTTCTTG</td>
</tr>
<tr>
<td>G. uraniireducens</td>
<td>Gura_0780</td>
<td>EB484</td>
<td>ctagatcgccgCCTCTGTGAATACAATGACC</td>
</tr>
<tr>
<td></td>
<td>(nikM1)</td>
<td>EB485</td>
<td>ctagatgtagacCGCGTTCGGTCAATGTGAGG</td>
</tr>
<tr>
<td></td>
<td>Gura_0772</td>
<td>EB482</td>
<td>gtatactaggccgATGTTGTGACGGAAAGGAG</td>
</tr>
<tr>
<td></td>
<td>(nikR)</td>
<td>EB483</td>
<td>ctagatgtagacGGTCTCTCCACTTGGTCAC</td>
</tr>
<tr>
<td></td>
<td>Gura_2762</td>
<td>EB609</td>
<td>cccccATGACATGCTCGAACCCCAAGTGC</td>
</tr>
<tr>
<td></td>
<td>(nikM2)</td>
<td>EB610</td>
<td>AAGCGCGTCTGCGCATATGCGATGTC</td>
</tr>
<tr>
<td></td>
<td>Gura_3002</td>
<td>EB611</td>
<td>cccccTCTTAAGAATCAAATTTGCCCAG</td>
</tr>
<tr>
<td></td>
<td>(fur)</td>
<td>EB612</td>
<td>GGAAGTCAATCGATTTTTTTTATTCC</td>
</tr>
<tr>
<td></td>
<td>Gura_1953</td>
<td>EB613</td>
<td>cccCGGACAATGCTGAATGCAGAACG</td>
</tr>
<tr>
<td></td>
<td>(hypE)</td>
<td>EB614</td>
<td>AAGTATGAGGTCCTTTGGTCAGGTT</td>
</tr>
</tbody>
</table>

310
<table>
<thead>
<tr>
<th>Organism</th>
<th>Accession</th>
<th>Restriction Site</th>
<th>Complementary Region</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. boonei</em></td>
<td>Mboo_1643</td>
<td>EB478</td>
<td>gtacatcggccgGTAAACTAAATAGCATACG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(nikR)</td>
<td>ctagatgctgacGATGCGGGAGAGATCGTTTTC</td>
</tr>
<tr>
<td></td>
<td>Mboo_1640</td>
<td>EB480</td>
<td>gtacatcggccgTTACCAGGACAAGATAGTCGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(nikQ)</td>
<td>ctagtagtgcagTTTCGATCTTCTGCATCCACTC</td>
</tr>
<tr>
<td></td>
<td>Mboo_2377</td>
<td>EB624</td>
<td>catgtacgagcctgCCTGAGGCTGCGGCGGTATCC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(nikA)</td>
<td>GAGAGTACTGTACTTTTTCATCAG</td>
</tr>
<tr>
<td></td>
<td>Mboo_1293</td>
<td>EB626</td>
<td>gtacatcggccgCCTGACCGGATCCCGGATAGTGCG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(nikM)</td>
<td>GAGAGTACTGTACTTTTTCATCAG</td>
</tr>
<tr>
<td><em>S. marinus</em></td>
<td>Smar_0002</td>
<td>EB451</td>
<td>gtacatcggccgGATTGTTGAAAGAAGTTTTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(nikM)</td>
<td>ctagtagtgcagTAAGTATGTTATAATACACCA</td>
</tr>
<tr>
<td></td>
<td>Smar_0364</td>
<td>EB453</td>
<td>catgtacgagcctgACGATATTTTGCTGTATCC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(sbp)</td>
<td>ctagtagtgcagTAATAACATGATAATGTATGC</td>
</tr>
</tbody>
</table>

\(^a\), Underlined bases correspond to restriction sites and capitalized bases correspond to regions complementary to genomic DNA sequence.
References


Chapter 7.

Conclusions and future directions
Conclusions

I have identified two linked, yet separable, mechanisms used by the bacterium *Helicobacter pylori* to control nickel homeostasis - differential DNA-binding and gene regulation of multiple nickel-related genes by NikR and a hierarchy of nickel trafficking to different nickel-dependent pathways in the cell. Although some specific details for both mechanisms remain to be elucidated, the identification of both in a single organism for a single metal establishes *H. pylori* nickel physiology as a tractable experimental system poised for detailed analyses of the interplay between multiple mechanisms mediating metal homeostasis.

The aspects of cellular metal homeostasis described in my work can be divided into two distinct components, which evolve depending on the specific metal physiology of a cell (Figure 7.1). The first is transition metal flux through distinct metal-dependent pathways, where the metalloenzyme content of a cell and the growth environment determine which pathways in the cell receive incoming metal ions and in what order (1 in Figure 7.1). The second is differential DNA-binding by a metal-dependent transcriptional regulator, which is modulated through amino acid changes of the protein that alter DNA affinity and specificity and through differences in gene regulatory sequences in the promoters from different metal-related genes (2 in Figure 7.1).

The detailed investigation of how NikR differentially regulates genes has identified novel aspects of DNA recognition that are relevant for the entire RHH family. The modulation of DNA-binding activity by a short, structural motif of the
Figure 7.1. Transition metal homeostasis involves two related, yet distinct, mechanisms. 1. Metal flux through different metal-dependent pathways controls the relative activity of the end targets of each pathway. 2. Differential gene regulation of multiple metal-related genes, which is mediated by the DNA-binding activity of the metalloregulator and sequence differences in the regulatory DNA of each gene.
protein (the N-terminal arm) and the ability of differences in DNA sequence and structure to alter NikR conformation and possibly function are aspects of protein-DNA interactions that have not been explored for the majority of prokaryotic regulatory proteins, and may represent more widespread, underappreciated components integral to sequence specific DNA-binding. Indeed, the modulation of DNA-binding activity by the N-terminal arm occurs in NikR proteins other than *H. pylori* NikR, suggesting that this is a commonly adapted structural motif.

**Nickel flux in *H. pylori***

NikR activation in *H. pylori* is controlled by a hierarchy of nickel trafficking. This result, while perhaps seeming obvious, is one of the only examples of studies examining the effects of disrupting one metal-dependent pathway on another, and while metal-dependent protein trafficking is a topic commonly investigated in eukaryotic cells which display metal-dependent metal-transporter re-localization, and multicellular organisms such as mammals that deploy iron throughout the body, little is known about the movement of metals in prokaryotes. In all domains of life, examination of metal movement independent of protein-binding is a difficult problem to address relative to tracking the localization of metal-binding proteins. In light of this fact, little data exists that directly measures metal localization.

Preferential trafficking of nickel is likely conserved in many microorganisms because a scenario similar to that observed for *H. pylori* occurs in *E. coli*, where
the nickel efflux protein RcnA inhibits NikR activation under low extracellular Ni$^{2+}$
conditions (26). The precise control of nickel in cells is consistent with the
generally accepted idea that at least some transition metals are highly toxic when
free to bind indiscriminately in cells (i.e., when the specific metal-binding sites of
a cell are saturated and excess metal remains), and implies that nickel belongs
to this subgroup of metals that also includes iron and copper. Each of these
metals require specific chaperones to shuttle metals directly to their designated
targets (38), presumably to preclude the deleterious reactions catalyzed by the
metals and to prevent non-specific binding to other macromolecules in the cell.

One impetus for studying the appropriation of nickel in cells was the relative
affinities of different nickel-binding proteins in bacteria, which include weakly
binding nickel chaperone proteins (e.g., $K_D \sim \mu$M) and tight binding nickel-
dependent regulators such as NikR ($K_D \sim \mu$M). Conclusions as to which proteins
in the cell bind more nickel or bind nickel more quickly are often drawn from
affinities that are determined in vitro, however the preferential trafficking of nickel
to urease in H. pylori argues against this simplistic view. Although the
mechanistic basis of hierarchical nickel trafficking in H. pylori remains unknown,
the identification of this property of intracellular nickel is an important finding on
its own.

The related question of how metal-binding proteins select the correct metal
inside cells containing multiple transition metals has been addressed by
Robinson and co-workers (4), and sheds some light on metal availability in the
intracellular milieu, which depends on metal trafficking in the cell. One study demonstrated that, in contrast to the metal-binding specificity observed \textit{in vitro}, the expression of the \textit{Mycobacterium tuberculosis} regulator NmtR in \textit{Synechococcus} was only activated by cobalt and not nickel, a ligand that activates NmtR in \textit{M. tuberculosis} \cite{4}. The authors determined this was likely due to \textit{Synechococcus} accumulating two orders of magnitude less nickel compared to \textit{M. tuberculosis}, a result that underscores the limitations to extrapolating \textit{in vitro} data to the complexity of cellular metal activities and also highlights the cell-specific nature of metal availability and trafficking.

The next step in understanding nickel flux in \textit{H. pylori} will be to identify what components of the urease assembly pathway are required to block NikR activation. One likely expectation is that nickel-binding by one or more chaperones is required for this effect, however it is also possible that direct metal transfer steps involving specific protein-protein interactions are important. Because multiple complex steps are possible \textit{in vitro} reconstitution of the system is difficult, so a complementary genetic approach to define the functional requirements of each urease pathway protein is likely to yield more useful information regarding the mechanistic details of nickel pathway competition.

Another intriguing question raised by the nickel trafficking observed in \textit{H. pylori} is how nickel is more widely allocated throughout an \textit{H. pylori} cell (i.e., pathways other than urease and NikR). The radioactive nickel isotope $^{63}\text{Ni}$ is a useful reagent that could be taken advantage of to monitor the localization and
kinetics of nickel-binding to proteins in the cell. A combination of pulse-chase with $^{63}\text{Ni}$ and cell fractionation to separate different proteins could reveal the kinetics and order of nickel trafficking. One potential problem is disassociation of $^{63}\text{Ni}$ from nickel-binding proteins during the cell fractionation step, however if these experiments are successful they would allow for the direct observation of time-dependent nickel trafficking in *H. pylori*, a feat that has not been achieved in any cell type for any metal.

* differential gene regulation by *H. pylori* NikR

Once NikR is activated by Ni$^{2+}$, the expression of multiple nickel-related genes can be regulated, ensuring that the amount of nickel imported into the cell is directly linked to the levels of nickel-utilizing enzymes and storage proteins. The autoregulation by NikR of its own gene similarly links levels of NikR protein to nickel import, utilization and storage. This would appear to suggest that independent of environmental nickel levels, *H. pylori* requires predetermined ratios of each nickel-related activity. However, closer inspection of NikR-dependent gene regulation demonstrated that NikR differentially regulates these genes in response to distinct concentrations of extracellular nickel. Consistent with this, NikR also binds to the promoters of these genes with a range of affinities, a result that was recently reproduced by an independent study (17). This capability of NikR results in all of the known nickel-dependent regulation in *H. pylori* being carried out by a single regulatory protein, while allowing for the
regulation of individual genes in response to very specific nickel levels that likely result in varying levels of Ni\textsuperscript{2+}-saturated (i.e., active) NikR in the cell.

The DNA-binding capabilities of \textit{H. pylori} NikR are distinct from the previously characterized NikR from \textit{E. coli}, however additional NikR family members are capable of binding to multiple promoters from different genes related to nickel physiology as well. The experimental determination of specific DNA-binding by metal-dependent or other signal-responsive transcriptional regulators often consists of DNA-binding reactions containing individual or few protein concentrations with one DNA fragment (5, 11, 22, 48, 54). More detailed analyses of the concentration dependence of protein-DNA interactions might reveal properties similar to those seen for \textit{H. pylori} NikR, namely a range of binding affinities for more than one promoter and differential gene regulation in response to a signal gradient.

The ability of \textit{H. pylori} NikR to differentially bind to promoters from multiple nickel-related genes is similar to observations made for the iron-dependent ferric uptake regulator (Fur) from \textit{H. pylori}. Fur was the first identified metalloregulator (in \textit{E. coli}), where it directly represses the expression of multiple iron transporter genes in response to increased extracellular iron (25) and indirectly activates iron storage and utilization genes via a small RNA (33, 34). In \textit{H. pylori}, Fur directly represses the expression of multiple iron transport genes and iron storage and utilization genes (2, 9, 13-15, 18, 19, 23, 36, 51, 52). The direct regulation of these different gene classes is a result of Fur displaying reduced affinity for
promoters from iron transporter genes in response to increasing iron concentrations (15) and reduced affinity for iron storage (15) and utilization (19) gene promoters in response to iron limitation. Despite extensive characterization of Fur regulation in vivo [in H. pylori and other organisms; (2, 9, 13-15, 18, 19, 23, 36, 51, 52)], structural studies have been more limited and produced contradictory results (40, 47). This contrasts with the significant amount of structural information available for NikR (3, 16, 39, 44, 45), which will greatly facilitate studies aimed at determining the structure-function relationships governing differential DNA-binding.

Additional NikR binding sites have also been identified in the promoters from the fur (12), exbBDtonB (12), fecA3 (21) and frpB4 (21) genes. Interestingly, the likely NikR recognition sequences present in each of these promoters also vary relative to nixA and ureA (12, 21), suggesting that NikR differentially regulates the expression of these genes. NikR-dependent repression of each gene has been demonstrated (1, 10, 12, 21, 50), however regulation was measured only in the presence or absence of high amounts of nickel, precluding comparison of the minimum nickel concentrations required to trigger regulation of each gene. A likely scenario is that NikR regulates genes with promoters that it binds more tightly in response to lower extracellular nickel concentrations and regulates genes with lower affinity promoters at higher extracellular nickel concentrations. Less overall intracellular nickel would result in lower levels of fully-saturated Ni$^{2+}$-
NikR, which would only be sufficient to occupy promoters for which NikR has higher affinity.

Others have studied the pH dependence of NikR-DNA interactions to understand how binding affinity might be affected by acidic pH (29). Interestingly, NikR binding to ureA under acidic pH conditions was Ni^{2+}-independent and binding to nikR was only observed at acidic pH with Ni^{2+}. However, *H. pylori* have robust mechanisms in place to buffer the periplasm from acidic conditions which prevents significant acidification of the cytoplasm (56), so it is not clear how relevant the *in vitro* studies at low pH are to regulation in the cell. Only some of the genes regulated by NikR at pH 7 are also NikR-regulated under acidic growth conditions. However, the nikR gene is strongly up-regulated under these conditions, suggesting that NikR is important in the acid response, although nickel-dependent regulation at acidic pH is clearly different from that which occurs at neutral pH.

The only study examining large scale NikR-dependent gene regulation in *H. pylori* compared gene expression in wild-type and a nikR mutant strain grown with a single high NiCl\(_2\) concentration (8), and attempts to validate the Ni\(^{2+}\)- and NikR-regulated genes were unconvincing (8). Indeed, one result was subsequently shown to be incorrect (20). There are several reasons to question the macroarray study, including a lack of appropriate controls for nickel-dependent NikR-independent regulation and careful consideration of growth conditions. Considering what we now know about NikR-DNA interactions, it will
be informative to repeat a global analysis in which wild-type and nikR transcripts are compared from cells grown over a range of nickel concentrations. This experiment would test various hypotheses generated from in vitro experiments. There is a good likelihood of identifying additional NikR-regulated genes, which would not only lead to a more complete understanding of NikR-dependent regulation in H. pylori, but may also identify genes encoding proteins of unknown function that are important in nickel physiology.

All of the studies measuring nickel- and NikR-dependent gene regulation in H. pylori have employed assays using populations of cells. An approach that takes advantage of multiple fluorescent reporters and fluorescence microscopy has been used to study the regulation of genes in single cells and has revealed novel aspects of transcriptional regulation (6, 30, 31). Similar studies examining Ni\textsuperscript{2+}- and NikR-dependent regulation of different genes in single H. pylori cells would reveal details of NikR-dependent gene regulation that are not detected by population-based assays. For example, measuring NikR-dependent regulation in individual cells in response to a range of nickel concentrations would reveal the precise extracellular nickel levels required to trigger regulation of each gene and the kinetics of gene regulation at each concentration. One expectation is that genes with promoters for which NikR has higher affinity would be regulated at lower nickel concentration and/or with faster kinetics compared to genes with lower-affinity promoters.
Evolution of the NikR regulon via protein and regulatory DNA sequence changes

To recognize and bind to different DNA sequences *H. pylori* NikR requires a small structural motif at its N-terminus (the N-terminal arm). In general, the addition of short, linear protein sequence motifs is a common way in which proteins adapt their functions throughout evolution (37, 53), and it is easy to envision similar scenarios occurring where DNA-binding by other transcriptional regulators is modulated by amino acid additions neighboring DNA-binding motifs. More subtle changes in protein sequence such as the addition or loss of the arm in NikR proteins, allow cells to alter gene regulation without gross changes in regulator function and independent of regulatory DNA sequences. Additionally, for regulators that control multiple genes, protein sequence changes affect all of the genes regulated by that transcription factor, whereas changes in the promoter regulatory sequences only affect the expression of individual genes or operons. This strategy can alter the expression of a complete regulon by changes in only one protein which may confer a significant fitness advantage to an organism, for example when a cell is adapting to a new environmental niche.

In future work it will be important to develop a robust assay for measuring the regulatory activity of *H. pylori* NikR. I have successfully re-integrated linear DNA containing different *nikR* mutants into the chromosome of a *nikR* mutant strain, although the preliminary results did not correlate with the biochemical analysis of the proteins. Additional experiments will be required to understand the discrepancy between the *in vitro* and *in vivo* studies (Appendix I). Repeated
attempts at detecting *H. pylori* NikR activity in *E. coli* cells were unsuccessful despite reproducible, relatively strong β-galactosidase activity detected from *H. pylori* promoter - *lacZ* fusions. One likely explanation considering the identification of highly controlled, cell-specific nickel trafficking is that nickel is trafficked differently in *E. coli* compared to *H. pylori*, so *H. pylori* NikR is unable to access intracellular nickel in this context. Notably, attempts at detecting other NikR family member activity in *E. coli* have also been unsuccessful.

An alternative strategy for measuring *H. pylori* NikR function is the development of an *in vitro* transcription assay to determine the ability of NikR to regulate the expression of various transcripts. Such a system does not currently exist for *H. pylori* but the information obtained from these experiments would be extremely useful. The availability of NikR mutants with alterations in DNA-binding properties would be useful reagents to compare models of NikR activity at different promoters.

At the same time the arm was identified as playing a critical role in *H. pylori* NikR DNA-binding, differences in the arm requirement when NikR is bound to different promoter fragments were observed. Further studies determined that NikR adopts distinct conformations when bound to different promoters. Attempts to identify the minimal 'nixA-like' DNA sequence determinants were unsuccessful, but revealed that, in contrast to the general belief that NikR DNA-recognition is limited to inverted repeat half sites (42, 43) or at a maximum a
DNase I footprint (17), sequences conveying promoter-specific information to NikR are also located outside of these regions.

This observation contrasts with the accepted model of RHH DNA recognition, in which the proteins are thought to recognize minimal DNA sites often consisting of inverted or direct repeats of < 10 bp. Despite co-crystal structures of 10 different RHH protein-DNA complexes (24, 28, 32, 35, 41, 45, 46, 49, 55, 57), no structures are available for any RHH protein bound to more than one DNA sequence. It will be interesting to determine if other RHH family members undergo similar differences in conformation as a result of differences in DNA binding site, in particular MetJ.

The only structures available of *H. pylori* NikR are in the absence of DNA and do not show any density for the residues N-terminal to the β-sheet (16), so the precise locations of the N-terminal arm and the orientation and DNA interactions of the β-sheet are unknown. Crystallization of NikR in the presence of DNA fragments corresponding to the *nixA* and the *ureA* promoters would directly identify the differential arm and RHH domain conformations that occur. The structures of NikR bound to two different DNA sequences would be the first structures of a RHH protein bound to multiple DNA fragments and would provide useful information regarding members of the RHH family that are known to recognize distinct DNA binding sites. These structures would also allow for the direct observation of any N-terminal arm-DNA interactions that occur in the two NikR-promoter contexts.
The DNA-dependent conformational differences of NikR parallel the observation of ‘DNA-dependent allostery’ in eukaryotic transcription factors (27), where a particular DNA-binding protein is triggered to bind in a specific conformation depending on the specific DNA sequence to which it is bound. However, no examples currently exist of similar conformational differences in prokaryotic transcriptional regulators. Given that many transcription factors, especially global regulators that regulate many genes, bind to degenerate DNA recognition motifs, it is likely that this allosteric control of protein conformation by DNA sequences will not be limited to *H. pylori* NikR and the NikR family.

It will also be interesting to define the changes in DNA recognition sequences that are necessary to convey promoter specific information to *H. pylori* NikR. Additional hybrid promoters can be constructed with increasing amounts of *nixA* sequence replacing the corresponding *ureA* promoter sequences and similarly increasing the amount of *ureA* replacing *nixA*. DNA-binding assays using the *nixA*-selective NikR mutant (Lys48Ala) will reveal the minimum sequence required to recover *ureA*-binding. The results from these experiments will allow for more detailed comparisons between all of the promoter sequences known to be recognized by NikR (i.e., *nixA*, *ureA*, *fur*, *nikR*, *frpB4*, *fecA3*, *exbB*, *hpn*) to determine if a particular sequence or structure is shared between different types of promoter (e.g., NikR-repressed or -activated promoters, or promoters regulated in response to a specific nickel concentration).
The N-terminal arm as an adaptable structural feature of the NikR family

The discovery that *H. pylori* NikR displayed DNA-binding activity that is dramatically different from that described for *E. coli* NikR raised the question of how diverse DNA-binding activity is throughout the entire NikR family. Results for a small number of NikR proteins from microorganisms with significantly different physiologies relative to *H. pylori* and *E. coli* suggested that DNA-binding properties, such as the number of distinct promoter sequences bound and the DNA recognition site architecture, are quite variable in the NikR family. The NikR proteins selected contained distinct DNA-contacting β-sheet and N-terminal arm sequences. Of those studied some bound to multiple promoters with different recognition sequences and others relied on N-terminal arms for aspects of DNA-binding similar, but not identical, to *H. pylori* NikR. This variability will likely make accurate predictions of NikR-regulated genes in different organisms more difficult, but also point to potentially important mechanistic details in how different NikR family members interact with DNA. The gain or loss of base contacts by β-sheet residues may change the stringency of DNA specificity, which would change the relative contributions of the β-sheet and N-terminal arm to DNA affinity and specificity. Differences in NikR DNA-binding could also reflect an organisms optimal growth environment, which would be apparent as specific requirements for DNA-binding, such as temperature, pH or salt concentration. Despite *E. coli* NikR being the first identified member of this RHH subfamily and the protein for which the most information is available, the differences in DNA-
binding between different family members indicate that generalizations may not be possible when comparing NikRs, particularly those with different β-sheet sequences.

The striking differences in DNA-binding by NikR homologs suggests that nickel-dependent gene regulation has evolved in response to variations in microbial physiology. Examining nickel- and NikR-dependent gene regulation in microbes with NikR proteins containing distinct DNA-binding properties (e.g., β-sheet sequences, N-terminal arms, DNA-binding to multiple promoters in vitro) would determine how NikR regulons change in organisms with NikRs of a particular amino acid sequence. This approach could also be used to compare organisms with significantly different nickel requirements (e.g., methanogenic archa expressing [Ni-Fe] hydrogenase, acetyl-CoA synthetase, carbon monoxide dehydrogenase and methyl-CoM reductase vs sulfate reducers expressing >5 [Ni-Fe] hydrogenases and carbon monoxide dehydrogenase) to understand how NikR function evolves in response to variations in physiology.

Another way in which to understand how NikR evolves in different microorganisms is to examine the potential for N-terminal arm evolution in vitro. During my initial work with H. pylori NikR I exchanged the N-termini of H. pylori and E. coli NikR to determine if these regions of the proteins were sufficient to confer DNA-binding specificity matching that of the parent protein. Interestingly, the H. pylori protein with the E. coli β-sheet bound to the E. coli nikA promoter and the H. pylori nixA promoter with high affinity, but was unable to bind to the
ureA promoter. Even more intriguing, the *E. coli* protein with the *H. pylori* N-terminus (arm and β-sheet) was completely non-functional. This suggests that *E. coli* NikR requires a very specific β-sheet, although other explanations are possible, and raises the question of whether this protein is capable of evolving an arm that could alter its DNA-binding properties. To explore what arm lengths and sequences are possible in the context of NikR DNA-binding *in vitro*, random amino acid sequences of increasing lengths could be added to *H. pylori* NikR followed by a functional screen, such as electrophoretic mobility shift assays, for the ability to recognize non-cognate regulatory DNA sequences. Results from these experiments would provide an alternative approach to examining the problem of the evolution of Ni$^{2+}$-dependent DNA-binding and gene regulation by NikR, as well as transcriptional regulators more generally.
Summary

When I began this project my expectations were limited to identifying activities of *H. pylori* NikR similar to those already known for *E. coli* NikR, and it was unclear if studying a second NikR protein would have implications outside of transcriptional regulation specific to *H. pylori*. Instead, I have uncovered novel aspects of NikR DNA-binding that are relevant for the entire RHH family. The regulation of multiple nickel-related activities by NikR, not just limited to nickel import, was an unexpected discovery and likely extends to additional NikR-containing organisms. The conformational differences of *H. pylori* NikR bound to different promoters is an exciting result, and can be used to pursue more general questions regarding protein-DNA recognition. The stark contrast with the single *E. coli* NikR-DNA interaction provides further opportunity for understanding how distinct DNA-binding properties evolve, and the initial discovery of novel properties among other NikR proteins suggests that a spectrum of DNA-binding activities is represented throughout the NikR family.

I was also very excited to pursue the broader problem of intracellular metal trafficking, and optimistic that *H. pylori* nickel physiology could be developed into a model with which to begin to understand this biological process. Even in the absence of detailed mechanistic explanations, the identification of cell-specific preferential nickel trafficking to different nickel-dependent pathways is a significant step towards understanding transition metal behavior in cells. Establishing this system provides a strong basis to identify the requirements for
proper nickel trafficking, with the hope of illuminating features of protein-metal interactions that are important for metal homeostasis in general.
References


Appendix 1.

*In vivo* analysis of *H. pylori* NikR gene regulation
Summary

Biochemical studies of *Helicobacter pylori* NikR identified a number of amino acids that are required for high-affinity DNA-binding, DNA specificity and differential interactions with two promoters. However, the effects of these mutations on NikR activity *in vivo* has not been addressed. A nikR mutant of *H. pylori* strain 26695 was successfully complemented using a counter-selection strategy for re-inserting genes into their endogenous loci by Berg and colleagues (4), and the results from this approach to analyzing NikR mutants *in vivo* are described below. It is important to note that no studies have reported the investigation of NikR mutants *in vivo*, with the one exception being the analysis of high-affinity and proposed intermediate Ni$^{2+}$-binding site mutants in conjunction with the solution of the NikR crystal structure, but this study only examined the Ni$^{2+}$ sensitivity of strains with different nikR mutants and did not measure Ni$^{2+}$-dependent gene regulation (5).

Consistent with what is known about Ni$^{2+}$-dependent activation of NikR, a high-affinity Ni$^{2+}$-binding site mutant of *H. pylori* NikR (His99Ala) was unable to repress nickel-transporter genes. Similarly, a N-terminal arm truncation mutant (nt9-NikR) was defective for nickel-dependent gene repression, most likely due to the increase in non-specific DNA-binding observed *in vitro*. In contrast, a RHH domain mutant (Lys48Ala) displayed a modest defect in the amount of repression of nickel-transporter genes. Finally, two N-terminal arm site mutants (Pro4Ala and Lys6Met) had increased NikR activity under low NiCl$_2$ conditions, which is
inconsistent with biochemical data that shows significant reductions in DNA affinity of these mutants, suggesting that the amino acid changes alter NikR activity in ways different from those previously described, or that the mutant proteins are present in higher concentrations than wild-type NikR in *H. pylori* cells.

Experiments measuring transcript levels in *H. pylori* indicated that cells exposed to increased extracellular NiCl$_2$ for a short time period do not up-regulate *ureA* (Chapter 3), which is in contrast to previously published data where cells were exposed to NiCl$_2$ for longer times (6). This is surprising given that two other Ni$^{2+}$- and NikR-regulated transcripts, *nixA* and *frpB4*, are repressed within 40 min of NiCl$_2$ exposure (Chapter 3). The reason for this discrepancy is not known, however because some NikR mutants are differentially affected in *nixA* and *ureA*-binding *in vitro* (Chapter 5) it will be important to establish experimental conditions that allow for the analysis of NikR-dependent repression of *nixA* and up-regulation of *ureA*. The kinetics of NikR-dependent gene regulation within short-term NiCl$_2$ exposure was also measured to determine if *ureA* up-regulation could be detected. Although no significant up-regulation of *ureA* was observed, an interesting increase in *nixA* and *frpB4* levels occurred.
Preliminary results and interpretation

Analysis of mutant NikR gene regulation in Helicobacter pylori

DNA-binding studies of *Helicobacter pylori* NikR determined that the N-terminal arm is required to maintain a hierarchy of affinities for different promoter DNA fragments and to inhibit non-specific DNA-binding [Chapter 4; (2)]. When the full-length arm is present in NikR, specific residues of the arm are also required for high-affinity DNA-binding. Additionally, differences in the binding requirements and conformations of NikR that are linked to the arm have been identified [Chapters 4 and 5; (2)]. Consistent with NikR interacting with the *nixA* and *ureA* promoters differently, RHH domain mutants have also been identified that are selectively impaired in *ureA*-binding, but not *nixA*-binding (Chapter 5).

Despite the identification of individual amino acids of NikR that are necessary for various aspects of NikR DNA-binding, the importance of each of these in NikR-dependent gene regulation has not been investigated.

To analyze the regulatory capabilities of different NikR mutants in *H. pylori* a counter-selection strategy that takes advantage of a *rpsL-*erm cassette, which confers streptomycin sensitivity in strains containing a *rpsL-*str*\(^r\)* allele and erythromycin resistance, was used (4). *nikR* genes encoding NikR mutants with a range of DNA-binding defects identified *in vitro* were recombined into the chromosome of a strain of *H. pylori* 26695 that was constructed previously (4) and contained the *rpsL-*str*\(^r\)* allele as well as the *rpsL-*erm cassette in place of the *nikR* gene. DNA sequencing of PCR products that amplified the re-inserted *nikR*
genes and immediate flanking regions confirmed the insertion of genes encoding the following mutants: His99Ala (high-affinity Ni\(^{2+}\) site ligand), Lys48Ala (RHH domain helix α2), Pro4Ala and Lys6Met (N-terminal arm site mutants) and nt9-NikR (arm deletion), as well as a wild-type copy of nikR.

The effect of each NikR mutation on nickel-dependent regulation in vivo was measured in 22 h cultures exposed to 100 µM dimethylglyoxime (DMG, a Ni\(^{2+}\) specific chelator) or 100 µM NiCl\(_2\) for 40 min. The levels of nixA and frpB4 transcripts in 10 µg of total RNA were determined using an S1 nuclease protection assay. As expected from studies with wild-type H. pylori strain 26695 and an isogenic nikR mutant [Chapter 3; (1)], the rpsL strain with wild-type NikR re-inserted into the chromosome displayed Ni\(^{2+}\)-dependent repression of nixA and frpB4, while the strain containing the rpsL-erm cassette in place of nikR had constitutively high levels of each transcript in the presence of both DMG and NiCl\(_2\) (Figure A1.1). The strain containing His99Ala NikR behaved similarly to the strain lacking the nikR gene, with constitutively high nixA and frpB4 levels, consistent with NikR activity requiring Ni\(^{2+}\)-binding to the high-affinity site.

The Lys48Ala NikR strain displayed Ni\(^{2+}\)-dependent repression of nixA and frpB4, however the decrease in both transcripts in response to 100 µM NiCl\(_2\) was less than for a strain with wild-type NikR (3.5- vs 5.3-fold for nixA, 2.2- vs 3.8-fold for frpB4, respectively; Figure A1.1). Together with the biochemical data that shows Lys48Ala NikR has a modest 2-fold decrease in affinity for nixA (Chapter 5) this data suggests that Lys48Ala and the conformation flexibility of the RHH
Figure A1.1. Mutant *H. pylori* NikR gene regulation as measured by S1 protection assays. *H. pylori* *rpsL* strains containing different *nikR* mutants recombined into the chromosome were exposed to DMG or NiCl$_2$ for 40 min and levels of *nixA* (a) or *frpB4* (b) transcript were measured using 10 µg input RNA. Data in each panel was normalized to the transcript level of wild-type cells exposed to DMG. Plotted data are the average of three independent cultures and error bars represent the calculated standard error. One representative gel for each probe is shown. P-values are indicated (*, <0.1; **, <0.05).
domain is required for wild-type levels Ni\(^{2+}\)-dependent gene repression \textit{in vivo}. Lys48Ala NikR displayed a more severe decrease in affinity for \textit{ureA} \textit{in vitro} (62-fold), however attempts to measure \textit{ureA} transcript levels in a Lys48Ala NikR strain were not successful. Only small changes in \textit{ureA} levels were observed under these experimental conditions [see below; Chapter 3; (1)], so whether this strain is differentially affected in \textit{nixA} and \textit{ureA} regulation remains to be determined. Experiments measuring \textit{nixA} and \textit{ureA} in cells grown in the presence of DMG or NiCl\(_2\) for longer times or overnight may reveal differences in the regulation of these two genes by Lys48Ala NikR.

Pro4Ala and Lys6Met NikR strains surprisingly showed reduced \textit{nixA} and \textit{frpB4} levels relative to the wild-type NikR strain after DMG exposure, although this was only significant for Pro4Ala \textit{nixA} levels (Figure A1.1). Both mutant proteins had significantly reduced affinities for the \textit{nixA} and \textit{ureA} promoters \textit{in vitro} (Chapter 4), so an increase in \textit{nixA} and \textit{frpB4} repression was unexpected. One explanation is that the DNA-binding affinity of Pro4Ala and Lys6Met is not sufficiently decreased to impair NikR activity \textit{in vivo}, although the decrease in affinities of Pro4Ala is much greater than that observed for Lys48Ala binding to \textit{nixA} and a Lys48Ala NikR strain did not have a reduction in \textit{nixA} in response to DMG (see above). It’s possible these mutations also affect the kinetics of NikR DNA-binding which results in increased promoter occupancy \textit{in vivo}, or the half-life or total concentrations of these mutants in \textit{H. pylori} cells is increased relative to wild-type NikR.
The nt9-NikR strain had constitutive high \textit{nixA} and \textit{frpB4} levels in the presence of DMG and NiCl\textsubscript{2} (Figure A1.1), similar to the strain lacking a \textit{nikR} gene and the Ni\textsuperscript{2+}-insensitive His99Ala NikR strain, suggesting that an arm-less NikR protein is unable to function \textit{in vivo}. This result is consistent with the significantly increased affinity of nt9-NikR for non-specific DNA \textit{in vitro}, and together these data suggest that an increase in non-specific DNA-binding by nt9-NikR impairs its ability to bind to specific promoter recognition sequences and regulate genes in response to Ni\textsuperscript{2+}. An alternative possibility is that the nt9-NikR protein is significantly less stable, or present at much lower concentrations than wild-type NikR in \textit{H. pylori} cells.

\textit{Kinetics of nickel- and NikR-dependent gene regulation in H. pylori}

As an additional way of detecting differences in NikR-dependent repression of \textit{nixA} and \textit{frpB4} as well as up-regulation of \textit{ureA}, the kinetics of Ni\textsuperscript{2+}- and NikR-dependent regulation were examined. The \textit{rpsL-str\textsuperscript{R}} \textit{H. pylori} strain (described above) and a mutant strain with a \textit{rpsL-erm} cassette replacing \textit{nikR} were exposed to 100 \textmu M NiCl\textsubscript{2} and \textit{ureA}, \textit{nixA} and \textit{frpB4} transcripts were measured at increasing times after NiCl\textsubscript{2} addition (Figure A1.2a).

\textit{ureA} levels decreased 1.3-fold in wild-type cells after 40' of NiCl\textsubscript{2} exposure, which is in contrast to what has been observed for long-term NiCl\textsubscript{2} exposure (3, 6). Furthermore, the most significant difference in \textit{ureA} levels between the \textit{nikR} and wild-type strains was 1.7-fold at time 0', indicating that Ni\textsuperscript{2+}- and NikR-
Figure A1.2. Kinetics of nickel- and NikR-dependent gene regulation in *H. pylori*. *H. pylori* 26695 *rpsL-stfr* (wild-type) and a mutant containing *rpsL-erm* in place of *nikR* were exposed to 100 µM NiCl₂ for increasing times and levels of *ureA* (a), *nixA* (b) or *frpB4* (c) transcript were measured using 10 µg input RNA. Bands on each gel were quantitated and each sample was normalized to wild-type transcript levels at time 0'.
dependent up-regulation of ureA does not occur under these experimental conditions or that up-regulation of ureA has occurred prior to the addition of NiCl₂ under these experimental conditions. frpB4 and nixA levels decreased 3.5- and 2-fold in wild-type cells after 40’ of NiCl₂ exposure (Figure A1.2b, c), consistent with the known Ni²⁺-dependent repression of these genes. The repression of frpB4 and nixA required NikR because the nikR mutant strain did not show decreased transcripts in response to NiCl₂. Interestingly, both frpB4 and nixA levels increased (3.5- and 4-fold, respectively) in the strain lacking NikR with longer exposure to NiCl₂. It is currently unclear why these transcripts increase, but suggests that transcription is somehow activated in response to NiCl₂ when NikR is not present.

To fully understand how NikR mediates Ni²⁺-dependent gene regulation in H. pylori it will be necessary to characterize the effect of different NikR mutations both biochemically and in cells. The results discussed above establish a genetic system with which to assess biochemically characterized H. pylori NikR mutants for nickel-dependent gene regulation, however because robust up-regulation of ureA is not observed under these conditions, additional growth conditions must be identified that allow for the assessment of all NikR target genes. The fact that NikR only regulates some target genes under these experimental conditions raises the question of why all target genes do not undergo nickel-dependent regulation, and suggests that it is possible to un-couple NikR-dependent regulation of different genes. Additionally, it will be important to assess total
NikR levels in cells expressing different mutants which may de-stabilize or stabilize NikR protein relative to wild-type NikR and could result in the incorrect interpretation of any observed effects.
References


Appendix 2.

Two separable pathways for nickel transport in *H. pylori*
Summary

*Helicobacter pylori* synthesize large quantities of the Ni\(^{2+}\)-dependent enzyme urease to buffer cells from acidic conditions that are prevalent in the gastric environment (3, 5, 7). However, a significant proportion of urease exists in the cell in an inactive, apo-form (12). Active urease requires Ni\(^{2+}\) as a cofactor making Ni\(^{2+}\) transport into the cell a critical aspect of the acid response. It has been suggested previously that Ni\(^{2+}\) import increases in response to a decrease in environmental pH (13, 14), which may help to explain the pool of inactive urease that exists at pH 7. The only known inner membrane Ni\(^{2+}\)-specific transporter in *H. pylori* is NixA, a member of the HoxN family of metal permeases (6), however the effect of pH on NixA function has not been investigated. Additionally, while it is known that the Ni\(^{2+}\)-dependent transcriptional regulator NikR represses *nixA* expression in response to increasing extracellular Ni\(^{2+}\), it is not clear if NikR regulates additional genes important for nickel transport under any condition. To determine if NixA activity increases in response to acidic pH and if NixA represents the only NikR-regulated nickel transporter I have measured long-term \(^{63}\)Ni accumulation in *H. pylori* strain 26695, *nixA* and *nikR* single mutants, and a *nikR-nixA* double mutant grown at pH 7 or pH 5.5 with urea. The results indicate that while NixA is responsible for almost all of the NikR-regulated Ni\(^{2+}\) transport at neutral pH, an additional NikR-regulated pathway(s) exists to import Ni\(^{2+}\) at acidic pH. Furthermore, measurements of short-term \(^{63}\)Ni uptake in intact cells in a minimal assay in the absence of rich
media revealed that under these conditions, NixA is the only active Ni\textsuperscript{2+}
transporter. \textsuperscript{63}Ni uptake by NixA under acidic conditions in a minimal assay
required urea and urease activity, and increased in a \textit{nikR} mutant strain. Finally,
similar to the one existing report on the energy requirements of a HoxN family
member, NixA required energy from the proton motive force for \textsuperscript{63}Ni uptake.
Preliminary results and interpretation

*NixA transports Ni\textsuperscript{2+} exclusively at pH 7.0*

One of the two Ni\textsuperscript{2+}-dependent enzymes produced by *Helicobacter pylori* is urease, which functions to protect cells against the acidic conditions that are commonly encountered in the gastric environment (1-3, 5, 8, 10, 11). At pH 7 urease has been observed to constitute up to 6% of the total cellular protein (3), but a significant proportion of the enzyme is in its apo, or Ni\textsuperscript{2+} free, form (12). Because a greater amount of urease activity is likely required in cells exposed to acidic conditions, one possibility is that nickel transport is increased in response to a decrease in pH (13, 14). To test this hypothesis two complementary assays were developed to measure nickel transport in *H. pylori*: a long-term \textsuperscript{63}Ni accumulation assay and a short-term, minimal \textsuperscript{63}Ni uptake assay. Initially these assays were also established to provide an indirect measure of NikR activity in *H. pylori* due to the nickel-dependent repression of nickel transporter genes by NikR.

\textsuperscript{63}Ni accumulation after overnight growth at pH 7 with either 10 nM \textsuperscript{63}Ni or a combination of 10 nM \textsuperscript{63}Ni with 10 µM NiCl\textsubscript{2} was measured in wild-type *H. pylori* strain 26695, nikR and nixA single mutant strains, and a nikR-nixA double mutant strain (Figure A2.1a). Relatively modest but significant 1.2- and 1.3-fold increases in accumulation were observed by the single nikR or double nikR-nixA mutants relative to the wild-type strain and a similarly small decrease of 1.4-fold was displayed by the nixA mutant under low Ni\textsuperscript{2+} conditions, demonstrating that
Figure A2.1. NixA-dependent nickel transport occurs exclusively at pH 7.0. $^{63}$Ni accumulation was measured in overnight cultures of *H. pylori* 26695 or the *nikR*, *nixA*, *nikR-nixA* mutant strains at pH 7.0 (a, b) or pH 5.5 (c, d) that were exposed to either 10 nM $^{63}$NiCl$_2$ (a, c) or 10 nM $^{63}$NiCl$_2$ with 10 µM NiCl$_2$ (b) or 5 µM NiCl$_2$ (d). Accumulation was measured in three independent cultures and reported with the calculated standard error. P-values are indicated (*) , <0.05; **, <0.01).
NixA is important in transporting a minor portion of the small amount of imported nickel under these conditions, however NixA is not the only NikR-regulated transporter contributing to nickel import. The \textit{nikR} mutant accumulated 5.1-fold more \textsuperscript{63}Ni compared to wild-type under high nickel (10 \( \mu \)M NiCl\(_2\)) conditions (Figure A2.1b), indicating the NikR is more active or represses nickel transport genes to a greater extent under this condition. The \textit{nixA} mutant accumulated 1.3-fold less \textsuperscript{63}Ni relative to wild-type, similar to the decrease observed at low Ni\(^{2+}\).

Interestingly, the \textit{nikR-nixA} mutant accumulated only 2-fold more \textsuperscript{63}Ni compared to wild-type under high Ni\(^{2+}\) conditions, which is less of an increase in accumulation relative to the \textit{nikR} mutant, and indicates that NixA is responsible for a significant proportion of total Ni\(^{2+}\) transport at neutral pH. Because the \textit{nikR-nixA} mutant accumulated more \textsuperscript{63}Ni relative to wild-type however, NikR likely regulates an additional gene(s) important for a small proportion of Ni\(^{2+}\) import under this condition.

Wild-type cells accumulated less \textsuperscript{63}Ni when grown with 10 nM \textsuperscript{63}Ni and 5 mM urea at pH 5.5 (1056 vs 1650 \textsuperscript{63}Ni/cell at pH 5.5 and pH 7, respectively; Figure A2.1), which argues against the hypothesis that \textit{H. pylori} Ni\(^{2+}\) transport is increased under acidic conditions. The single and double mutant strains displayed similar trends at pH 5.5 with 10 nM \textsuperscript{63}Ni as those observed at pH 7 (Figure A2.1c). This was not the case for the high (5 \( \mu \)M NiCl\(_2\)) condition, however, where both the \textit{nikR} and the \textit{nikR-nixA} mutants accumulated 2-fold more \textsuperscript{63}Ni compared to wild-type cells. These data indicate that at pH 5.5 NixA is
not required for Ni\(^{2+}\) import. Considering that this was not the case for cells grown at pH 7 these data imply that distinct nickel-uptake pathways are active in *H. pylori* under neutral and acidic conditions.

*Short-term \(^{63}\)Ni uptake in a minimal assay is mediated by NixA*

To more closely examine Ni\(^{2+}\) uptake in *H. pylori* under minimal conditions (i.e., in the absence of rich media components that can potentially bind nickel ions), a short-term \(^{63}\)Ni uptake assay was developed in which cells were washed three times with 900 \(\mu\)l 50 mM HEPES (pH 7.0) after overnight growth, resuspended in the same buffer or 50 mM MES (pH 5.5) and pre-incubated at 37° C for 10 min. \(^{63}\)NiCl\(_2\) (specific activity 9.87 mCi mg\(^{-1}\); Perkin Elmer, Boston, MA) was added at time 0 and uptake was quenched at increasing times by incubating cells in an ice water bath for 2 min. Cells were harvested, rinsed once with 900 \(\mu\)l 50 mM HEPES (pH 7), 50 mM EDTA, resuspended in 200 \(\mu\)l 10 \(\mu\)M acetic acid and scintillation counted for total \(^{63}\)Ni content. Total \(^{63}\)Ni/cell was calculated using the following equation:

\[
\text{Total } ^{63}\text{Ni/cell} = \frac{(\text{Total counts/O}\text{D}_{600}\times\text{culture volume})}{2.13\times10^{-9}}
\]

where 2.13 \(\times\)10\(^{-9}\) is the specific activity of \(^{63}\)Ni. At pH 7 wild-type *H. pylori* strain 26695 took up approximately 4000 \(^{63}\)Ni/cell in this assay, however no uptake was observed at pH 5.5 (Figure A2.2a). Surprisingly, given the long-term
Figure A2.2. NixA-dependent uptake in a minimal uptake assay requires the proton motive force at pH 7 and urea and urease activity at pH 5.5. (a) wild-type *H. pylori* 26695 (circles) vs a nixA mutant (squares): at pH 7 (black), at pH 5.5 (dark grey) and at pH 5.5 with 5 mM urea (light grey). (b) Uptake at pH 5.5 with 5 mM urea in wild-type (solid black), nixA (dark grey), *ureE* (light grey) and *nikR* (open black) strains. (c) Uptake at pH 7 in wild-type in the absence (solid black) or presence (open black) of 10 µM CCCP, a proton motive force inhibitor. The data plotted is the average of three experimental replicates and is reported with the calculated standard error.
accumulation data, a nixA mutant displayed no $^{63}\text{Ni}$ uptake under any of the conditions tested during the time course of this assay, indicating that all uptake measured in this minimal assay is mediated by NixA.

Considering that urease plays a major role in cell survival under acidic conditions, I tested the ability of urea (the urease substrate) to rescue $^{63}\text{Ni}$ uptake at pH 5.5. The addition of 5 mM urea to wild-type cells at pH 5.5 did indeed resulted in $^{63}\text{Ni}$ uptake comparable to that measured at pH 7 (Figure A2.2a), suggesting that urea hydrolysis by urease is required for cellular nickel uptake. Consistent with urease activity being required to rescue the defect in uptake at pH 5.5, a ureE mutant which lacks the chaperone responsible for inserting Ni$^{2+}$ into the urease active site, displayed no significant $^{63}\text{Ni}$ uptake at pH 5.5 (Figure A2.2b).

A nikR mutant strain was measured for $^{63}\text{Ni}$ uptake to determine if NikR activity can be indirectly monitored by this assay. The nikR mutant displayed increased $^{63}\text{Ni}$ uptake relative to wild-type cells at pH 5.5 with urea, which is consistent with a role for NikR in the repression of nixA expression and demonstrates that short-term $^{63}\text{Ni}$ uptake can be used to monitor NikR activity.

To better understand the mechanism of NixA-dependent nickel transport in H. pylori, I examined the energy requirement for $^{63}\text{Ni}$ uptake in the short-term assay. A previous study has demonstrated that the cobalt transporter NhlF from Rhodococcus rhodochrous is dependent on the proton motive force (pmf) for activity (4). To determine if the pmf is similarly required for NixA activity, I added
10 μM carbonyl cyanide-m-chlorophenylhydrazone (CCCP, Sigma, Saint Louis, MO), a pmf inhibitor, to wild-type *H. pylori* cells at pH 7. CCCP completely abolished $^{63}$Ni uptake (Figure A2.2c), demonstrating that NixA uses the pmf as an energy source for Ni$^{2+}$ transport.

Since the onset of these studies an outer membrane, TonB-dependent transporter of *H. pylori* that is homologus to iron-siderophore transporters, FrpB4, has been shown to be regulated by NikR in response to increased extracellular Ni$^{2+}$ (9). In addition, FrpB4, as well as the TonB-ExbB-ExbD system that provides energy for outer membrane transporters, were required for Ni$^{2+}$ accumulation at pH 5 but not at pH 7 (9). These results are consistent with the long-term $^{63}$Ni accumulation data that showed that NixA is not important for Ni$^{2+}$ transport under acidic conditions, and indicate that NixA likely makes the most significant contribution to Ni$^{2+}$ transport at neutral pH, whereas FrpB4 (and an unknown inner membrane transporter) are the most significant Ni$^{2+}$ transporters at acidic pH. It is interesting to speculate that, given the homology of FrpB4 to iron-siderophore transporters, Ni$^{2+}$ is likely transported across the outer membrane in complex with an unidentified small molecular. The fact that NixA transports Ni$^{2+}$ alone suggests that *H. pylori* transports isolated Ni$^{2+}$ at pH 7 and an unidentified Ni$^{2+}$-complex(es) at lower pH.

The implication that Ni$^{2+}$ is transported in a complex by FrpB4 does not invalidate the short-term $^{63}$Ni uptake results presented here because it is likely that *H. pylori* are still capable of transporting Ni$^{2+}$ alone at either pH, especially in
a minimal buffer that lacks any potential Ni$^{2+}$ chelators. This assay was extremely useful in determining the pmf requirement of NixA-dependent Ni$^{2+}$ transport, and will also be important for identifying physiologically relevant Ni$^{2+}$-complexes that are transported by *H. pylori*.

The demonstration that additional NikR-regulated genes are important for Ni$^{2+}$ transport, most notably at pH 5.5, suggests one way in which to identify additional Ni$^{2+}$ transporters in *H. pylori*. A comparative analysis of transcript levels in wild-type and nikR mutant strains grown under acidic conditions would likely identify candidate genes important for Ni$^{2+}$ transport through the inner membrane.
References


