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MINI-REVIEW

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Nickel transport systems in microorganisms

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Abstract The transition metal Ni is an essential cofactor for a number of enzymatic reactions in both prokaryotes and eukaryotes. Molecular analyses have revealed the existence of two major types of high-affinity Ni^{2+} transporters in bacteria. The Nik system of *Escherichia coli* is a member of the ABC transporter family and provides Ni^{2+} ion for the anaerobic biosynthesis of hydrogenases. The periplasmic binding protein of the transporter, NikA, is likely to play a dual role. It acts as the primary binder in the uptake process and is also involved in negative chemotaxis to escape Ni overload. Expression of the *nik* operon is controlled by the Ni-responsive repressor NikR, which shows functional similarity to the ferric ion uptake regulator Fur. The second type of Ni^{2+} transporter is represented by HoxN of *Ralstonia eutropha*, the prototype of a novel family of transition metal permeases. Members of this family have been identified in gram-negative and gram-positive bacteria and recently also in a fission yeast. They transport Ni^{2+} with very high affinity, but differ with regard to specificity. Site-directed mutagenesis experiments have identified residues that are essential for transport. Besides these uptake systems, different types of metal export systems, which prevent microorganisms from the toxic effects of Ni^{2+} at elevated intracellular concentrations, have also been described.

Key words Metal ion transport · ABC transporter · Permease family · Metalloregulatory protein · Nickel-containing enzymes · Metal resistance

Introduction

Nickel-containing enzymes are involved in at least five metabolic processes, including the production and consumption of molecular hydrogen, hydrolysis of urea, reversible oxidation of carbon monoxide under anoxic conditions, methanogenesis, and detoxification of superoxide anion radicals. The active sites of the relevant enzymes harbor unique metallocenters which are assembled by auxiliary proteins (reviewed by Hausinger 1997; Ermler et al. 1998; Maroney 1999). Different ligand environments are involved in the coordination of Ni in the various metalloenzymes.

[NiFe] hydrogenases contain a heterobimetallic center in the large subunit. Four cysteine ligands coordinate the Ni, two of which bridge to a Fe ion. Fe is also bound by two cyanides and one molecule of CO.

Ureases hydrolyze urea to form ammonium ion and carbamate. The latter hydrolyzes spontaneously to form another ammonium ion and carbonate. Each active site in urease contains two Ni ions, which are coordinated by a bridging carbamylated lysine as well as two histidine residues at Ni-1 and two histidine residues and an aspartate residue at Ni-2.

Anaerobic carbon monoxide dehydrogenases (CODH) reversibly convert CO and H_2O into CO_2 and reducing equivalents. More complex forms of CODH are the acetyl-coenzyme A synthetases (ACS), which catalyze the reversible formation of acetyl-CoA from CO_2, coenzyme A, and a methyl group donated by a corrinoid-[FeS] protein. Both types of CODH harbor a Ni ion that is linked to a [4Fe-4S] cluster by a component X, which has not yet been classified. ACS also contain a second, structurally similar Ni center.

Less information is available on Ni-containing superoxide dismutases, which have been identified only in

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Streptomyces species. X-ray absorption-, EPR- and UV/VIS-spectroscopic data suggest that in the oxidized enzyme Ni is present in a five-coordinate site composed of three S-donor, one N-donor, and one O- or N-donor ligand.

The only example of an enzyme containing Ni in the form of a complex organic cofactor is methyl-coenzyme M reductase of methanogenic archaea. Its enzymatic activity, the reduction of methyl-CoM to methane, is dependent on the presence of coenzyme F_{430}, a Ni-containing, highly saturated, cyclic tetrapyrrole that is bound noncovalently.

Additional Ni-dependent enzymes are likely to be discovered. Maximal activation by Ni of purified *Escherichia coli* peptide deformylase and glyoxalase I suggests that Ni could be a cofactor of these enzymes in vivo. They catalyze the deformylation of nascent polypeptides, and the isomerization reaction during the conversion of toxic α-keto aldehydes into their corresponding nontoxic 2-hydroxycarboxylic acids, respectively.

Nickel-dependent enzymes have been identified in bacteria, archaea, fungi, algae, higher plants and a few animals (Hausinger 1993). These organisms have to satisfy their requirements for Ni by adequate transport systems. In natural environments soluble transition metal ions like Ni^{2+} are usually present only in trace amounts. Thus, the affinity of the transporters for their substrates must be very high. On the other hand, cells have to avoid the hazardous effects of metal ions at elevated intracellular concentrations, e.g. the production of toxic free radicals. This problem can be overcome by the production of uptake systems with a very low capacity, by repression of transcription of the transporter genes in response to elevated metal concentrations, by activating a negative chemotactic response, and by the expression of resistance determinants designed for metal expulsion from the cell. The intention of the present review is to summarize the data on the major types of bacterial high-affinity Ni uptake systems and to provide preliminary insight into their regulation.

Microbial nickel transport

The average concentration of Ni is as low as 2 ppb in sea water and 0.3 ppb in fresh water, corresponding to a molarity of approximately 30 nM and 5 nM, respectively. The most commonly occurring oxidation state of Ni is Ni(II), and thus Ni^{2+} ion is the substrate for transport. Under artificial conditions, i.e. at concentrations that are much higher than in the natural environment, Ni^{2+} can be taken up by microbial Mg^{2+} transport systems (reviewed by Eitinger and Friedrich 1997; Kehres et al. 1998; Smith and Maguire 1998). Since Mg^{2+} is a competitive inhibitor of Ni^{2+} transport by these systems and since the natural concentration of Mg^{2+} is at least three orders of magnitude above that of Ni^{2+}, Mg^{2+} transport systems probably contribute little to Ni^{2+} uptake under physiological conditions.

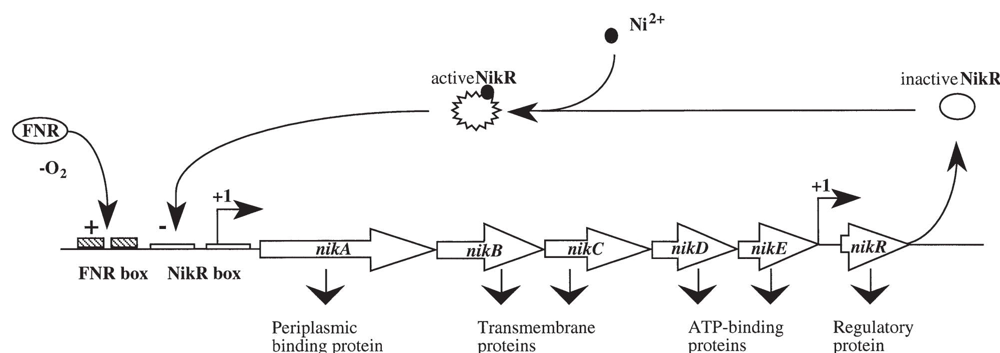
How do prokaryotes satisfy their demands for Ni? Transport assays have identified Ni^{2+} uptake in a variety of bacteria and archaea in the presence of several thousand-fold excess of Mg^{2+} (reviewed by Hausinger 1993). These studies took advantage of the radioactive isotope ^{63}Ni, a low-energy (67 keV) β^- emitter that decays with a physical half-life of 100 years to the stable isotope ^{63}Cu. The characterization of Ni-deficient mutants of *E. coli* and *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) were milestones for the analysis of the underlying transport mechanisms. Complementation of the mutants and sequence analysis of the genes revealed that a transporter of the ATP-binding cassette family is responsible for Ni^{2+} uptake in *E. coli*. An unrelated single-component permease that probably acts as a uniporter operates in *R. eutropha*. These two major types of bacterial high-affinity Ni^{2+} transport systems are discussed in detail below.

The nickel-specific ABC transporter of *E. coli*

The discovery of a highly specific Ni transport system in *E. coli* originates from the characterization of a novel category of mutants isolated by Mu*d*I transposon mutagenesis that were totally impaired in hydrogenase activity (Wu and Mandrand-Berthelot 1986). These mutants contain significantly reduced levels of H_2-related activities, fermentative formate hydrogenlyase and respiratory fumarate-dependent H_2 uptake. Insertion mutations (formerly called *hydC* and subsequently designated *nik*), map at 77 min on the *E. coli* chromosome, defining a new locus that is different from the structural *hya*, *hyb* and *hyc* operons, which encode the main three hydrogenase isoenzymes, or from the pleiotropic *hyp* region, involved in the maturation or processing of hydrogenases (Sawers 1994). Interestingly, addition of high concentrations (0.5 mM) of Ni salts leads to a complete recovery of hydrogenase activity and to the concomitant restoration of normal H_2 uptake activities. This strictly Ni-dependent restoration was taken as the first suggestion of a defect in Ni transport.

Further investigation of the mutants has been performed using both biochemical and genetic approaches. When grown in a rich medium, the cellular Ni content of the Nik^- mutants is found to be abnormally low, less than 1% of that of the parental strain (Wu et al. 1989). Therefore these mutants are unable to acquire sufficient Ni for hydrogenase biosynthesis during normal growth. However, gradually increasing external Ni concentration increases both cellular Ni content and hydrogenase activity to attain normal wild-type levels. Under these circumstances, Ni can be readily taken up by the low-affinity high-capacity magnesium transport system CorA (Smith and Maguire 1998). This behavior is further substantiated by the fact that the *nik* hydrogenase phenotype can be fully suppressed by growth in media containing very low (0.01 mM) added magnesium, which would reduce Mg competition for Ni entry by the Mg uptake system (Wu et al. 1989). Finally, a direct demonstration for a defect in Ni uptake came from examination of the ability of the Nik^- mutants to take up $^{63}Ni^{2+}$ (Navarro et al. 1993). Assays have been conducted in the presence of a high amount (10 mM) of Mg salt to saturate the nonspecific Ni uptake via the

Fig. 1 The Ni transport operon of *Escherichia coli* and its regulation. The initiation sites (+*1*) and the direction of transcription are indicated by *arrows*; (+) and (–) correspond to the activation and repression of the expression of the *nikABCDE* operon, respectively



Mg system and under low concentration (0.15 μM) of Ni chloride. The rate of Ni transport in the mutant is markedly altered compared to that observed in the parental strain. Almost no difference is detected when a higher amount (0.5 μM) of Ni was used, presumably because the physiological function of the specific transport system is masked by the presence of the nonspecific carrier.

The *nik* locus has been cloned and its nucleotide sequence has been determined (Wu et al. 1991; Navarro et al. 1993). It encodes five proteins, NikA–NikE, which display significant sequence similarity to the components of periplasmic binding protein-dependent transport systems for dipeptides or oligopeptides of several gram-negative and gram-positive species (Navarro et al. 1993). A recent systematic analysis of the complete *E. coli* genome sequence regarding the distribution of the ATP-binding cassette proteins has assigned the Nik system to ABC subfamily 2, which contains seven different members, three of which have been unambiguously identified as peptide transporters (Linton and Higgins 1998).

Nik is an archetypal ABC transporter. It consists of two hydrophobic transmembrane proteins, NikB and NikC (314 and 277 amino acids long, respectively), each harboring six membrane-spanning regions and both assumed to form a pore for the translocation of Ni, and two membrane-associated components, NikD and NikE (253 and 268 amino acids long, respectively), which contain the conserved Walker A and B motifs, involved in the binding and hydrolysis of ATP, and likely to be responsible for coupling energy to the transport process (Navarro et al. 1993; Figs. 1, 5). A periplasmic binding protein NikA (524 amino acids long) provides the primary substrate receptor for Ni uptake. Interestingly, it has been shown to belong to a family of homologous substrate-binding proteins with a broad range of substrate specificity. This family includes, besides dipeptide- and oligopeptide-binding proteins, hemin- and pheromone-binding proteins as well as the enzyme hyaluronate synthase (Wu and Mandrand-Berthelot 1995). These homologous proteins, which are able to perform distinct biological functions, are found in both proteobacteria and gram-positive bacteria, indicating the presence of a common ancestor before the split between the two eubacterial phyla.

The soluble periplasmic Ni-binding protein NikA has been investigated further. Overproduction from the cloned *nikA* gene enables the purification of a homogenous preparation of NikA from the periplasm by hydrophobic and ion-exchange chromatography (De Pina et al. 1995). Mature NikA is a monomer of 56 kDa lacking, as expected, the N-terminal 22-amino acid signal sequence predicted from the nucleotide sequence of the *nikA* gene (Navarro et al. 1993). About 23,000 molecules of NikA are present per cell, which corresponds to the levels of other periplasmic proteins and of the cytoplasmic protein HypB, involved in the insertion of Ni into the large subunits of hydrogenases (Maier et al. 1993). Quenching of intrinsic tryptophan fluorescence and equilibrium dialysis reveal that NikA binds a single Ni^{2+} ion per molecule of protein with a dissociation constant (K_d) of less than 0.1 μM. This K_d value reflects a high specificity for Ni^{2+} since the other divalent metal ions tested (Co^{2+}, Cu^{2+}, Fe^{2+}) are bound at least tenfold less tightly (De Pina et al. 1995). This high specificity is also demonstrated by high-performance immobilized-metal affinity chromatography. The NikA protein is completely retained by a HiTrap column charged with Ni^{2+}, whereas it is bound very weakly to Co^{2+}- or Zn^{2+}-charged columns. In addition to its function in Ni uptake at limited Ni concentrations, NikA was also found to sense Ni at higher extracellular concentrations. Ni is known to act as a repellent for *E. coli* and its action is mediated by the Tar chemoreceptor. In the presence of 0.3 mM Ni^{2+}, a *nikA* mutant behaves as the *tar* mutant and, in contrast to the wild-type strain, is still able to swim in minimal swarm agar plates (De Pina et al. 1995). From these results, it seems possible to consider that binding of Ni to NikA induces a conformational change in the protein that allows the substrate-binding protein complex to interact with the Tar signal transducer.

Purification of NikA in sufficient quantity has opened the way to a detailed structural analysis. Crystals of NikA were previously obtained and a preliminary X-ray diffraction study achieved at a maximum resolution of 3.0 Å (Charon et al. 1994). Attemps to solve the crystal structure of NikA by the molecular replacement method using the OppA oligopeptide-binding protein model (Tame et al. 1994) remained unsuccessful because the sequence identity between the two proteins was too low (21%; M.-H. Charon and J. Fontecilla-Camps, personal communication). However, NikA, like OppA, is almost twice the size of other periplasmic binding proteins; it is proposed to contain a third domain of about 120 amino acids in length, which accounts for the larger size, but the function of this domain is not yet known (Tame et al. 1994). The compar-

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ZUR_BACSU    --MNVQ--------EALNLLKGNGYKYTNKREDMLQLFADS---DRYLTAKNVLSALNDDYPG--LSFDTIYRNLSLYEELGILETTELS   75
FUR_STAEP    --MNTN--------DAIKILKDNGLKYTDKRKDMLDIFVEE---DKYLNAKHIQQKMDKDYPG--ISFDTVYRNLHLFKDLGIIESTELE   75
FUR_CAMJE    -MLIENVEYDVLLERFKKILRQGGLKYTKQREVLLKTLYHS---DTHYTPESLYMEIKQAEPDLNVGIATVYRTLNLLEEAEMVTSISFG   86
FUR_CAMUP    -MLMENLEYDVLLEKFKKILREGGLKYTKQREVLLKTLYHS---DTHYTPESLYMEIKQAEPDSNVGIATVYRTLNLLEEAEMVTSLSLD   86
FUR_NEIME    MEKFSN----------IAQLKDSGLKVTGPRLKILDLFETH--AEEHLSAEDVYRILLEEGVE--IGVATIYRVLTQFEQAGILQRHHFE   76
FUR_NEIGO    MEKFSN----------IAQLKDSGLKVTGPRLKILDLFEKH--AEEHLSAEDVYRILLEEGVE--IGVATIYRVLTQFEQAGILQRHHFE   76
FUR_BORPE    ---MSD----------QSELKNMGLKATFPRLKILDIFRKS--DLRHLSAEDVYRALIAENVE--IGLATVYRVLTQFEQAGILTRSQFD   73
FUR_RALEU    ---MPS----------PADLKNIGLKATVPRLKILEIFQTS--EQRHLSAEDVYRILLNEHMD--IGLATVYRVLTQFEQAGLLSRNNFE   73
FUR_PSEPU    ---MVE----------NSELRKAGLKVTLPRVKILQMLDST--EQRHMSAEECIKALMEAGED--VGLATVYRVLTQFEAAGLVVRHNFD   73
FUR_PSEAE    ---MVE----------NSELRKAGLKVTLPRVKILQMLDSA--EQRHMSAEDVYKALMEAGED--VGLATVYRVLTQFEAAGLVVRHNFD   73
FUR_LEGPN    ---MEE----------SQQLKDAGLKITLPRIKVLQILEQS--RNHHLSAEAVYKALLESGED--VGLATVYRVLTQFEAAGLVSRHNFE   73
FUR_KLEPN    --MTDN----------NTALKKAGLKVTLPRLKILEVLQEP--DNHHVSAEDLYKRLIDMGEE--IGLATVYRVLNQFDDAGIVTRHNFE   74
FUR_ECOLI    --MTDN----------NTALKKAGLKVTLPRLKILEVLQEP--DNHHVSAEDLYKRLIDMGEE--IGLATVYRVLNQFDDAGIVTRHNFE   74
FUR_YERPE    --MTDN----------NKALKNAGLKVTLPRLKILEVLQNP--ACHHVSAEDLYKILIDIGEE--IGLATVYRVLNQFDDAGIVTRHNFE   74
FUR_VIBAN    --MSDN----------NQALKDAGLKVTLPRLKILEVLQQP--ECQHISAEELYKKLIDLGEE--IGLATVYRVLNQFDDAGIVTRHHFE   74
FUR_VIBCH    --MSDN----------NQALKDAGLKVTLPRLKILEVLQQP--ECQHISAEELYKKLIDLSEE--IGLATVYRVLNQFDDAGIVTRHHFE   74
FUR_VIBVU    --MSDN----------NQALKDAGLKVTLPRLKILEVLQQP--DCQHISAEDLYKKLIDLGEE--IGLATVYRVLNQFDDAGIVTRHHFE   74
FUR_VIBPA    --MSDN----------NQALKDAGLKVTLPRLKILEVLQQP--DCQHISAEDLYKKLIDLGEE--IGLATVYRVLNQFDDAGIVTRHHFE   74
FUR_HAEDU    -MSEEN----------TKLLKSVGLKVTEPRLTILALMQQYREEMQHFSAEDIYKLLLEQSSD--IGLATVYRVLNQFEEVGILLRHNFD   77
FUR_HELPY    MKRLETLES--ILERLRMSIKKNGLKNSKQREEVVSVLYRS---GTHLSPEEITHSIRQKDKN--TSISSVYRILNFLEKENFICVLETS   83
FUR_RHILV    --MTDVAKT------LEELCTERGMRMTEQRRVIARILEDS---EDHPDVEELYRRSVKVDAK--ISISTVYRTVKLFEDAGIIARHDFR   77
FUR_SYNP7    --MTYTAAS------LKAELNERGWRLTPQREEILRVFQNLP-AGEHLSAEDLYNHLLSRNSP--ISLSTIYRTLKELNQP--------   70
PerR_BACSU   --MAAHELK-----EALETLKETGVRITPQRHAILEYLVNS---MAHPTADDIYKALEGKFPN--MSVATVYNNLRVFRESGLVKELTYG   78
FUR_BACSU    -MENRIDR-------IKKQLHSSSYKLTPQREATVRVLLEN--EEDHLSAEDVYLLVKEKSPE--IGLATVYRTLELLTELKVVDKINFG   78
NIKR_ECOLI   -------------------MQRVTITLDDDLLETLDSLSQR--RGYNNRSEAIRDILRSALAQ----EATQQHGTQGFAVLSYVYEHEKR   65
                                                 :              .                    :  .

ZUR_BACSU    GEKLFRFKC---SFTHHHHHFICLACGKTKEIESCPMDKLCDDLD---GYQVSGHKFEIYGTCP---DCTAENQENTTA----------   145
FUR_STAEP    GEMKFRIAC---TN-HHHHHFICENCGDTKVIDFCPIEQIKQYLP---NVTIHTHKLEVYGVCE---SCQKNA----------------   138
FUR_CAMJE    SA-GKKYEL---ANKPHHDHMICKNCGKIIEFENPIIERQQALIAKEHGFKLTGHLMQLYGVCG---DCNNQKAKVKI-----------   157
FUR_CAMUP    SA-GKKYEL---SNKPHHDHMICKVCGKIIEFENPIIERQQSLIANEHHFKLTGHLMQLYGICS---DCN-HKTKVKI-----------   156
FUR_NEIME    TG-KAVYEL---DKGDHHDHIVCVKCGEVTEFHNPEIEALQDKIAEENGYRIVDHALYMYGVCS---DCQ-AKGKR-------------   144
FUR_NEIGO    TG-KAVYEL---DKGDHHDHIVCVKCGEVTEFHNPEIEALQDKIAEENGYRIVDHALYMYGVCS---DCQ-AKGKR-------------   144
FUR_BORPE    TG-KAVFEL---NDGDHHDHLICTNCGTVFEFSDPDIEKRQYKVAKDNGFVLESHAMVLYGICG---NC--QKGR--------------   139
FUR_RALEU    SG-KAIFEL---NEGKHHDHLVCLDCGRVEEFFDADIEQRQQSIARERGFALQEHALSLYGNCT-KDDCP-HRPRR-------------   143
FUR_PSEPU    GG-HAVFEL---ADGGHHDHMVNVETSEVIEFMDAEIEKRQREIVAEHGFELVDHNLVLY-VRK--KK---------------------   134
FUR_PSEAE    GG-HAVFEL---ADSGHHDHMVCVDTGEVIEFMDAEIEKRQKEIVRERGFELVDHNLVLY-VRK--KK---------------------   134
FUR_LEGPN    GG-HSVFEL---SQGEHHDHLVCVKCGRVEEFVDEIIEQRQKAIAERAHFKMTDHALNIYGICP--QCQ--------------------   136
FUR_KLEPN    GG-KSVFEL---TQQHHHDHLICLDCGKVIEFSDDSIELRQREIASRHGIRLTNHSLYLYGHC---AEGD-CRETNTPTTRWKNNSPFR   155
FUR_ECOLI    GG-KSVFEL---TQQHHHDHLICLDCGKVIEFSDDSIEARQREIAAKHGIRLTNHSLYLYGHC---AEGD-CREDEHAHEGK-------   148
FUR_YERPE    GG-KSVFEL---TQQHHHDHLICLDCGKVIEFSNESIESLQREIAKQHGIKLTNHSLYLYGHC---ETGN-CREDESAHSKR-------   148
FUR_VIBAN    GG-KSVFEL---STQHHHDHLVCLDCGEVIEFSDEVIEQRQREIAEQYNVQLTNHSLYLYGKC---ADGS-CKQNPNAHKSKR------   149
FUR_VIBCH    GG-KSVFEL---STQHHHDHLVCLDCGEVIEFSDDVIEQRQKEIAAKYNVQLTNHSLYLYGKCG--SDGS-CKDNPNAHKPKK------   150
FUR_VIBVU    GG-KSVFEL---STQHHHDHLVCLDCGEVIEFSDDIIEERQKEIAAAYNVQLTNHSLYLYGKC---GDGS-CKGNPDAHKRKS------   149
FUR_VIBPA    GG-KSVFEL---STQHHHDHLVCLDCGEVIEFSDDIIEERQREIAAKYNVTLTNHSLYLYGKC---SDGG-CKENPDAHKPAK------   149
FUR_HAEDU    SN-KAVFEL---NVDHEHDHIICMDCGKVFEFKDPDIERRQREISEQHGMKLATHSLYLYAKCSDISHCD-SKDKKD------------   149
FUR_HELPY    KS-GRRYEI---AAKEHHDHIICLHCGKIIEFADPEIENRQNEVVKKYQAKLISHDMKMFVWCK---ECQESEC--------------   150
FUR_RHILV    DG-GSRYET---VPEEHHDHLIDLKTGTVIEFRSPEIEALQERIAREHGFRLVDHRLELYGVP-----LK--KEDL------------   142
FUR_SYNP7    -------------L-KHHHHLICVSCSKTIEFKSDSVLKIGAKTSEKEGYHLLDCQLTIHGVCP---TCQ--RSLV------------   127
PerR_BACSU   DA-SSRFDF---VT-SDHYHAICENCGKIVDFHYPGLDEVEQLAAHVTGFKVSHHRLEIYGVCQ---ECS-KKENH------------   145
FUR_BACSU    DG-VSRYDLRKEGAAHFHHHLVCMEFGAVDEIEGDLLEDVEEIIERDWKFKIKDHRLTFHGICH---RCNGKETE-------------   149
NIKR_ECOLI   DLASRIVST------QHHHHDLSVATLHVHINHDDCLEIAVLKGDMGDVQHFADDVIAQRGVRHGHLQCLPKED--------------   133
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Fig. 2 Multiple sequence alignment of NikR and Fur-like proteins. Sequences of the Fur proteins of *Staphylococcus epidermis*, *Campylobacter jejuni*, *C. uppsaliensis*, *Neisseria meningitidis*, *N. gonorrhoeae*, *Bordetella pertussis*, *Ralstonia eutropha*, *Pseudomonas putida*, *P. aeruginosa*, *Legionella pneumophila*, *Klebsiella pneumoniae*, *Escherichia coli*, *Yersinia pestis*, *Vibrio anguillarum*, *V. cholerae*, *V. vulnificus*, *V. parahaemolyticus*, *Haemophilus ducreyi*, *Helicobacter pylori*, *Rhizobium leguminosarum*, *Synechococcus* PCC7942, and *Bacillus subtilis*, the Zur and PerR proteins of *B. subtilis*, are compared with the sequence of *E. coli* NikR. Conserved identical amino acids are indicated by a *star* and similar ones by a *double point* or a *single point*. Potential helix-turn-helix motif in the N-terminal domain and a stretch of histidines likely to be important for metal binding in the C-terminal part are *overlined*. The sequences were compared using the computer program CLUSTAL W(1.74) available at http://pbil.ibcp.fr.

ative study of the structures of NikA and OppA will provide very important information on the function and ligand specificity in this group of proteins. Recent results from an extended X-ray absorption fine-structure investigation revealed that the Ni site in NikA is six- to seven-coordinate and comprises five to six O- or N-donor ligands and a single S-donor ligand (Allan et al. 1998). The finding clearly distinguishes the Ni environment in NikA from the Ni site typical of redox enzymes, like hydrogenases, which are dominated by cysteinate ligation.

Regulation of *nikABCDE* operon expression

Expression of the *nik* locus has been monitored following measurement of β-galactosidase activity from a chromosomal *nikA-lacZ* operon fusion. Transcription is induced under anoxic conditions and is strictly dependent on the global regulatory protein FNR, which is known to control several anaerobic respiratory and fermentative metabolic activities (Wu and Mandrand-Berthelot 1986). Accordingly, mutations in the *fnr* gene abolish *nik* expression and give rise to a defective hydrogenase phenotype similar to that of *nik* mutants in all respects examined (Wu et al. 1989; see above). Furthermore, introduction of the functional *nik* locus borne on a multicopy plasmid restores hydrogenase activity in *fnr* mutants. This study establishes that the effect of *fnr* on hydrogenase activity is only mediated via *nik*, which is responsible for the cellular Ni content. This clearly distinguishes the indirect role of FNR on hydrogen metabolism from its proposed direct interaction with the structural genes of a number of anaerobic respiratory enzymes (Sawers 1994).

When supplied at high external concentration (from 0.3 mM in a rich medium), Ni totally represses expression of the *nik* operon (Wu and Mandrand-Berthelot 1986; Wu et al. 1989), therefore blocking the entrance of metal ion by the high-affinity Ni transport system. Such elevated concentrations are toxic to the cells since Ni, as already outlined above, can catalyze the formation of dangerously reactive oxygen species that can damage virtually all cellular constituants (Wu et al. 1994). This explains why specific transport is so tightly regulated.

The *nikR* gene responsible for repression of the *nikABCDE* operon in the presence of Ni has been recently identified (De Pina et al. 1999). By using random transposon Tn*10* insertion, mutants in which the *nik* operon is still expressed, even in the presence of high Ni concentration, have been isolated. Complementation studies, as well as cloning and sequencing experiments, revealed the presence of an ORF located 5 bp downstream of the last (*nikE*) gene of the *nik* operon. This ORF encodes a hydrophilic polypeptide of 133 amino acids with a molecular weight of 15 kDa (Fig. 1). NikR was used as a query sequence to scan the nr database (nonredundant Genbank CDS translations+PDB+SwissProt+SPupdate+PIR) and

the unfinished genome sequences using the respective BLASTP or TBLASTN programs available at NCBI. The highest scoring sequences are found in the enteric bacteria *Salmonella typhi, S. typhimurium, S. paratyphi* and *Klebsiella pneumoniae*. The following best-scoring sequences are present, in decreasing order, in the genomes of *Plasmodium falciparum*, *Chlorobium tepidum*, and, interestingly, in the five archaea *Pyrococcus horikoshii*, *P. furiosus*, *P. abyssi*, *Methanococcus jannaschii*, *Archaeoglobus fulgidus*, the gastric pathogen *Helicobacter pylori* and the methanogen *Methanobacterium thermoautotrophicum.* The last seven bacterial species are known to have a high demand for Ni in order to synthesize one or several enzymes, namely hydrogenase, methyl-coenzyme M reductase, CODH and urease. However, all these NikR-like sequences are conserved hypothetical proteins.

A thorough computer-assisted investigation indicates that the NikR sequence is also functionally related to the ferric ion uptake regulation (Fur) proteins from the four *Vibrio* species, *V. cholerae*, *V. anguillarum*, *V. vulnificus*, *V. parahaemolyticus* – although the Smallest Sum Probability is too low to establish sequence relatedness between them (DePina et al 1999). The Fur proteins act as negative regulators of the expression of bacterial operons for high-affinity iron uptake pathways and thus display a similar regulatory role to that of NikR. Other Fur homologues have been identified in numerous other bacteria (Hantke and Braun 1997; Helmann 1997), and Fur-like regulators that control other functions have been described. *B. subtilis* PerR regulates the peroxide stress response (Bsat et al. 1998), while *B. subtilis* Zur and *E. coli* Zur control the transcription of zinc uptake operons (Gaballa and Helmann 1998; Patzer and Hantke 1998). Comparison of NikR with 24 Fur and Fur-like proteins of known function show conserved amino acids in N-terminal or C-terminal regions presumed important for either DNA-binding or metal-binding selectivity, respectively (Fig. 2). Remarkably, a cluster of histidines in Fur around positions 86–90, which is thought to be involved in metal sensing, is conserved in *E. coli* NikR and in *B. subtilis* Zur and PerR (but not in *E. coli* Zur which has not been aligned; Patzer and Hantke 1998). Mutagenesis of the two His-96 and His-97 residues in *B. subtilis* Fur reveals that they are likely candidates for Fe^{2+} ligands (Bsat and Helmann 1999). Identification of critical residues providing ligands for metal coordination and necessary for metal specificity would be of primary importance to allow comparison of the metal ion binding sites in the three types of proteins.

Monitoring of *nikR* operon fusions under various genetic and environmental conditions suggests that *nikR* can be transcribed from two promoters (Fig. 1). The first one is responsible for constitutive *nikR* transcription; it ensures a basal level in the presence of oxygen and is independent of the Ni concentration (De Pina et al. 1999). The second, the promoter of the *nikABCDE* operon, contains a partially conserved FNR box located immediately upstream of an inverted repeated sequence composed of a 14-base dyad that could serve as a binding site for the NikR regulatory protein (Navarro et al. 1993; V. Desjardin and M.-A. Mandrand-Berthelot, unpublished results). Therefore, the partial autoregulation and the FNR-mediated activation of *nikR* expression are the consequence of transcriptional regulation of *nikR* at the level of the promoter of the *nik* operon. Our model implies that NikR becomes active after binding Ni in order to repress the transcription of *nik*. Tight control by NikR is achieved through the permanent, basal level synthesis of the repressor from the *nikR* promoter, enabling the cells to respond immediately to changes in the Ni status.

A novel family of transition metal permeases

The first structural gene for a Ni^{2+}-specific permease has been identified by sequencing a fragment of *R. eutropha* DNA that restores Ni deficiency of an *R. eutropha* mutant (reviewed by Eitinger and Friedrich 1997). This strain is unable to grow on hydrogen as the energy source, and produces only low activity of urease under standard conditions. The deduced amino acid sequence revealed an integral membrane protein (HoxN) that did not show any similarities to available database entries. Transport assays indicate that HoxN has an extremely high affinity for Ni^{2+} ion, but a very low capacity. The K_t value for Ni^{2+} is estimated to 20 nM and the maximal velocity is 1.5 pmol $Ni^{2+} \times min^{-1} \times (mg\ total\ cell\ protein)^{-1}$. These values represent, however, a rough estimation rather than an exact determination, because it is very difficult to distinguish transport from binding to the cell envelope at very low substrate concentrations. At high Ni^{2+} concentrations and although the assays were conducted in the presence of high amounts of Mg^{2+}, interference of Mg^{2+} uptake systems cannot be excluded. Expression of *hoxN* in *E. coli* allows the reproducible measurement of HoxN activity using a physiological assay system (Wolfram et al. 1995). The motive force behind transport is unclear. Accumulation experiments with recombinant *E. coli* growing in the presence of $^{63}Ni^{2+}$ indicate that HoxN concentrates its substrate only tenfold (Wolfram et al. 1995; Degen et al. 1999). This result suggests a very slow uniport mechanism. The membrane topology of HoxN has been analyzed by fusing N-terminal segments to alkaline phosphatase and β-galactosidase, which serve as reporters for periplasmically and cytoplasmically located sites, respectively. These studies revealed that the N- and C-termini of the Ni permease are located in the cytoplasm and that the protein contains eight membrane-spanning segments (Eitinger and Friedrich 1994; Eitinger et al. 1997).

In the past 5 years, sequences related to HoxN were reported for gram-negative and gram-positive bacteria. The list includes: *Bradyrhizobium japonicum* (BjHupN; Fu et al. 1994), *Helicobacter pylori* (HpNixA; Mobley et al. 1995), *Mycobacterium tuberculosis* (MtNicT; Cole et al. 1998) and *Rhodococcus rhodochrous* (RrNhlF; Komeda et al. 1997). Database searches with the BLASTP program at http://www.ncbi.nlm.nih.gov/BLAST and the TBLASTN and TBLASTX programs at http://www.ncbi.nlm.nih.gov/BLAST/unfinishedgenome.html have identi-

fied related sequences in *Mycobacterium avium*, *Salmonella paratyphi*, *S. typhi*, *S. typhimurium*, *Staphylococcus aureus*, *Yersinia pestis* and in the fission yeast *Schizosaccharomyces pombe*. Fragments producing high similarity scores are also present in the genomes of *Klebsiella pneumoniae* and *Mycobacterium bovis*.

The role of BjHupN, HpNixA, and RrNhlF has been experimentally investigated. In the absence of BjHupN, *B. japonicum* produces only low levels of hydrogenase activity under Ni limitation (Fu et al. 1994). HpNixA plays a crucial role in *H. pylori*, a human pathogen that is dependent on a highly active urease for initial colonization of the gastric mucosa. At very low concentrations of noncomplexed Ni^{2+}, reflecting the situation in human serum, HpNixA is essential for urease activity. A K_t value of 11 nM Ni^{2+} has been determined for this permease (Mobley et al. 1995). Preliminary cross-linking studies suggest that HpNixA operates as a monomer (J. Fulkerson and H. Mobley, personal communication). RrNhlF was originally identified as a Co^{2+} transporter in the actinomycete *R. rhodochrous* J1 (Komeda et al. 1997). This organism produces two types of Co-containing nitrile hydratases, which are used as industrial catalysts for the kiloton-scale production of acrylamide and nicotinamide (Kobayashi and Shimizu 1998). A recent reinvestigation of its substrate specificity demonstrates that RrNhlF is able to transport Ni^{2+} with high affinity, although a slight preference for Co^{2+} ion is observed (Degen et al. 1999).

Pairwise amino acid sequence alignments of the aforementioned permeases show 27–77% identity. Based on this similarity and the presence of conserved motifs that are essential for transport activity (see below), some of these transporters have been grouped into a phylogenetic family, the Ni^{2+}-Co^{2+} transporter family (Saier et al. 1999). A different approach to searching for structural homologues of transmembrane proteins has recently been developed by Lolkema and Slotboom (1998a; 1998b). The rationale of this method is the statistical analysis of multiple hydropathy profile alignments of membrane proteins. Since the hydropathy profile represents a link between the amino acid sequence and the three-dimensional structure, the profiles are much better conserved among related membrane proteins than the amino acid sequences themselves. The hydropathy profiles of HoxN and nine relatives have been aligned and the result is presented in Fig. 3. It is obvious that the hydropathy profiles of the individual sequences are very similar. The structure divergence score (SDS) provides a measure to describe the divergence of the profiles of related proteins. The SDS of the HoxN-type permeases (0.126) is a typical value for a structurally homologous family. The profiles of the *S. typhimurium* and *Y. pestis* permeases are the most closely related to the family profile, as represented by profile divergence scores (PDS) of 0.091 and 0.098, respectively. The largest distance to the family profile was observed for RrNhlF (PDS = 0.158) and BjHupN (PDS = 0.160).

A Ni^{2+} transport system has been reported for the thermophilic *Bacillus* sp. strain TB-90 (Maeda et al. 1994). It is encoded by the two distal genes (*ureH*, *ureI*) of the urease operon. When the operon is expressed in *E. coli*, urease activity is strongly diminished in the absence of UreH or UreI. UreH shows approximately 15% amino acid sequence identity to members of the HoxN family, but contains most probably less than eight transmembrane segments. UreI consists of only 65 amino acid residues and has no counterparts in the databases. The *Methanococcus jannaschii* genome sequencing project revealed an ORF (ID number MJ1092) that is related to the *Bacillus* UreH. Two similar sequences were also reported for the plant *Arabidopsis thaliana* (ID numbers AL022023 and AC005825). *M. jannaschii* requires Ni for methanogenesis, and *A. thaliana* for incorporation into urease. Nevertheless, the available data do not allow additional conclusions on the mechanism of Ni^{2+} transport in these organisms.

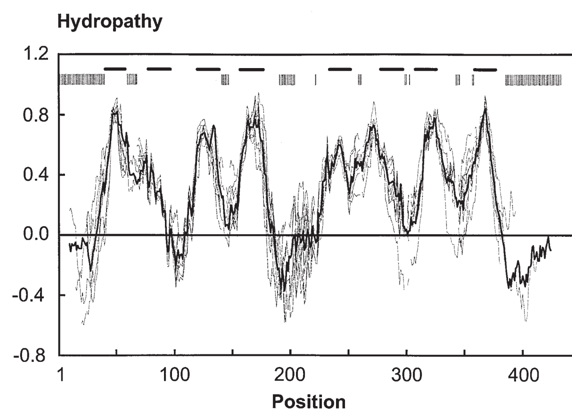

Fig. 3 Hydropathy profile alignment of HoxN-type Ni permeases. The individual hydropathy profiles of 10 members of the HoxN family were calculated and are shown as *thin lines*. The *bold line* represents the family profile. *Horizontal bars* represent putative transmembrane segments and *vertical bars* show the gaps that occur at any place in the alignment

Critical residues of nickel permeases

In addition to the common topology of HoxN-type Ni permeases, a few amino acid sequence motifs are conserved (Fig. 4). Four of these motifs are located within transmembrane segments. Site-directed mutagenesis experiments have been performed to obtain more detailed information on the significance of these residues for affinity and specificity of the permeases. In a study with HoxN of *R. eutropha* (Eitinger et al. 1997), replacements were introduced into the HX_4DH motif, located in the unusually polar transmembrane segment II. This motif is fully conserved among the HoxN-type permeases, but is also present in the unrelated NikC, one of the two integral membrane proteins of the *E. coli* ABC-type Ni^{2+} transporter. In the latter case, however, the signature is located in the first periplasmic loop connecting the putative transmembrane segments I and II. Exchanges of Asp-67 and His-68 (shown in solid boxes in Fig. 4) in HoxN abolish transport activity completely without affecting membrane topology. On the other hand, replacement of His-62 (grey box) by

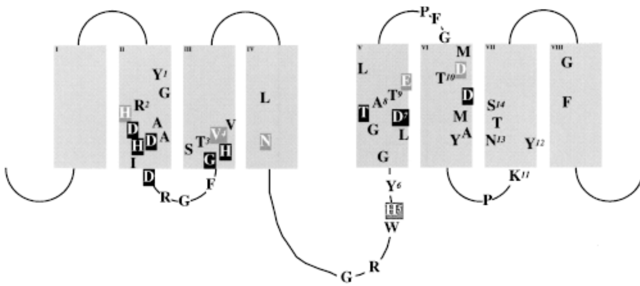

Fig. 4 Conserved amino acid residues and structural motifs in HoxN-type Ni permeases. Residues which are conserved in the permeases of *Bradyrhizobium japonicum* (BjHupN), *Helicobacter pylori* (HpNixA), *Mycobacterium avium* (M.avi), *M. tuberculosis* (MtNicT), *Ralstonia eutropha* (HoxN), *Rhodococcus rhodochrous* (RrNhlF), *Salmonella typhimurium* (Sa.typhim), *Schizosaccharomyces pombe* (Sc.pom), *Staphylococcus aureus* (St.aur), and *Yersinia pestis* (Y.pes) are shown and exceptions are indicated. *Solid boxes* represent residues which have been replaced in at least one of the proteins and replacement of which inactivated the respective permease(s). Alterations at the positions of *grey boxes* led to a strong decrease of activity. The data were taken from Eitinger et al. (1997), O. Degen and T. Eitinger (unpublished results) for HoxN; Fulkerson et al. (1998), L. Wolfram and P. Bauerfeind (personal communication) for HpNixA; O. Degen and T. Eitinger (unpublished results) for RrNhlF. [1] W in BjHupN, Sc.pom; [2] K in HpNixA; [3] S in BjHupN, HpNixA, HoxN, St.aur; [4] I in Sa.typhim, Y.pes; [5] K in Sc.pom; [6] L in St.aur; [7] E in RrNhlF; [8] S in Sc.pom; [9] S in HpNixA, RrNhlF, St.aur; [10] S in Sa.typhim, Y.pes; [11] R in Sc.pom; [12] F in RrNhlF; [13] S in Sc.pom; [14] T in RrNhlF

Ala, Ile, and Ser results in permeases that are still capable of transporting Ni^{2+} ion, although with a dramatically reduced affinity. The strongest effect among the His-62 mutants is observed for the H62I replacement. The affinity is less impaired in the H62S mutant suggesting that a hydrophilic residue is required at position 62. Investigation of the HX_4DH motif in HpNixA revealed similar results (Fulkerson et al. 1998). In addition, a number of Asp, Arg, Glu, His, and Lys residues have been replaced by Ile. Alterations affecting the two His and the three Asp residues of transmembrane segment II (Fig. 4) abolish transport activity. The negatively charged residues within transmembrane segments V and VI were identified as important, although considerable transport activity is observed for the E198I and D234I mutants. Unpublished results demonstrate the significance of transmembrane segment III and of the large hydrophilic loop connecting transmembrane segments IV and V. Replacement of Gly, His, and Val in the GHSTVV motif (Fig. 4) abolishes or strongly affects activity of HpNixA. The permease is inactivated by deletion of Phe-138 to Ser-179 in the hydrophilic loop (L. Wolfram and P. Bauerfeind, personal communication; Fulkerson et al. 1998). Deletions have been introduced into the corresponding loop of HoxN removing Arg-160 to Ser-194, Leu-173 to Ser-194, and Ile-183 to Ser-194. Although none of the alterations prevent HoxN from insertion into the membrane, all three deletions abolish transport activity (O. Degen and T. Eitinger, unpublished result). With the exception of the permease of *Schizosaccharomyces pombe* (Sc. pom), HoxN-type permeases contain the signature WH(M/I)(Y/L) close to transmembrane segment V. In Sc. pom, a variation of this signature is present with a lysine instead of the histidine. Exchange of the His residue to Ala leads to a 50% inhibition of HpNixA activity (L. Wolfram and P. Bauerfeind, personal communication). A HoxN variant harboring an Ile at the corresponding position is inactive (O. Degen and T. Eitinger, unpublished result).

A very important point in the analysis of membrane transporters is the question regarding how substrate specificity is mediated. Members of the HoxN-type permeases differ in ion selectivity. HoxN, for instance, is highly specific for Ni^{2+} and unable to transport Co^{2+}. Ni^{2+} uptake is not inhibited by a tenfold excess of divalent Co, Mn, and Zn ions (Degen et al. 1999). RrNhlF is less selective and transports both Ni^{2+} and Co^{2+}. Ni^{2+} uptake is strongly inhibited by a tenfold excess of Co^{2+}, but not by Mn^{2+} and Zn^{2+} (Degen et al. 1999). Ni^{2+} transport by HpNixA is moderately sensitive to the presence Co^{2+} (Fulkerson et al. 1998). Direct measurements of Co^{2+} transport have not been reported, and thus conclusions with regard to specificity of HpNixA remain speculative. Attempts have been made to analyze the molecular basis of the different selectivity of HoxN and RrNhlF. Transmembrane segment I is the most divergent membrane-spanning helix among HoxN-type permeases. Since transmembrane segment I of RrNhlF resembles a transmembrane segment of a Co^{2+} transporter of yeast (*Saccharomyces cerevisiae*) intracellular membranes, it has been suggested that this segment is involved in Co^{2+} recognition (Komeda et al. 1997). Presently available data do not conclusively support or discard this hypothesis. A number of chimeras of HoxN and RrNhlF have been constructed and analyzed. Unfortunately, most of them are devoid of any transport activity. A hybrid containing transmembrane segment I of RrNhlF and transmembrane segments II–VIII of HoxN transports both Ni^{2+} and Co^{2+} ion, albeit with a very low activity (O. Degen and T. Eitinger, unpublished result).

Nickel homeostasis

Microorganisms have developed a number of devices to maintain appropriate intracellular concentrations of transition metal ions. Fig. 5 schematically illustrates uptake and export systems which are involved in Ni homeostasis in different bacteria. At high extracellular concentrations, Ni^{2+} enters the cells by Mg^{2+} transport systems. Under physiological conditions, Ni^{2+} uptake is mediated by the Nik system in *E. coli*, and by members of the HoxN family in many other organisms. Metal resistance determinants like the Cnr, Czc and Ncc export systems of *Alcaligenes* and *Ralstonia* species (reviewed by Silver and Phung 1996) or the P-type ATPases of *H. pylori* (Melchers et al. 1998) export either excess Ni^{2+} (Cnr, Ncc) or other transition metal cations (Czc, P-type ATPases). The role of an ABC transporter of *H. pylori* (Hendricks and Mobley 1997), which potentially lacks a periplasmic binding protein, could be similar to that of the P-type AT-

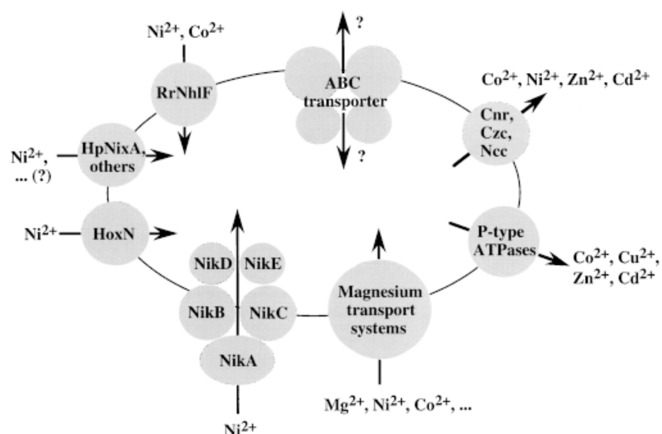

Fig. 5 Bacterial transport systems involved in Ni homeostasis. (See text for details)

Pases, i.e. prevention of misincorporation of "false" transition metals into Ni enzymes by exporting excess amounts of these ions.

Additional mechanisms of homeostasis include the chemotactic response towards metal ions in the environment and the regulation of transcription of transporter genes, as discussed for the Nik system of *E. coli*. Very limited information is available regarding regulation of the HoxN-type Ni permeases. *hoxN* is located on the 450-kb megaplasmid pHG1 in *R. eutropha* strain H16, downstream of a large transcriptional unit comprising at least 18 hydrogenase structural, accessory, and regulatory genes. This unit controls itself by a positive feedback loop under hydrogenase-derepressing conditions (Schwartz et al. 1999), and it is likely that transcription of *hoxN* is affected by this regulatory system. Regulation of *hoxN* in response to the extracellular Ni^{2+} concentration has not been reported.

Perspectives

The transcription of *nik* genes is stimulated by the global regulator FNR under anoxic conditions, and negatively controlled by a new repressor protein, NikR, responding specifically to Ni. Further biochemical and biophysical characterization of NikR, and its interactions with both operator DNA and metal ions, will help clarify the molecular basis of Ni homeostasis in *E. coli*. Since a protein homologous to NikR has recently been identified in the gastric pathogen *H. pylori* (De Pina et al. 1999) whose urease activity acts as a virulence factor, the possibility that NikR participates in bacterial pathogenesis should also be considered. Understanding the molecular basis of ion specificity of the widespread HoxN-type permeases is also important. Additional site-directed mutagenesis, the isolation of second-site revertants, and the construction of chimeric permeases will be helpful towards this goal. Structural information on the permeases that could be obtained by electron microscopic analyses of 2D crystals is required to understand metal ion coordination.

Yeast is frequently used as a model organism to investigate transition metal transport in eukaryotes, and parallels between the mechanisms employed by yeast and higher eukaryotes, including humans, are obvious. While the transport of Fe, Cu, Zn and Mn is under intensive investigation (Nelson 1999; Radisky and Kaplan 1999), the mode of Ni uptake remains unclear. The finding that a HoxN-type permease is encoded in the genome of *Schizosaccharomyces pombe* may stimulate further research in this field.

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