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A novel transporter involved in cobalt uptake

(transport/metal/nitrile hydratase/membrane protein/nickel)

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ABSTRACT **Cobalt is an essential component of a low molecular-mass nitrile hydratase (L-NHase) from *Rhodococcus rhodochrous* J1. We have found a new gene, *nhlF*, in the DNA region sandwiched between *nhlBA* encoding L-NHase and *amdA* encoding amidase, which are involved in the degradation of nitriles. The product of *nhlF*, NhlF, shows a significant sequence similarity with those of *hoxN* from *Alcaligenes eutrophus*, *hupN* from *Bradyrhizobium japonicum*, *nixA* from *Helicobacter pylori*, and *ureH* from *Bacillus* sp., which are considered to be involved in nickel uptake into these cells. Sequence and hydropathy plot analyses have shown that NhlF encodes a 352-amino acid (aa) protein with eight hydrophobic putative membrane-spanning domains. *nhlF* expression in *R. rhodochrous* ATCC 12674 and *Escherichia coli* JM109 confers uptake of ^{57}Co in their cells, but not of ^{63}Ni. The expression of both *nhlF* and *nhlBA* in *R. rhodochrous* ATCC 12674 exhibited higher NHase activity than *nhlBA* expression. These findings together with the inhibitory effect by uncouplers (CCCP and SF6847) for the cobalt uptake suggest that NhlF mediates the cobalt transport into the cell energy-dependently finally to provide L-NHase.**

Cobalt is necessary as a trace element for all cells but is toxic at higher concentrations, a fact of considerable environmental importance. It is the central metal cofactor in the corrin ring of vitamin B_{12} (1) and also plays crucial roles in biological functions. Methionyl aminopeptidase, which catalyzes the removal of the initiator methionine from nascent polypeptide chains, contains cobalt ions in both prokaryotes and eukaryotes; the N-terminal modification caused by this enzyme appears to be involved in functional regulation, intracellular targeting, and protein turnover (2). Methylmalonyl-CoA carboxytransferase from *Propiobacterium shermanii* (3), glucose isomerase from *Streptomyces albus* (4), and lysine 2,3-aminomutase from *Clostridium subterminale* (5) are also cobalt-containing enzymes.

Several transition metals, which play an essential role as cofactors in many biochemical processes, must be transported into cells against concentration gradients—i.e., trace concentrations outside and substantial amounts within the cells. Divalent cations of Zn^{2+}, Co^{2+}, Ni^{2+}, and Cd^{2+} are transported into the cells by a broad-substrate-range Mg^{2+} transport system in *Alcaligenes eutrophus* (6). The transport of a broad range of metal ions by the relatively unspecific uptake system is an economical solution for most cells and allows the accumulation of trace elements inside the cells for future needs. On the contrary, there seem to be other transport systems for Zn^{2+}, Co^{2+}, and Ni^{2+} with higher ion selectivity (7, 8). A Ni-specific transporter has been identified as a part of the plasmid-encoded hydrogenase (a Ni-containing enzyme) gene cluster in *A. eutrophus* (9). However, there are no reports on the structure and function of a transporter involved in cobalt-specific uptake.

Cobalt is an essential component of two kinds of nitrile hydratase (NHase; EC 4.2.1.84), in the "practical" actinomycete *Rhodococcus rhodochrous* J1 (10), which catalyze the hydration of nitriles to the corresponding amides followed by their conversion to the acids and ammonia by amidase. One is a low molecular-mass NHase (L-NHase) (11), and the other is a high molecular-mass NHase (H-NHase) (12); the former is scheduled to be used for the industrial production of nicotinamide from 3-cyanopyridine, and the latter has been in use for the industrial production of acrylamide from acrylonitrile in Japan. In the presence of cobalt ions, L-NHase and H-NHase are selectively induced by cyclohexanecarboxamide and urea, respectively. Both L- and H-NHases exhibit different physicochemical properties and substrate specificities, and they are composed of α- and β-subunits (α differs in size from β in each case, and the α- and β-subunits of L-NHase differ from those of H-NHase) (10).

We have previously cloned and sequenced L- and H-NHase genes (*nhlBA* and *nhhBA*) from *R. rhodochrous* J1 (13). In both of *nhlBA* and *nhhBA*, an ORF for the β-subunit (*nhlB* and *nhhB*) is located just upstream of that for the α-subunit (*nhlA* and *nhhA*). However, the gene organization including *nhlBA* differs from that including *nhhBA* (11, 12). In the L-NHase gene cluster, we have found two ORFs (*nhlC* as a positive regulator and *nhlD* as a negative regulator required for the amide-dependent induction of *nhlBA*) (11). An amidase gene (*amdA*) (14) is located 1.9 kb downstream of *nhlA* (Fig. 1), and the expression of *nhlBA* and *amdA* is coordinately regulated (11).

In the present study, we report the identification of a gene, *nhlF*, which is situated between *nhlBA* and *amdA* and is similar to the bacterial genes encoding nickel transporters reported previously (9, 18–20). Furthermore, we present evidence that the product of *nhlF*, NhlF transports cobalt ions into *R. rhodochrous* and *Escherichia coli* host cells. Using a host-vector system in *Rhodococcus*, we have also characterized the transporter specific for cobalt ions.

MATERIALS AND METHODS

Strains, Plasmids, and Media. *E. coli* JM109 (15) was used as the host strain for recombinant plasmids. *R. rhodochrous* ATCC 12674 was the host for a *Rhodococcus–E. coli* shuttle vector pK4 (16) and its derivatives, and was used for the expression of the L-NHase gene (*nhlBA*) and the presumed cobalt transporter gene (*nhlF*). *R. rhodochrous* ATCC 12674 and the plasmid pK4 were kindly provided by Beppu's group (The University of Tokyo). *E. coli* transformants were grown

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Abbreviations: L-NHase, low molecular-mass nitrile hydratase; H-NHase, high molecular-mass nitrile hydratase; aa, amino acid.
Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. D83695).
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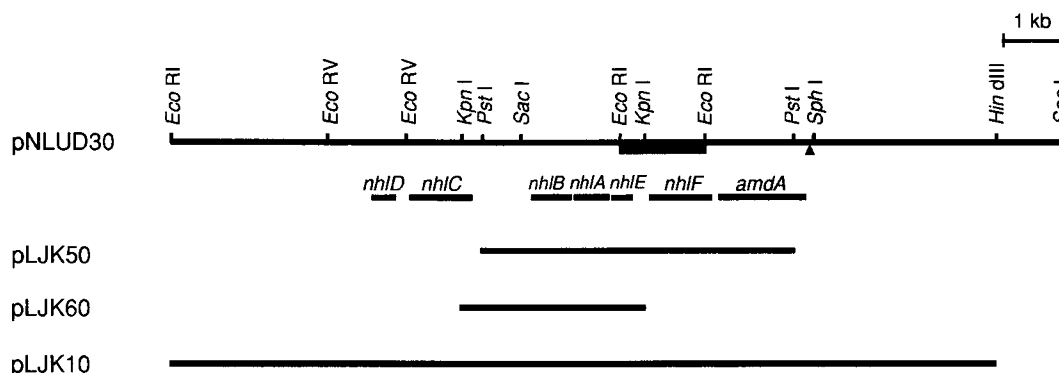

FIG. 1. Genetic organization of the L-NHase gene cluster and the constructed fragments. *nhlB* and *nhlA* are genes encoding L-NHase β- and α-subunit proteins, respectively (13). *nhlC* and *nhlD* have recently been found to be responsible for the amide-dependent L-NHase induction (11). *amdA* encodes the amidase (14). *nhlE* and *nhlF* are the newly identified genes described in this paper. ▲ , Putative transcription terminator (14). The thick line demonstrates the region corresponding to the sequence determined here.

in Luria–Bertani medium (15). *R. rhodochrous* ATCC 12674 transformants were grown in a medium which consisted of 10 g of glycerol, 5 g of polypeptone, 3 g of yeast extract, 3 g of malt extract, 1 g of KH_2PO_4, and 1 g of K_2HPO_4 (pH 7.0)/liter (MYP) (16).

Enzymes and Chemicals. Restriction endonucleases, calf intestine alkaline phosphatase, and T4 DNA ligase were purchased from Takara Shuzo (Kyoto). Isopropyl β-D-thiogalactopyranoside was obtained from Wako Pure Chemical (Osaka). [α-^{32}P]dCTP (110 TBq/mmol), [γ-^{32}P]ATP (180 TBq/mmol), $^{57}CoCl_2$ (17.3 TBq/mmol), and $^{63}NiCl_2$ (26.2 GBq/mmol) were from Amersham. Carbonylcyanide *m*-chlorophenylhydrazone was from Nakalai (Kyoto, Japan). 3,5-Di-*tert*-butyl-4-hydroxybenzilidenemalononitrile (SF6847) was kindly provided from H. Miyoshi (Kyoto University). All other chemicals were of the highest purity commercially available.

DNA Manipulation. DNA manipulation was performed essentially as described by Sambrook *et al.* (15). The DNA sequence was determined by the dideoxynucleotide chain termination method (17). [α-^{32}P]dCTP and Sequenase (United States Biochemical) or [γ-^{32}P]ATP and a *Tth* Sequence kit (Toyobo, Osaka) were used for sequencing.

Transformation of *R. rhodochrous* ATCC 12674 by Electroporation. A mid-exponential culture of *R. rhodochrous* ATCC 12674 was centrifuged at 6500 $\times$ *g* for 10 min at 4°C and washed three times with demineralized cold water. Cells were then concentrated 20-fold in demineralized cold water and kept on ice. Ice-cold cells (100 μl) were mixed with 1 μg DNA in 1 μl of TE buffer (10 mM Tris/1 mM EDTA, pH 8.0) in a 1-mm-gapped electrocuvette (Bio-Rad), and subjected to a 2.0-kV electric pulse from a Gene Pulser (Bio-Rad) connected to a pulse controller (25-μF capacitor; external resistance, 400 Ω). Pulsed cells were diluted immediately with 1 ml of MYP medium (16) and incubated for 2 h at 26°C. They were then spread on MYP medium containing 75 μg kanamycin/ml.

Preparation of Cell Suspension and Enzyme Assay. *R. rhodochrous* ATCC 12674 transformants were grown at 28°C for 24 h in MYP medium containing $CoCl_2 \cdot 6H_2O$ at several concentrations, harvested by centrifugation at 6500 $\times$ *g* at 4°C, and washed twice with 0.15 M NaCl. The washed cells were suspended in 0.1 M Hepes/KOH buffer (pH 7.2) containing 44 mM *n*-butyric acid. NHase activity was assayed as described (13).

Cobalt Uptake Experiment. Assay for cobalt uptake was performed using *R. rhodochrous* ATCC 12674 transformant cells grown in MYP medium without cobalt ions for 24 h at 28°C as described above. Cells were centrifuged at 6500 $\times$ *g* for 10 min at 4°C and washed twice with 150 mM NaCl. The washed cells were suspended in buffer A [50 mM Tris·HCl (pH 7.5) containing 10 mM $MgCl_2$] to a concentration of about 10 mg dry cell mass/ml. The reaction mixture (in 10 ml of buffer A) consisted of 10 nM $^{57}CoCl_2$ and the cell suspension (0.5 mg dry cell mass). Cells were preincubated for 5 min at 30°C before addition of $^{57}CoCl_2$ in a 30 ml Erlenmeyer flask. Cobalt uptake assays were initiated by the addition of $^{57}CoCl_2$ and performed at 30°C with shaking. To determine the cobalt content of the cells, 0.1 ml of the assay volume was taken at appropriate times and immediately passed through a membrane filter (pore size 0.45 μm; diameter 2.5 cm; Millipore). Cells were immediately washed on the filter thrice with 3 ml of buffer A. The filters were dried and counted in a γ counter (Packard).

Construction of *nhlF* Expression Plasmid. To express *nhlF* in *E. coli*, we improved the sequence upstream from the putative start codon (TTG, nucleotides 744–746 in the sequence registered by DDBJ/EMBL/GenBank accession number D83695) by PCR with pLJK50 as a template and two oligonucleotides (primers 1 and 2) as primers. Primer 1 (5′-CTGCAAGCTTTAAGGAGGAATAGCGTATGACC-AGCACCACCATTACAC-3′) contained a *Hin*dIII recognition site, a ribosome-binding site, a TAG stop codon in frame with the *lacZ* gene in pUC19 and 22 nucleotides (nucleotides 744–765 in the sequence of D83695) of *nhlF* with the ATG start codon instead of the TTG codon. Primer 2 (5′-GTAT-CTCGGTGGCTGCAGTGATCGTG-3′) contained 26 nucleotides of the gene (complementary to nucleotides 1810–1835 in the sequence of D83695) 8 nucleotides downstream from the end of the reading frame and a *Pst*I recognition site. DNA was amplified by PCR using a thermal cycler (Perkin–Elmer). Reaction mixtures contained 1 μg of template DNA, 100 pmol of each oligonucleotide pool, and *Thermus thermophilus* DNA polymerase (Toyobo) in a volume of 100 μl. Thirty thermal cycles consisted of 94°C for 1 min, 55°C for 1 min, and 75°C for 3 min each. The plasmids designated as pLCO10 and pLCO20 were constructed by ligation of the gel-purified and *Hin*dIII–*Pst*I-digested PCR product with pUC19/*Hin*dIII–*Pst*I and pSTV29/*Hin*dIII–*Pst*I, respectively, and were transformed into *E. coli* JM109.

Computer Analysis of Amino Acid Sequences. The DNA sequence was analyzed using the GENETYX sequence analysis program (Software Development, Tokyo). A search of the National Biomedical Research Foundation protein sequence data bank for sequence similarities was carried out with the BLAST algorithm.

RESULTS

Primary Structure of the Intervening Region Among *nhlBA* and *amdA*. We previously cloned and sequenced a 1.73-kb *Sac*I–*Eco*RI region containing *nhlBA* (13) and a 1.96-kb

*Eco*RI–*Sph*I region containing *amdA* (14) from *R. rhodochrous* J1; *nhlA* and *amdA* are separated by the 1.9-kb intervening region (Fig. 1). Both enzyme genes are coordinately regulated by the positive regulator (*nhlC*) and the negative regulator (*nhlD*), which are located upstream of *nhlB* (11). Here, we determined the nucleotide sequence of the 1.5-kb *Eco*RI intervening region between *nhlA* and *amdA*, and found two adjacent ORFs (*nhlE* and *nhlF*) in the region (Fig. 1). The presumptive ATG start codon was found for *nhlE*, but the TTG initiation codon was for *nhlF*. *nhlE* and *nhlF* were preceded by Shine–Dalgarno sequences located within reasonable distances from the respective presumptive start sites. The first ORF (*nhlE*) located just downstream from *nhlA* is 447 nucleotides long, and would encode a protein of 148 aa (16,887 Da). NhlE showed a low similarity of amino acid sequence with NhhG (12) from *R. rhodochrous* J1 (35.6% identity) (data not shown); *nhhG* is also located just downstream from *nhhA* encoding the H-NHase α-subunit protein, and its function has not yet been determined. The second ORF named *nhlF*, is 1059 nucleotides long, and would encode a highly hydrophobic polypeptide of 352 aa (37,187 Da); the deduced amino acid sequence of NhlF includes a substantial number of hydrophobic residues (63%). A search of the protein sequence databases revealed weak but significant sequence similarity between NhlF and nickel transporters such as HoxN (9) from *A. eutrophus* (36.1% identity), HupN (18) from *Bradyrhizobium japonicum* (37.8% identity), NixA (19) from *Helicobacter pylori* (37.8% identity), and UreH (20) from *Bacillus* sp. (16.9% identity) (Fig. 2). *hoxN* and *hupN* are located in each nickel-containing hydrogenase gene cluster, and *ureH* is located in the nickel-containing urease gene cluster. *nixA* is isolated as the gene complementing urease activity in *E. coli* harboring urease structural genes under nickel limitation; this gene is not closely linked to the urease gene cluster.

NHase Activity Under Cobalt Limitation. We examined the effect of *nhlF* on the activity of cobalt-dependent L-NHase using the *Rhodococcus–E. coli* host-vector system. As shown in Fig. 1, plasmid pLJK50 contains *Pst*I fragment (5.5 kb) covering intact *nhlBAEF* and a part of *amdA* on the *Rhodococcus–E. coli* shuttle vector pK4, and pLJK60 contains *Kpn*I fragment (3.1 kb) covering a part of *nhlC* and intact *nhlBAE* on pK4. We transformed each plasmid into *R. rhodochrous* ATCC 12674 as a host strain and cultured the resultant transformants in the medium changing final concentrations of $CoCl_2$. NHase assays using benzonitrile as a substrate for each cell suspension prepared as described in *Materials and Methods* demonstrated that the presence of *nhlF* yields catalytically active NHase even at very low cobalt concentrations (1–5 μM) (Fig. 3); in particular, *nhlF* increased NHase activity 3.7-fold, in the case of 1 μM of $CoCl_2$. These findings, together with the position of *nhlF* close to the cobalt-containing L-NHase gene (*nhlBA*) and the similarity in the amino acid sequence between NhlF and the bacterial nickel transporters, suggest that NhlF would be a transport protein that mediates uptake of cobalt ions into the cell.

In the case of 5 μM of $CoCl_2$, however, NHase activity with the pLJK50-containing transformant was only 2-fold compared with the pLJK60-containing transformant. Furthermore, both transformants showed almost the same NHase activities when they were cultured in the medium supplemented with 0.001% $CoCl_2$ (wt/vol) (data not shown), corresponding to 42 μM of $CoCl_2$, which is the optimum concentration for the NHase formation in *R. rhodochrous* J1 (23). This indicates the presence of nonspecific transport system with low affinity for cobalt ions in the *R. rhodochrous* ATCC 12674 host strain.

Cobalt Uptake of *R. rhodochrous* ATCC 12674 Transformants. The synthesis of catalytically active NHase by the transformant harboring pLJK50 led us to examine whether NhlF could function as a cobalt transporter. We measured $^{57}Co^{2+}$ uptake of the *Rhodococcus* transformants. Cell suspensions of *R. rhodochrous* ATCC 12674 containing either pLJK50, pLJK60, or pK4 were prepared. Uptake of Co^{2+} was determined by the addition of $^{57}CoCl_2$ (final concentration, 10 nM) to the cell suspension followed by vacuum filtration after 5, 10, 15, 20, and 25 min. Identical assay without the cells showed that nonspecific binding of $^{57}Co^{2+}$ to the membrane filter was negligible. As illustrated in Fig. 4*A*, the presence of *nhlF* significantly increased cobalt uptake.

Effects of Uncouplers and Divalent Cations on Cobalt Uptake. The effects of uncouplers on the *nhlF*-conferred cobalt uptake were examined. Carbonylcyanide *m*-chlorophenylhydrazone as a protonophore was added to the cell suspension of the *Rhodococcus* transformant harboring pLJK50, 10 min prior to the addition of $^{57}CoCl_2$. Carbonylcyanide *m*-chlorophenylhydrazone at the final concentration of 1 μM and

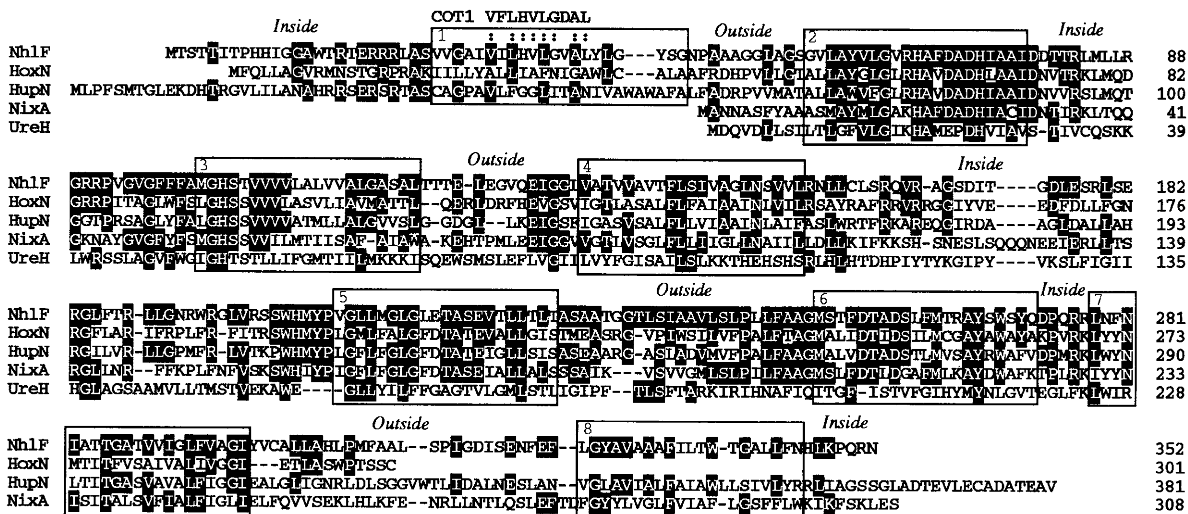

FIG. 2. Alignment of amino acid sequences among NhlF from *R. rhodochrous* J1 and homologous proteins. Amino acid sequences of NhlF from *R. rhodochrous* J1, HoxN from *A. eutrophus* (9), HupN from *B. japonicum* (18), NixA from *H. pylori* (19), and UreH from *Bacillus* sp. (20) were aligned by introducing gaps (hyphens) to achieve maximum homology. Residues in solid boxes indicate identical sequences. Putative transmembrane or membrane-associated domains (1–8) is enclosed by boxes. The orientation (inside, internal; outside, external) of the nonmembrane loop regions, predicted by the "positive inside rule" (21) is shown above the sequence. Highly conserved residues between NhlF and COT1 from *S. cerevisiae* (22) are marked with colons (:).

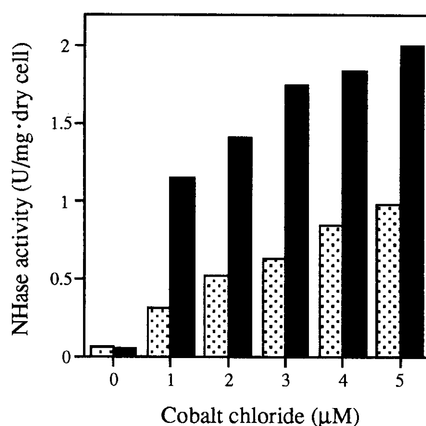

FIG. 3. Effect of *nhlF* on NHase activity of the *R. rhodochrous* ATCC 12674 transformants. Each transformant was grown for 24 h at 28°C in MYP medium containing $CoCl_2$ as indicated. The NHase activity was measured as described. Solid boxes, *R. rhodochrous* ATCC 12674/pLJK50; dotted boxes, *R. rhodochrous* ATCC 12674/pLJK60.

10 μM in the reaction mixture inhibited the uptake by 25% and 85%, respectively, after 25 min of the reaction time. Since carbonylcyanide *m*-chlorophenylhydrazone is known to exhibit side effects—i.e., blockage for sulfhydryl groups in membrane proteins (24, 25)—the effect of an uncoupler 3,5-di-*tert*-butyl-4-hydroxybenzilidenemalononitrile (SF6847) on the cobalt uptake was investigated. 3,5-Di-*tert*-butyl-4-hydroxybenzilidenemalononitrile at the final concentration of 0.1 μM, 1 μM, and 10 μM inhibited the uptake by 55%, 85%, and 85%, respectively, after the reaction time of 25 min. These findings demonstrate that proton gradients are involved in the cobalt uptake conferred by *nhlF*.

Effect of other divalent cations such as Mn^{2+}, Fe^{2+}, Ni^{2+}, and Cu^{2+} on the cobalt uptake of the *R. rhodochrous* ATCC 12674 transformant containing pLJK50 was examined. The measurement of the cobalt uptake in each condition showed that none of each Mn^{2+}, Fe^{2+}, or Cu^{2+} affected the cobalt uptake, while the addition of Ni^{2+} led a marked decrease of the cobalt uptake (Fig. 4*B*).

We next examined $^{63}Ni^{2+}$ uptake activity in the *Rhodococcus* transformants by the same method as in the case of the $^{57}Co^{2+}$ uptake experiments with the exception that $^{63}NiCl_2$ replaced $^{57}CoCl_2$. However, the pLJK50-containing transformant did not show this activity; and no difference was observed between this transformant and the control (the *Rhodococcus* transformants harboring pLJK60 or pK4).

Expression of the Cobalt Transporter in *E. coli*. Plasmid pLJK50 containing *nhlBAEF* and a part of *amdA* conferred the energy-dependent cobalt uptake upon the *Rhodococcus* host, whereas pLJK60 containing a part of *nhlC* and *nhlBAE* did not. These observations suggest that NhlF is a single component responsible for the cobalt uptake. To test this possibility, *nhlF* modified in the sequence upstream from its presumptive start codon was introduced into *E. coli* JM109, and cobalt uptake activity in the resultant *E. coli* transformants was investigated by the same method as in the case of the *R. rhodochrous* ATCC 12674 transformants with the exception that *E. coli* transformants replaced *Rhodococcus* transformants.

To enhance *nhlF* expression in *E. coli*, we at first altered the sequence upstream from TTG start codon by PCR as described in *Materials and Methods*. The resultant *nhlF* was introduced into a high-copy-number plasmid pUC19 or a low-copy-number plasmid pSTV29, resulting in the construction of pLCO10 or pLCO20, respectively. The *E. coli* transformants harboring each pLCO10, pLCO20, and pUC19 were cultured in Luria–Bertani medium supplemented with 1 mM of isopropyl β-D-thiogalactopyranoside for 12 h at 28°C. Cells were harvested, and the cell suspensions were prepared by the method as in the case of the *Rhodococcus* transformants. The cobalt uptake experiments revealed that each of the *nhlF*-containing plasmids (pLCO10 and pLCO20) confers significantly cobalt uptake activity upon the *E. coli* strain (Fig. 4*C*), suggesting that only *nhlF* is required for the functional cobalt uptake and that the NhlF polypeptide folded in a functionally active form. The uptake activity seemed to be independent of the copy number of the plasmids within the *E. coli* cells.

DISCUSSION

The present study on the nucleotide sequence of the intervening region between *nhlBA* and *amdA* suggests that *nhlF* located in this region possesses significant similarities to the previously known genes encoding potential nickel transporters from Gram-negative and Gram-positive bacteria. We also found that *nhlF* significantly enhances *nhlBA*-derived L-NHase activity in *Rhodococcus* transformants in the cobalt-limiting conditions and that *nhlF* on the plasmid confers the cobalt uptake activity upon *Rhodococcus* and *E. coli* hosts. The studies using the uncouplers revealed that proton gradients are

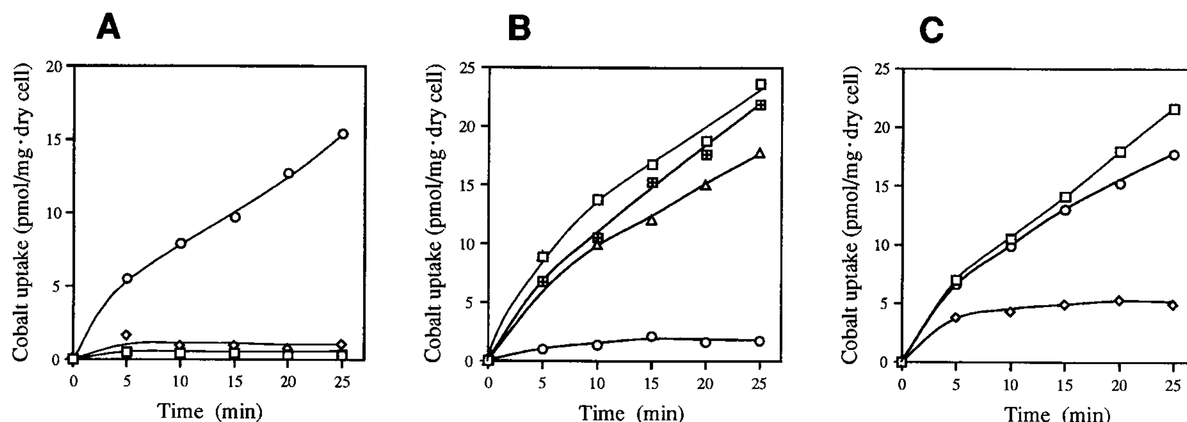

FIG. 4. Examination of cobalt uptake in the *R. rhodochrous* or *E. coli* transformants. (*A*) Cobalt uptake of the *R. rhodochrous* ATCC 12674 transformants. The reaction mixture consisted of 10 nM $^{57}CoCl_2$, 10 mM $MgCl_2$, and the cells in a 50 mM Tris·HCl buffer (pH 7.5). ○, *R. rhodochrous* ATCC 12674/pLJK50; ◇, *R. rhodochrous* ATCC 12674/pLJK60; □, *R. rhodochrous* ATCC 12674/pK4. (*B*) Effect of other transient metals on the cobalt uptake of the *R. rhodochrous* ATCC 12674/pLJK50. Each metal was added at the final concentration of 5 μM, 10 min before the addition of $^{57}CoCl_2$ into the cell suspension. □, $MnCl_2$; △, $FeSO_4$; ○, $NiCl_2$; ⊞, $CuSO_4$. (*C*) Cobalt uptake by the *E. coli* JM109 transformants. The reaction mixture consisted of 10 nM $^{57}CoCl_2$, 10 mM $MgCl_2$ and the cells in a 50 mM Tris-hydrochloride buffer (pH 7.5). □, *E. coli* JM109/pLCO10; ○, *E. coli* JM109/pLCO20; ◇, *E. coli* JM109/pUC19.

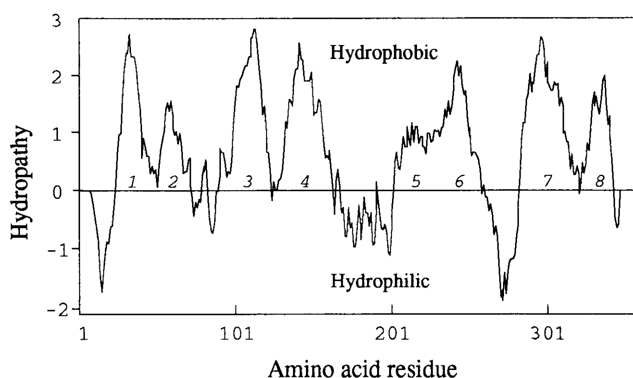

FIG. 5. Hydropathy plot of NhlF. Hydropathy was calculated for NhlF by using the algorithm of Kyte and Doolittle (27) with a window of 15-aa residues.

involved in the cobalt transport. These findings suggest that NhlF energy-dependently mediates the transport of cobalt ions into the cell and therefore facilitates its incorporation into the L-NHase enzyme.

It is interesting to note that *nhlF* is a part of the L-NHase gene cluster, which contains the structural genes (*nhlBA* and *amdA*) involved in sequential nitrile metabolism by the combination of L-NHase and the amidase (10), together with the regulatory genes (*nhlC* and *nhlD*) responsible for the amide-dependent induction of both enzymes (11). The cobalt uptake experiments using the *Rhodococcus* transformant harboring pLJK10 (see Fig. 1) cultured in the medium supplemented with or without an inducer crotonamide for the formation of L-NHase and the amidase showed that NhlF activity appeared only in the presence of crotonamide (data not shown). These findings suggest the coordinate regulation of *nhlBA*, *nhlF*, and *amdA*, which is probably due to a cotranscription of these genes in a single mRNA, consistent with the presence of ρ-independent potent transcriptional terminator found in the downstream region of *amdA* (14) and with the absence of such a sequence between *nhlA* and *nhlF* or *nhlF* and *amdA*.

The proteins homologous to NhlF are directly or indirectly shown to be involved in the uptake of nickel ions. Among these nickel transporters, HoxN from *A. eutrophus* is most intensively characterized. In *A. eutrophus*, two transport systems for nickel ions exist (7): a nonspecific high-capacity magnesium transport system and a high-affinity low-capacity nickel transporter (HoxN). Nickel uptake by the magnesium transport system was competitively inhibited by Mg^{2+}, Mn^{2+}, Zn^{2+}, and Co^{2+}, whereas the activity of the HoxN-mediated transport system was inhibited only by Co^{2+} (26). In this study, we demonstrated that the *nhlF*-dependent cobalt uptake activity is markedly inhibited by the addition of excess Ni^{2+} to the cell suspension; the other metals tested—i.e., Mn^{2+}, Fe^{2+}, and Cu^{2+} had no effect on the cobalt uptake. These observations suggested the possibility that NhlF might be responsible for nickel uptake as well as cobalt uptake and also that HoxN is involved in cobalt uptake as well as nickel uptake; unfortunately, HoxN has never been reported to be involved in the cobalt uptake in *A. eutrophus*. However, we have never been able to detect nickel uptake by the pLJK50-containing *Rhodococcus* using radioactive $^{63}NiCl_2$, which indicates that nickel ion is not a substrate for NhlF. Consequently, NhlF is a cobalt-specific transporter.

A hydropathy plot (27) of the amino acid sequence of NhlF and the topological model by the "positive inside rule" (21) revealed that NhlF is a markedly hydrophobic protein with the orientation of locating N terminus in the cytoplasm (Fig. 2) and contains eight transmembrane helices (Fig. 5). Alignment of amino acid sequences of NhlF and its related nickel transporter proteins (9, 18–20) (Fig. 2) provides some information about the structure of NhlF. Wolfram *et al.* (28) presented two segments conserved among the nickel transporters as potent domains involved in the high-affinity nickel binding or in the translocation process; their positions are from aa 44–72 and from aa 89–99 of HoxN. Both segments include histidine residues (aa 62, 68, and 97 of HoxN), which are generally considered to be potential metal ligands. The regions corresponding to both segments are highly conserved in NhlF, and the above-mentioned histidine residues also exist in NhlF (aa 68, 74, and 103 of NhlF). Compared with the nickel transporters, the following distinct different amino acid residues appear to be unique to the corresponding sequence of NhlF; histidine (aa 10), tryptophan (aa 15), tyrosine (aa 44), alanine (aa 50), leucine (aa 87), threonine (aa 143, 254, and 284), arginine (aa 179), serine (aa 200). On the other hand, the different set of amino acids are found among the nickel transporters. Therefore, these amino acid residues may be involved in the cobalt-specificity.

nhlF also showed very little sequence similarity with *COT1* (22, 29), which has been isolated as a suppressor of cobalt toxicity by sequestration or compartmentalization within the mitochondria of cobalt ions that cross the plasma membrane in *Saccharomyces cerevisiae*. As shown in Fig. 2, helix 1 of NhlF contains a segment highly similar to a segment in helix 5 of COT1 with six membrane-spanning domains (8 of 10 residues are identical); but, there is no similarity throughout the sequence except this segment. In this homologous region, both NhlF and COT1 contain a histidine residue that is a potential metal-binding amino acids, but neither HoxN nor HupN contains histidine at the corresponding site, suggesting the functional role for the cobalt-specific recognition. On the other hand, there is no sequence similarity between NhlF and COT2 (presently GRRI): the latter has recently been reported not to be responsible for the cobalt transport but may play a more general role in yeast physiology that indirectly controls the permeability of the membrane to cobalt ions or the driving force for the uptake in *S. cerevisiae* (29, 30).

NhlF showed no sequence similarity to CorA Mg^{2+} transport system, which mediates influx of Co^{2+} as well as those of Mg^{2+} and Ni^{2+} (31). NhlF also exhibited no sequence similarity to the proteins involved in the active efflux system of broad specificity for metals of Ca^{2+}, Zn^{2+}, and Co^{2+}, which have been characterized in detail by genetic analyses on resistance of these metals in *A. eutrophus* (18, 32, 33).

L- and H-NHases are selectively produced in *R. rhodochrous* J1 cultured only in the presence of cobalt ions with each inducer (10). Both purified enzymes contain cobalt atoms as a prosthetic metal and require this divalent cation for the catalytically active enzyme; these cobalt atoms bind tightly to the enzyme and are not released from the protein even after dialysis for 5 days (34). No other metals such as Ni and Fe, the latter of which is a cofactor of NHases from *Pseudomonas chlororaphis* B23 (35) and *Brevibacterium* sp. R312 (36), can replace cobalt ions in both NHases. To provide the NHase enzyme with sufficient cobalt, the metal ions are actively transported into the *R. rhodochrous* J1 cells by NhlF. Further characterization of NhlF will be invaluable for analyzing not only nitrile metabolism, which has received increasing broad interest in both academic and applied fields such as biosynthesis of plant hormone (37), biotransformation (10), and bioremediation, but also the molecular basis of cobalt homeostasis in all forms of living organisms.

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