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Cloning of pMOL28-Encoded Nickel Resistance Genes and Expression of the Genes in *Alcaligenes eutrophus* and *Pseudomonas* spp.

ROMAN A. SIDDIQUI, KARIN BENTHIN, AND HANS G. SCHLEGEL*

Institut für Mikrobiologie der Universität Göttingen, Grisebachstrasse 8, D-3400 Göttingen, Federal Republic of Germany

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The 163-kilobase-pair (kb) plasmid pMOL28, which determines inducible resistance to nickel, cobalt, chromate, and mercury salts in its native host *Alcaligenes eutrophus* CH34, was transferred to a derivative of *A. eutrophus* H16 and subjected to cloning procedures. After Tn*5* transposon mutagenesis, restriction endonuclease analysis, and DNA-DNA hybridization, two DNA fragments, a 9.5-kb *Kpn*I fragment and a 13.5-kb *Hin*dIII fragment (HKI), were isolated. HKI contained EK1, the *Kpn*I fragment, as a subfragment flanked on both sides by short regions. Both fragments were ligated into the suicide vector pSUP202, the broad-host-range vector pVK101, and pUC19. Both fragments restored a nickel-sensitive Tn*5* mutant to full nickel and cobalt resistance. The hybrid plasmid pVK101::HKI expressed full nickel resistance in all nickel-sensitive derivatives, either pMOL28-deficient or -defective, of the native host CH34. The hybrid plasmid pVK101::HKI also conferred nickel and cobalt resistance to *A. eutrophus* strains H16 and JMP222, *Alcaligenes hydrogenophilus*, *Pseudomonas putida*, and *Pseudomonas oleovorans*, but to a lower level of resistance. In all transconjugants the metal resistances coded by pVK101::HKI were expressed constitutively rather than inducibly. The hybrid plasmid metal resistance was not expressed in *Escherichia coli*. DNA sequences responsible for nickel resistance in newly isolated strains showed homology to the cloned pMOL28-encoded nickel and cobalt resistance determinant.

Resistance to heavy metal salts is widespread in bacteria isolated from polluted environments (7, 34, 35, 45). In many cases the resistance properties are plasmid determined. Resistance to mercuric ions and mercurial compounds is the best-studied example of plasmid-mediated heavy metal resistance. It functions by conversion of inorganic mercury to elemental mercury (Hg^0), which is volatilized and thus released from the cell (35, 43, 47). Resistance to copper was reported for *Pseudomonas syringae* pv. *tomato* (2, 5) and for *Escherichia coli* (28). Resistance to cadmium was studied in *Staphylococcus aureus* (46; G. Nucifora, L. Chu, T. K. Misra, and S. Silver, Proc. Natl. Acad. Sci. USA, in press), and resistance to thallium was studied in *Nocardia opaca* (30). Plasmid-mediated resistances to arsenic and antimony compounds, tellurite, tellurate, chromate, cadmium, silver, copper, mercury, thallium, bismuth, cobalt, nickel, and lead have recently been reviewed (35). So far two mechanisms have been found for specific metal resistances, an ATP-dependent pump mechanism (36; Nucifora et al., in press) and volatilization (35). However, studies of mechanisms are quite preliminary. Molecular biology and genetic experiments have sometimes run ahead of physiological and biochemical understanding (35).

Plasmid-mediated resistance to nickel in bacteria was first reported by Smith (41). Clinical isolates of *E. coli* harbored R factors which conferred resistance to mercury, nickel, and cobaltous ions in addition to commonly known antibiotic tolerance determinants. In *Alcaligenes eutrophus* CH34, originally isolated from a zinc decantation tank (21), nickel and cobalt resistance determinants were found to be located on the 163-kilobase-pair (kb) plasmid pMOL28. This plasmid determines resistance to 3 mM Ni^{2+}, 5 mM Co^{2+}, 0.7 mM Hg^{2+}, and 0.2 mM chromate (22, 24). The pMOL28-encoded nickel resistance mechanism is inducible (32). Compared with cured plasmid-free cells, induced pMOL28-harboring cells accumulate negligible amounts of $^{63}Ni^{2+}$ (32). When transferred to various susceptible wild-type strains of *A. eutrophus*, resistance was expressed in the same manner as in its original strain, CH34. From a transconjugant of *A. eutrophus* N9A, constitutive mutants were derived whose plasmids pMOL28.1 and pMOL28.2 confer constitutive nickel resistance to various *A. eutrophus* recipient strains. The constitutively resistant transconjugants tolerate up to 8 mM $NiCl_2$ (33).

Studies using pMOL28.1-harboring cells demonstrated that the pMOL28-encoded resistance is due to a specific energy-dependent nickel efflux system (31).

Our interest is focused on the molecular biology of pMOL28-encoded nickel resistance. We are aware that the nickel resistance determinant includes cobalt resistance as well. The experimental results presented in this and the accompanying paper (24) demonstrate that nickel and cobalt resistances are probably linked to the same determinants on plasmid pMOL28. The present study was aimed at localizing the pMOL28-encoded nickel resistance genes and cloning the DNA fragments involved in the resistance mechanism. We investigated the expression of cloned DNA fragments in the cured strains and nickel-sensitive mutants of *A. eutrophus* CH34 as well as in various other wild-type *Alcaligenes* and *Pseudomonas* strains.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *A. eutrophus*, *Pseudomonas*, and *E. coli* strains, mutants, and transconjugants isolated and the plasmids used in this study are listed in Table 1.

Media and growth conditions. *A. eutrophus* and *Pseudomonas* strains were grown in nutrient broth (8 g/liter; Difco

* Corresponding author.

TABLE 1. Bacterial strains, transconjugants, mutants, and plasmids used in this study

Strains, transconjugants, mutants, and plasmids	Relevant characteristics	Origin or reference[a]
A. eutrophus		
H16	Nic^s Cob^s; wild type	9
HF33	Nic^s Cob^s; plasmid-free mutant of H16	13
M219	Nic^r CobA^r; transconjugant of HF33	This study
KB1T	Nic^s CobA^s; Tn*5* mutant of M219	This study
KB6T	Nic^s CobA^s; Tn*5* mutant of M219	This study
KB8(5)N	Nic^s CobA^s Aut$^-$; nitrite-induced mutant of M219	H. G. Schlegel, unpublished
CH34	Nic^r CobA^r Cad^r Zin^r CobB^r; wild type	22
AE126	Nic^r CobA^r Cad^s Zin^s CobB^s; curing mutant of CH34	22
AE128	Nic^s CobA^s Cad^r Zin^r CobB^r; curing mutant of CH34	22
AE104	Nic^s CobA^s Cad^s Zin^s CobB^s; curing mutant of CH34	22
MO2	Nic^s CobA^s Cad^r Zin^r CobB^r; novobiocin-induced mutant of CH34	H. G. Schlegel, unpublished
MO3	Nic^s CobA^s Cad^r Zin^r CobB^r; deletion mutant of CH34	11
MO5	Nic^s CobA^s Cad^r Zin^r CobB^r; mutant of CH34 induced by growth at 37°C	H. G. Schlegel, unpublished
JMP222	Nic^s CobA^s	10
A. hydrogenophilus	Nic^s Cob^s; wild type	27
P. putida	Nic^s Cob^s; wild type	DSM 291
P. oleovorans	Nic^s Cob^s; wild type	ATCC 29347
E. coli		
S17-1	*recA tra* genes of plasmid RP4 are integrated into the chromosome; auxotrophic for proline and thiamine	38, 39
XL-1 Blue	*recA1 lac* (F′ *proAB lacI*^{q} *lacZ*ΔM15 Tn*10*)	4
Plasmids		
pHG1	Megaplasmid conferring autotrophy, present in H16	9, 11
pHG21-a	Megaplasmid conferring autotrophy to *A. hydrogenophilus*	9, 11
pHG21-b	Cryptic megaplasmid in *A. hydrogenophilus*	8
pHG13c	Megaplasmid pMOL28, formerly called pHG13b, with 40-kb deletion responsible for Nic^s and CobA^s in MO3	11
pMOL28	Megaplasmid conferring inducible resistance to nickel and cobalt in M219, AE126, and CH34	22
pMOL30	Megaplasmid conferring resistance to cadmium, cobalt, and zinc in CH34, AE128, MO2, MO3 and MO5	22
pMOL28::Tn*5-mob*	Megaplasmid conferring kanamycin resistance and sensitivity to nickel and cobalt in KB1T and KB6T	This study
pSUP202	Tc^r Ap^r Cm^r	39
pSUP202::EK1	Plasmid conferring Tc^s Ap^r Cm^r; with 9.5-kb *Eco*RI insert (formerly PA57 from pPA57) from pMOL28	This study
pSUP202::HKI	Plasmid conferring Tc^s Ap^r Cm^r; with 13.5-kb *Hin*dIII insert from pMOL28	This study
pSUP5011	Cm^r Ap^r Km^r; harbors Tn*5-mob*	38
pUC19	Ap^r	48
pPA27	pUC19::PA27; with 9.5-kb *Kpn*I fragment of pMOL28	This study
pPA56	pUC19::PA56; with 8.8-kb *Kpn*I fragment of pMOL28	This study
pPA57	pUC19::PA57; with 9.5-kb *Kpn*I fragment of pMOL28	This study
pPA87	pUC19::PA87; with 8.8-kb *Kpn*I fragment of pMOL28	This study
pVK101	Tc^r Km^r	18
pVK101::EK1	Plasmid conferring Tc^r Km^r; leaky resistance to nickel and cobalt in *Alcaligenes* and *Pseudomonas* strains due to 9.5-kb *Eco*RI fragment (formerly fragment PA57 from pPA57)	This study
pVK101::HKI	Plasmid conferring Tc^r Km^s; resistance to nickel and cobalt in *Alcaligenes* and *Pseudomonas* strains due to 13.5-kb *Hin*dIII fragment	This study

[a] ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen.

Laboratories, Detroit, Mich.). Metal tolerance and growth inhibition by $NiCl_2$ or $CoCl_2$ were studied with Tris-mineral medium as described previously (33). For heterotrophic growth, filter-sterilized sodium gluconate (3 g/liter) or fructose (5 g/liter) was added unless otherwise stated. Growth rates and doubling time under heterotrophic conditions in the absence or presence of nickel and cobalt salts were measured as previously described (33).

Metal salts were added to the liquid agar before the solidified agar was poured. Growth media were solidified with 15 g of agar per liter.

Analytical-grade salts of $NiCl_2 \cdot 6H_2O$ and $CoCl_2 \cdot 6H_2O$ (E. Merck AG, Darmstadt, Federal Republic of Germany) were prepared as 1.0 M stock solutions and sterilized by autoclaving. *E. coli* was grown in complex Luria-Bertani medium (20) at 37°C. For maintenance of plasmid markers, mutant selection, or isolation of transconjugants, the medium was supplemented with the desired concentration of

filter-sterilized solutions of the necessary antibiotic. Clones of *E. coli* XL-1 Blue harboring recombinant pUC plasmids were identified on Luria-Bertani agar plates which contained 50 mg of ampicillin, 12.5 mg of tetracycline, 40 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside per liter, and 0.2 mM isopropyl-β-D-thiogalactopyranoside.

Tn*5* mutagenesis and selection of nickel-sensitive mutants. To isolate transposon-induced nickel-sensitive mutants, the nickel-resistant transconjugant *A. eutrophus* M219 was mated on nutrient broth with *E. coli* S17-1 harboring pSUP5011 as described below. Kanamycin-resistant transconjugants of *A. eutrophus* M219 were screened on selective plates (Tris-mineral medium containing 700 μg of kanamycin sulfate per ml).

Single kanamycin-resistant colonies were transferred to Tris-mineral medium containing 700 μg of kanamycin sulfate per ml and 1.0 mM $NiCl_2$. Tranconjugants which formed no colonies in the presence of 1.0 mM $NiCl_2$ were collected and tested for maintenance of pMOL28. Mutants which were nickel sensitive and kanamycin resistant but still harbored the megaplasmid pMOL28 were subjected to further analysis.

Isolation of DNA. Megaplasmid DNAs (160 to 300 kb) of *A. eutrophus* strains were isolated from cells grown in Tris-mineral medium containing 2 g of fructose or 3 g of gluconate per liter at 30°C. The procedure described previously (16, 25) was used with slight modifications.

After the first ethanol precipitation, the DNA solution was subjected to extraction and phase separation once with 1 volume of Tris-saturated phenol, twice with 1 volume of Tris-saturated phenol-(chloroform-isoamylalcohol) (1:1), and twice with chloroform-isoamylalcohol (24:1) as described for the alkaline lysis procedure (3, 20). The purified DNA was precipitated with ethanol a second time at 0°C for 30 min, washed once with 70% ethanol, pelleted, vacuum dried, and dissolved in sterile water or TE buffer (10 mM Tris hydrochloride [pH 7.6], 1 mM disodium EDTA). Small plasmid DNA (2.7 to 50 kb) was isolated as described previously (3).

DNA fragments were isolated from agarose gels by using an electroeluating apparatus (Biometra, Göttingen, Federal Republic of Germany), following the protocol of the manufacturer.

Analysis of plasmid DNA. Crude lysates were separated by electrophoresis in horizontal slab gels of 0.8% (wt/vol) agarose in TBE buffer (50 mM Tris hydrochloride, 50 mM boric acid, 1.25 mM disodium EDTA [pH 8.5]) at 150 V and 40 mA for 6.5 h. Isolated plasmid DNA was digested with various restriction endonucleases as described by the manufacturer. Endonuclease-digested DNA fragments were separated in horizontal slab gels with TBE containing 0.8 to 1.5% (wt/vol) agarose or in vertical polyacrylamide gels (8% [wt/vol] acrylamide) as described previously (21). The migration of *Eco*RI-, *Hin*dIII-, and *Pst*I-digested DNA fragments from bacteriophage λ served as a standard for estimating the molecular weights of unknown DNA fragments. DNA bands were stained with ethidium bromide and visualized on a UV transilluminator.

Hybridization of Southern filters. Southern blots (42) with nitrocellulose filters (BA85; pore size, 0.45 μm; Schleicher & Schuell, Dassel, Federal Republic of Germany) were hybridized with biotinylated probes as described previously (19), with the following exception: DNA fragments blotted to nitrocellulose were fixed routinely by UV treatment (17).

DNA ligation. To prevent religation, vector DNA was routinely dephosphorylated as recommended by the manufacturer and restriction enzymes were removed by phenol-chloroform extraction before ligation. DNA fragments with sticky ends were ligated with T4 DNA ligase to vector DNA at 25°C for 4 h in 50 mM Tris hydrochloride (pH 7.6), 10 mM $MgCl_2$, 5% (wt/vol) polyethylene glycol 8000, 1 mM ATP, and 1 mM dithioerythritol according to the protocol of the manufacturer. Blunt-end ligation was performed as described above for ligation of sticky ends but in threefold excess of T4 DNA ligase.

Transformation. For transformation, *E. coli* was grown aerobically in LB medium supplemented with 20 mM $MgCl_2$ at 37°C (12). Competent cells were transformed by using the calcium chloride procedure (20). Transformants were selected as described in Results.

Conjugation. Matings of *A. eutrophus* and other Gram-negative bacteria with *E. coli* S17-1 harboring hybrid donor plasmids were performed on solidified nutrient broth medium as described previously (9). After 20 h of incubation at 30°C, the cells were washed from the agar and plated onto selective media to isolate the desired transconjugants.

Manipulation of restriction sites by T4 DNA polymerase. In order to clone *Kpn*I-generated fragments into the *Eco*RI cloning site of the broad-host-range vehicle pVK101 (18), the *Kpn*I-cut ends were modified by using the following procedure. First the 3′-protruding *Kpn*I ends were blunted in the presence of the deoxynucleoside triphosphates dATP, dGTP, dCTP, and dTTP by the 3′-5′ exonuclease activity of T4 DNA polymerase (20). The *Eco*RI-generated 5′-protruding ends of vector DNA were then filled in the presence of deoxynucleoside triphosphates by the 5′-3′ polymerase function of the same T4 DNA polymerase (20). Vector and insert sites were joined by blunt-end ligation. After redigestion of the joined molecules by *Eco*RI, the former *Kpn*I sites of insert DNA were converted to *Eco*RI sites.

Assay of $^{63}Ni^{2+}$ uptake. The uptake assay of $^{63}Ni^{2+}$ with nickel-sensitive strains and resistant recombinant cells was performed as previously described (33).

Chemicals. Restriction endonucleases, Biotin-11-dUTP, the nick translation kit, the DNA detection kit, and T4 DNA ligase were obtained from GIBCO/Bethesda Research Laboratories GmbH, Eggenstein, Federal Republic of Germany. Agarose type NA and T4 DNA polymerase were purchased from Pharmacia, Uppsala, Sweden. Lambda DNA and calf intestinal phosphatase were obtained from C. F. Boehringer & Soehne, Mannheim, Federal Republic of Germany. Antibiotics and ethidium bromide were obtained from Sigma Chemical Co., St. Louis, Mo. $^{63}NiCl_2$ (0.73 Ci/mmol) was purchased from the Radiochemical Centre, Amersham, England. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside and isopropyl-β-D-thiogalactopyranoside were obtained from Biomol, Ilvesheim, Federal Republic of Germany. Complex media were obtained from Difco Laboratories. All other chemicals of pro analysis quality were obtained from E. Merck AG, Darmstadt, Federal Republic of Germany.

RESULTS

Isolation of Tn*5*-*mob*-induced nickel-sensitive mutants. Transposon mutagenesis was applied to generate nickel-sensitive mutants. The plasmid pMOL28 (163 kb) was mutagenized by using the transposable element Tn*5*-*mob* by mobilization of the suicide vehicle pSUP5011 from *E. coli* S17-1 into the nickel-resistant transconjugant *A. eutrophus* M219. Approximately 22,000 kanamycin-resistant transconjugants were screened on Tris-mineral medium containing 1.0 mM $NiCl_2$ (see Materials and Methods) for nickel sensi-

tivity. Among 128 nickel-sensitive, kanamycin-resistant mutants, only two nickel-sensitive strains (referred to as KB1T and KB6T) which still harbored plasmid pMOL28 were isolated.

Localization of Tn*5-mob* insertion and characterization of transposon mutants. Biotinylated plasmid pSUP5011 was used as a specific probe in hybridization analysis to localize the Tn*5-mob* insertion. As Tn*5-mob* contains no restriction sites for the endonucleases *Eco*RI, *Kpn*I, *Pvu*I, and *Bal*I (15), single fragments harboring the Tn*5* insertion were expected when these restriction enzymes were used. Upon digestion of the pMOL28::Tn*5-mob* DNA from KB1T and KB6T with *Eco*RI and after hybridization with biotinylated pSUP5011 in both mutants, a single 52-kb *Eco*RI fragment showing a signal was detected. Considering the size of Tn*5-mob* (7.5 kb) (37), this signal corresponded to a native 44-kb *Eco*RI fragment of pMOL28. In Tn*5* mutant KB6T, an additional 8-kb *Eco*RI signal of unknown origin was observed. This 8-kb fragment could not be related to any *Eco*RI fragment of the native pMOL28 DNA. The native pMOL28 did not show a hybridization signal (data not shown).

Compared with the resistant strain *A. eutrophus* M219, the Tn*5*-induced nickel-sensitive derivatives KB1T and KB6T were unable to grow in the presence of 1 to 3 mM $NiCl_2$. Both mutants were also sensitive to cobaltous ions (1 to 5 mM $CoCl_2$). No nickel-resistant revertants of KB1T appeared upon incubation on Tris-mineral medium in the presence of 1.0 to 3.0 mM $NiCl_2$ or $CoCl_2$. In contrast, spontaneous nickel-resistant revertants of KB6T which had acquired full resistance to nickel and cobalt appeared. Further experiments were done with mutant KB1T exclusively.

Cloning of the Tn*5-mob*-labeled fragment. Digestion of pMOL28::Tn*5-mob* from KB1T with *Eco*RI and *Kpn*I and subsequent hybridization with biotinylated pSUP5011 resulted in a single 17-kb *Kpn*I signal. The 17-kb *Kpn*I fragment named K10 was cloned into pUC19. *E. coli* XL-1 Blue served as the recipient for transformation, and 12 kanamycin-resistant clones were selected. All hybrid plasmids contained an identical 17-kb *Kpn*I fragment (data not shown). The cloned Tn*5-mob*-carrying DNA fragment K10 was electroeluted and used as a biotinylated probe to identify the corresponding native 9.5-kb fragment from *Kpn*I-digested pMOL28 DNA.

Cloning of the nickel resistance gene(s). In order to clone the wild-type nickel resistance gene(s) from pMOL28, the 44-kb *Eco*RI fragment of pMOL28 was isolated by electroelution and digested with *Kpn*I. The *Kpn*I fragments were ligated into pUC19 and transformed into *E. coli* XL-1 Blue. The recombinant plasmids were collected and screened for fragments of 8 to 10 kb. Four plasmids (pPA27, pPA56, pPA57, and pPA87) were cleaved with *Kpn*I. The respective digests, the *Kpn*I-digested native pMOL28 DNA, and pMOL28::Tn*5-mob* from mutant KB1T were hybridized with biotinylated K10 (Fig. 1). Specific fragment signals were obtained as expected. The K10 probe hybridized to the 17-kb *Kpn*I fragment of pMOL28::Tn*5-mob* from KB1T and to the native 9.5-kb *Kpn*I fragment from M219. Furthermore, specific signals due to homology were observed with two fragments named PA27 and PA57 from the *Kpn*I gene bank of pMOL28. Thus these clones were shown to contain the native fragments which correspond to the Tn*5-mob*-labeled *Kpn*I fragment (Fig. 1).

The position of Tn*5-mob* insertion within *Kpn*I fragment K10 was located 1.1 kb from one end (Fig. 2). This asymmetric position of Tn*5* in PA27 and PA57 suggested that these fragments might not contain the full nickel resistance determinant. As the Tn*5-mob*-carrying fragment K10 had only two *Hin*dIII cleavage sites, both within Tn*5*, a *Hin*dIII fragment which included the *Kpn*I fragment PA57 should have been present in pMOL28. This was examined by probing a *Hin*dIII digest of the native pMOL28 DNA with labeled PA57 DNA, and a single 13.5-kb *Hin*dIII fragment was identified. The metal resistance genes of this fragment were cloned by constructing a *Hin*dIII gene library of pMOL28. The fragments were ligated into the Tc^r gene of the mobilizable suicide vector pSUP202 (39) in *E. coli* S17-1. A total of 438 Cm^r Ap^r Tc^s transformants were selected and mated with the mutant strain KB1T as the indicator. Tc^s transconjugants were tested for metal resistance by replica plating to Tris-mineral medium containing 1 mM Ni^{2+}. Three

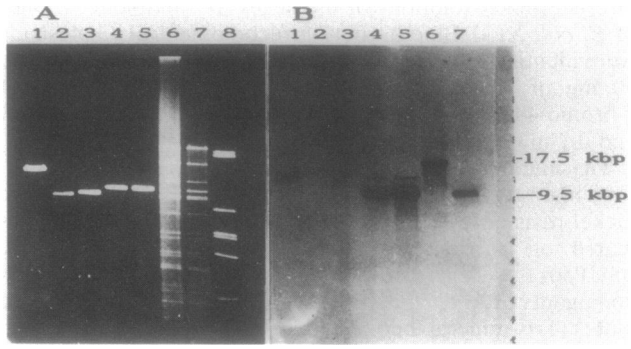

FIG. 1. Hybridization of *Kpn*I-digested pMOL28, pMOL28::Tn*5*, and cloned *Kpn*I fragments from pMOL28 with the biotinylated DNA fragment K10. Restriction fragments were separated by electrophoresis in 0.8% agarose and blotted onto a nitrocellulose filter (A) and then were probed with the biotinylated DNA fragment K10 as described in Materials and Methods (B). Hybridization signals of *Kpn*I fragments PA57 and PA27 (lanes 4 and 5) and *Kpn*I-restricted pMOL28::Tn*5-mob* DNA from KB1T (lane 6) are shown. Lane 7 shows the homology signal of *Kpn*I-restricted pMOL28 DNA from *A. eutrophus* M219. No hybridization was found with the *Kpn*I fragments PA87 and PA56 (lanes 2 and 3). pSUP5011 served as a positive control for hybridization (lane 1). Lane 8 shows *Eco*RI-cut lambda DNA. The cloning vector pUC19 is not visible in the gel because of prolonged electrophoresis.

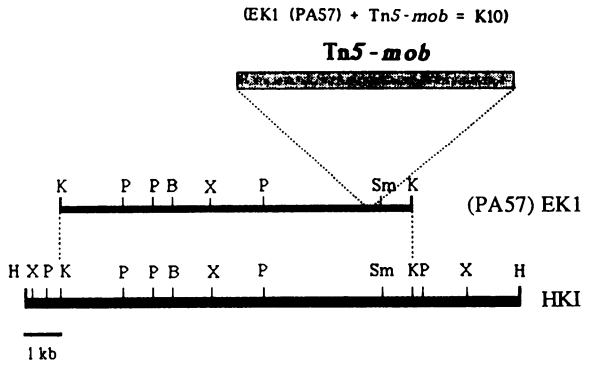

FIG. 2. Physical map of the 13.5-kb *Hin*dIII fragment HK1 coding for resistance to nickel and cobalt. The upper line shows the originally cloned *Kpn*I fragment PA57, which was converted by T4 DNA polymerase treatment (see Materials and Methods) into a fragment with *Eco*RI sites named EK1, which is internal to fragment HK1 (lower line). Restriction sites: H, *Hin*dIII; B, *Bam*HI; P, *Pst*I; X, *Xho*I; K, *Kpn*I; Sm, *Sma*I. The position of Tn*5-mob* insertion is shown within EK1 (PA57).

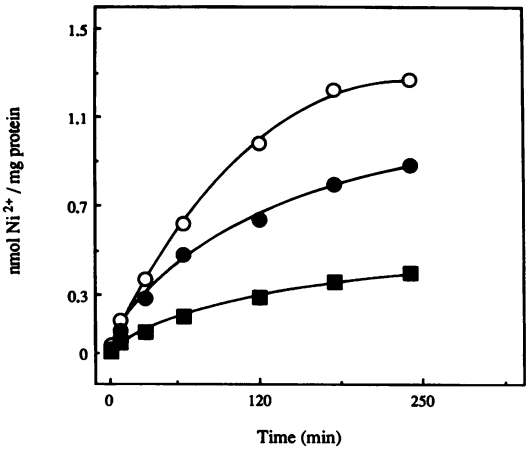

FIG. 3. Uptake of $^{63}Ni^{2+}$ (1.0 μM) by the sensitive cured strain AE104 (○), its pVK101::EK1-harboring derivative (●), and the resistant strain AE104 harboring pVK101::HKI (■).

independently isolated transconjugants had recovered the phenotype of M219 and were resistant to 3 mM $NiCl_2$ and 5 mM $CoCl_2$. The hybrid plasmids were characterized by *Hin*dIII and *Kpn*I endonuclease digestion. All three hybrids contained the same 13.5-kb *Hin*dIII fragment. It was named HKI. As shown by restriction enzyme mapping, HKI contained the previously mentioned *Kpn*I fragment PA57 as a subfragment (Fig. 2).

Complementation of nickel-sensitive mutants. Fragments PA57 and HKI were tested for their abilities to confer nickel and cobalt resistance. Therefore, the suicide vector pSUP202 was used. Complementation was studied with the IncP1 broad-host-range vector pVK101. Since neither pSUP202 nor pVK101 contains single cloning sites for *Kpn*I fragments, the *Kpn*I-generated 3′-protruding ends of fragment PA57 were converted into *Eco*RI-compatible ends. The former *Kpn*I fragment PA57, which was converted to an *Eco*RI fragment, was named EK1. This was ligated into the Cm^r inactivation site of pSUP202 and into the *Eco*RI site of pVK101; HKI was cloned into the *Hin*dIII-site of pVK101. The hybrid plasmids pSUP202::EK1, pVK101::EK1, pSUP202::HKI, and pVK101::HKI were transformed to *E. coli* S17-1, and the resulting strains were used as donors in mating experiments with a variety of sensitive recipients (Table 1).

The fragments EK1 and HKI conferred resistance to nickel and cobalt but exhibited considerable differences in the levels of expression. Both fragments restored the Tn*5* mutant KB1T to full nickel and cobalt resistance, i.e., to tolerate 3 mM $NiCl_2$ and 6 mM $CoCl_2$. In contrast, the mutant KB8(5)N was complemented by HKI but not by EK1. Conjugative transfer of pVK101::EK1 to strains AE104 or AE128 resulted in only poor expression of nickel resistance (i.e., growth up to 1.2 mM $NiCl_2$). In contrast, transfer of pVK101::HKI to AE104 and AE128 resulted in full nickel resistance (Table 2).

$^{63}Ni^{2+}$ uptake assays with AE104 harboring the recombinant plasmids showed that plasmid pVK101::HKI reduced the uptake of nickel to a greater extent than pVK101::EK1 (Fig. 3). These $^{63}Ni^{2+}$ uptake assays were in accordance with data of growth experiments (Table 2). The poor growth of AE104(pVK101::EK1) in the presence of 1 mM $NiCl_2$ compared with that of AE104(pVK101::HKI) is due to a higher level of nickel uptake, which impairs metabolism

TABLE 2. Maximum tolerable concentrations of nickel and cobalt in wild-type strains and transconjugants harboring hybrid plasmids pVK101::EK1 and pVK101::HKI

Mutants and strains	Maximum tolerable concentration[a] (mM) of:	
	Ni^{2+}	Co^{2+}
A. eutrophus		
CH34(pMOL28, pMOL30)	3.0	20.0
AE126(pMOL28)	3.0	5.0
HF33	0.3	0.3
M219(pMOL28)	3.0	6.0
AE104	0.5	0.07
AE104(pVK101)	0.5	0.07
AE104(pVK101::HKI)	3.0	5.0
AE104(pVK101::EK1)	1.5	1.0
AE128	0.5	20.0
AE128(pVK101)	0.5	20.0
AE128(pVK101::HKI)	3.0	20.0
AE128(pVK101::EK1)	1.2	20.0
KB1T	0.1	0.3
KB1T(pVK101)	0.1	0.3
KB1T(pVK101::HKI)	4.0	5.0
KB1T(pVK101::EK1)	4.0	5.0
KB8(5)N	0.3	0.3
KB8(5)N(pVK101)	0.3	0.3
KB8(5)N(pVK101::HKI)	3.0	3.0
KB8(5)N(pVK101::EK1)	0.3	0.3
MO3	0.5	20
MO3(pVK101)	0.5	20
MO3(pVK101::HKI)	3.0	20
MO2	0.5	20
MO2(pVK101)	0.5	20
MO2(pVK101::HKI)	3.0	20
MO5	0.5	20
MO5(pVK101)	0.5	20
MO5(pVK101::HKI)	3.0	20
H16	0.3	0.2
H16(pVK101)	0.3	0.2
H16(pVK101::HKI)	1.0	0.8
A. hydrogenophilus	0.5	0.5
A. hydrogenophilus(pVK101)	0.5	0.5
A. hydrogenophilus(pVK101::HKI)	3.0	1.5
A. eutrophus		
JMP222	0.5	0.8
JMP222(pVK101)	0.5	0.8
JMP222(pVK101::HKI)	2.0	1.5
P. oleovorans	0.5	0.5
P. oleovorans(pVK101)	0.5	0.5
P. oleovorans(pVK101::HKI)	1.5	1.5
P. putida	0.5	0.5
P. putida(pVK101)	0.5	0.5
P. putida(pVK101::HKI)	1.0	1.0

[a] Conditions, 4 days of growth on Tris-gluconate mineral medium containing nickel and cobalt salts at 30°C.

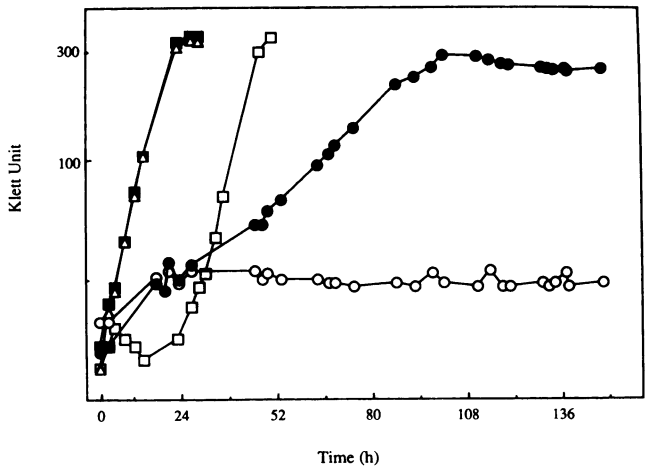

FIG. 4. Growth of the wild-type *A. eutrophus* CH34 and its cured derivative AE104 harboring different recombinant plasmids in Tris-gluconate medium containing $NiCl_2$. Symbols: ○, plasmid-free AE104 or AE104 containing pVK101 in the presence of 1 mM $NiCl_2$; △, AE104(pVK101::HKI) in the presence of 1 mM $NiCl_2$; ●, AE104 (pVK101::EK1) in the presence of 1 mM $NiCl_2$; ■, AE104(pVK101:: HKI) in the presence of 3 mM $NiCl_2$; □, wild-type *A. eutrophus* CH34 in the presence of 3 mM $NiCl_2$.

(Fig. 4). Transfer of the plasmid pVK101 alone to sensitive recipients did not cause nickel resistance. Thus pVK101:: EK1 did not contain all the genetic information for expression of heavy metal resistance, whereas pVK101::HKI did (24).

Previously, three nickel-sensitive mutants with defective pMOL28 were isolated from the multiple-metal-resistant wild-type strain *A. eutrophus* CH34. The nickel-sensitive mutant MO2 was isolated after growth of *A. eutrophus* CH34 in the presence of novobiocin (50 μg/ml) and selected for nickel sensitivity (H. G. Schlegel, unpublished results). The nickel-sensitive mutant MO3 was isolated after treatment of cells of wild-type CH34 with 4 μg of mitomycin per ml and shown to carry pMOL28 with a deletion of about 40 kb (11). MO3 was unable to grow in the presence of 1 mM $NiCl_2$. The nickel-sensitive strain MO5 was isolated after growth at an elevated temperature of 37°C, which was used for curing purposes (21) (H. G. Schlegel, unpublished results). pVK101::HKI restored full nickel resistance to all three mutants MO2, MO3, and MO5 (Table 2).

However, pVK101::HKI-encoded nickel resistance differed from pMOL28-encoded resistance (32, 33). While pMOL28 determined inducible nickel resistance in all transconjugants, the hybrid plasmid in strain AE104 determined constitutive nickel resistance (Fig. 4).

Heterologous expression of nickel resistance. The hybrid plasmid pVK101::HKI was transferred to several wild-type strains (Table 1). The plasmid conferred resistance to *A. eutrophus* H16 and JMP222, *Alcaligenes hydrogenophilus*, *Pseudomonas putida*, and *Pseudomonas oleovorans*. The degree of resistance varied from strain to strain (Table 2). Transconjugants of *A. hydrogenophilus*, *P. putida*, and *P. oleovorans* were resistant to 1 to 3 mM $NiCl_2$ (Table 2). Transconjugants of H16 (Table 2) or HF33 (data not shown) exhibited poor expression of nickel and cobalt resistance. No expression of nickel resistance was observed with *E. coli* and *Pseudomonas stutzeri* (data not shown).

DISCUSSION

We report here the first genetic analysis of a nickel resistance determinant. We have shown recently that the nickel resistance plasmid pMOL28 and the constitutive mutant pMOL28.1 are mobilizable to the cured, plasmid-free, nickel-sensitive strain AE104 derived from CH34 and to other wild-type strains of *A. eutrophus* (H16, N9A, G29, and their derivatives) in which nickel resistance is wild type-like (33). The plasmid pMOL28 was transferred by conjugation to *E. coli*, but was not expressed effectively (H. G. Schlegel, unpublished results).

In this study we isolated two DNA fragments of pMOL28 which carry nickel and cobalt resistance genes, the 9.5-kb fragment EK1 and the 13.5-kb *Hin*dIII fragment HKI. EK1 is contained within HKI. Analysis by marker rescue and complementation in *trans* showed that both fragments were able to restore full nickel resistance in the nickel-sensitive Tn*5* mutant KB1T. Furthermore, pVK101::HKI restored full nickel resistance in nickel-sensitive mutants MO2, MO3, and MO5 of strain CH34, which carry defective pMOL28 plasmids. Finally, HKI conferred full resistance to the cured, pMOL28-free derivatives AE104 and AE128 of strain CH34. These results indicate that fragment HKI carries the complete information required for the expression of full nickel resistance in the native host.

Both DNA fragments EK1 and HKI were also transferred to various other strains of *A. eutrophus*. Although pMOL28 is fully expressed in these strains, fragment HKI was only incompletely expressed (tolerating up to approximately 1 mM $NiCl_2$). The expression of EK1 was even less. Heterologous expression of HKI occurred to about the same extent in *A. hydrogenophilus*, *P. oleovorans*, and *P. putida*.

The failure to express the cloned DNA fragment in *E. coli* may be due to the failure of the genes to be transcribed or translated or to the inability of the gene products to function in *E. coli*. Some *A. eutrophus* genes coding for the synthesis of poly-β-hydroxybutyric acid are readily expressed in *E. coli* (29, 40), and recently a plasmid-encoded inducible tellurite resistance determinant has been transferred from an *Alcaligenes* strain to *E. coli*, in which an even higher level of resistance was expressed than in the native host (14).

The low level of expression of nickel resistance by fragment HKI in *A. eutrophus* H16 has a parallel in the expression of pMOL30 DNA fragments coding for cadmium, cobalt, and zinc resistances. The hybrid plasmid pDN7 containing a pMOL30 DNA fragment was fully expressed in metal-sensitive derivatives of the native host CH34 but only poorly in *A. eutrophus* H16 or *A. hydrogenophilus* (25). Furthermore, all three resistances (Cd^r, Zn^r, and Co^rB) coded by pDN7 were expressed constitutively, whereas the resistance properties in CH34 are inducible (26).

The complete expression of fragment HKI in the native host bacterium and the incomplete expression in closely related strains or species raises the question of whether an additional host cell gene product is required for expression. We are currently searching for an additional DNA fragment of pMOL28 which enhances nickel resistance in transconjugants of *A. eutrophus* H16.

Our studies so far suggest that nickel and cobalt resistances are coded for by the same determinants. Subinhibitory concentrations of nickel and cobalt can induce the metal resistances in the wild-type strain CH34 and in pMOL28-carrying transconjugants (H. G. Schlegel, unpublished data). The mutants constitutive for nickel resistance are also constitutive for cobalt (33). The fragment HKI

conferred both nickel and cobalt resistances (Table 2). Whether resistance to both metals is due to the same efflux protein is unknown. DNA-DNA hybridization has been used to study homology among arsenate (23), cadmium (6), copper (2), and mercury (1) resistance determinants. It was shown recently that a second cobalt resistance system ($CobB^r$) from pMOL30, which also confers cadmium and zinc resistance, lacks homology to pMOL28-encoded heavy metal resistance genes (25). However, by probing with nickel resistance-specific DNA sequences from pMOL28 cloned within fragment HKI, we found homology with nickel resistance genes of a recently isolated strain, 4a-2, and of a nickel- and cobalt-resistant isolate, KTO2 (44; P. Kaur, K. Ross, H. G. Schlegel, and R. A. Siddiqui, manuscript in preparation). The results for pMOL28-encoded metal resistances are in contrast to the behavior of two plasmids, pTOM8 and pTOM9, both of which code for nickel and cobalt resistance when transferred to strain AE104 but which express only nickel resistance when transferred to strain H16 (T. Schmidt and H. G. Schlegel, FEMS Microbiol. Ecol., in press). These well-documented results indicate that nickel and cobalt resistances can be due to different gene products and can be regulated differently.

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