Identification of Functional Amino Acids in the Nramp Family by a Combination of Evolutionary Analysis and Biophysical Studies of Metal and Proton Cotransport *in Vivo*†

Roman Chaloupka,‡,§,|| Pascal Courville,‡,§ Frédéric Veyrier,‡ Bjarne Knudsen,⊥ Thomas A. Tompkins,@ and Mathieu F. M. Cellier*,‡

Institut National de la Recherche Scientifique, INRS-Institut Armand-Frappier, 531, Bd des prairies, Laval, Quebec, Canada H7V 1B7, Department of Zoology, University of Florida, Gainesville, Florida 32611-8525, and Institut Rosell-Lallemand Inc., 8480, boulevard Saint-Laurent, Montréal, Quebec, Canada H2P 2M5

Received September 14, 2004; Revised Manuscript Received October 14, 2004

ABSTRACT: The natural resistance-associated macrophage protein (Nramp) family is functionally conserved in bacteria and eukarya; Nramp homologues function as proton-dependent membrane transporters of divalent metals. Sequence analyses indicate that five phylogenetic groups comprise the Nramp family, three bacterial and two eukaryotic, which are distinct from a more distantly related group of microbial sequences (Nramp outgroup). The Nramp family and outgroup share many conserved residues, suggesting they derived from a common ancestor and raising the possibility that the residues invariant in the Nramp family that correspond to residues which are different but also conserved in the outgroup represent candidate sites of functional divergence of the Nramp family. Four Nramp family-specific residues were identified within transmembrane domains 1, 6, and 11, and replaced by the corresponding invariant outgroup residues in the *Escherichia coli* Nramp ortholog (the proton-dependent manganese transporter, MntH of group A, EcoliA). The resulting mutants ($Asp^{34}Gly$, $Asn^{37}Thr$, $His^{211}Tyr$, and $Asn^{401}Gly$) were tested for both divalent metal uptake and proton transport; quasi-simultaneous analyses of uptake of metals and protons revealed for the first time protons and metals cotransport by a bacterial Nramp homologue. Additional mutations were studied for comparison ($Asp^{34}Asn$, $Asn^{37}Asp$ and $Asn^{37}Val$, $Asn^{401}Thr$, $His^{211}Ala$, $His^{216}Ala$, and $His^{216}Arg$). EcoliA activity was impaired after each of the Nramp/outgroup substitutions, as well as after more conservative replacements, showing that the tested sites are all important for metal uptake and metal-dependent H^+ transport. It is proposed that co-occurrence of these four Nramp-specific transmembrane residues may have contributed to the emergence of this family of metal and proton cotransporters.

The Nramp family is required to maintain divalent metal (Me^{2+}) homeostasis in eukaryotes (*1*). The murine Nramp1 protein is needed for innate resistance to some intracellular parasites, including mycobacteria, which reside within a phagocytic vacuole (*2*). Mutation of the murine *Nramp2* gene (also known as divalent metal transporter 1) results in microcytic anemia (*3*). Similarly, yeast homologues are also necessary for metal uptake either directly at the plasma membrane or by mobilization of metals from endosomes or the lysosomal vacuole (*4*). These eukaryotic Nramp proteins are energized by the proton electrochemical gradient (*5*, *6*) and were reported to cotransport metal ions and protons (Me^{2+} and H^+, symport).

Major goals in the study of Nramp permeases include understanding their function in health and diseases and their role during infection. Eukaryotic Nramp family members were likely derived from bacterial precursors thought to have emerged prior to the separation of Gram-positive and Gram-negative clusters (*7*). Bacterial Nramp homologues were characterized as proton-dependent manganese (and iron) transporters (*8*, *9*), which are regulated by repressors using manganese and/or iron as a corepressor (*10*, *11*). The MntH proteins characterized so far belong to one of three bacterial phylogenetic groups, MntH A (Supporting Information), including the *Escherichia coli* homologue, named herein EcoliA,[1] which constitutes an attractive model for structure−function studies.

Recognition of the phylogenetic diversity within a protein family allows identification of site-specific rate shifts between groups, which may improve functional predictions and provide a rational approach for identifying key residues

† This work was supported by a research grant from the Canadian Institutes of Health Research (MOP-78014-MI). M.F.M.C. is a Scholar of the Fonds pour la Recherche en Santé du Québec. B.K. was supported by The Carlsberg Foundation.

* To whom correspondence should be addressed: INRS-Institut Armand-Frappier, 531, Bd des prairies, Laval, QC, Canada H7V 1B7. E-mail: mathieu.cellier@inrs-iaf.uquebec.ca. Telephone: (450) 687-5010, ext. 4681. Fax: (450) 686-5301.

‡ INRS-Institut Armand-Frappier.

§ These authors contributed equally to this work.

|| Present address: Institute of Physics, Charles University in Prague, Ke Karlovu 5, 121 16 Prague 2, Czech Republic.

⊥ University of Florida.

@ Institut Rosell-Lallemand Inc.

[1] Abbreviations: pHL, pHluorin (pH-dependent ratiometric GFP); EcoliA, *E. coli* MntH A.

10.1021/bi048014v CCC: $30.25
Published on Web 12/16/2004

(*12*, *13*). Bacterial MntH groups (MntH A−C) differ in sequence (26−29 ± 3% identical) and in site-specific evolutionary rates, both among sites within one group and between respective sites of different groups (*14*). Such differences likely reflect distinct origins and modes of evolution, e.g., horizontal versus vertical gene transmission; hence, it was suggested that the precursor of eukaryotic *Nramp* genes was likely derived from a *mntH* A ancestor, whereas *mntH* C homologues could result from the horizontal transfer of a eukaryotic *Nramp* gene.

Nevertheless, structural and functional similarities among MntH A−C proteins suggest a common transmembrane topology, with 11−12 transmembrane domains (depending on the length of the C-terminus) and the N-terminus that is cytoplasmic (*14*, *15*). In addition, heterologous expression of MntH B and C proteins in *E. coli* resulted in Me^{2+}-dependent H^+ uptake and increased metal sensitivity suggestive of metal intracellular influx. Such functional conservation suggests that analyses of site-specific amino acid replacement rate shifts between groups of MntH homologues may reveal critical steps in the functional evolution of Nramp proteins, and contribute to a better understanding of some mechanisms that are important for metal ion homeostasis in bacteria and eukaryotes.

To establish the usefulness of this approach, we initially searched for residues corresponding to Nramp family-specific rate shifts. Such residues are invariant in the Nramp family, and thus expected to fulfill key structural or functional roles. They correspond to residues that are different but also invariant in a group of distantly related sequences [phylogenetic outgroup (Supporting Information)]. Our hypothesis is that these Nramp site-specific rate shifts identify residues that are essential to H^+-dependent Me^{2+} transport function.

The Nramp phylogenetic outgroup (Supporting Information) contains sequences that encode predicted membrane proteins that are up to 30% identical over a span of 385 residues with sequences from either the MntH A, B, or C group. Members of the Nramp outgroup were found in both archaea (euryarchaeota) and bacteria, including planctomycetes and Gram-negative and Gram-positive bacteria. These sequences are not highly related to each other (Supporting Information), consistent with a possible ancestral origin for the Nramp outgroup (*14*).

Approximately 30 residues are highly conserved among more than 145 Nramp homologues, including those from divergent eukaryotic taxa (e.g., plasmodia). It is generally accepted that the corresponding sites evolve slowly under strong purifying selection due to structural and/or functional constraints. The outgroup sequences share most of these conserved Nramp residues, supporting the possibility that the phylogenetic outgroup and the Nramp family were derived from a common ancestral transporter.

However, among eight residues that are invariant in the Nramp family, four are identical in the outgroup and four correspond to different residues, which are also invariant in the outgroup (Asp, Asn, His, and Asn and Gly, Thr, Tyr, and Gly, respectively). These evolutionary rate shifts may correspond to type II functional divergence, implying that the corresponding residues may play important but different roles in the Nramp and outgroup families (Supporting Information and ref *13*). These four rate-shifted sites are located within the C-terminal half of transmembrane domains 1, 6, and 11; three of them are predicted to be potentially exposed to the outward face of the plasma membrane (*16*).

Table 1: Sequences of the Oligonucleotide Primers (mutated codon in italics)

Primer	Sequence
WT	A I G Y I D P G N F A T
	5'GCG ATT GGT TAT ATC GAT CCC GGT AAC TTT GCG
D34G	5'GCC ATT GGT TAT ATC *GGG* CCC GGT AAC TTT GCG
D34N	5'GCG ATT GGT TAT ATC *AAC* CCC GGT AAC TTT GCG ACC
WT	I D P G N F A T N
	5'ATC GAT CCC GGT AAC TTT GCG ACC AAT
N37T	5'ATC GAT CCC GGT *ACC* TTT GCG ACC AAT
N37V	5'ATC GAT CCC GGT *GTC* TTT GCG ACC AAT
N37D	5'ATC GAT CCC GGT *GAC* TTT GCG ACC AAT
WT	A T I M P H V I Y L H S
	5' G GCG ACG ATT ATG CCG CAT GTG ATT TAT TTG CAC TCC
H211Y	5' G GCG ACG ATT ATG CCG *TAC* GTG ATT TAT TTG CAC TCC
H211A	5'GG GCG ACG ATT ATG CCG *GCC* GTG ATT TAT TTG CAC TCC
WT	H V I Y L H S S L T
	5' CAT GTG ATT TAT TTG CAC TCC TCG CTC ACT C
H216A	5' CAT GTG ATT TAT TTG *GCG* TCC TCG CTC ACT C
H216R	5' CAT GTG ATT TAT TTG *AGG* TCC TCG CTC ACT C
WT	V V A L N I W L L V G
	5'G GTC GTC GCG CTG AAT ATC TGG TTG TTG GTG GGG
N401G	5'G GTC GTC GCG CTG *GGG* ATC TGG TTG TTG GTG GGG
N401T	5'G GTC GTG GCG CTG *ACC* ATC TGG TTG TTG GTG GGG

If the four Nramp-specific residues identified herein contributed to the functional emergence of the Nramp family, they should be specifically involved in H^+-dependent Me^{2+} transport. Studies of different transporters revealed that Asp and His residues can be part of proton cotransport pathways and participate directly in proton coupling mechanisms (*17*−*19*). It was also reported that conserved transmembrane Asn residues can influence both channel conductance and cation transport (*20*, *21*).

Therefore, to determine the functional importance of the four Nramp-specific residues, we substituted EcoliA residues Asp^{34}, Asn^{37}, His^{211}, and Asn^{401} for the corresponding outgroup residues. The resulting mutant activities were compared to that of the wild type and confirmed with c-Myc-tagged proteins for which membrane expression levels were determined.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis of MntH. Site-directed mutagenesis was carried out using the Quickchange mutagenesis kit (Stratagene, La Jolla, CA) and the oligonucleotide primers shown in Table 1. The full-length DNA sequence was determined for each mutant (CEQ 2000XL, Beckman Coulter, Mississauga, ON).

Functional Assays of Metal Uptake. The expression of MntH proteins in *E. coli* increases the sensitivity of growth

to Me^{2+}, measured by disk assay using a metallo-dependent Ts strain. Overaccumulation of metals is toxic for cells and can inhibit growth, whereas in some metal-dependent mutant strain, intracellular accumulation of iron or manganese can restore growth at restrictive temperature (*9*). Disk assays were performed as previously described in DH11S *mntH* or SL93 *mntH* strains (*16*) using two independent clones for each mutant (except N37V). Briefly, a small volume (10 μL) of metal solution is used to impregnate a 7 mm filter that is placed in the center of a plate of medium containing an amount of inducer for protein expression, and onto which bacteria are plated. High concentrations of metals are used to facilitate diffusion around the disk; expression of EcoliA in the presence of a concentration gradient of a metal substrate for transport affects bacterial growth, measured after incubation for 16 h, depending on the metal affinity for EcoliA, metal cytotoxicity, and EcoliA expression level. Metal uptake was assessed by inductively coupled plasma mass spectrometry (ICP-MS) by comparing counts generated by the sample to those generated by standard solutions of a known concentration (*22*).

Western Blot Analyses. Membrane fractions were prepared with clones expressing EcoliA and each mutant as c-Myc-tagged proteins (*16*). The samples were solubilized in 4% (w/v) SDS, 40% (v/v) glycerol, 0.5 M β-mercaptoethanol, and 40 mM Tris-HCl (pH 7.2) by incubation at either 4 or 37 °C for 30 min; 80 μg of membrane extracts was separated by electrophoresis in 12% polyacrylamide baby gels before semidry electrotransfer on polyvinylidene fluoride membranes (Bio-Rad, Mississauga, ON) at 70 mA for 1 h. Membranes were revealed as previously described (*16*) using a mouse anti-c-Myc mAb (1/200), a secondary Ab from rabbit (anti-IgG1-HRP, 1/1250; Perkin-Elmer, Woodbridge, ON), and the ECL Western blotting reagent (Roche, Laval, PQ). Protein concentrations were determined using the Non-Interfering protein assay (Genotech, St. Louis, MO).

Proton Transport Assay. The pH-dependent ratiometric GFP (pHluorin, pHL; *23*, *24*) was used to follow the MntH- and Me^{2+}-dependent intracellular accumulation of protons as previously described (*16*). Briefly, *E. coli* DH11S *mntH* cells were transformed with pGBM6-pHL and pBAD plasmids encoding different MntH mutants. Clones were subcultured at 37 °C and 220 rpm in LB broth containing ampicillin and spectinomycin (100 μg/mL each) until an OD_{600} of 0.4 was reached, and 0.06% L-arabinose was added for a further 1 h incubation to allow the expression of MntH proteins. Cells were then harvested by centrifugation, washed once in 25 mM citric acid and 50 mM K_2HPO_4 at pH 5.0 or 4.7 (*25*), and resuspended in the same buffer at room temperature and a final OD_{600} of 0.2. Fluorescence was measured immediately on a Cary Eclipse fluorescence spectrophotometer (Varian, Inc.) equipped with a xenon lamp; The pHL excitation ratio *R*(410/470) was monitored for 1000 s (emission detected at 520 nm). The ratio was then transformed into the intracellular concentration of protons or the pH value according to the calibration curve obtained by use of carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP; *24*). Manganese, cadmium, iron, or cobalt was added to the samples 25 s after the beginning of acquisition. The metal-dependent proton transport by wild-type MntH was evaluated in *E. coli mntH unc* cells lacking the F_1F_0-ATPase as well. At extracellular pH values ranging from 5.5 to 6.5, an ~30% increase in the pHL response was observed as compared to that of *E. coli* DH11S *mntH*. However, the pHL response was not sufficient for measuring the functional differences between wild-type and mutant transporters at higher extracellular pH values, even in *unc mntH E. coli* cells. The proton transport assays were therefore performed in whole cells of *E. coli* DH11S *mntH* at an acidic extracellular pH.

Assessment of MntH-Dependent Proton and Metal Cotransport. The MntH-dependent accumulation of Mn or Cd and protons was observed quasi-simultaneously using spectroscopic measurement of intracellular pH changes and ICP-MS measurement of metal uptake as follows. Twenty milliliters of a cell suspension (OD_{600} of 0.2) was prepared in a citrate−phosphate buffer (pH 4.7, proton transport assay). The initial intracellular pH and metal content were measured (0 s), and 100 μM metal was then added to the sample. The pH changes were subsequently monitored for 500 s (Cd) or 1000 s (Mn), and transport was stopped by addition of a chelator mix (50 μM bathophenanthrolinedisulfonic acid and 50 μM dipivaloyl-L-tartaric acid) and a rapid filtration through a membrane filter (Metricel, 0.45 μm). Fifteen milliliters of 5 mM Tris (pH 8.0) containing 0.2 mM EDTA and 0.1 mM nitrilotriacetic acid was quickly filtered to wash out extracellular metal traces from the membrane which was then disposed to dry. The ICP-MS analysis was performed on the dried membranes wet-ashed with nitric acid (HNO_3) using closed vessel microwave digestion (Covance Laboratories Inc., Madison, WI). The negative controls included in these experiments measured the bacterial metal and proton background levels prior to the addition of metal (0 s) and the levels of metal and proton uptake by cells that did not possess EcoliA (vector alone, pBAD) and by EcoliA-expressing cells that were depleted of energy using CCCP.

RESULTS AND DISCUSSION

The metal and proton transport activity of wild-type and mutant EcoliA proteins was determined by three independent methods. Bacterial Me^{2+} sensitivity due to MntH proteins that are functionally expressed in *E. coli* was paralleled by MntH-dependent and Me^{2+}-induced H^+ transport, measured with a bacterially expressed pH-dependent ratiometric GFP. However, increased bacterial Me^{2+} sensitivity to highly toxic cations (Cd^{2+} and Co^{2+}) in the absence of detectable proton transport indicated that periplasmic binding of Cd^{2+} or Co^{2+} to truncated MntH proteins could induce bacteriotoxicity (*16*). Intracellular Cd^{2+} or Mn^{2+} was quantified by ICP-MS, which combined with the fluorescence proton transport assay revealed proton and metal cotransport by a bacterial Nramp homologue.

E. coli MntH A (EcoliA) Asp^{34}. Replacement of this Nramp-specific acidic residue with the corresponding outgroup residue in the EcoliA protein (D34G mutation) diminished the MntH-dependent sensitivity of bacterial growth to Cd^{2+} and Co^{2+} and abrogated the sensitivity of growth to Fe^{2+} and Mn^{2+} (Figure 1A); it also reduced the level of EcoliA-dependent Me^{2+}-induced H^+ transport to background levels (Figure 1B, compare with vector alone). The epitope-tagged EcoliA protein D34G-L412-c-Myc was detected in membrane preparations at levels similar to that of wild-type EcoliA-L412-c-Myc (Figure 2A); it also conferred minimal bacterial sensitivity of growth to Me^{2+} and

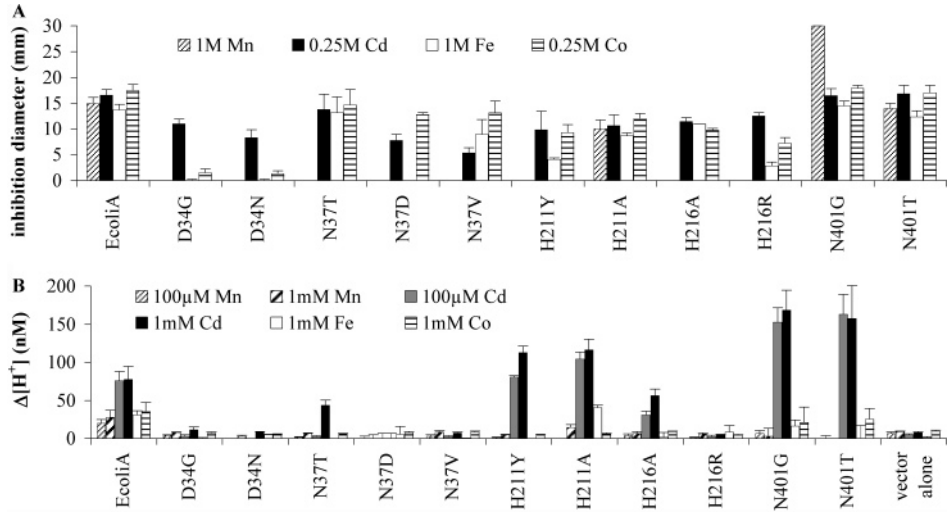

FIGURE 1: Functional characterization of EcoliA mutants. A disk assay of the MntH-dependent sensitivity of bacterial growth to divalent metals (Mn, Cd, Co, and Fe) was carried out for every mutant and wild-type EcoliA; values obtained with the vector alone were subtracted (A). Fluorescence assay of MntH-dependent H^+ uptake at pH 4.7 in response to divalent metals. Values obtained with the vector alone show the proton entry in bacteria devoid of EcoliA (B).

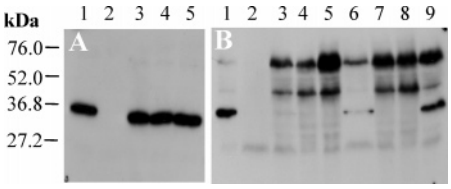

FIGURE 2: Western blot analysis of c-Myc-tagged wild-type EcoliA and mutant proteins. Membrane extracts were incubated for 30 min in solubilization buffer prior to loading on a 12% polyacrylamide gel either at 37 °C (A) [lane 1, EcoliA; lane 2, pBAD (vector alone); lane 3, D34G; lane 4, D34N; and lane 5, N401G] or at 4 °C (B) [lane 1, D34N; lane 2, pBAD (vector alone); lane 3, N37T; lane 4, N37V; lane 5, N37D; lane 6, H211A; lane 7, H211Y; lane 8, H216A; and lane 9, N401T].

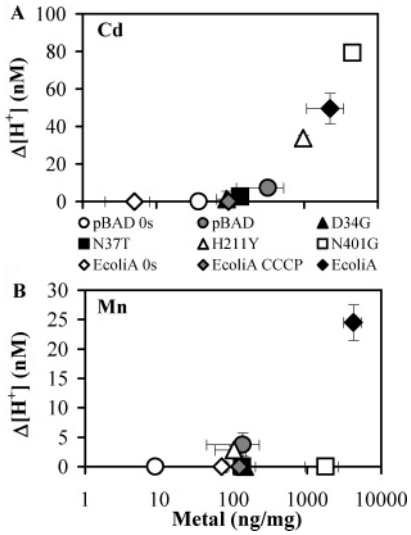

FIGURE 3: Cotransport of divalent metals and protons by EcoliA is impaired by exchanging a Nramp-specific residue with the corresponding outgroup residue. Quasi-simultaneous assessment of the intracellular accumulation of H^+ and Cd^{2+} (A) or Mn^{2+} (B) by fluorescence proton transport assay and ICP-MS, respectively, as described in Experimental Procedures.

was not associated with significant H^+ transport under the conditions that were tested (Supporting Information). To determine whether the residual sensitivity of bacterial growth to Cd^{2+} corresponded to Me^{2+} influx in the absence of H^+ transport by the mutant protein D34G, ICP-MS and fluorescence measurements were performed quasi-simultaneously; neither Me^{2+} nor H^+ transport was detected compared to the experimental controls (Figure 3A,B). The results demonstrate that the EcoliA D34G mutation prevents both Me^{2+} and H^+ transport.

To evaluate whether Asp^{34} was involved in the sensitivity of growth to Cd^{2+} that was conferred by membrane expression of an EcoliA fusion construct (L324-Cat) in the absence of Cd^{2+}-dependent H^+ uptake (*16*), the mutant EcoliA D34G L324-Cat was constructed. Expression of this construct conferred chloramphenicol resistance as expected, indicating that the D34G mutation did not alter EcoliA transmembrane topology, but also sensitivity of bacterial growth to Cd^{2+} similar to that of the wild-type fusion. The results suggest that EcoliA residue Asp^{34} is not merely a Cd^{2+} binding site but likely participates in the Me^{2+} uptake process (and possibly proton binding).

Since the different biochemical properties of Asp and Gly residues could explain the D34G mutant phenotype, a more conservative change was performed (D34N) to determine whether it would preserve MntH function. The double mutant D34N-L412-c-Myc was expressed in a manner similar to that of the wild-type protein (Figure 2A); it conferred some bacterial sensitivity to Cd^{2+} and did not facilitate any Me^{2+}-dependent H^+ uptake (Supporting Information). Consistently, the simple mutant D34N exhibited transport properties most similar to those of the D34G mutant (Figure 1). Minimal bacterial metal sensitivity was also observed after expression of the mutants D34E and D34E-EcoliA-c-Myc, despite normal membrane protein expression levels (data not shown). Therefore, the effects of the D34G mutation are due to the requirement of an Asp residue at this position.

An Nramp2 mutant resembling EcoliA D34G (Nramp2 D86A) was previously shown to be well expressed at the plasma membrane of eukaryotic cells and not to mediate Me^{2+} uptake (*26*). Such similar effects obtained by replacing the homologous Asp residues in MntH and Nramp proteins with Gly or Ala residues imply that this Nramp-specific Asp residue is mandatory for transport activity. However, it is

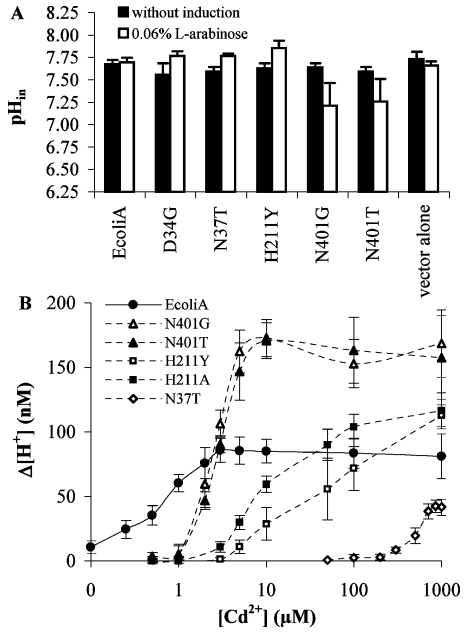

FIGURE 4: Replacement of Nramp-specific residues impairs EcoliA-dependent H^+ uptake. Fluorescence assay of the intracellular pH in *E. coli* cells expressing or not expressing wild-type EcoliA and different mutants (A). Cd^{2+} dose-dependent H^+ uptake mediated by wild-type EcoliA and selected mutants (B). The difference in intracellular pH due to expression of the N401G and -T mutants is highly significant ($p < 0.00001$, in paired Student's *t* tests).

dispensable for insertion of the protein in the membrane. Abrogation of transport activity despite normal protein expression has previously been observed after replacement of conserved transmembrane Asp residues (*27*). These data are consistent with the hypothesis that a Gly to Asp mutation could have contributed to the functional divergence of the ancestor of the Nramp family.

EcoliA Asn^{37}. The second exchange of Nramp for an outgroup residue (N37T) preserved the close to wild-type EcoliA-dependent sensitivity of bacterial growth to Fe^{2+}, Co^{2+}, and Cd^{2+} but not to Mn^{2+} (Figure 1A). This mutation also strongly limited Me^{2+}-induced H^+ transport, which was detected in the presence of high concentrations of Cd^{2+} only (Figures 1B and 4B); neither H^+ nor Mn^{2+} or Cd^{2+} uptake was detected in quasi-simultaneous measurements using 100 μM metal (Figure 3A,B). Both the absence of Mn^{2+}-induced growth inhibition (Figure 1A) and the requirement for a Cd^{2+} concentration above 200 μM to observe H^+ transport via EcoliA N37T (Figure 4B) suggested this mutant protein may have a lower affinity for Cd^{2+} and Mn^{2+}.

Alternatively, this mutant protein may not be sufficiently well-expressed in the membrane to appear to be active in cation transport assays unless a large Cd^{2+} dose was used. The amount of N37T-L412-c-Myc protein in membrane extracts was notably reduced compared to the amount of EcoliA L412-c-Myc and corresponded to bands of altered mobility (Figure 2B); it was also sensitive to the temperature used to solubilize membrane extracts (Supporting Information). In a manner similar to that of N37T, expression of the N37T-L412-c-Myc protein conferred substantial sensitivity of growth to Fe^{2+}, Co^{2+}, and Cd^{2+}, but not to Mn^{2+}, and did not induce detectable H^+ uptake under the conditions that were tested (Supporting Information). These data therefore suggest that the N37T mutation results in both a low level of stable protein expression in the membrane and a reduced affinity for Cd^{2+} and Mn^{2+}.

Interestingly, the N37T mutant lacked both Mn^{2+} uptake and Mn^{2+}-dependent H^+ transport, while another mutant obtained serendipitously (N37V) lost in addition Cd^{2+}-dependent H^+ transport and exhibited a reduced sensitivity of growth to Cd^{2+} compared to the N37T mutant (Figure 1). However, EcoliA N37V-L412-c-Myc protein levels were similar to that of the N37T-L412-c-Myc mutant (Figure 2) and less sensitive to the temperature used for solubilization (Supporting Information). Since N37V-L412-c-Myc abrogated the sensitivity of growth to Fe^{2+} compared to N37T-L412-c-Myc (Supporting Information), it can be concluded that the N37V mutation affected more negatively EcoliA transport activity than its expression level. The N37V mutation seems to be less conservative than the N37T mutation since replacing a hydroxyl with a methyl group renders this site apolar. The data thus suggest that EcoliA Asn^{37} is important for Me^{2+}-induced H^+ transport.

To determine whether a more conservative mutation at position 37 would preserve EcoliA function, we also studied the N37D mutant. Sensitivity to Fe^{2+} was lost, whereas sensitivity to Cd^{2+} and Co^{2+} remained similar to that of the N37V mutant (Figure 1A). Similar levels of sensitivity to Cd^{2+} were also detected for both N37D-L412-c-Myc and N37V-L412-c-Myc, whereas the former conferred higher sensitivity to Co^{2+} (Supporting Information) and was detected at higher levels in membrane preparations (Figure 2). No Me^{2+}-dependent H^+ uptake was detected after expression of N37D-L412-c-Myc (Supporting Information) or N37D (Figure 1B).

On the basis of the sensitivity of growth to Cd^{2+} and Cd^{2+}-induced H^+ proton uptake, EcoliA activity was better preserved by introducing the outgroup residue in place of Asn^{37} (N37T vs N37D and -V, Figures 1 and 4B). In addition, the conservative N37Q mutation did not restore levels of bacterial metal sensitivity better than the N37T mutation, despite improved membrane protein expression levels detected with N37T-EcoliA-c-Myc (data not shown). This Nramp-specific Asn residue is thus also required for the functional expression of MntH. A dual role in activity and structural integrity was previously reported for the strictly conserved Asn residue in the globular proteins cysteine proteases (*28*). It is thus reasonable to conclude that EcoliA Asn^{37} is required for both stable membrane insertion and Me^{2+}-dependent H^+ transport activity.

A mutation corresponding to N37R is present in the N-terminal moiety of plant Ein2 proteins, which are weakly related to Nramp proteins and lack metal transport activity (*29*). The transport defects resulting from the mutagenesis of EcoliA Asn^{37} imply that exchange for a positively charged residue should also impair transport. The presence of an Arg residue in place of the Nramp-specific Asn may be functionally significant for Ein2 proteins.

EcoliA Asn^{401}. Finding an Nramp-specific residue close to the periplasmic C-terminus of EcoliA, a region otherwise not conserved in the Nramp family, suggested a possible functional role. Moreover, MntH activity is affected when this region is mutated (*16*). Hence, the levels of Me^{2+}-induced H^+ uptake measured for L412-c-Myc-tagged EcoliA proteins differed from those of the corresponding proteins

devoid of the C-terminal tag (Figure 1B and Supporting Information).

The metal transport activity of the N401G mutant resembled that of wild-type EcoliA, including suppression of the *hflB1* Ts growth defect, but with some variations such as increased sensitivity of bacterial growth to Mn^{2+} (Figure 1A). Me^{2+}-dependent H^+ transport was more affected, with a strong increase in Cd^{2+}-dependent but a decrease in Mn^{2+}-, Fe^{2+}-, and Co^{2+}-dependent H^+ uptake rates (Figures 1B and 3A,B). This selective inhibition in Me^{2+}-dependent H^+ uptake was less pronounced when an external pH of 5 was used (Supporting Information). The increase in the rate of Cd^{2+}-dependent H^+ uptake was paralleled by an increased rate of Cd^{2+} uptake (Figure 3A), indicating that both cation transport capacities are enhanced in the EcoliA N401G mutant. Under identical conditions, the rate of Mn^{2+} uptake appeared to be significantly decreased and H^+ uptake completely inhibited, suggesting that Mn^{2+} accumulation might be possible in the absence of H^+ cotransport (Figure 3B). These results implied that the EcoliA N401G mutation alters both H^+ uptake and protein Me^{2+} interactions; this was confirmed by further observations.

First, as opposed to the mutants created at other positions, expression of the EcoliA N401G mutant protein diminished the intracellular pH of bacteria growing in rich broth (Figure 4A), suggesting that either metals in the culture medium might induce MntH-dependent H^+ uptake or H^+ entry might be independent of metals. Second, the comparison of H^+ uptake in response to the Cd^{2+} dose showed important differences between wild-type EcoliA and the mutant N401G, in their apparent affinity for Cd^{2+} and the resulting H^+ entry (Figure 4B). Last, the double mutant N401G-L412-c-Myc was expressed at levels similar to that of EcoliA-c-Myc (Figure 2A); it conferred a slightly decreased and increased sensitivity to Mn^{2+} and Fe^{2+}, respectively, and significantly lower levels of Me^{2+}-dependent H^+ uptake except in the presence of 1 mM Cd^{2+} (Supporting Information). These results thus indicate that replacing the Nramp invariant residue Asn^{401} with the corresponding outgroup residue affects primarily EcoliA Me^{2+}-dependent H^+ uptake.

To establish that the properties of the N401G mutant revealed some functional constraints exerted on Asn^{401} versus differences in the properties of Asn and Gly side chains, another mutation was studied. N401T is a more conservative mutation than N401G, introducing an alcohol side chain with a polar and acid character, and of intermediate size, which should thus better preserve MntH activity, except in a case in which Asn^{401} was required for transport.

The expression of EcoliA mutant N401T resulted in sensitivity of growth to metals that was similar to wild-type EcoliA activity (Figure 1A), including suppression of the *hflB1* Ts growth defect, and in Me^{2+}-dependent H^+ uptake resembling that measured for the N401G mutant at pH 4.7 (Figure 1B) and pH 5 (Supporting Information). These results were confirmed by quasi-simultaneous measurements of Cd^{2+} and H^+ uptake (data not shown) and by measuring the intracellular pH resulting from the expression of N401T in growing cells and after addition of various amounts of Cd^{2+} (Figure 4A,B); the data obtained were very similar for N401G and N401T. However, N401T-L412-c-Myc was significantly less stable than N401G-L412-c-Myc (Figure 2B and Supporting Information); it conferred a sensitivity of growth to metals similar to that of N401G-L412-c-Myc but significantly more variable Me^{2+}-dependent H^+ uptake (Supporting Information).

Together, the data demonstrate that substituting EcoliA Asn^{401} with Thr or Gly has a similar impact on cation transport, which implies that Asn^{401}, or an amide group, is required for EcoliA function. The N401Q mutant induced bacterial metal sensitivity levels similar to that of wild-type EcoliA, but the double mutant N401Q-L412-c-Myc, which was expressed well in the membrane, did not confer Mn sensitivity anymore and suppressed the *hflB1* Ts defect less efficiently than the N401G and -T mutants (data not shown). The data suggest that Asn^{401} is strictly required for proper EcoliA-dependent transport. The different outcome of Asn to Thr or Gln mutations at EcoliA positions 37 and 401 demonstrates unique functional constraints exerted at these sites.

One possibility for explaining the clear phenotypes observed after substituting EcoliA residues Asp^{34}, Asn^{37}, and Asn^{401} is their exofacial location, suggesting possible direct contacts with the substrates, which might affect the rate of transport. Since more conservative mutations at these sites did not preserve EcoliA function, the phenotypes observed as a result of the substitutions of EcoliA residues Asp^{34}, Asn^{37}, and Asn^{401} for the corresponding outgroup residues indicate that the targeted Nramp-specific residues are required for metal and proton cotransport.

EcoliA His^{211}. The fourth predicted site of type II functional divergence differs from the others since it is located within the predicted C-terminal half of transmembrane domain 6, closer to the cytoplasmic face of the membrane, and its replacement with the corresponding outgroup residue seems to be rather conservative in terms of size and hydrophobicity [H211Y, LRT values of 1.40−1.76 (Supporting Information)]. However, this mutation should affect a protein active site because Tyr exposes an acidic proton and His has a basic amino group.

Compared to the EcoliA control, the H211Y mutant conferred a notably reduced sensitivity of growth to all metals that were tested, including Mn^{2+}, while H^+ uptake was detected exclusively in the presence of Cd^{2+} and was seemingly unaltered (Figure 1A,B). This was confirmed by detection of cotransport of Cd^{2+} and H^+, albeit at a level reduced compared to that of wild-type EcoliA (Figure 3A), whereas no transport at all was detected with Mn^{2+} (Figure 3B). In addition, Cd^{2+} dose-dependent H^+ uptake mediated by H211Y indicated a reduction of the apparent affinity for this metal ion compared to wild-type EcoliA (Figure 4B). A general decrease in metal sensitivity could explain the weak effect of the expression of EcoliA H211Y on bacterial growth in the presence of metals (Figure 1A). However, substantial expression level and a relative protein stability similar to that observed with N37D-L412-c-Myc were detected with H211Y-L412-c-Myc (Figure 2B and the Supporting Information); this double mutant also conferred little sensitivity of bacterial growth to metals and metal-dependent H^+ uptake activity (Supporting Information). Thus, though it does not abrogate MntH activity, the H211Y mutation affects significantly EcoliA structure and is not functionally conservative. This suggests that the basic amino group of His^{211} may be required for EcoliA function or that the phenotype resulting

from the mutation H211Y could be due to antagonistic acid−base properties of His and Tyr residues.

To address this issue, another nonconservative but more neutral mutation was performed (H211A). This mutation preserved bacterial sensitivity to all the metals that were tested, albeit at levels inferior to that of wild-type EcoliA (Figure 1A), and facilitated H^+ uptake in the presence of Cd^{2+}, Fe^{2+}, and Mn^{2+} at pH 4.7 (Figure 1B) and of Cd^{2+} at pH 5 (Supporting Information). EcoliA transport properties were thus better preserved in the H211A mutant than in the H211Y mutant, as confirmed by measuring the rate of Cd^{2+} dose-dependent H^+ uptake (Figure 4B). In addition, H211A-L412-c-Myc protein levels in membrane preparations indicated moderate alterations that resembled those observed with the N401T-L412-c-Myc mutant (Figure 2B). Nevertheless, the expression of H211A-L412-c-Myc was superior to that of H211Y-L412-c-Myc in mediating metal-dependent H^+ uptake (Supporting Information). In contrast, H211R-L412-c-Myc did not induce metal sensitivity above negative controls, suggesting impaired expression (data not shown). The results indicate thus that changing the outgroup Tyr residue for the Nramp-specific His likely implied some divergence in transport function.

Two other mutants were studied for comparison (H216A and H216R) since the homologous mutations in Nramp2 impaired the uptake of Fe^{2+} and Co^{2+}, and metal uptake was partly restored at an acidic external pH with the Ala mutant only (*26*). Consistently, higher levels of both intracellular metal accumulation and H^+ uptake were preserved with the EcoliA H216A mutant compared to H216R. Indeed, only bacteria expressing the H216R, D34G or -N, and N37V or -D mutants, and those devoid of the EcoliA protein, did not exhibit upregulation of Me^{2+}-dependent H^+ uptake when measured at pH 4.7 versus pH 5.0 (Figure 1B and the Supporting Information), indicating that these mutations affect the H^+ transport pathway.

Despite possibly lower membrane protein levels, H211A-L412-c-Myc mediated higher rates of Cd^{2+}-dependent H^+ uptake compared to H216A-L412-c-Myc, whereas both conferred similar levels of sensitivity of bacterial growth to metals (Supporting Information). Comparison of the transport properties of the corresponding single mutants demonstrated that H211A was more active than H216A (Figure 1). Consistent differences were observed with the Nramp2 protein after replacement of the corresponding His residues with Ala (*26*). The relatively well-preserved activity of EcoliA H211A thus suggests that the H211Y substitution impaired MntH transport due to the different acid−base properties of His and Tyr residues. The data thus support the proposition that exchange of the outgroup residue for the corresponding Nramp-specific residue could have contributed to the functional divergence of the Nramp family.

In conclusion, the four rate-shifted sites that were predicted to have contributed to the functional divergence of the Nramp family are important for EcoliA transport activity. This functional evolutionary approach identified separate residues with key roles in metal and proton transport.

Each of the four replacements of Nramp-specific residues with the corresponding outgroup residues resulted in mutant EcoliA proteins that were expressed in the plasma membrane, albeit at different levels, some showing shifted electrophoretic mobility and/or stability and different levels of functional activity. In one instance, H211Y, the outgroup residue impaired specifically the transport activity. The data support the notion that outgroup and MntH proteins share similar transmembrane features and can accommodate each other's residues.

Quasi-simultaneous measurements of the rates of Me^{2+} and H^+ uptake indicate that MntH proteins cotransport these cations (symport). The relative functional impairment of the D34G and N401G mutants on one hand, and the N37T and N401T mutants on the other hand, revealed that EcoliA Asp^{34} is strictly required for Me^{2+} and H^+ cotransport and that Asn^{401} is required for proper Me^{2+}-dependent H^+ transport. Detailed kinetic studies of the more conservative changes (D34E and N401Q) will likely reveal insight into the mechanism of transport via MntH.

This study reveals both structural and functional roles for EcoliA Asn^{37} and His^{211} residues, and suggests possible interactions with metal ions consistent with a direct role in cation permeation pathway. The data also demonstrate a role in proton transport for the residues located within transmembrane domains 1 and 6 (Asp^{34} and His^{211} and His^{216}, respectively), which were previously shown to affect Nramp2 metal or cation transport (*26*, *30*). EcoliA Asn^{401} mutant data, and previously published data obtained with C-terminally truncated and tagged EcoliA proteins (*16*), indicate that transmembrane domain 11 is important for Me^{2+}-dependent H^+ transport.

The functional significance of the four Nramp invariant residues suggests that they represent sites of type II functional divergence, which may have co-evolved in relation to the emergence of the Nramp family. This study shows the usefulness of evolutionary rate shift analyses via application of a transmembrane protein similarity matrix (*31*) to the study of transmembrane residues likely involved in transport function by site-directed mutagenesis. This evolutionary-based approach will be useful in evaluating the significance of sequence diversity in the Nramp family, including laterally transferred *mntH* C genes, as well as in other conserved families of membrane transporters.

ACKNOWLEDGMENT

The expert assistance of L. Wilson is gratefully acknowledged.

SUPPORTING INFORMATION AVAILABLE

Phylogenetic groups of the Nramp family, defining Nramp function using rate shift analysis, functional characterization of C-terminally c-Myc tagged EcoliA wild-type and mutant proteins, temperature sensitivity of c-Myc-tagged EcoliA mutant proteins revealed by Western blot analysis, and fluorescence assay of MntH-dependent proton uptake at pH 5 in response to divalent metals. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Mackenzie, B., and Hediger, M. A. (2004) SLC11 family of H^+-coupled metal-ion transporters NRAMP1 and DMT1, *Pfluegers Arch. 447*, 571−579.
2. Vidal, S., Tremblay, M. L., Govoni, G., Gauthier, S., Sebastiani, G., Malo, D., Skamene, E., Olivier, M., Jothy, S., and Gros, P. (1995) The Ity/Lsh/Bcg locus: Natural resistance to infection with

intracellular parasites is abrogated by disruption of the Nramp1 gene, *J. Exp. Med. 182*, 655−666.
3. Fleming, M. D., Trenor, C. C., III, Su, M. A., Foernzler, D., Beier, D. R., Dietrich, W. F., and Andrews, N. C. (1997) Microcytic anaemia mice have a mutation in Nramp2, a candidate iron transporter gene, *Nat. Genet. 16*, 383−386.
4. Portnoy, M. E., Liu, X. F., and Culotta, V. C. (2000) *Saccharomyces cerevisiae* expresses three functionally distinct homologues of the nramp family of metal transporters, *Mol. Cell. Biol. 20*, 7893−7902.
5. Gunshin, H., Mackenzie, B., Berger, U. V., Gunshin, Y., Romero, M. F., Boron, W. F., Nussberger, S., Gollan, J. L., and Hediger, M. A. (1997) Cloning and characterization of a mammalian proton-coupled metal-ion transporter, *Nature 388*, 482−488.
6. Chen, X. Z., Peng, J. B., Cohen, A., Nelson, H., Nelson, N., and Hediger, M. A. (1999) Yeast SMF1 mediates H^+-coupled iron uptake with concomitant uncoupled cation currents, *J. Biol. Chem. 274*, 35089−35094.
7. Cellier, M. F., Bergevin, I., Boyer, E., and Richer, E. (2001) Polyphyletic origins of bacterial Nramp transporters, *Trends Genet. 17*, 365−370.
8. Agranoff, D., Monahan, I. M., Mangan, J. A., Butcher, P. D., and Krishna, S. (1999) *Mycobacterium tuberculosis* expresses a novel pH-dependent divalent cation transporter belonging to the Nramp family, *J. Exp. Med. 190*, 717−724.
9. Makui, H., Roig, E., Cole, S. T., Helmann, J. D., Gros, P., and Cellier, M. F. (2000) Identification of the *Escherichia coli* K-12 Nramp orthologue (MntH) as a selective divalent metal ion transporter, *Mol. Microbiol. 35*, 1065−1078.
10. Patzer, S. I., and Hantke, K. (2001) Dual repression by Fe^{2+}-Fur and Mn^{2+}-MntR of the mntH gene, encoding an NRAMP-like Mn^{2+} transporter in *Escherichia coli*, *J. Bacteriol. 183*, 4806−4813.
11. Kehres, D. G., Janakiraman, A., Slauch, J. M., and Maguire, M. E. (2002) Regulation of *Salmonella enterica* serovar *Typhimurium* mntH transcription by H_2O_2, Fe^{2+}, and Mn^{2+}, *J. Bacteriol. 184*, 3151−3158.
12. Gaucher, E. A., Gu, X., Miyamoto, M. M., and Benner, S. A. (2002) Predicting functional divergence in protein evolution by site-specific rate shifts, *Trends Biochem. Sci. 27*, 315−321.
13. Knudsen, B., Miyamoto, M. M., Laipis, P. J., and Silverman, D. N. (2003) Using Evolutionary Rates to Investigate Protein Functional Divergence and Conservation. A case study of the carbonic anhydrases, *Genetics 164*, 1261−1269.
14. Richer, E., Courville, P., Bergevin, I., and Cellier, M. F. (2003) Horizontal gene transfer of "prototype" Nramp in bacteria, *J. Mol. Evol. 57*, 363−376.
15. Cellier, M., Prive, G., Belouchi, A., Kwan, T., Rodrigues, V., Chia, W., and Gros, P. (1995) Nramp defines a family of membrane proteins, *Proc. Natl. Acad. Sci. U.S.A. 92*, 10089−10093.
16. Courville, P., Chaloupka, R., Veyrier, F., and Cellier, M. F. (2004) Determination of transmembrane topology of the *Escherichia coli* natural resistance-associated macrophage protein (Nramp) ortholog, *J. Biol. Chem. 279*, 3318−3326.
17. Abramson, J., Smirnova, I., Kasho, V., Verner, G., Kaback, H. R., and Iwata, S. (2003) Structure and mechanism of the lactose permease of *Escherichia coli*, *Science 301*, 610−615.
18. Phelps, A., Briggs, C., Mincone, L., and Wohlrab, H. (1996) Mitochondrial phosphate transport protein. Replacements of glutamic, aspartic, and histidine residues affect transport and protein conformation and point to a coupled proton transport path, *Biochemistry 35*, 10757−10762.
19. Buch-Pedersen, M. J., and Palmgren, M. G. (2003) Conserved Asp684 in transmembrane segment M6 of the plant plasma membrane P-type proton pump AHA2 is a molecular determinant of proton translocation, *J. Biol. Chem. 278*, 17845−17851.
20. Nakazawa, K., Inoue, K., and Ohno, Y. (1998) An asparagine residue regulating conductance through P2X2 receptor/channels, *Eur. J. Pharmacol. 347*, 141−144.
21. Lin, F., Lester, H. A., and Mager, S. (1996) Single-channel currents produced by the serotonin transporter and analysis of a mutation affecting ion permeation, *Biophys. J. 71*, 3126−3135.
22. AOAC INTERNATIONAL (2000) Method 993.14, *Official Methods of Analysis of AOAC INTERNATIONAL*, 17th ed., AOAC INTERNATIONAL, Gaithersburg, MD.
23. Miesenbock, G., De Angelis, D. A., and Rothman, J. E. (1998) Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins, *Nature 394*, 192−195.
24. Olsen, K. N., Budde, B. B., Siegumfeldt, H., Rechinger, K. B., Jakobsen, M., and Ingmer, H. (2002) Noninvasive measurement of bacterial intracellular pH on a single-cell level with green fluorescent protein and fluorescence ratio imaging microscopy, *Appl. Environ. Microbiol. 68*, 4145−4147.
25. Siegumfeldt, H., Rechinger, K. B., and Jakobsen, M. (1999) Use of fluorescence ratio imaging for intracellular pH determination of individual bacterial cells in mixed cultures, *Microbiology 145*, 1703−1709.
26. Lam-Yuk-Tseung, S., Govoni, G., Forbes, J., and Gros, P. (2003) Iron transport by Nramp2/DMT1: pH regulation of transport by 2 histidines in transmembrane domain 6, *Blood 101*, 3699−3707.
27. Merickel, A., Kaback, H. R., and Edwards, R. H. (1997) Charged residues in transmembrane domains II and XI of a vesicular monoamine transporter form a charge pair that promotes high affinity substrate recognition, *J. Biol. Chem. 272*, 5403−5408.
28. Vernet, T., Tessier, D. C., Chatellier, J., Plouffe, C., Lee, T. S., Thomas, D. Y., Storer, A. C., and Menard, R. (1995) Structural and functional roles of asparagine 175 in the cysteine protease papain, *J. Biol. Chem. 270*, 16645−16652.
29. Thomine, S., and Schroeder, J. I. (2004) Plant metal transporters with homology to proteins of the Nramp family, in *The Nramp family* (Cellier, M. F. M., and Gros, P., Eds.) pp 114−124, Landes Bioscience/Kluwer Academic, Georgetown, TX.
30. Cohen, A., Nevo, Y., and Nelson, N. (2003) The first external loop of the metal ion transporter DCT1 is involved in metal ion binding and specificity, *Proc. Natl. Acad. Sci. U.S.A.*
31. Jones, D. T., Taylor, W. R., and Thornton, J. M. (1994) A mutation data matrix for transmembrane proteins, *FEBS Lett. 339*, 269−275.

BI048014V