JOURNAL OF BACTERIOLOGY, Mar. 1989, p. 1340–1345
0021-9193/89/031340-06$02.00/0

Vol. 171, No. 3

Genetic Determinants of a Nickel-Specific Transport System Are Part of the Plasmid-Encoded Hydrogenase Gene Cluster in *Alcaligenes eutrophus*

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Received 6 September 1988/Accepted 26 November 1988

Nickel-deficient (Nic^-) mutants of *Alcaligenes eutrophus* requiring high levels of nickel ions for autotrophic growth with hydrogen were characterized. The Nic^- mutants carried defined deletions in the hydrogenase gene cluster of the indigenous pHG megaplasmid. Nickel deficiency correlated with a low level of the nickel-containing hydrogenase activity, a slow rate of nickel transport, and reduced activity of urease. The Nic^+ phenotype was restored by a cloned DNA sequence (*hoxN*) of a megaplasmid pHG1 DNA library of *A. eutrophus* H16. *hoxN* is part of the hydrogenase gene cluster. The nickel requirement of Nic^- mutants was enhanced by increasing the concentration of magnesium. This suggests that the Nic^- mutants are impaired in the nickel-specific transport system and thus depend on the second transport activity which normally mediates the uptake of magnesium.

Bartha and Ordal (1) were the first to report a specific nickel requirement for chemolithoautotrophic growth of *Hydrogenomonas* strains. Later it was shown that nickel is essential for catalytically active hydrogenase of *Alcaligenes eutrophus* (formerly *Hydrogenomonas eutropha*) (6) and is incorporated into the enzyme during protein synthesis (8). Subsequently, nickel was shown to be an essential trace element for many microorganisms, in which it is involved in at least four biological processes: hydrolysis of urea, hydrogen uptake and hydrogen evolution, methanogenesis, and acetogenesis (9).

In order to provide the cells with sufficient nickel, the metal ion has to be actively transported into the bacterial cell. Two energy-dependent processes have been reviewed recently (9). Nickel ion transport may be catalyzed by a magnesium transporter and by a high-affinity, nickel-specific system. The latter was first shown to exist in strains of *A. eutrophus* (26). Lohmeyer and Friedrich (17) demonstrated that both transport components are active in this bacterium.

A. eutrophus contains three nickel-containing enzymes: soluble NAD^+-reducing hydrogenase (22) (EC 1.12.1.2), membrane-bound hydrogenase which is linked to the respiratory chain and does not reduce NAD^+ (19), and urease (16). The four structural genes of soluble hydrogenase (*hoxS*) and the two structural genes of membrane-bound hydrogenase (*hoxP*) are located on a large 450-kilobase-pair (kb) plasmid of *A. eutrophus* H16. A regulatory gene (*hoxC*) whose product is essential for *hox* gene expression is a constituent of the *hox* gene cluster (4).

We have described plasmid deletion mutants which are devoid of *hoxC* and *hoxP* genes but retain the *hoxS* DNA sequence (15). From analysis of mutants bearing structural gene mutations in *hox*, it was concluded that the presence of soluble hydrogenase (HoxS) is sufficient to allow autotrophic growth with hydrogen (10). Thus, by introducing the *hoxC* DNA sequence into *hoxS*-retaining *hoxP* deletion mutants, the resulting transconjugants should be able to grow autotrophically with hydrogen due to the HoxS protein. Unexpectedly, this was not the case under standard conditions (15).

In the present communication, we demonstrate that the deletion mutants have a higher nickel ion requirement (Nic^-) than the wild type. They immediately start growing and expressing HoxS activity when the nickel ion concentration in the medium is increased. This nickel deficiency is shown to be caused by the deletion of a plasmid-located nickel transport-associated DNA sequence.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids are listed in Table 1.

Growth conditions. Strains of *Alcaligenes eutrophus* were grown lithoautotrophically in mineral salts medium as described before (20) with the following modifications: ferric ammonium citrate was replaced by ferric chloride, and the trace element solution was prepared without adding nickel chloride. $NiCl_2$ and $MgSO_4$ were supplied at concentrations indicated in the text. The final sulfate concentration in the medium was kept constant by the addition of K_2SO_4. The gaseous atmosphere consisted of hydrogen, oxygen, and carbon dioxide in a ratio of 8:1:1 (vol/vol/vol). For heterotrophic cultivation of the cells, the mineral salts medium contained 0.4% (wt/vol) fructose (FN-medium) or 0.2% (wt/vol) fructose and 0.2% glycerol (FGN-medium). The cells were incubated under air on a rotary shaker at 30°C. Growth was monitored with a Klett-Summerson colorimeter equipped with a 520- to 540-nm filter.

The characterization of nickel-deficient mutants and their derivatives was conducted as follows. $NiCl_2$-free FN-medium (10 ml) was inoculated with cells from an agar plate. The medium for growing transconjugants was routinely supplied with 7.5 μg of tetracycline per ml. The cells were grown overnight and diluted in 30 ml of FN-medium as specified above to an optical density (OD) of approximately 35 Klett units. After growth to mid-exponential phase, the cells were washed once with 36 mM sodium potassium phosphate buffer, pH 7.0. Mineral salts medium (10 ml) with additions of Ni^{2+} and Mg^{2+} at concentrations specified in the

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TABLE 1. Strains used

Strain	Plasmid[a]	Relevant characteristics[b]	Reference or source
A. eutrophus			
H20	pHG7	HoxS HoxP Nic	ATCC 17700
H16	pHG1	HoxS HoxP Nic	ATCC 17699
HF47	pHG1Δ1	$HoxS^-$ $HoxP^-$ Nic^-	24
H20F10	pHG7Δ1	$HoxS^-$ $HoxP^-$ Nic^-	15
H20F13	pHG7Δ1::pCH125, pGE4	HoxS HoxP Nic	This study
H20F11	pHG7Δ2	$HoxS^-$ $HoxP^-$ Nic^-	This study
E. coli S17-1	None	*pro thi hsdS rpsL*, RP4-Tra functions	23
	pVK101	Tc^r Km^r	14
	pSUP202	Tc^r Cm^r Ap^r	23
	pGE4	*hoxC*, Tc^r Km^r	4
	pCH125	*hoxN*, Tc^r Ap^r Cm^s	This study

[a] pGE4 is a pVK101 derivative, and pCH125 is a pSUP202 derivative.
[b] HoxS, Soluble hydrogenase; HoxP, particulate hydrogenase; Nic, high-affinity Ni^{2+} transport; Tc, tetracycline; Km, kanamycin; Cm, chloramphenicol; Ap, ampicillin; *hoxC*, hydrogenase DNA of regulatory function; *hoxN*, nickel transport DNA.

text was inoculated to an OD of 30 Klett units and incubated lithoautotrophically for 2 days.

Complementation and marker rescue experiments. Vector and hybrid plasmids were transferred from *Escherichia coli* S17-1 to strains of *A. eutrophus* by conjugation. Spot matings were performed as described previously (4). Transconjugants were selected either heterotrophically on FN-agar supplemented with 15 μg of tetracycline per ml or lithoautotrophically on mineral salts agar with 0.08 μM $NiCl_2$.

Enzyme assays. The activity of soluble hydrogenase (EC 1.12.1.2) was determined with whole cells as described before (6). Units are defined as micromoles of $NADH_2$ formed per minute per milligram of protein. The cells were cultivated as specified in the Results section. For urease (EC 3.5.1.5) assays, the cells were grown heterotrophically in mineral salts medium with 0.5% (wt/vol) sodium pyruvate as the carbon source. Instead of ammonium chloride, 0.1% (wt/vol) potassium nitrate was used as the nitrogen source. The concentration of Mg^{2+} was 1.6 mM; Ni^{2+} was added as indicated. The cells were harvested, washed twice with 50 mM potassium phosphate buffer, pH 7.5, and concentrated 10-fold to yield an approximate protein concentration of 4 to 5 mg/ml. The reaction mixture contained 0.8 ml of 50 mM potassium phosphate buffer, pH 7.5, 0.01 ml of 0.5% (wt/vol) cetyl-*N*,*N*,*N*-trimethylammonium bromide, and 0.1 ml of cell suspension. After 5 min of incubation at 30°C, the reaction was started with 0.1 ml of a freshly prepared solution of 50 mM urea. Urease activity was determined by measuring the amount of ammonium produced from urea within a defined period of time. Ammonium was determined by the method of Fawcett and Scott (5). Urease activity was expressed as micromoles of urea consumed per minute per milligram of protein. Protein was determined by the method of Lowry et al. (18) with bovine serum albumin as the standard.

Nickel transport. Cells were grown heterotrophically in the standard mineral salts medium with 0.08 μM $NiCl_2$ and 0.81 mM $MgSO_4$. Fructose and glycerol were the carbon sources. After 48 h of incubation, the cells were washed once with test buffer (50 mM Tris hydrochloride, pH 7.5) and suspended in the buffer to yield a final protein concentration of 0.3 to 0.5 mg/ml. After shaking for 5 min at 30°C, 10 ml of the cell suspension was added under standard conditions to a mixture of 5 μl of $^{63}NiCl_2$ (691 Ci/mol, 25.5 μCi/ml) and 100 μl of 100 mM $MgCl_2$ in a 50-ml Erlenmeyer flask, and shaking was continued. The final Ni^{2+} concentration was 18 nM. To determine the amount of nickel taken up by the cells, 0.5 ml of the reaction mixture was passed through a membrane filter (pore size, 0.45 μm; diameter, 25 mm; Schleicher & Schuell, Dassel, Federal Republic of Germany [FRG]). Nonspecific binding of Ni^{2+} to the cell surface and the filter membrane was kept at a minimum by two washes with test buffer. After this procedure, approximately 3% of the total $NiCl_2$ was found to bind nonspecifically, and this value was thus subtracted from the measured values. Washing the filters with EDTA did not decrease the amount of nonspecifically bound nickel chloride. Addition of cold nickel chloride caused a rapid exchange reaction and prohibited kinetic analysis. Filters were transferred to 5 ml of scintillation fluid (Quickszint 402; Zinsser, Frankfurt, FRG) and acidified with 50 μl of 5 N hydrochloric acid by the method of Lohmeyer and Friedrich (17). Samples were counted with a Beckman LS 3801 liquid scintillation counter equipped with a specific program for ^{63}Ni. Protein was determined by the method of Lowry et al. (18) with bovine serum albumin as the standard. The standard solution was prepared with test buffer to correct for Tris hydrochloride interference in the assay. The rate of nickel transport is expressed in picomoles nickel ions taken up per milligram of protein.

Chemicals. $^{63}NiCl_2$ was obtained from Amersham Buchler (Braunschweig, FRG), biochemicals were from C. F. Boehringer & Soehne (Mannheim, FRG), and tetracycline was purchased from Sigma (Munich, FRG). All other chemicals were from E. Merck (Darmstadt, FRG). Gases were obtained from Messer-Griesheim (Frankfurt, FRG).

RESULTS

Lithoautotrophic growth and hydrogenase activity in nickel-deficient mutants. The plasmid deletion mutants used in this study were obtained either spontaneously (H20F11) or in the course of transposon mutagenesis (24). The extent of deleted DNA in the mutant plasmids is indicated by open bars in Fig. 1. The deletion maps were deduced from the restriction pattern of purified megaplasmid DNA and constructed as described previously (15). The map positions of the structural genes of soluble hydrogenase (*hoxS*), particulate hydrogenase (*hoxP*), and the regulatory DNA sequence (*hoxC*) are marked as dark sections (Fig. 1).

Mutant HF47(pGE4), which contains *hoxC* on a broad-host-range plasmid, was chosen as a representative to demonstrate the elevated nickel requirement for autotrophic growth with hydrogen (Fig. 2). The other mutants used in this study behaved similarly (data not shown). Although

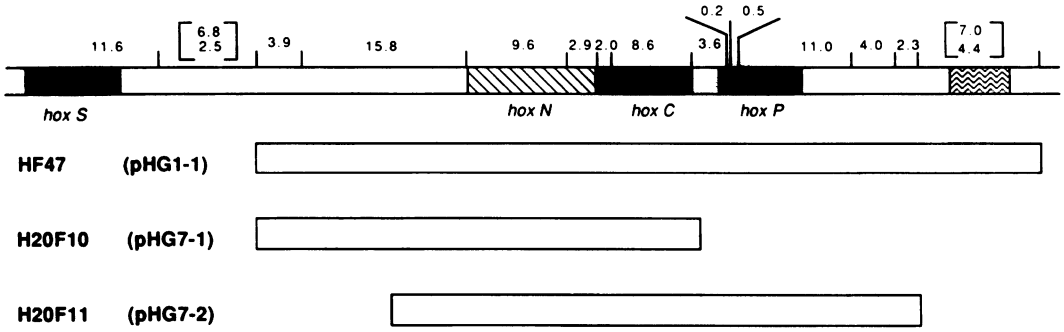

FIG. 1. Genetic map of the hydrogenase gene cluster of megaplasmid pHG1 from *A. eutrophus* H16. *hoxS*, Structural genes of soluble hydrogenase; *hoxC*, regulatory *hox* gene sequence; *hoxP*, structural genes of membrane-bound hydrogenase; *hoxN*, nickel transport. The open bars represent the extent of deletions in the mutants. The numbers above the bar indicate the sizes of *Eco*RI fragments in kilobase pairs; those in brackets indicate the sizes of fragments whose order is still unknown. The wavy-hatched bar is the site of homologous recombination.

traces of nickel in the mineral salts medium were sufficient to reach maximal OD with the wild-type strain H16, the activity of soluble hydrogenase (HoxS) clearly responded to nickel starvation (Fig. 2). However, HoxS activity in mutant HF47(pGE4) was even more reduced under these conditions, allowing almost no growth with hydrogen.

Nickel transport in Nic$^-$ mutants. Nickel uptake in *A. eutrophus* has been shown to occur via two pathways (17). Thus, nickel deficiency in the plasmid deletion mutants may result from a defect in one of the two transport components. To test this hypothesis, a metal competition experiment was conducted. *A. eutrophus* H20 and the two deletion mutants H20F10(pGE4) and H20F11(pGE4) derived therefrom were grown lithoautotrophically in mineral salts medium supplemented with 0.1 μM $NiCl_2$ and various concentrations of $MgSO_4$. The results (Fig. 3) clearly demonstrate that growth of the mutants with hydrogen was inhibited by Mg^{2+}. As expected, the level of active HoxS protein was low. Mutant H20F11(pGE4) appeared to be less sensitive to Mg^{2+}; mutant HF47(pGE4) revealed the same inhibition pattern (data not shown) as strain H20F10(pGE4) (Fig. 3).

The rate of nickel ion transport was determined by measuring the amount of $^{63}Ni^+$ taken up by the cells within 15 min. The kinetic data obtained with *A. eutrophus* H16 and its mutant derivative HF47(pGE4) are shown in Fig. 4A. It is evident that the rate of nickel transport in the mutant was lower than that in the wild type. Consistent data were obtained with *A. eutrophus* H20 and the two Nic$^-$ mutants derived from this parent strain (Fig. 4B). It was reproducibly found that nickel transport by strain H20 was less active than that by the H16 wild type, although the strains were otherwise very much alike. Lohmeyer and Friedrich (17) reported an affinity constant of 0.34 μM nickel chloride for magnesium-independent transport in *A. eutrophus* H16. This value was obtained with phosphate-containing mineral salts medium; the assay used in the present study was conducted in a Tris hydrochloride buffer in the presence of magnesium chloride. Under these conditions, the K_m for the high-affinity transport in strain H16 was 20 nM nickel chloride.

Restoration of the Nic$^-$ phenotype by a cloned DNA insert of megaplasmid pHG1. The deletion mutants were characterized by lack of a DNA sequence of approximately 20 kb which maps in the internal region of the *hox* gene cluster (Fig. 1). Up to this stage, no specific function could be assigned to this particular plasmid DNA sequence. Part of this region was detected in the megaplasmid pHG1 library of

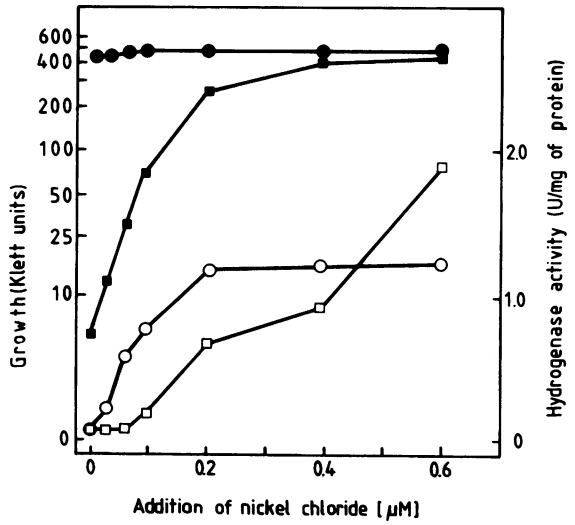

FIG. 2. Lithoautotrophic growth (solid symbols) and hydrogenase activity (open symbols) in response to the addition of nickel chloride to the medium. Circles, *A. eutrophus* H16 wild type; squares, mutant HF47(pGE4) derived therefrom. The concentration of $MgSO_4$ was 0.81 mM.

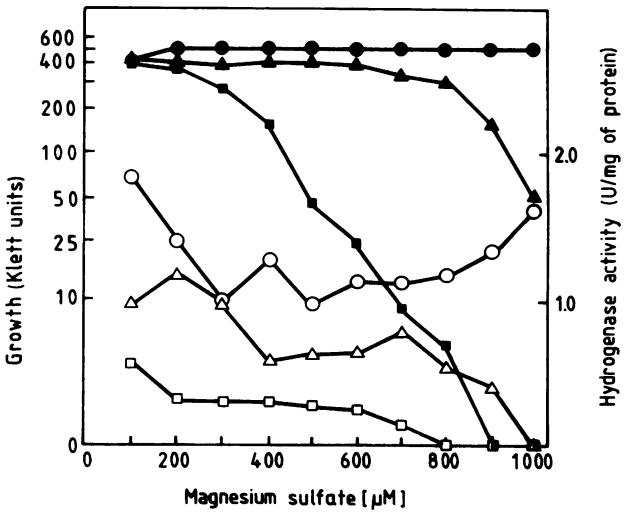

FIG. 3. Inhibition of lithoautotrophic growth (solid symbols) and hydrogenase activity (open symbols) by magnesium sulfate. Circles, *A. eutrophus* H20 wild type; squares, mutant H20F10(pGE4); triangles, mutant H20F11(pGE4). The concentration of $NiCl_2$ was 0.1 μM.

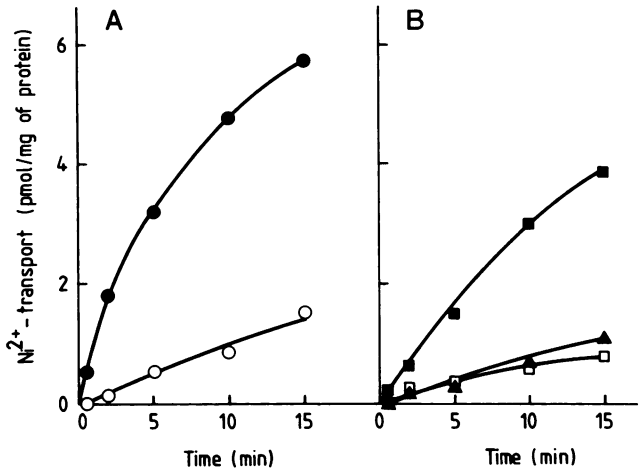

FIG. 4. Ni^{2+} transport activity of two *A. eutrophus* wild-type strains and Nic^- mutants derived therefrom. (A) H16 (●) and HF47(pGE4) (○). (B) H20 (■), H20F10(pGE4) (□), and H20F11(pGE4) (▲). The experimental details are described in Materials and Methods.

A. eutrophus H16 constructed with the vector pSUP202 (23) and cloned in *E. coli*. This procedure has been described previously (4). The hybrid plasmid pCH125 (Fig. 5) contained two *Eco*RI fragments of 9.6 and 2.9 kb and an additional 4.4-kb fragment which maps outside the *hox* gene cluster (Fig. 1). Plasmid pCH125, which can be mobilized into *A. eutrophus* via conjugation but cannot be maintained in this host as an independent replicon, was introduced into H20F10 by homologous recombination. The only homologous site in the recipient DNA was the 4.4-kb fragment, which was absent in the Nic^- mutant HF47, and thus further experiments were restricted to strain H20 and its derivatives.

The resulting heterogenote recombinant H20F13(pGE4) was tested for lithoautotrophic growth and hydrogenase activity under conditions of nickel limitation and nickel excess. The data in Table 2 show that wild-type characteristic growth and hydrogenase activity were recovered under nickel starvation. pCH125 DNA also restored the rate of nickel transport to at least 50% of the wild-type level.

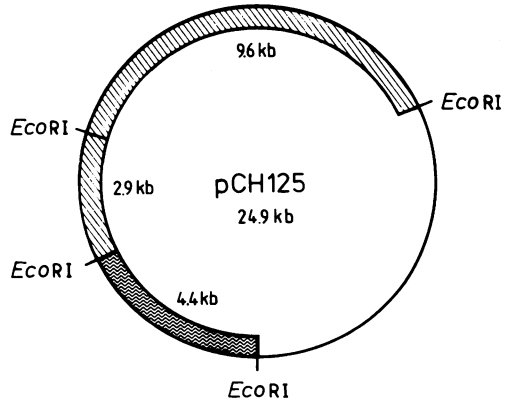

FIG. 5. Recombinant plasmid with Nic-restoring activity. DNA inserts were derived from a megaplasmid pHG1 DNA library of *A. eutrophus* H16. The hatched bar specifies the DNA region which is identical with *hoxN* in Fig. 1. The wavy-hatched bar is the site of homologous recombination.

TABLE 2. Lithoautotrophic growth and hydrogenase activity in Nic^- mutants and transconjugants

Strain	t_d[a] (h) at $NiCl_2$ concn:		Hydrogenase activity[b] (U/mg of protein) at $NiCl_2$ concn:	
	0.1 μM	1 μM	0.1 μM	1 μM
H20	4.1	4.7	1.25	2.33
H20F10(pGE4)	10.0	5.3	0.38	1.56
H20F13	NG	NG	0	0
H20F13(pGE4)	5.4	6.4	1.44	1.09

[a] t_d, Doubling time (tested with H_2, O_2, and CO_2 atmosphere). NG, No growth.
[b] Soluble hydrogenase activity was determined with whole cells cultivated heterotrophically with fructose and glycerol as the carbon sources.

However, nickel transport deficiency and recovery could only be demonstrated in the presence of magnesium salt and a low concentration (18 nM) of nickel chloride (Fig. 6B). Under conditions of nickel excess (520 nM), there was a barely detectable difference in transport activity between the wild type, the Nic^- mutant, and the corresponding recombinant (Fig. 6A), which is in good agreement with the growth characteristics presented in Table 2. Thus, to keep interference by the low-affinity, high-capacity nickel transport activity as low as possible, we used nonsaturating nickel concentrations in the mutant analysis.

Interestingly, the activity of the nickel-containing urease was also affected in the Nic^- mutants. The putative lack of the nickel-specific transport system in mutant H20F10 (pGE4) was associated with a threefold decrease of urease activity when the cells were grown without nickel in the medium. Urease activity was restored in the Nic^+ recombinant H20F13(pGE4). Moreover, this enzyme activity appeared to be stimulated in the absence of hydrogenase, which was not formed in pGE4-free cells of H20F10 and H20F13 (Table 3).

DISCUSSION

A new class of hydrogenase mutants of *Escherichia coli* impaired in the *hydC* gene has been reported (28). Addition of 500 μM nickel chloride to the growth medium led to a complete recovery of hydrogenase activity. It was suggested that the *hydC* product is involved in either transport or processing of nickel in the cell. Mutants with a similar phenotype were described by Chaudhuri and Krasna (3) and Waugh and Boxer (27). In this study we have demonstrated the presence of nickel-deficient hydrogenase mutants (Nic^-) of *A. eutrophus* which carry defined deletions in the *hox* gene cluster of megaplasmid pHG. The nickel chloride concentrations necessary to suppress the effects of the mutation on hydrogenase in *A. eutrophus* were significantly lower (0.5 μM) than those required to restore hydrogenase activity in *E. coli*. This is conceivable, since nickel chloride concentrations higher than 10 μM have been shown to be toxic to cells of *A. eutrophus* (6). Hydrogenase activity was recovered to the same extent in the wild-type and Nic^- mutants, indicating that there are no functional differences among the strains when nickel ions are present in excess. From these results, we conclude that the *hoxN* mutation must affect an early step in hydrogen metabolism. This may imply effects on a nickel-processing pathway, nickel transport, or even both components concomitantly.

It is not known yet how nickel is incorporated and bound to the hydrogenases of *A. eutrophus*. Protein synthesis was

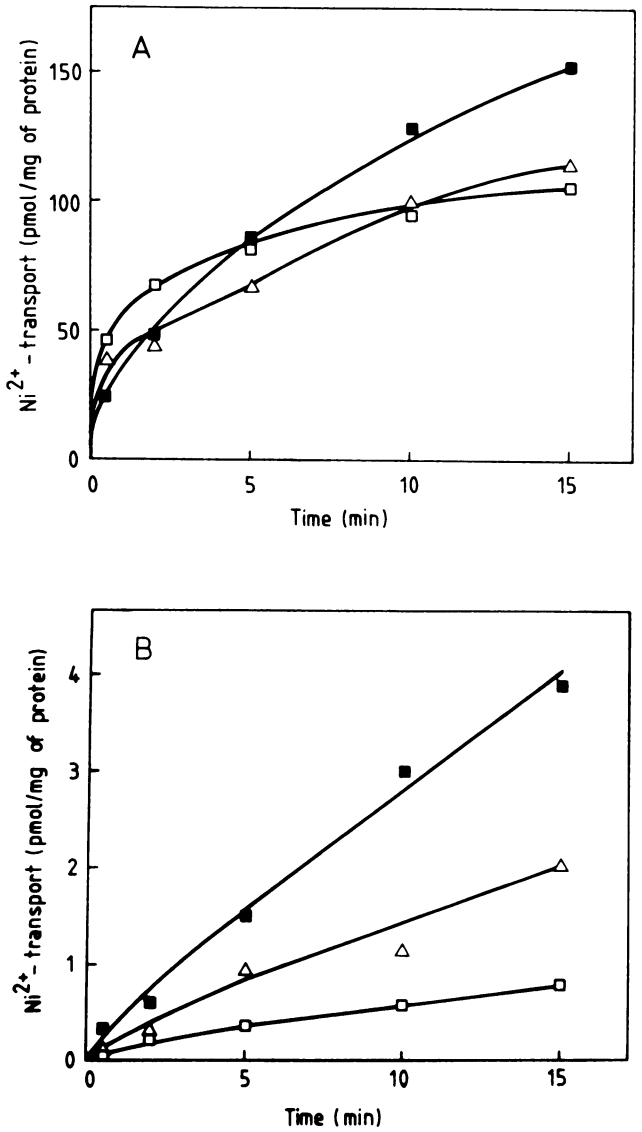

FIG. 6. Restoration of Ni^{2+} transport activity by *hoxN*. Symbols: ■, *A. eutrophus* H20; □, H20F10(pGE4); and △, H20F13(pGE4). (A) 520 nM nickel chloride, without magnesium chloride; (B) 18 nM nickel chloride, with 1 mM magnesium chloride.

shown to be required for the incorporation of nickel into soluble hydrogenase. Striking similarities between nickel and iron incorporation were found. Since the latter is bound to acid-labile sulfur, a corresponding configuration was proposed for nickel binding (8). There is evidence that nickel is associated with a single subunit (M_r 56,000) of the heterotetrameric soluble hydrogenase (11) and to the large peptide (M_r 63,000) of the heterodimeric membrane-bound hydrogenase (21).

TABLE 3. Response of urease to nickel limitation

Strain	Urease activity (U/mg of protein)	
	No Ni^{2+}	0.5 μM Ni^{2+}
H20F10	0.033	0.036
H20F10(pGE4)	0.010	0.034
H20F13	0.033	0.037
H20F13(pGE4)	0.025	0.031

Although the megaplasmid DNA may well code for a nickel-processing pathway, we favor the assumption that the *hoxN* mutation discussed in this communication is specifically associated with nickel transport. This conclusion is based on the following results. (i) Nickel deficiency of Nic^- mutants was enhanced by increasing the concentration of magnesium ions in the medium. This effect was expected to occur in a strain which transports nickel via a single magnesium pathway. It has been shown before that the activity of this low-affinity (K_m, 17 μM) nickel transport system is competitively inhibited by the divalent cations Mg^{2+}, Mn^{2+}, Zn^{2+}, and Co^{2+} (17). Nickel transport in several organisms has been attributed to a system which normally takes up magnesium (13). With high nickel concentrations in the absence of magnesium chloride, nickel transport activity in the Nic^- mutants resembled that in the wild type, suggesting that the magnesium system was effective. This implied that in order to demonstrate nickel deficiency, it was necessary to use low, nonsaturating nickel concentrations. Thus, all kinetic data have to be evaluated critically because of interference between the two coexisting transport activities. Attempts should be made to eliminate the magnesium-specific nickel transport by mutation. (ii) The rate of $^{63}Ni^{2+}$ uptake in Nic^- mutants was reduced to approximately 20% of the wild-type level and correlated with a low amount of hydrogenase activity. Introduction of megaplasmid-derived *hoxN* DNA via recombination restored more than 50% of the parental $^{63}Ni^{2+}$ uptake activity. Since the cloned DNA sequence contained three *Eco*RI fragments, two of which mapped adjacent to each other (9.6 and 2.9 kb), we assume that the third distantly located 4.4-kb fragment, present in the hybrid plasmid pCH125, is not directly involved in Nic activity. (iii) Although the *hoxN* DNA maps in the middle of the *hox* gene cluster, its function is apparently not restricted to supplying hydrogenase with nickel ions, since the *hoxN* mutation also had a pleiotropic effect on urease activity. However, urease activity in the Nic^- mutants only responded to nickel limitation in cells containing hydrogenase. This was only the case when the *hox* activator gene, *hoxC*, was present. The latter has been shown to be absolutely necessary for *hox* gene expression (4). Under these conditions, hydrogenase is undoubtedly the major sink for nickel ions. A similar nickel-accumulating activity was assigned to the hydrogenase of *Bradyrhizobium japonicum*. Strains of this bacterium were shown to contain a single nickel-specific transport system with K_m values of 26 to 50 μM for nickel (25). Jarrell and Sprott (12) reported a magnesium-independent specific nickel transport in *Methanobacterium bryantii*. The K_m of this uptake system was determined to be 3.2 μM. An extremely low affinity constant of 17 nM nickel chloride was demonstrated in cells of *Anabaena cylindrica*, which contains hydrogenase and urease (2). *A. eutrophus* H16 resembles this cyanobacterium with respect to the low K_m value (20 nM) determined for its specific transport system.

The *hoxN* sequence described in this report provides an attractively simple genetic system for studying nickel transport at the molecular level. The plasmid-encoded function is dispensable under conditions of nickel excess. However, in an environment where only traces of nickel are available, the specific high-affinity transport system is advantageous to the cells.

ACKNOWLEDGMENTS

We thank C. G. Friedrich for helpful discussions and D. Matzkuhn for assistance in preparing the manuscript.

The project was supported by a grant from the Deutsche Forschungsgemeinschaft.

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