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Inducible and Constitutive Expression of pMOL28-Encoded Nickel Resistance in *Alcaligenes eutrophus* N9A

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The nickel and cobalt resistance plasmid pMOL28 was transferred by conjugation from its natural host *Alcaligenes eutrophus* CH34 to the susceptible *A. eutrophus* N9A. Strain N9A and its pMOL28-containing transconjugant M220 were studied in detail. At a concentration of 3.0 mM $NiCl_2$, the wild-type N9A did not grow, while M220 started to grow at its maximum exponential growth rate after a lag of 12 to 24 h. When grown in the presence of subinhibitory concentrations (0.5 mM) of nickel salt, M220 grew actively at 3 mM $NiCl_2$ without a lag, indicating that nickel resistance is an inducible property. Expression of nickel resistance required active growth in the presence of nickel salts at a concentration higher than 0.05 mM. Two mutants of M220 were isolated which expressed nickel resistance constitutively. When the plasmids, pMOL28.1 and pMOL28.2, carried by the mutants were transferred to strains H16 and CH34, the transconjugants expressed constitutive nickel resistance. This indicates that the mutation is plasmid located. Both mutants expressed constitutive resistance to nickel and cobalt. Physiological studies revealed the following differences between strain N9A and its pMOL28.1-harboring mutant derivatives. (i) The uptake of $^{63}NiCl_2$ occurred more rapidly in the susceptible strain and reached a 30- to 60-fold-higher amount than that in the pMOL28.1-harboring mutant; (ii) in intact cells of the susceptible strain N9A, the cytoplasmic hydrogenase was inhibited by 1 to 5 mM $NiCl_2$, whereas 10 mM Ni^{2+} was needed to inhibit the hydrogenase of mutant cells; (iii) the minimal concentration of nickel chloride for the derepressed synthesis of cytoplasmic hydrogenase was lower in strain N9A (1 to 3 μM) than in the constitutive mutant (8 to 10 μM).

Hydrogen-oxidizing bacteria such as *Alcaligenes eutrophus* require nickel as a trace element for autotrophic growth (1, 22). Nickel is needed for the synthesis of hydrogenase (6) and is a constituent of the hydrogenases of *A. eutrophus* H16 (9) in both the cytoplasmic NAD-reducing and the membrane-bound forms (16, 17). The uptake of nickel as a trace element has been studied in *A. eutrophus* (11), *Bradyrhizobium japonicum* (20), and *Rhodobacter capsulatus* (23).

A. eutrophus CH34 (12) tolerates 3.0 mM Ni^{2+}, 20 mM Co^{2+}, 12 mM Zn^{2+}, or 2.5 mM Cd^{2+}. It contains two plasmids, pMOL28 (163 kilobase pairs) coding for Ni and Co resistance and pMOL30 (238 kilobase pairs) coding for Cd, Zn, and Co resistance. The plasmids are self-transmissible at low frequencies (10^{-8} per donor cell). Transfer was boosted to 10^{-3} by IncP1 plasmids such as RP4, pUZ4, and pUL B113 (13).

The pMOL28-encoded Ni resistance mechanism in strain CH34 is inducible, with resistance to 3 mM $NiCl_2$ being acquired during growth in the presence of 0.5 mM $NiCl_2$. Uptake of $^{63}NiCl_2$ by induced pMOL28-harboring cells is much lower than that by cured plasmid-free cells (18). Strain CH34 is resistant to several antibiotics and refractory to lysozyme treatment. These properties are shared by the cured, plasmid-free mutant *A. eutrophus* AE104 and thus are chromosomally determined. Physiological studies on induction and expression of nickel resistance as well as on the uptake and target sites of nickel require the use of well-characterized bacteria. Therefore, conjugational transfer of pMOL28 from derivatives of strain CH34 to wild-type strains *A. eutrophus* H16 and N9A, which have been studied in great detail (3), was investigated. The successful transfer of plasmid pMOL28 to these strains and the isolation of mutants of the transconjugants which express nickel and cobalt resistance constitutively facilitated physiological investigations, especially those which require the absence of the inducer. These investigations concern the uptake of nickel by the wild-type strain and the transconjugants and the concentration of nickel in the medium which is required to saturate the requirement for the synthesis of nickel-containing hydrogenases.

The present paper reports on studies on the expression of plasmid pMOL28-encoded nickel resistance in transconjugants of the wild-type strains *A. eutrophus* N9A, H16, and CH34 and constitutive mutants.

MATERIALS AND METHODS

Bacterial strains and plasmids. All bacteria, mutant derivatives, and transconjugants used in this study are listed in Table 1.

Growth conditions. Growth and growth inhibition by $NiCl_2$ was studied with Tris-mineral medium as described previously (13) supplemented with trace element solution SL7 (2). This medium did not contain chelating or precipitating compounds and provided conditions of maximum inhibitory effects exerted by heavy metal salts. For heterotrophic growth, 0.3% (wt/vol) sodium gluconate was added, unless otherwise stated.

Growth rates and doubling times under heterotrophic conditions in the absence or presence of nickel salts and various additives were measured in 300-ml Klett sidearm flasks containing 50 ml of cell suspension on a rotary shaker at 30°C. The optical density was monitored with a Klett-Summerson colorimeter (filter no. 54). Klett units were converted to optical density values (at 546 nm) by using a standard curve.

To determine resistance, we chose the following concentrations of metals in solidified Tris-mineral medium: 1.0 and 3.0 mM Ni^{2+}, 2.0 mM Co^{2+}, 1.0 mM Cd^{2+}, and 2.5 mM

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TABLE 1. Bacterial strains

Strain, mutant, or transconjugant	Derived from[a]:	Resistance to[b]:				Other relevant marker(s)	Plasmid(s)	Origin or reference
		Ni	Co	Zn	Cd			
Alcaligenes eutrophus								
H16	WT	s	s	s	s	Aut^+	pHG1	7
N9A	WT	s	s	s	s	Aut^+	pHG3	8
M220	N9A	r	r	s	s	Aut^+	pHG3, pMOL28	This study
G29	WT	s	s	s	s	Aut^+	pHG22	8
M241	G29	r	r	s	s	Aut^+	pHG22, pMOL28	This study
M219	H16	r	r	s	s	Aut^-	pMOL28	This study
HF33	H16	s	s	s	s	Aut^-	None	
M243	M220	rc	rc	s	s	Aut^+	pHG3, pMOL28.1	This study
M244	M220	rc	rc	s	s	Aut^+	pHG3, pMOL28.2	This study
M245	HF33	rc	rc	s	s	Aut^-	pMOL28.1	This study
M246	HF33	rc	rc	s	s	Aut^-	pMOL28.2	This study
CH34	WT	r	r	r	r	Aut^+	pMOL28, pMOL30	13
AE128	CH34	s	r	r	r	Aut^+	pMOL30	13
AE126	CH34	r	r	s	s	Aut^+	pMOL28	13
AE104	AE128	s	s	s	s	Aut^+	None	13
AE81	CH34	r	r	r	r	Aut^+ *trp leu met*	pMOL28, pMOL30	13
AEM813	AE81	r	r	r	r	Aut^+ *trp leu met* Km^r Ap^r Tc^r	pMOL28, pMOL30, pULB113	This study
AEM247	AE128	rc	rc	r	r	Km^r Ap^r Tc^r	pMOL28.1, pMOL30, pULB113	This study
Escherichia coli								
K-12	WT	s	s	s	s			
CM214	K-12					Km^r Ap^r Tc^r	pULB113	24

[a] WT, Wild type.
[b] s, Sensitive; r, resistant; rc, constitutively resistant.

Zn^{2+}. The metal salts were added to the liquid agar before pouring or onto the solidified agar. Solidified growth media contained 15 g of agar per liter. Analytical grade salts of $CdCl_2 \cdot H_2O$, $CoCl_2 \cdot 6H_2O$, $NiCl_2 \cdot 6H_2O$, and $ZnCl_2 \cdot 6H_2O$ (E. Merck AG, Darmstadt, Federal Republic of Germany) were prepared as 1.0 M stock solutions and sterilized by autoclaving.

Matings. Donor and recipient strains were grown in liquid nutrient broth at 30°C. Spot mating occurred on nutrient broth agar overnight. The Ni^{2+} selective agar medium contained Tris medium supplemented with 0.2% (wt/vol) sodium gluconate and 1 mM $NiCl_2$. The selective medium for transconjugants harboring pULB113 from *Escherichia coli* CM214 as a donor contained ampicillin, kanamycin, and tetracycline (20 μg of each per ml). The selection of transconjugants and tests for the absence of donor or recipient cells involved was based on the ability to use various organic compounds as well as hydrogen and carbon dioxide as growth substrates.

Isolation of plasmid DNA and agarose gel electrophoresis. Crude lysates of plasmid DNA were prepared as described previously (10). Electropherograms were prepared in 0.8% (wt/vol) agarose in 89 mM Tris-borate buffer (pH 8.5) plus 2.5 mM disodium EDTA and run as horizontal slab gels for 6 h at 150 V. DNA bands were stained with ethidium bromide and visualized by UV transillumination.

Selection of constitutive mutants. Cell suspensions of M220 were treated with nitrite or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Mutants which expressed resistance to 3 mM $NiCl_2$ constitutively were selected by applying alternating growth periods: the cells were grown for 20 h in liquid Tris-gluconate medium in the presence of 3 mM $NiCl_2$ and for 72 h in nickel-free Tris-gluconate medium. After 10 or 20 growth periods, the cell suspension, grown for 3 days in nickel-free medium, was spread on agar containing 3 mM $NiCl_2$. The constitutive mutants formed large colonies after 2 days, while the M220 parent colonies were still scarcely visible. The constitutive expression of nickel resistance was confirmed by measuring growth rates in liquid culture.

Manometric measurements. The uptake of gas ($H_2 + O_2 \pm CO_2$) was measured as described before (15).

Determination of MICs. Tris-gluconate agar plates containing 20 to 0.039 mM metal ion concentrations (in 2:1 dilution steps) were routinely used as described previously (13); intermediate concentrations were used to resolve fine differences.

Whole-cell assay to measure NAD-dependent hydrogenase. NAD-dependent hydrogenase (EC 1.12.1.2) in whole cells was assayed in Tris hydrochloride buffer (pH 8.0) saturated with H_2 and containing 0.005% cetyltrimethylammonium bromide (6).

Assay of $^{63}Ni^{2+}$ uptake. The cells were grown in Tris-gluconate medium, harvested, washed with ice-cold 50 mM sodium phosphate buffer (pH 7.0), and resuspended (0.25 mg of cell protein per ml) in the same buffer containing 200 μg of chloramphenicol per ml. The cell suspensions were shaken in 100-ml Erlenmeyer flasks at 30°C, and radioactive $^{63}NiCl_2$ (0.1 μCi/ml) diluted with appropriate concentrations of nonlabeled salt was added. Samples (0.2 to 0.5 ml) were withdrawn at intervals and filtered through cellulose-acetate membranes (0.45-μm pore size); the filters were washed twice with 2 to 5 ml of 50 mM potassium phosphate buffer (pH 3.0), dried, and dissolved in Packard emulsifier scintillator (special Mi96), and the radioactivity was counted in a Beckman scintillation counter (18).

Chemicals. $^{63}NiCl_2$ (0.73 mCi/μmol) was purchased from the Radiochemical Centre, Amersham, England. Carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone, *N,N'*-dicyclohexylcarbodiimide, valinomycin, and agarose type V were obtained from Sigma Chemie GmbH, Munich, Federal Republic of Germany. NAD was obtained from C. F. Boehringer Söhne, Mannheim, Federal Republic of Germany. All

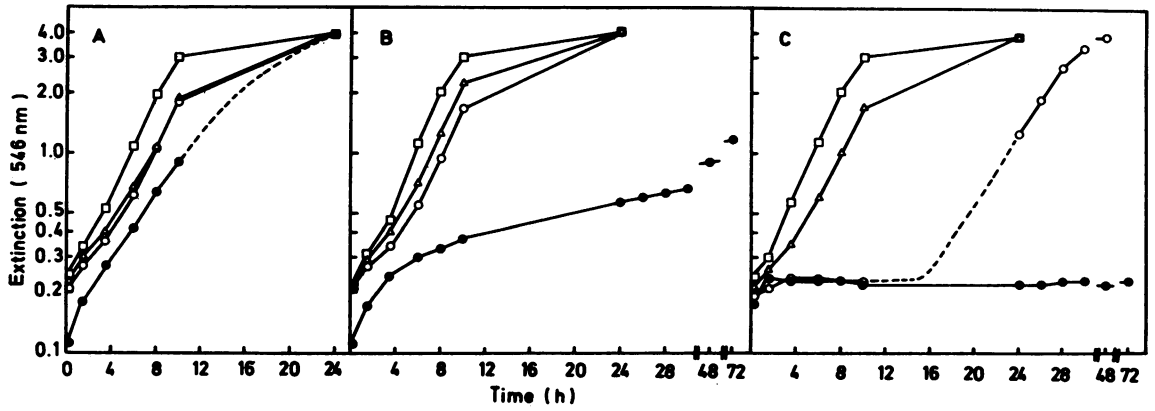

FIG. 1. Growth of *A. eutrophus* derivatives without or in the presence of $NiCl_2$ after antecedent growth in the absence of nickel salts. At zero time, the Tris-gluconate media containing no (A), 0.5 mM (B), and 3.0 mM (C) $NiCl_2$ were inoculated with suspensions of cells grown in Tris-gluconate medium for 16 h. Symbols: ●, wild-type strain N9A; ○, its pMOL28-containing transconjugant M220; △, constitutive mutant M243; □, constitutive mutant M244. An extinction of E (546 nm) = 1.0 corresponds to 0.19 g (dry weight) per liter.

complex media were from Difco Laboratories, Detroit, Mich. All other chemicals of pro analysis quality were obtained from Merck, and the gases used were from Messer Griesheim, Kassel, Federal Republic of Germany.

RESULTS

Transfer of pMOL28 to other *A. eutrophus* strains. To transfer pMOL28 to other strains of *A. eutrophus*, we constructed a donor strain from AE81, a triplicate auxotrophic mutant of CH34, by the introduction of pULB113. The transfer of pULB113 from *E. coli* CM214 (24) resulted in strain AEM813, which was shown to contain the native plasmids and in addition pULB113 (data not shown). Matings of AEM813 with wild-type strains of *A. eutrophus* such as H16, N9A, G29, and the type strain TF93 as recipients resulted in conjugational transfer. Transconjugants were selected under heterotrophic conditions on agar media containing 1 mM $NiCl_2$. However, all transconjugants could also grow in 3 mM $NiCl_2$. Plasmid profiles showed that the transconjugants obtained with N9A and G29 as recipients contained their native plasmids, pHG3 and pHG22, respectively (7, 8), in addition to pMOL28 and thus were able to grow autotrophically. These plasmids enabling the cells to grow autotrophically on hydrogen and carbon dioxide as energy and carbon sources (Aut^+) were maintained even in the absence of selective conditions. Transconjugants of strain H16 and of the type strain had lost the native plasmid and were Aut^-.

Mutants of M220 were selected which expressed nickel resistance constitutively. Enrichment of these mutants was achieved by alternating growth conditions, one period in the $NiCl_2$-containing medium and the other in nickel-free medium. The inoculum for this mutant enrichment culture had been mutagenized with nitrite or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Mutant M243 originated from the nitrite-treated inoculum after 10 alternating cycles, whereas mutant M244 originating from the *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-treated inoculum was obtained after 20 cycles. Growth of mutants in 0.5 and 3.0 mM $NiCl_2$ was tested in liquid culture (Fig. 1B and C). The plasmids carried by both mutants (pMOL28.1 and pMOL28.2) were transferred to several recipients to examine whether constitutivity was due to a mutation on the plasmid or on the chromosome and also to construct a transconjugant containing plasmids pMOL28.1 and pMOL28.2 alone. Plasmid pULB113 was transferred from *E. coli* CM214 to both constitutive mutants M243 and M244. Transconjugants were used as donors in crosses with streptomycin- and nalidixic acid-resistant derivatives of AE128 and HF33. Three transconjugants which expressed resistance to 3 mM $NiCl_2$ constitutively were selected for further characterization: M245 derived from HF33 and containing pMOL28.1 from M243; M246 derived from HF33 and containing pMOL28.2 from M244; and AEM247 derived from AE128 and containing pMOL28.1 from M243 in addition to pULB113 and the native plasmid pMOL30. The constitutive expression of nickel resistance in these transconjugants indicated that the mutation was located on pMOL28 of M220 rather than on the chromosome.

Nickel salts exert no killing effect. The question was raised whether nickel salts exert a killing effect on the cells at growth inhibitory concentrations. Cell suspensions of strain N9A, the transconjugant M220, and its constitutive derivative M244 which had been grown in nickel-free medium were exposed to 0.1 to 6 mM $NiCl_2$ under conditions suitable for growth. The viable count was determined after 1, 7, and 24 h. Strain N9A did not grow but retained its full viability, whereas M220 and M244 could grow. Thus, exposure of cells to high growth-suppressing concentrations of $NiCl_2$ was not bactericidal.

Inducibility of nickel resistance. When strain N9A was inoculated into Tris-gluconate liquid medium containing 3 mM $NiCl_2$, no growth occurred (Fig. 1C). When M220 grown in the absence of nickel was transferred to the same medium, growth occurred, but only after 16 to 18 h (Fig. 1C). This indicates that pMOL28-encoded resistance to 3 mM $NiCl_2$ is an inducible property. The concentration at which nickel is normally added to nutrient media as a trace element (0.1 μM Ni^{2+}) had no inductive effect.

The inductive expression of full nickel resistance occurred in gluconate medium or nutrient broth or under autotrophic conditions. Only growing cells expressed nickel resistance. Expression of nickel resistance required protein synthesis and was suppressed by chloramphenicol (200 μg/ml) or nalidixic acid (200 μg/ml). Induction with nickel resulted in the expression of full resistance to cobalt. If the induced cells were inoculated into nickel-free media and grown for two subcultures, the resistance to the metals was lost.

TABLE 2. Inducing effect exerted by various subinhibitory concentrations of nickel chloride on the cell doubling time of M220 and M244 during growth in the presence of 3 mM $NiCl_2$

Antecedent growth at $NiCl_2$ concn (mM) of:	Doubling time during growth on Tris-gluconate medium in the presence of 3.0 mM $NiCl_2$ (h)[a]	
	M220	M244
0	46	2.2
0.05	5	2.2
0.1	3.2	2.2
0.5	3.2	2.2
1	3.2	2.2
3	3.2	

[a] Average doubling times during the first 7 h after transfer of cells to the 3 mM $NiCl_2$ medium were calculated from three independent experiments. Cells were shaken under air in Klett flasks at 30°C. Strain N9A did not grow under these conditions.

Constitutive resistance to nickel salts. The constitutive mutants, M243 and M244, were similar and had the following properties. (i) They were able to grow in the presence of 3 mM $NiCl_2$ without a lag period (Fig. 1C). (ii) In the presence of 3 mM $NiCl_2$, they exhibited a lower doubling time than the induced inducible strain M220 (Table 2). (iii) The constitutive mutants formed larger colonies than uninduced cells of parent strain M220 on agar media containing 3 mM $NiCl_2$. (iv) The MICs of nickel and cobalt salts (Table 3) were higher compared with those for M220.

Nickel uptake. To determine whether nickel resistance coded by pMOL28 is due to decreased uptake, we incubated cells in the presence of $^{63}Ni^{2+}$ (Fig. 2). The sensitive wild-type strain N9A took up about threefold more nickel (3.3 nmol of nickel per mg of protein) than the noninduced pMOL28-harboring transconjugant M220 (1.1 nmol of nickel per mg of protein) and 40 to 60 times more nickel than induced cells of M220 or uninduced cells of M243. These results confirm previous reports on nickel uptake with strain CH34 and its cured derivative AE104 (18). However, induction of CH34 could have been due to carryover and saturation of the cells with nonradioactive nickel ions. The data with the constitutive mutant M243 excluded this suspicion.

Effects of inhibitors on Ni uptake. Inhibitors of nickel uptake were tested. Cells were exposed to the inhibitor 15 min prior to the addition of $^{63}Ni^{2+}$ (1.0 μM) at 30°C, and nickel uptake was monitored over 45 min. Dimethyl sulfoxide exhibited no significant effect on nickel uptake (data not shown). The protonophore carbonyl-cyanide-*p*-trifluoromethoxyphenylhydrazone (12 μM) inhibited nickel uptake by strain N9A and by induced cells of M220. The terminal oxidase inhibitor azide (40 mM) and the membrane potential dissipator valinomycin (6 μM in presence of 20 mM K^+) exerted different degrees of inhibition on both N9A and M220. The ATPase inhibitor *N,N'*-dicyclohexylcarbodiimide (10 μM) did not impair nickel uptake in N9A significantly, but increased uptake in induced cells of M220. A dicyclohexylcarbodiimide-sensitive mechanism is presumably not involved because of the lack of effect on N9A (11). The results of these preliminary studies suggest that nickel uptake as well as the resistance to Ni^{2+} are due to energy-requiring processes.

TABLE 3. MICs of nickel, cobalt, cadmium, and zinc ions

Strain	MIC (mM)			
	Ni^{2+}	Co^{2+}	Cd^{2+}	Zn^{2+}
N9A	0.2	0.1	0.2	2.0
M220	3	6	0.3	3.0
M243	9	10	0.3	3.0
M244	9	10	0.3	3.0

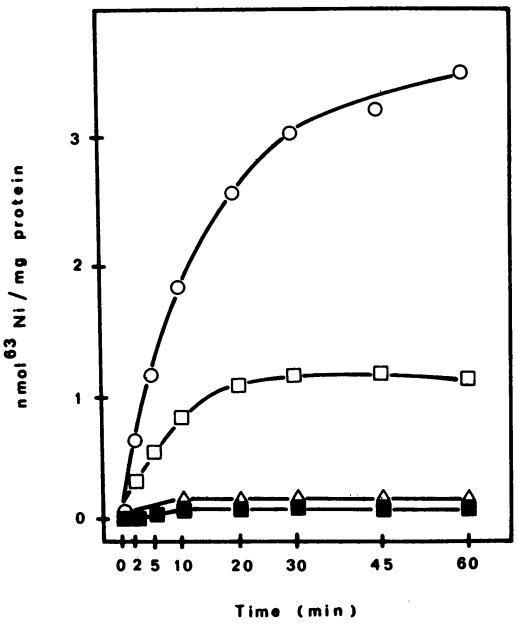

FIG. 2. Uptake of $^{63}Ni^{2+}$ (1.0 μM) by the sensitive wild-type N9A (○), its pMOL28-harboring uninduced (□) and induced (△) transconjugant M220, and the constitutively resistant mutant M243 (■).

Inhibition of cytoplasmic enzymes. Cell suspensions of autotrophically grown cells of strains N9A and M243 oxidized hydrogen at about the same rate. When $NiCl_2$ was added to the suspensions, the rates of hydrogen oxidation decreased, but to different degrees (Fig. 3). In the presence of 5 mM $NiCl_2$, for example, N9A was inhibited by 60% while M243 continued unimpaired. At 10 mM $NiCl_2$, both strains were inhibited by about 75%. The specific hydroge-

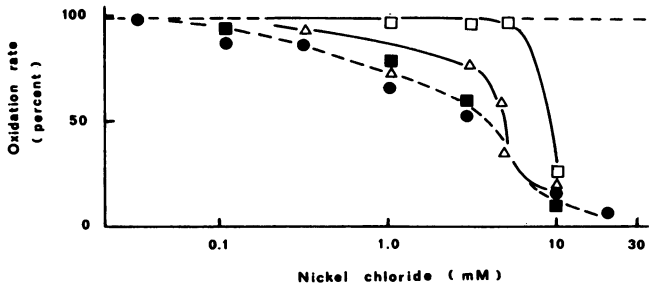

FIG. 3. Inhibition of the cytoplasmic hydrogenase and of hydrogen oxidation in intact cells of the wild-type strain *A. eutrophus* N9A and the constitutively resistant mutant M243 at varied concentrations of nickel chloride. The supernatants of crude extracts of lithoautotrophically grown cells of both strains were used to measure NAD reduction by H_2 in a photometric assay. Symbols: ●, N9A; ■, M243. Intact lithoautotrophically grown cells were used to measure the hydrogen-oxygen reaction manometrically under an atmosphere of 10% oxygen, 10% carbon dioxide, and 80% hydrogen. Symbols: △, N9A; □, M243.

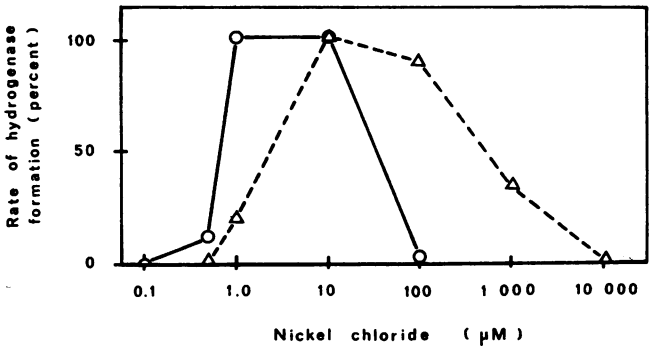

FIG. 4. Dependence of the derepressed formation of hydrogenase in growing cells of N9A (○) and M243 (△) on the concentration of nickel chloride in the fructose-glycerol medium. The cells were grown in fructose (0.2%, wt/vol)-glycerol (0.2%, vol/vol) growth medium containing 10 μM EDTA. After fructose had been consumed (after a 21-h growth period), $NiCl_2$ was added and samples were withdrawn for 30 h at intervals to measure hydrogenase activity by the whole-cell assay (see Materials and Methods). The rate of hydrogenase formation was plotted against the Ni^{2+} concentration added.

nase activities of the crude cell extracts of both N9A and M243 were similar and equally sensitive to nickel ions. The results indicate that in the intact cells, the cytoplasmic hydrogenase was inhibited by nickel ions, although at different concentrations.

In contrast, under anaerobic conditions there was no difference in susceptibility of hydrogenase activities to nickel. In both strains, the hydrogenase activities were equally impaired at low nickel concentrations. Thus, under anaerobic conditions, the pMOL28-encoded resistance mechanism failed to protect the function of the cytoplasmic enzyme against inhibition by 5 mM $NiCl_2$.

Nickel requirement for hydrogenase formation and autotrophic growth. When N9A and M243 were grown in a fructose-glycerol medium containing 10 μM EDTA, the growth rate decreased after the consumption of fructose (6). When nickel was added, hydrogenase was formed at a rate which depended on the nickel concentration. In the absence of added nickel, hydrogenase activity was not detectable (6).

In our experiments, 1 to 3 μM $NiCl_2$ was required to achieve maximum rates of hydrogenase formation in N9A (Fig. 4). For hydrogenase formation by strain M243, 8 to 10 μM concentrations of nickel salts were required to saturate the hydrogenase-synthesizing system. The tolerance to further increasing concentrations of Ni^{2+} was different.

DISCUSSION

In this study, Tris-buffered synthetic media were chosen because in these media copper, cobalt, nickel, cadmium, and zinc ions exerted growth inhibitory effects at a micromolar level, while in various complex media high concentrations of toxic heavy metals are tolerated, probably owing to complexation by metabolites, peptides, and nucleic acids. Defined synthetic media were also required because ion gradients over the cytoplasmic membrane have considerable energy-storing functions (4).

The study of the mechanism, the enzyme proteins, and the genetics of plasmid-mediated resistance has made the greatest progress in the area of mercuric and arsenic compounds (5, 21).

Inducibility of plasmid-mediated metal resistance was reported for several systems. Resistance to Hg^{2+} and organomercurial compounds in *Staphylococcus aureus* and *Pseudomonas aeruginosa* was found to be inducible (26). Plasmid-carrying *S. aureus* showed a lag of 5 to 7 h when exposed to 3 or 6 mM arsenate in nutrient broth, but commenced growth immediately when pregrown in the presence of 1.0 mM arsenate, which is subinhibitory (19). Inducible plasmid-mediated metal resistance was also reported for copper resistance in *E. coli* K-12 determined by the conjugative plasmid pRJ1004 (14).

The resistance to nickel determined by plasmid pMOL28 is inducible in the native host strain *A. eutrophus* CH34 (18) as well as in the transconjugant M220. Full resistance was reached after growth for 18 to 24 h at 3 mM $NiCl_2$ or after 5 to 6 h of growth at 0.1 mM $NiCl_2$. The availability of the constitutively resistant mutants M243 and M244 made possible physiological experiments which required the absence of nickel. With inducible strains, the fully expressed gene, the gene product, and gene function can only be studied in the presence of the inducer(s) or after the cells have grown in the presence of the inducer(s). The presence of the inducing metal ions may saturate the cells with the ions or cause a carryover into the experimental suspension media and thus obscure phenomena such as storage of metal ions, uptake or efflux rates, amounts taken up, enzyme activities, and measurements of trace element requirements.

Thus, with the help of the constitutive mutants of *A. eutrophus* we could elaborate the following facts. (i) The lag time (about 20 h) of growth of strain M220 in the presence of 3 mM $NiCl_2$ is not due to growth of rare mutants but is a regulatory phenomenon. When starting these studies, we were tempted to assume that the lag was caused by killing of most of the cells and then rapid onset of growth by surviving Ni-resistant mutants. But we were not able to obtain mutants resistant to nickel from the plasmid-free wild-type strain N9A. We also failed to pick constitutive mutants of M220 directly from agar plates without prior mutant enrichment. These results are in accordance with the observation that Ni-resistant mutants have never been obtained from plasmid-free strains of *A. eutrophus*. (ii) The existence of the constitutive mutants and the cotransfer of the plasmid pMOL28.1 and constitutive nickel resistance to plasmid-free Ni-sensitive recipients indicated that the regulatory gene(s) is located on the plasmid. Further information will come from current genetic analysis. (iii) The resistance to nickel is not caused by the binding and accumulation of the metal by binding proteins such as metallothionein but is caused by the exclusion of nickel. This is indicated by the very small amount of nickel taken up by strain M243 compared with that taken up by the plasmid-free strain N9A. (iv) The resistance mechanism leads to protection of the cytoplasm from penetration by toxic nickel ions. (v) The constitutively expressed state results in the requirement for a higher concentration of nickel as a trace element for hydrogenase synthesis compared with the plasmid-free strain N9A.

Aside from the genetic location, the gene products, and the regulation of resistance expression, the problem of the mechanism of resistance is the most fascinating. The results of the present study define the cytoplasm as the target site of the toxic effects of nickel and the cytoplasmic membrane as the decisive barrier. The failure of the plasmid pMOL28.1-encoded resistance mechanism to protect the cells under anaerobic conditions suggests that the resistance mechanism involves an energy-dependent process such as an ATP- or Δp-driven efflux system instead of a static alteration of the cell envelope properties. Previous attempts to obtain proof for an efflux mechanism failed owing to the low uptake of

^{63}Ni-labeled nickel chloride by the resistant cells and to erratic results when the cells were depleted of reserve materials and preloaded with high concentrations of radioactive Ni^{2+}.

The use of *A. eutrophus* and its pMOL28.1-containing derivatives provoked another strategy to demonstrate nickel efflux directly. Unlike *E. coli* and *S. aureus*, which are facultatively fermentative bacteria, *A. eutrophus* is a strictly aerobic, respiratory bacterium which can neither grow nor form enzymes or poly-β-hydroxybutyrate under anoxic conditions (25). When $^{63}Ni^{2+}$ was added to cell suspensions of N9A and M243 under anaerobic conditions after preincubation under these conditions for several hours, the uptake of nickel was affected in both strains with different effects: in N9A the uptake was decreased and in M243 it was drastically increased, compared with the time course under aerobic conditions. Subsequent aeration had a dramatic effect also: strain N9A responded with a renewed increase of the uptake rate, and strain M243 responded with an instantaneous efflux of $^{63}Ni^{2+}$ ions (C. Sensfuß, unpublished data). The time course of deenergization is highly dependent on the choice of buffers and the content of potassium and sodium ions and may reflect the transient stabilization of Δp by Na^+ and K^+ gradients (4). Thus, the efflux experiments explain the physiological effects of plasmid pMOL28.1-encoded nickel resistance and are compatible with the assumption that in M243 under aerobic conditions two constitutive energy-dependent cation transport systems may be working concomitantly, a chromosomally determined nickel uptake system and a plasmid-mediated nickel efflux system.

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