Conserved Residues and Motifs in the NixA Protein of *Helicobacter pylori* Are Critical for the High Affinity Transport of Nickel Ions*

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NixA, the high affinity nickel transport protein of *Helicobacter pylori*, imports Ni\(^{2+}\) ions across the cytoplasmic membrane for insertion into the active site of the urease metalloenzyme, which is essential for colonization of the gastric mucosa. Twelve conserved aspartate (aspartates 47, 49, 55, 194, 231, and 234), glutamate (glutamates 106, 198, and 274), and histidine (histidines 44, 50, and 79) residues were identified by alignment of NixA with homologous transporters. Polymerase chain reaction-generated site-directed mutants of these residues were expressed in *E. coli* along with the *H. pylori* urease gene cluster. Mutations in residues within the predicted periplasmic domains of NixA maintained near wild type levels of Ni\(^{2+}\) uptake and urease activity, as did control mutations of conserved positively charged residues (lysines 140 and 268; arginines 162 and 167). Mutations in highly conserved motifs in predicted helices II and III of NixA abolished Ni\(^{2+}\) uptake and urease activity. Mutations in helices V and VI and the cytoplasmic domains decreased Ni\(^{2+}\) transport rates by >90%. Reduction in rates of Ni\(^{2+}\) transport correlated with reduction in urease activities (\(r = 0.77\)). Ni\(^{2+}\) transport was inhibited in the presence of Co\(^{2+}\), Cu\(^{2+}\), and Zn\(^{2+}\), indicating that these ions may also be bound or transported by NixA. We conclude that conserved Asp, Glu, and His residues in the transmembrane domains of NixA are critical for the transport of the divalent cations Ni\(^{2+}\), Co\(^{2+}\), Cu\(^{2+}\), and Zn\(^{2+}\) into the cytoplasm of *H. pylori*.

*Helicobacter pylori*, a Gram-negative, spiral-shaped, microaerophilic bacterium is a well established etiologic agent of gastritis and peptic ulcer disease (1–3). More serious sequelae of infection, the development of gastric adenocarcinoma and MALT\(^{1}\) lymphoma, are also strongly associated with chronic atrophic gastritis caused by *H. pylori* infection (4, 5).

*H. pylori* produces virulence factors that include flagella cholomact for gastric epithelial determinants (6), a vacuolating cytotoxin (7), and numerous putative adherence factors (8). Possibly the most significant virulence factor is an abundant urease (7), and numerous putative adherence factors (8). Possibly the most significant virulence factor is a very high affinity Ni\(^{2+}\) importer with a \(K_r = 11.3\) nM, well suited to physiological conditions, and allowed synthesis of catalytically active urease independent of growth conditions (17).

NixA is a 331-amino acid, 37-kDa integral membrane protein consisting of eight predicted transmembrane domains. Three one-component Ni\(^{2+}\)-importing homologs have been identified: the HoxN protein of *Alcaligenes eutrophus* (18), the HupN protein of *Bradyrhizobium japonicum* (19), and the UreH protein of the thermophilic *Bacillus* TB90 (20). NixA, HoxN, HupN, and UreH share the amino acid sequence motif HX\(_{X}\)DH located in transmembrane helix II of NixA with the NikC component of the nonhomologous Nik ABC type Ni\(^{2+}\) transporter of *E. coli* (18). Significantly, the amino acid sequence motif GX\(_{X}\)GHSSVV, located in the sequence spanning the second periplasmic loop and helix III of NixA, is also conserved in HoxN, HupN, and UreH (18).

In this report, we present the results of specific site-directed mutagenesis of 12 potential metal-binding residues conserved among NixA and homologous transporters by direct measurement of Ni\(^{2+}\) uptake and the synthesis of catalytically active urease.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Culture Conditions—*E. coli* DH5α (supE44 ΔlacU169 (Δ80 lacZ delta M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1), E. coli M15pRep4 (Qiagen), and E. coli SE5000 (araD139Δ(argF-lac) U169 rpsL150 relA1 F′ lacZΔM15 endA1 tct86 rbsR recA46) were used as recipients of recombinant plasmids (21). Plasmid pUE2304 encoding the *H. pylori* nixA gene cloned in pBluescript II SK+ (Stratagene) and plasmid pH808 encoding the *H. pylori* urease operon (*ureABIEFGH*) in pACYC184 have been described previously (17, 22). Strains were maintained on Luria-Bertani agar containing the appropriate antibiotics and were stored at −70 °C in Luria broth supplemented with 15% (v/v) glycerol. Other media included M9 minimal salts medium and Luria broth supplemented with 1 μM NiCl\(_2\).
Recombinant DNA Techniques—Recombinant DNA methods including restriction endonuclease digestion, ligation, and transformation were performed according to standard protocols (21, 23). Plasmid DNA was purified by rapid alkaline lysis (24). Large scale preparations were isolated using Qiagen DNA purification columns according to the manufacturer's instructions.

Site-directed Mutagenesis—Site-directed mutations were PCR-generated by the overlap extension method of Ho (25), using the mutagenic and flanking primers listed in Table I. First round PCR products were agarose gel-purified to prevent the amplification of wild type nixA. PCR reactions were performed using cloned PfDNA polymerase (Stratagene). PCR-generated mutants were cloned into pBluescript II SK + as XbaI/HindIII fragments.

Nucleotide Sequencing—Plasmid DNA was sequenced by the dideoxy chain termination method (26). Reactions were run on an Applied Biosystems model 373A DNA sequencer.

Preparation of a Polyclonal Antiserum to NixA—A 149-base pair PCR-amplified fragment corresponding to gene sequences encoding NixA amino acids 133–180 was subcloned into pBluescript SK + (Stratagene), excised at PCR-generated Ncol and BglII sites, and ligated into the His6a tail vector pQE60 (Qiagen). The resulting construct, pQNS99, was transformed into E. coli M15pRep4, and the NixA-His6a polypeptide was overexpressed by induction of exponential (A600 = 0.5) phase Luria broth cultures (1 liter) containing 5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) and 30°C, 17 h. Bacteria were harvested by centrifugation (8000 × g, 10 min, 4°C) and resuspended in 30 ml of buffer B (50 mM Tris-HCl, pH 7.5, 0.1 M NaH2PO4, 0.01 M Tris-HCl, pH 8.0). Cells were ruptured in a French press at 20,000 p.s.i., and the resultant lysate was centrifuged (8000 × g, 10 min, 4°C). The supernatant was loaded onto a 4-ml Ni2+–nitrilotriacetic acid column (Qiagen) pre-equilibrated with buffer B. The column was washed with 200 ml of buffer B then washed with buffer C (buffer B, adjusted to pH 6.3) until the A600 of the eluent was <0.01. The NixA-His6a polypeptide was eluted with 10 ml of buffer E (buffer B, adjusted to pH 4.5) collected in 1-ml fractions, which were analyzed by SDS-polyacrylamide gel electrophoresis in a 20% polyacrylamide gel. NixA-His6a-containing fractions were dialyzed overnight against 1 mM Tris-HCl, 0.5 mM EDTA, pH 8.0.

The purified polypeptide was trichloroacetic acid-precipitated, and 100 μg was emulsified in Freund’s complete adjuvant and used to immunize two New Zealand White rabbits by subcutaneous injection. Three 30-μg boosts were administered subcutaneously in Freund’s incomplete adjuvant at 3-week intervals. NixA-specific antibodies were affinity-purified by immobilizing the NixA-His6a polypeptide on a Pierce Aminolink column according to the manufacturer’s instructions. Serum (1 ml) was diluted 1:1 in 10 ml Tris-HCl, pH 7.5, and loaded onto the column followed by 200 μl of 10 mM Tris-HCl, pH 7.5, and allowed to stand at room temperature for 1 h. The column was washed with 40 ml of 10 mM Tris-HCl, pH 7.5, and then with 40 ml of 0.5 mM NaCl, 10 mM Tris-HCl, pH 7.5. Antibodies were eluted with 10 ml of 100 mM glycine, pH 2.5, into a tube containing 1 ml of 1 M Tris-HCl, pH 8.

Western Blotting—Membranes of E. coli SE5000 (pHP808) cotransformed with pUPEF204, pBluescript, or each of the site-directed mutants of nixA were isolated from 100-ml overnight cultures. Bacteria from Luria broth cultures (100 ml) of each strain were harvested by centrifugation (8000 × g, 5 min, 4°C) upon reaching an A600 of 0.5. Cell pellets were resuspended in and washed with transport buffer (50 mM Tris, 1 mM MgCl2, pH 7.5). Cells were pelleted and resuspended in the same buffer to an A600 of 0.5, and 60NiCl2 (specific activity, 63.5 μCi/ml; 0.46 mg of nickel/ml); Amersham Corp.) was added to a final concentration of 50 mM at 37°C. Radioactive 60Ni2+ incorporation was assayed by vacuum filtration as described previously (17). For 60Ni2+ transport inhibition experiments, washed cells were resuspended in transport buffer to a final A600 of 1.0 and added to an equal volume of transport buffer containing 100 mM 60NiCl2 and from 0 to 500 mM CaCl2, CuCl2, CoCl2, MgCl2, MnCl2, NiCl2, or ZnCl2.

Urease Assays—E. coli SE5000 (pHP808) cotransformed individually with pUPEF204, pBluescript, or each of the site-directed mutants was grown overnight in 100 ml of Luria broth supplemented with 1 mM NiCl2. Cells were harvested by centrifugation (8000 × g, 5 min, 4°C), resuspended in 2 ml of ice-cold 10 mM NaH2PO4, pH 6.8, and lysed by passing through a French pressure cell at 20,000 p.s.i. Lysates were centrifuged (8000 × g, 10 min, 4°C), and urea hydrolysis by supernatants was measured by the phenol red assay of Hamilton-Miller and Gargan (27) as previously calibrated for quantitation (28). Protein concentrations were determined by the bicinchoninic acid method using the Pierce BCA assay kit according to the manufacturer’s instructions.

RESULTS

Conservation of Amino Acid Residues among NixA and Homologs—A word search of the Swissprot and PIR data bases with the NixA amino acid sequence revealed three significant homologs of NixA that are also single component Ni2+ importers (17). The 38.9-kDa HoxN protein of the Gram-negative soil and aquatic bacterium A. eutrophus shares 40% amino acid sequence identity with NixA (18). HoxN is responsible for the high affinity (Kd = 20 nM) import of Ni2+ for incorporation into at least three enzymes including a cytoplasmic NAD-reducing hydrogenase, an electron transport-coupled hydrogenase, and a urease (29). Topological mapping of this integral inner membrane protein suggests eight transmembrane domains, with both the amino and carboxyl termini located in the cytoplasm (29, 30). The second homolog, HupN, is a 40-kDa integral membrane protein that is encoded as part of a three-gene operon involved in Ni2+ incorporation into the H2-recycling membrane of the N2-fixing Gram-negative soybean symbiont B. japonicum (19). The HupN protein shares 41% amino acid identity with NixA; charge distribution and amino acid sequence similarity suggest eight membrane-spanning domains as in HoxN (31). The third homolog, the 25-kDa UreH protein of the thermophilic Bacillus TB90 (20), shares only 13% amino acid identity with NixA but contains sequence signatures of other high affinity Ni2+ transporters (18) and is the only known example of a bacterial Ni2+ transporter encoded within a urease operon (17).

Alignment of the sequence of these four homologous transporters, Kyte and Doolittle hydrophathy predictions, and the results of NixA-LacZ fusions suggest an eight-transmembrane domain topology for NixA. Furthermore, sequence alignment produced an overall consensus (at least three out of four residues identical) sequence identity of 30%. Among the 95 amino acids of the consensus sequence, we identified 12 conserved Asp, Glu, and His residues (Fig. 1), including residues in the HX2DH motif in helix II and the GX2GHSSVV motif in helix III (18), which we postulated might be involved in Ni2+ transport. Additionally, 2 conserved Lys and 2 conserved Arg residues were identified to serve as controls in mutagenesis experiments.

Site-directed Mutagenesis—To evaluate the contribution of conserved residues in Ni2+ uptake, single amino acid mutations of NixA were generated by PCR overlap extension (25) using pUPEF204 as a template and the primers listed in Table I. Amino acids His44, Asp47, Asp49, His50, Asp52, His79, Glu106, Asp194, Glu198, Asp231, Asp234, Glu274, Lys140, Arg162, Arg167, Asp194, Glu198, Asp231, Asp234, and Gln286 were individually replaced by Ile, which has a negative ΔG of formation for both α-helices and β-sheets (32) and is abundant in the wild type protein. First round PCR

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products were agarose gel-purified to eliminate amplification of wild type sequences in the second round of PCR. Second round PCR products were subcloned into the EcoRV site of pBlue-script SK1 for sequencing and isolation of plasmids carrying the mutant constructs. The nixA gene carrying each mutation was excised at PCR-generated XbaI and HindIII sites and religated into pBlue-script SK+ at these sites to regenerate modified plasmid pUEF204 encoding each mutation.

Ni²⁺ Transport—NixA site-directed mutations H44I, D49I, and H50I, which lie in the conserved HX₄DH motif of helix II, and H79I, which lies in the conserved GX₅GHSSVV motif, showed complete abolishment of ⁶³Ni²⁺ uptake when co-expressed in E. coli with the urease operon encoded on plasmid pH808 (Table II). Transport was also abolished by or was not significantly different from background levels (E. coli transformed with pBluescript) for mutations D47I, D55I, D194I, and D231I, all of which lie within or immediately adjacent to putative transmembrane domains. Mutations E106I (predicted to be located in the periplasm), K140I, R162I, R167I, and K286I (conserved positively charged residues mutated as controls) imported Ni²⁺ at rates not significantly different from wild type NixA. Mutations E198I, D234I, and E274I, all located within or immediately adjacent to putative transmembrane domains, maintained consistently low (<10% of wild type) rates of transport that were not statistically different from vector controls.

Urease Activities Confirm Transport Results—To confirm the Ni²⁺ transport results and investigate the mutations that demonstrated low but measurable transport activity, urease assays were conducted. Cultures were grown overnight in Luria broth containing 1 μM NiCl₂, as opposed to the transport conditions of 50 nM ⁶³NiCl₂ sampled over 2.5 min to allow constructs that might import Ni²⁺ at low rates to accumulate sufficiently high ion concentrations to yield synthesis of statistically significant urease activity. Mutations that abolished measurable Ni²⁺ uptake at 50 nM also lowered urease activity to <1% of wild type, with the exceptions of D55I and D231I, which retained 5 and 16% of wild type urease activity, respectively (Table II). Mutations that demonstrated wild type uptake yielded wild type or greater urease activities. Interestingly, mutations E198I, D234I, and E274I, which demonstrated low but consistent levels of transport, did have greater than 50% of wild type urease activity in the assay. Significantly, all mutations that retained any measurable transport, with the exception of E106I, which gave inconsistent results, correlated directly (r = 0.77) with observed urease activities (Table II, Fig. 2).

Western Blots Confirm the Presence of Full-length NixA in the Membrane—To show that changes in Ni²⁺ transport and urease activity of NixA containing site-directed mutations were not the result of synthesis of a truncated protein, failure of the protein to be inserted in the membrane, or improper insertion or misfolding of the protein that might render it more susceptible to proteolysis, membrane preparations of E. coli transformed with pBluescript, pUEF204, or each site-directed mutation were assayed by Western blot with affinity-purified polyclonal antiserum raised against an internal polypeptide of NixA (Fig. 3). All NixA site-directed mutants produced a band of the appropriate size, at least equal in intensity to pUEF204
Role of Conserved Residues in the NixA Protein of H. pylori

TABLE I
PCR primers used in site-directed mutagenesis of nixA

| Mutation | Mutagenic primers (5’→3’) |
|----------|---------------------------|
| H44I     | Upper: TACATGCCTAGGGCGGAAAAGATTGCGTTTGTACGCGGATCAC Lower: GGTGATCCGATCACCAACCTTTGGCCTTCAACATGTA |
| D47I     | Upper: AAAAGACATGGCAAATATTGCGGATCATACTG |
| D49I     | Upper: GCGTGTGATGCAGTCATACATCGCAGGTAATAAAG |
| H50I     | Upper: CATCGTGTTAGCGGATCTACGCTTCGATACGATCG |
| D55I     | Upper: GATCAGATGCGTTTCGTAATTACACAAACATTTAGAAGCTT |
| H79I     | Upper: TTTTACCTTTATTGCGGATCTGGATACGATG |
| E106I    | Upper: AGCCCGATCTAGGAAATGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA
NixA. While the UreH protein is probably too small to possess eight transmembrane domains and shares only 13% homology with NixA (17), it does contain two sequence motifs (HX4DH and GXXGHSSVV), which are present in the other high affinity Ni\(^{2+}\) permeases (18). It is also the only known example of a Ni\(^{2+}\)-specific transporter encoded within a bacterial urease operon. Interestingly, HoxN and HupN are both encoded within hydrogenase clusters (17), while NixA is not included in any operon structure, and its gene (nixA) is found over 400,000 base pairs away from the urease operon in the recently published genome sequence of \(H.\) pylori (34).

To investigate the role of 12 conserved negatively charged or histidine residues in the transport of Ni\(^{2+}\) across the periplasmic membrane, we constructed specific site-directed mutations of these residues and measured the effect of these single amino acid changes directly on Ni\(^{2+}\) uptake and on the resultant urease activities of recombinant constructs. MgCl\(_2\) (10 mM) was included in the transport buffer to inhibit the nonspecific binding and transport of Ni\(^{2+}\) by the CorA, MgtA, and MgtB Mg\(^{2+}\) transport systems. The \(E.\) coli NikABCD transporter has a significantly lower affinity for Ni\(^{2+}\) and a lower rate of Ni\(^{2+}\) transport (35) compared with NixA and would not be relevant at the low Ni\(^{2+}\) concentrations used in this study. As expected, mutations H44I, D49I, and H50I, which comprise the HX4DH motif of helix II abolished Ni\(^{2+}\) uptake and urease activity. Recent studies of the two corresponding His residues in HoxN (30) confirm the importance of these residues in Ni\(^{2+}\) binding and transport. Mutation of Asp47, which is conserved in three of the four homologs and lies in the variable region of the helix II motif in NixA, also abolished Ni\(^{2+}\) transport and urease activity.

Since the helix II motif is highly conserved among NixA and its homologs, as well as in the NikC protein of the \(E.\) coli Nik nickel-importing ABC transporter and the Ni\(^{2+}\) binding site of human serum albumin (18), it is very tempting to assign the role of Ni\(^{2+}\) specificity determinant to this motif. However, the recent discovery of a cobalt-importing transporter NhlF in \(Rhodococcus rhodochrous\) (36), which is similar in size and topology to NixA and also contains this motif, implies that the HX4DH motif is not the sole determinant of specificity. As further support for this concept, we found that the presence of Co\(^{2+}\) in 10–100-fold excess produced 50% inhibition of Ni\(^{2+}\) transport by NixA. The presence of 500-fold excess Ni\(^{2+}\) decreased Co\(^{2+}\) transport by approximately 90% in NhlF, although direct uptake of \(^{63}\)Ni\(^{2+}\) by NhlF could not be demonstrated (36).

NixA mutation H79I in the GXXGHSSVV motif also abolished Ni\(^{2+}\) uptake and urease activity. While this motif is conserved perfectly among NixA and homologous transporters, it is present as a slight variant (GXXGHSTVV) in helix III of the cobalt importer of \(R.\) rhodochrous (36). Despite this slight change in consensus, it also seems unlikely that this sequence alone is sufficient for ion specificity.

The fact that mutation D194I completely abolished Ni\(^{2+}\) transport and urease activity, as did the mutations in the helix II and III motifs, implies that these residues are directly involved in binding and translocation of nickel ions. Notably, Western blotting confirmed that none of these mutations truncated the synthesis of the protein or impaired the insertion of the transporter into the membrane, and no significant increase in proteolytic degradation products were observed in relation to total amount of NixA.

Mutations of residues that are located in predicted periplasmic or cytoplasmic loops (E106I, K140I, R162I, R167I, and K286I) all exhibited Ni\(^{2+}\) transport rates not significantly different than the wild type. Similarly, they also exhibited wild

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**Fig. 2.** Urease activities of NixA site-directed mutants correlate directly with Ni\(^{2+}\) transport. Maximum rates of Ni\(^{2+}\) transport (at 50 nm) were measured as described in Table II for each mutation, as well as for wild type and vector control constructs. Urease activities of whole cell lysates of each construct grown in Luria broth supplemented with 1 \(\mu\)M NiCl\(_2\) were measured as described in Table II. The percentage of wild type urease activity of each mutant correlates \(r = 0.77\) with percentage of wild type Ni\(^{2+}\) transport for each mutant.
Type or greater than wild type urease activity. Mutation of residues located in or immediately adjacent to predicted transmembrane domains (D55I, D231I, E198I, D234I, and E274I), but not in the helix II or III motifs, reduced $^{\text{63}}$Ni$^{2+}$ transport to <10% of wild type. These constructs, however, showed significant urease activities due to the sensitivity of the urease assay conditions (1 μM NiCl$_2$). It appears, therefore, that the mutated conserved residues outside of the helix II and III motifs are not absolutely essential for Ni$^{2+}$ transport.

Urease activities correlated directly with observed rates of transport, with the exception of mutation E106I, which gave 150% of the wild type. These constructs, however, showed significant urease activities due to the sensitivity of the urease assay conditions (1 μM NiCl$_2$). It appears, therefore, that the mutated conserved residues outside of the helix II and III motifs are not absolutely essential for Ni$^{2+}$ transport.

Alternatively, it may be the combination of one or more of these residues with residues that clearly abolish all transport and urease activity when mutated that defines the Ni$^{2+}$ specificity determinant of NixA and the other high affinity one-component transporters. The possibility of additional periplasmic binding proteins or highly ion specific surface proteins that interact with NixA and other transporters also cannot be ruled out (17).

The fact that the high affinity NixA permease may also transport lower levels of Co$^{2+}$, Cu$^{2+}$, and Zn$^{2+}$ is not altogether surprising. While each of these ions would be toxic in high concentration, specific exporters of these ions (37) that could serve as compensatory mechanisms have been described in the literature.

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REFERENCES

1. Blaser, M. J. (1990) J. Infect. Dis. 161, 626–633
2. Marshall, B. J., and Warren, J. R. (1984) Lancet 1, 1311–1315
3. Marshall, B. J., McCollie, D. R., Rogers, P. A., and Glancy, R. J. (1985) Med. J. Australia 142, 439–444
4. Forman, D., Newell, D. G., Fullerton, F., Yarnell, J. W. G., Stacey, A. R., Wald, N., and Sitas, F. (1991) Br. Med. J. 302, 1302–1305
5. Parsonnet, J., Hansen, S., Rodriguez, L., Gelb, A., Warner, R., Jellum, E., Orentreich, N., Vogelman, J., and Friedman, G. (1994) N. Engl. J. Med. 330, 1267–1271
6. Hazell, S. L., and Lee, A. (1985) Campylobacter III: Proceedings of the Third International Workshop on Campylobacter Infections, Ottawa, Canada (Pearson, A. D., Skirrow, M. B., Lion, H., and Rowe, B., eds) pp. 189–191, Public Health Laboratory Service, London
7. Figura, N., Gualielmetti, P., Rossolini, A., Barberi, A., Cusi, G., Musmanno, R. A., Russi, M., and Quarantha, S. (1989) J. Clin. Microbiol. 27, 225–226
8. Lee, A., and Mitchell, H. (1984) in Helicobacter pylori: Basic Mechanisms to Clinical Cure (Hunt, R. H., and Tytgat, G. N. J., eds) Kluwer Academic Publishers, Lancaster, UK
9. Hu, L.-T., and Mollby, H. L. T. (1990) Infect. Immun. 58, 992–998
10. Mollby, H. L. T., Island, M. D., and Hausinger, R. P. (1990) Microbiol. Rev. 59, 451–480
11. Marshall, B. J., Barett, L. J., Prakash, C., McCallum, R. W., and Guerrat, R. L. (1990) Campylobacter IV, pp. 492–493, University of Goteborg, Goteberg, Sweden
12. Blaser, M. J. (1990) Rev. Infect. Dis. 12, Suppl. 1, 99–106
13. Eaton, K. A., and Markowitz, S. (1990) Infect. Immun. 58, 3604–3607
14. Teuda, M., Karita, M., Morshed, M. G., Okita, K., and Nakazawa, T. (1994) Infect. Immun. 62, 3586–3589
15. Hawtin, P. R., Delves, H. T., and Newell, D. G. (1995) FEMS Microbiol. Lett. 77, 51–54
16. Sundermann, F. W., Jr. (1993) Scand. J. Work Environ. Health 19, Suppl. 1, 34–38
17. Mollby, H. L. T., Garner, R. M., and Bauerfeind, P. G. (1995) Mol. Microbiol. 16, 97–109
18. Wolfram, L., Friedrich, B., and Eiting, T. (1995) J. Bacteriol. 177, 1840–1843
19. Fu, C., Javedan, S., Moshiri, F., and Maier, R. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 5099–5103
20. Maeda, M., Hidaka, M., Nakamura, A., Masaki, S., and Voxumi, T. (1994) J. Bacteriol. 176, 432–442
21. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York

Table III

| Ion          | Concentration | Inhibition |
|--------------|---------------|------------|
| CoCl$_2$     | 500 μM        | 99.41      |
|              | 500 μM        | 99.41      |
|              | 50 μM         | 90.86      |
|              | 5 μM          | 62.17      |
|              | 0.5 μM        | 33.11      |
|              | 0.05 μM       | 12.21      |
| CuCl$_2$     | 500 μM        | 96.80      |
|              | 50 μM         | 69.27      |
|              | 5 μM          | 15.67      |
|              | 0.5 μM        | 0          |
|              | 0.05 μM       | 0          |
| NiCl$_2$     | 500 μM        | 98.35      |
|              | 50 μM         | 97.99      |
|              | 5 μM          | 92.04      |
|              | 0.5 μM        | 49.32      |
|              | 0.05 μM       | 33.88      |
| ZnCl$_2$     | 500 μM        | 99.88      |
|              | 50 μM         | 91.41      |
|              | 5 μM          | 44.81      |
|              | 0.5 μM        | 13.10      |
|              | 0.05 μM       | 0.30       |
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A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold
Spring Harbor, New York
22. Hu, L.-T., and Mobley, H. L. T. (1993) Infect. Immun. 61, 2563–2569
23. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Smith, J. A., Seidman,
J. G., and Struhl, K. (1987) Current Protocols in Molecular Biology, Greene
Publishing Associates and Wiley Interscience, New York
24. Birnboim, H. C., and Doly, J. (1979) Nucleic Acids Res. 7, 1513–1523
25. Hu, S., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) Gene
Amst. 77, 51–59
26. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A.
74, 5463–5467
27. Hamilton-Miller, J. M. P., and Gargan, R. A. (1979) Invest. Urol. 16, 327–328
28. Mobley, H. L. T., Jones, B. D., and Jerse, A. E. (1986) Infect. Immun. 54,
161–169
29. Eitinger, T., and Friedrich, B. (1994) Mol. Microbiol. 12, 1025–1032
30. Eitinger, T., Wolfram, L., Degen, O., and Anthon, C. (1997) J. Biol. Chem. 272,
17139–17144
31. Eitinger, T., and Friedrich, B. (1997) in Transition Metals in Microbial
Metabolism (Winkelmann, G., and Carrano, C., eds) pp. 235–256, Harwood
Academic Publishers, London
32. Creighton, T. E. (1993) Proteins: Structures and Molecular Properties 2nd Ed.,
pp. 186 and 256, W. H. Freeman, New York
33. Bauerfeind, P. G., Mobley, H. L. T., and Garner, R. M. (1996) Infect. Immun.
64, 2877–2880
34. Tomb, J. F., Tomb, J.-F., White, O., Kerelabage, A. R., Clayton, R. A., Sutton,
G. G., Fleischmann, R. D., Ketchum, K. A., Klenk, H. P., Gill, S., Dougherty,
B. A., Nelson, K., Quackenbush, J., Zhou, L., Kirkness, E. F., Peterson, S.,
Loftus, B., Richardson, D., Dodson, R., Khalak, H. G., Glodek, A., McKen-
ney, K., Fitzgerald, L. M., Lee, N., Adams, M. D., Hickey, E. K., Berg, D. E.,
Gocayne, J. D., Utterback, T. R., Peterson, J. D., Kelley, J. M., Cotten,
M. D., Weidman, J. M., Fujii, C., Bowman, C., Watthey, L., Wallin, E.,
Hayes, W. S., Borodersky, M., Karp, P. D., Smith, H. O., Fraser, C. M., and
Venter, J. C. (1997) Nature 388, 534–547
35. De Pina, K., Navarro, C., McWalter, L., Boxer, D. H., Price, N. C., and Kelly,
S. M. (1996) Eur. J. Biochem. 227, 857–865
36. Komeda, H., Kobayashi, M., and Shimizu, S. (1997) Proc. Natl. Acad. Sci.
U. S. A. 94, 36–41
37. Ge, Z., Hirasuka, K., and Taylor, D. (1995) Mol. Microbiol. 15, 97–106