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The *Alcaligenes eutrophus* Protein HoxN Mediates Nickel Transport in *Escherichia coli*

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HoxN, an integral membrane protein with seven transmembrane helices and a molecular mass of 33.1 kDa, is involved in high-affinity nickel transport in *Alcaligenes eutrophus* H16. From genetic analyses, it has been concluded that HoxN is a single-component ion carrier. To investigate this assumption, *hoxN* was introduced into *Escherichia coli*. The recombinant strain showed significantly enhanced nickel uptake in a short-interval assay. Likewise, growth in the presence of $^{63}NiCl_2$ yielded a more than 15-fold-increased cellular nickel content. The HoxN-based nickel transport activity could also be demonstrated in a physiological assay: an *E. coli* strain coexpressing *hoxN* and the urease operon of *Klebsiella aerogenes* exhibited urease activity 10-fold greater than that in the strain lacking a functional *hoxN*. These results strongly suggest that HoxN is sufficient to operate as a nickel permease. Multiple sequence alignment of HoxN and four other bacterial membrane proteins implicated in nickel metabolism revealed two conserved signatures which may play a role in the nickel translocation process.

Nickel is an essential trace element for at least four biological processes: (i) oxidation and evolution of molecular hydrogen, (ii) hydrolysis of urea, (iii) carbon monoxide dehydrogenase-mediated acetate metabolism in methanogens and in homoacetogenic bacteria, and (iv) reduction of methyl coenzyme M to methane in methanogenic archaea (6). Uptake of nickel ions is a prerequisite for those organisms catalyzing nickel-dependent reactions. The microbial transport of the divalent Ni^{2+} cation, the most commonly occurring oxidation state of nickel, has been investigated with regard to physiology, ion specificity, and very recently, the molecular structure of the transport proteins (3, 18). Nickel uptake is mediated by nonspecific Mg^{2+} transport systems and by high-affinity systems specific for the transport of nickel (for a review, see reference 7).

In *Alcaligenes eutrophus*, a gram-negative aquatic and soil bacterium which can utilize molecular hydrogen as an energy source, nickel uptake occurs by Mg^{2+} transporters and by a high-affinity nickel transporter, the product of gene *hoxN* (2, 11). The phenotype of a HoxN-negative mutant manifested itself in the inability to grow on hydrogen as the energy source under nickel limitation (below 100 nM) in the presence of 0.8 mM Mg^{2+}. As expected for a mutant lacking the specific nickel transporter, this nickel deficiency was physiologically compensated for by either increasing the Ni^{2+} concentration to 1 μM or decreasing the Mg^{2+} concentration (1, 2). The nucleotide sequence of *hoxN* has been determined, and the two-dimensional membrane topology of the HoxN nickel transport protein has been investigated. While computer analyses of potential membrane-spanning segments gave ambiguous results, the construction of a set of fusions to alkaline phosphatase and β-galactosidase clearly allowed the identification of segments located in the periplasm and in the cytoplasm, respectively. Using these data, a topological model for HoxN predicting seven transmembrane helices with the amino terminus in the cytosol and the carboxyl terminus facing the periplasm was developed (3).

On the basis of uptake experiments and genetic analyses, it was concluded that HoxN is a single-component permease as has been described for a number of secondary carriers (for reviews, see references 19 and 20). To examine this assumption, we purified HoxN and subjected it to reconstitution experiments in proteoliposomes. For this purpose, a gene fusion encoding a tripartite protein consisting of β-galactosidase, an endoproteolytically cleavable peptide linker, and HoxN was constructed and overexpressed in *Escherichia coli* (22). The nickel transporter could be purified by affinity chromatography and subsequent site-specific proteolysis. Nickel uptake experiments with the reconstituted system, however, were unsatisfactory since nonspecific binding of Ni^{2+} to phospholipids hindered kinetic analyses (21).

To overcome these problems, we developed a physiological assay. We now present evidence that the expression of *hoxN* of *A. eutrophus* in *E. coli* results in a functional nickel permease which increases the intracellular content of nickel and therefore facilitates its incorporation into urease. In addition, by sequence comparison of HoxN and related proteins, we identified conserved signatures which may play a role in nickel recognition or in the translocation pathway.

MATERIALS AND METHODS

Materials. Analytical-grade chemicals were obtained from E. Merck or from Sigma. $^{63}NiCl_2$ was purchased from Amersham. Nitrocellulose membrane filters with a pore size of 0.45 μm were from Schleicher & Schuell. Zinsser Aquasafe 300 Plus was the cocktail for liquid scintillation analyses in a Packard 1600 TR counter.

Bacterial strains and plasmids. *E. coli* CC118 [*araD139* Δ(*ara leu*) Δ*lacX74 phoA*Δ*20 galE thi rpsB rpoB argE*(Am) *recA1*] (14) was used as the host strain for recombinant plasmids. Plasmids pCH231-Sm and pCH231-P47 were described recently (3). pCH231-Sm is a derivative of vector pBluescript KS+ containing the *A. eutrophus* gene *hoxN* under control of a *lac* promoter. A streptomycin resistance cassette replaces a major part of the *bla* gene. pCH231-P47 is derived from plasmid pCH231-Sm and harbors an insertion of the transposon Tn*phoA* in the 48th codon of *hoxN*. Plasmid pKAU17 (17) contains the urease operon of *Klebsiella aerogenes* under control of the *lac* promoter and was a gift from R. P. Hausinger (Michigan State University, East Lansing).

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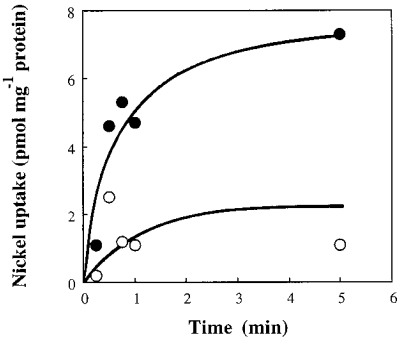

FIG. 1. Nickel uptake of recombinant *E. coli* CC118. Solid circles, CC118(pCH231-Sm) containing functional *hoxN*; open circles, CC118(pCH231-P47) harboring an inactivated *hoxN*. The assay mix consisted of 100 nM $^{63}NiCl_2$ and 10 mM $MgCl_2$ in a 50 mM Tris-hydrochloride buffer (pH 7.5).

Nickel uptake. Cells were grown in the absence of nickel in Luria-Bertani broth (LB) supplemented with streptomycin (50 μg ml^{-1}) to the mid-exponential phase, washed once with 50 mM Tris-hydrochloride (pH 7.5) containing 10 mM $MgCl_2$, and resuspended to a protein concentration of 0.25 to 0.3 mg ml^{-1}. Glucose was added to a final concentration of 10 mM, and after shaking for 5 min at 30°C, uptake experiments were initiated by the addition of $^{63}NiCl_2$ (883 Ci/mol) to a final concentration of 100 nM. Aliquots of 150 μl were passed through membrane filters, and the filters were washed twice with 3 ml of 100 mM LiCl. The radioactivity was determined by liquid scintillation counting. Nickel uptake activity is expressed as picomoles of Ni^{2+} taken up per milligram of protein at 30°C. Protein was estimated by the method of Lowry et al. (12).

Nickel accumulation. Strains were grown overnight at 37°C in LB with the appropriate antibiotics in the presence of 500 nM $^{63}NiCl_2$ (883 Ci/mol), washed twice with 50 mM Tris-hydrochloride (pH 7.5), and concentrated 10-fold. One hundred microliters of the cell suspension was subjected to liquid scintillation analysis. The cellular content of ^{63}Ni is expressed as picomoles per milligram of protein.

Urease assay. *E. coli* CC118(pKAU17; pCH231-Sm or pCH231-P47) was grown aerobically overnight in LB in the presence of ampicillin (100 μg ml^{-1}), streptomycin (50 μg ml^{-1}), and $NiCl_2$ as indicated. Cells were washed twice in 35 mM potassium phosphate buffer (pH 7.0) and resuspended to a protein content of approximately 4 mg ml^{-1}. Urease activity was measured with permeabilized cells by quantitating the rate of ammonium ion released from urea by formation of indophenol (16). The assay mix consisted of the cell suspension in 35 mM potassium phosphate (pH 7.0) and 0.15 mM *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide. The reactions were initiated by the addition of a urea solution to a final concentration of 5 mM, and the released ammonium ion was determined in timed aliquots. One unit of urease activity is defined as the amount of enzyme required to form 2 μmol of ammonium ion per min at 37°C.

Sequence alignments. For multiple sequence alignments, the CLUSTAL program (version 1.20) of the PC/GENE software package (version 6.80; IntelliGenetics Inc.) was used.

RESULTS AND DISCUSSION

Functional expression of the nickel transporter in *E. coli*. In previous reports, we demonstrated that HoxN is a major component for high-affinity nickel transport in *A. eutrophus* (1, 2, 22). Complementation analyses of $HoxN^-$ mutants suggested that DNA regions flanking the gene *hoxN* are not essential for the transport activity. We could not exclude the possibility, however, that genetically unlinked determinants are important for nickel uptake. Therefore, we developed a new strategy of investigation. *hoxN* was introduced on a high-copy-number plasmid into an *E. coli* strain, and the nickel uptake activity of the recombinant strain was investigated. As illustrated in Fig. 1, the presence of an intact *hoxN* gene led reproducibly to a fivefold increase in nickel uptake in a 5-min interval.

To examine the relevance of this effect, we assayed the cellular nickel content upon growth in liquid medium containing 500 nM $^{63}NiCl_2$. The results are summarized in Table 1. The *hoxN*-harboring strain *E. coli* CC118(pCH231-Sm) accumulated approximately 25 pmol of Ni per mg of protein, compared to 1.4 pmol of Ni per mg of protein for the control strain

TABLE 1. HoxN-dependent nickel accumulation of *E. coli* cells

Strain[a] (plasmid[s])	Phenotype	cpm[b]	Nickel accumulation (pmol/mg of protein)
CC118 (pCH231-P47)	$HoxN^-$ $Urease^-$	1,300	1.4
CC118 (pCH231-Sm)	$HoxN^+$ $Urease^-$	22,800	24.6
CC118 (pCH231-P47, pKAU17)	$HoxN^-$ $Urease^+$	2,900	2.9
CC118 (pCH231-Sm, pKAU17)	$HoxN^+$ $Urease^+$	15,700	14.7

[a] Strains were grown aerobically in LB with 500 nM $^{63}NiCl_2$ overnight.
[b] Radioactivity of an aliquot of 10-fold-concentrated washed cells.

which contained an inactivated *hoxN*. These values lie within the same order of magnitude as those obtained with the uptake assays, confirming that the transport process has a very low capacity.

The high-affinity low-capacity uptake of nickel may meet the physiological requirements of the organism. On the one hand, nickel is an essential trace element; on the other hand, supplied at elevated concentrations, nickel acts as a toxic transient metal. In *A. eutrophus* CH34, for example, a nickel resistance determinant (*cnr*) whose products export Ni^{2+} from the cytoplasm and therefore protect the organism against toxic effects was discovered (10). This ambivalent role of nickel could explain the existence of uptake systems with an outstanding affinity but a very low capacity. These properties, however, make a straightforward biochemical analysis rather difficult.

A nickel-specific high-affinity transporter has been analyzed in detail in *E. coli*. This system is completely different from that in *A. eutrophus*. It consists of the five proteins NikA, -B, -C, -D, and -E. NikA is a periplasmic binding protein, NikB and NikC are integral membrane components, and NikD and NikE contain ATP-binding sites. The system has been classified as a member of the ATP-binding cassette family of transporters (18). The *nik* operon is expressed under anaerobic growth conditions and provides nickel for the three hydrogenases involved in anaerobic metabolism (23).

Interestingly, the presence of an intracellular nickel-scavenging pathway did not increase the HoxN-dependent nickel accumulation in *E. coli*. Strain CC118(pKAU17, pCH231-Sm)

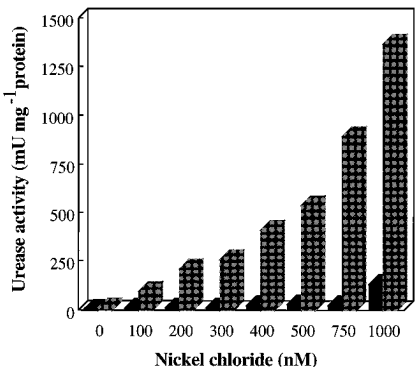

FIG. 2. Effect of *hoxN* on the urease activity of *E. coli* CC118 expressing the *K. aerogenes ure* operon. Solid boxes, CC118(pKAU17, pCH231-P47); shaded boxes, CC118(pKAU17, pCH231-Sm). Strains were grown overnight in LB supplemented with $NiCl_2$ as indicated. The urease activity was measured with permeabilized cells by quantitating the amount of ammonium ion released from urea.

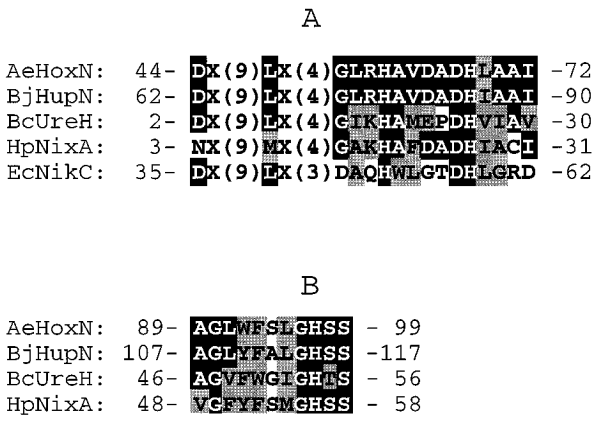

FIG. 3. Multiple sequence alignment of nickel transport-related proteins. Signatures with high similarity were detected in five (A) or four (B) of the following proteins: AeHoxN, *A. eutrophus* high-affinity nickel transporter (2, 3); BjHupN, *B. japonicum* protein HupN (5); BcUreH, urease accessory protein of a thermophilic *Bacillus* sp. (13); HpNixA, *H. pylori* nickel transporter (15); EcNikC, integral membrane protein of the *E. coli* nickel-specific ATP-binding cassette transport system (18). Identical residues are shown in black boxes; similar residues are shaded. Numbers represent positions in the sequence.

coexpressing *hoxN* and the urease operon of *K. aerogenes* contained about 15 pmol of Ni per mg of protein (Table 1). pKAU17 directs the synthesis of seven urease-related polypeptides, the urease subunits UreA, UreB, and UreC, and the accessory proteins UreD, UreE, UreF, and UreG (8). The coexpression of the urease operon as a sink for nickel did not enhance the long-term nickel uptake. It should be considered in this context that both plasmids pKAU17 and pCH231-Sm contain an origin of replication derived from plasmid ColE1. Thus, introduction of pKAU17 into a strain already harboring pCH231-Sm reduces its copy number. The plasmid content of the strains used in this study was analyzed. Growth with antibiotic selection for both plasmids resulted in a 1:1 stoichiometry (data not shown). This can explain the lower nickel accumulation of strain CC118(pKAU17, pCH231-Sm) than of the strain lacking pKAU17 (Table 1).

HoxN increases urease activity under nickel limitation. The nickel uptake and accumulation assays indicated that HoxN increases the cellular nickel content of *E. coli*. However, these experiments did not completely rule out the possibility that HoxN binds Ni^{2+} specifically and tightly on the cell surface. Therefore, we monitored the effect of *hoxN* on the activity of a cytoplasmic nickel-dependent enzyme. We introduced the urease operon of *K. aerogenes* on plasmid pKAU17 into *E. coli* CC118 containing plasmid pCH231-Sm or pCH231-P47. The functional expression of the *K. aerogenes* urease in *E. coli* has been shown by Lee et al. (8) to be strictly nickel dependent. The results presented in Fig. 2 clearly demonstrate that the presence of a functional HoxN allows the formation of catalytically active urease even at extremely low nickel concentrations. This finding is compatible with the view that HoxN not only binds Ni^{2+} but also transports the divalent cation into the cytoplasm and therefore facilitates its incorporation. At elevated nickel concentrations in the range of 1 mM, the HoxN effect was much less obvious and urease activities exceeded 10 U/mg of protein even in the absence of HoxN (data not

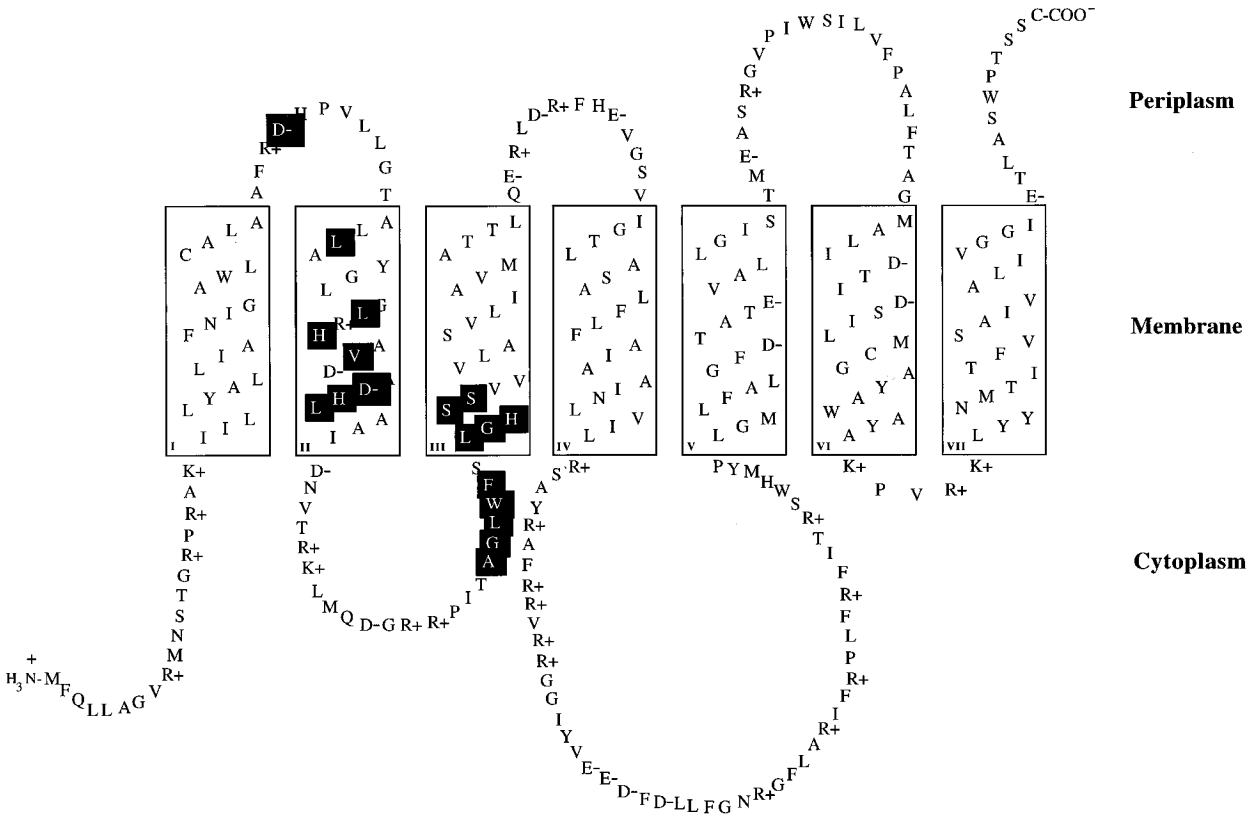

FIG. 4. Localization of putative nickel translocation motifs in the *A. eutrophus* protein HoxN. The topological model was reported previously (3). Identical or similar residues which were detected in related proteins are shown in black boxes.

shown). This was probably due to nickel uptake by nonspecific magnesium transport systems.

Sequence alignment of HoxN and related proteins. Very recently, the cloning and sequencing of *hoxN*-related genes of other bacteria whose products are essential under nickel-limiting conditions have been described. HupN of *Bradyrhizobium japonicum* is an integral membrane protein, exhibits high similarity to *A. eutrophus* HoxN (56% amino acid identity), and is required for the activity of hydrogenase (5). The homology between HoxN and HupN strongly suggests a function in nickel transport for *B. japonicum* HupN, but its exact role has not yet been established (5). The NixA protein mediates high-affinity nickel transport in *Helicobacter pylori* (15), a gastrointestinal pathogen producing a potent urease. NixA is highly similar to HoxN (41% identity). Sequence determination of the urease operon of thermophilic *Bacillus* sp. strain TB-90 revealed two accessory genes (*ureH* and *ureI*) which are required for urease activity under nickel limitation. Nickel transport was not further investigated, but since UreH shows significant similarity to *A. eutrophus* HoxN (23% identity), a role in nickel uptake has been postulated (13).

Potential nickel recognition motifs have not yet been reported for these transport proteins. To search for common motifs, we conducted a sequence comparison by multiple alignments. The result is shown in Fig. 3. Two conserved signatures were found to occur with very similar distances in HoxN, HupN, NixA, and UreH. Both signatures include histidine residues. Histidine motifs are generally considered potential Ni ligands. This has been shown for proteins with a function in nickel incorporation into hydrogenases and urease (4, 9). Thus, the stretches of amino acids shown in Fig. 3 may be involved in high-affinity nickel binding or in the translocation process. Interestingly, one of the conserved signatures was also detected in NikC, an integral membrane component of the *E. coli* nickel transport system (Fig. 3A). The localization of these putative nickel translocation motifs in *A. eutrophus* HoxN is illustrated in Fig. 4. One of the histidine-containing signatures lies within a transmembrane helix. The localization of that motif might be surprising if it were involved in nickel recognition. However, examination of well-characterized transport proteins such as the lactose (LacY) or melibiose (MelB) carrier of *E. coli* shows that important residues for sugar specificity and cation coupling are located in a narrow area in the middle of the transmembrane helices (LacY) or in cytoplasmic loops (MelB) (19). The identification of the putative nickel translocation motif in HoxN of *A. eutrophus* invites experiments to exchange specific amino acids which may lead to a more detailed insight into nickel ion transport.

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