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High-Level Nickel Resistance in *Alcaligenes xylosoxydans* 31A and *Alcaligenes eutrophus* KTO2

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Two new nickel-resistant strains of *Alcaligenes* species were selected from a large number (about 400) of strains isolated from ecosystems polluted by heavy metals and were studied on the physiological and molecular level. *Alcaligenes xylosoxydans* 31A is a heterotrophic bacterium, and *Alcaligenes eutrophus* KTO2 is an autotrophic aerobic hydrogen-oxidizing bacterium. Both strains carry—among other plasmids—a megaplasmid determining resistance to 20 to 50 mM $NiCl_2$ and 20 mM $CoCl_2$ (when growing in defined Tris-buffered media). Megaplasmids pTOM8, pTOM9 from strain 31A, and pGOE2 from strain KTO2 confer nickel resistance to the same degree to transconjugants of all strains of *A. eutrophus* tested but were not transferred to *Escherichia coli*. However, DNA fragments carrying the nickel resistance genes, cloned into broad-host-range vector pVDZ'2, confer resistance to *A. eutrophus* derivatives as well as *E. coli*. The DNA fragments of both bacteria, TBA8, TBA9, and GBA (14.5-kb *Bam*HI fragments), appear to be identical. They share equal size, restriction maps, and strong DNA homology but are largely different from fragment HKI of nickel-cobalt resistance plasmid pMOL28 of *A. eutrophus* CH34.

Generally, the concentrations of the ions of heavy metals in soils and waters are very low and allow unimpaired growth of "normal" bacteria, i.e., those not carrying metal resistance properties. Ecosystems polluted by toxic concentrations of heavy metals are inhabited by bacteria which are resistant to one or another or a whole series of metal ions. Many of these bacteria carry plasmids (20, 28, 36). Metal ions are present in the wastewaters of the metal-working industry in galvanization tanks, decantation tanks, and the corresponding sediments and composts. A metal decantation tank of a zinc factory in Liège, Belgium, was the location where *Alcaligenes eutrophus* CH34 was isolated. This strain represents a type of a metal-resistant bacterium which was later repeatedly encountered in various other habitats. It is resistant to a series of heavy metals, such as nickel, cobalt, zinc, cadmium, mercury, and chromate (21, 22, 24). Reisolations occurred near Göttingen, Germany (31, 39), in industrial regions in Belgium, and from mining areas in Zaire (8, 9).

In *A. eutrophus* CH34, metal resistance is determined by two plasmids (13), pMOL28 (163 kilobase pairs [kb]) for resistance to cobalt, nickel, chromate, and mercury and pMOL30 (238 kb) for resistance to cadmium, zinc, and cobalt (22, 24, 25). These plasmids are self-transmissible to cured derivatives of the native host bacterium CH34 and were transferred to other bacteria by helper plasmid-assisted conjugation. The resistance properties carried by either one of both plasmids have been studied in some detail. Plasmid pMOL28-encoded nickel and cobalt resistances are inducible properties (34, 35), and nickel resistance was shown to be due to an energy-dependent specific efflux mechanism (32, 40). In rare mutants nickel and cobalt resistance is expressed constitutively (35).

The pMOL30-encoded resistance to cadmium, zinc, and cobalt turned out to be inducible and due to an efflux mechanism, too (26). The resistance genes have been cloned from pMOL28 by Siddiqui et al. (33) and Nies et al. (23, 24) and from pMOL30 by Nies et al. (25).

The present paper deals with two strains of metal-resistant bacteria which have been isolated from polluted ecosystems and concentrates predominantly on nickel resistance. Like CH34, both bacteria are gram negative, but in contrast to CH34, which tolerates only 3 mM $NiCl_2$ when grown on or in Tris-buffered defined media, the new strains tolerate about 40 mM $NiCl_2$. One of them is a chemolithoautotrophic bacterium growing aerobically on H_2 as an energy source and CO_2 as a carbon source and is a knallgas bacterium like CH34 (39), whereas the other grows heterotrophically only (31). There is also another reason for paying special attention to the two new isolates. Cloned DNA fragments carrying the nickel resistance genes are expressed not only in *A. eutrophus* strains and derivatives of CH34 but also in *Escherichia coli* K-12 and derivatives therefrom. This difference facilitates the application of the methods of molecular analysis and raises further questions with regard to the regulation and genetic localization of expression of nickel resistance.

MATERIALS AND METHODS

Strains and transconjugants used in this study are summarized in Table 1; cloning vectors are listed in Table 2.

Growth conditions. *Alcaligenes* strains were grown at 30°C in Tris mineral medium (22) supplemented with gluconate (0.4%) or fructose (0.4%). Heavy metal chlorides were added before autoclaving, and the pH was adjusted to 7.0. *E. coli* strains were grown at 37°C in Luria-Bertani medium (29) containing appropriate antibiotics. Nickel resistance of *E. coli* strains was examined in Tris mineral medium or in *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)-buffered (50 mM) mineral medium of the same composition; both mineral media were supplemented with gluconate.

Estimation of highest tolerable metal concentrations. Cells were grown on liquid or solidified TES-gluconate medium and spread on agar plates containing various concentrations

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TABLE 1. Bacterial strains, mutants, and transconjugants

Species and strain	Relevant characteristics	Plasmid(s) (kb)	Reference or source
A. xylosoxydans 31A	Nic^r Cob^r Zin^r Cad^r	pTOM8 (340), pTOM9 (200)	31
A. eutrophus			
KTO2	Nic^r Cob^r Zin^r Cad^r	pGOE1 (250), pGOE2 (210), pGOE3 (170)	39
CH34	Nic^r Cob^r Zin^r Cad^r	pMOL28 (163), pMOL30 (238)	22
AE104	Metal sensitive	Curing mutant of CH34	22
H16	Metal sensitive	pHG1 (458)	41
HF33	Metal sensitive	Curing mutant of H16	15
N9A	Metal sensitive	pHG3 (458)	11
N9AF06	Metal sensitive	Curing mutant of N9A	15
G29	Metal sensitive	pHG 22 (458)	DSM517
E. coli			
K12	Metal sensitive		DSM496
HB101	Metal sensitive, *leu pro thi* mutant, *recA2*		3
S17-1	Metal sensitive, *pro thi* mutant, *recA2 tra*$^+$		38
JM109	Metal sensitive, *thi recA* Δ(*lac-proAB*) F′ [*traD36 proAB lacI*^{q}ZΔM15]	F′	42

of metal salts. The plates were incubated at 30°C (*Alcaligenes* strains) or 37°C (*E. coli* strains) and were inspected at intervals for up to 5 days. Instead of measuring the MICs, which inhibit the formation of colonies on plates, we in many instances measured the highest tolerable concentration of metal salts which allows growth without decreasing the CFU. This concentration is lower than the MIC by a factor of about 2 (20).

Inducibility of nickel resistance genes. Precultures of *Alcaligenes* strains were grown at 30°C in Tris-gluconate medium without added $NiCl_2$ (uninduced cells) or with 2 to 3 mM $NiCl_2$ (induced cells). Two cultures containing high nickel concentrations (10 to 20 mM $NiCl_2$) were inoculated with induced or uninduced cells, respectively. As a positive control, a culture without added nickel was inoculated; as a negative control, a comparable strain lacking nickel resistance genes was incubated in the presence of 10 to 20 mM $NiCl_2$. All cultures were incubated on a rotary shaker at 30°C. Growth curves were based on turbidity measurements in 300-ml Klett flasks by using a Klett-Summerson photometer. *E. coli* strains were grown in the same way in TES-gluconate medium at 37°C.

Conjugation. Conjugation donors and recipients were grown in nutrient broth at 30°C for 12 to 14 h. *E. coli* strains were grown in Luria-Bertani broth at 37°C for 4 to 6 h. Tenfold-concentrated cell suspensions of donor and recipient strains were mixed in a ratio of 1:1. The mixture was dropped on nutrient broth agar (spot mating) and incubated at 30°C for 16 to 20 h. After mating, the cells were suspended in saline and transferred to appropriate selective agar media.

Transformation of *E. coli*. *E. coli* cells were grown in Luria-Bertani medium containing 20 mM $MgCl_2$ at 37°C to 30 to 40 Klett units. To make cells competent for DNA uptake, the calcium chloride procedure was used as described previously (14, 29).

TABLE 2. Cloning vectors and broad-host-range plasmids

Vector	Antibiotic resistance(s)	Reference or source
pSUP202	Ap^r Cm^r Tc^r	37
pVDZ'2	Tc^r	7
pUC19	Ap^r	42
Bluescript	Ap^r	Stratagene
pULB113	Ap^r Km^r Tc^r	18

Isolation of plasmid DNA. Vector DNA was isolated by the alkaline lysis method (29). For isolation of megaplasmids, cells were grown in Tris-gluconate medium at 30°C for at least 24 h. Crude lysates of megaplasmid DNA were prepared by the method of Kado and Liu (16) as modified by Nies et al. (25). Plasmid DNA was purified by extraction with 1 volume of Tris-saturated phenol-(chloroform-isoamyl alcohol) (1:1) and by extraction with chloroform-isoamyl alcohol (24:1). Ethanol precipitation was carried out as described previously (29).

Hybridization experiments. Fragments to be used as probes were purified from agarose gels by electrophoresis into a 3 mM sodium acetate buffer, according to the instructions of Biometra (Göttingen, Germany). Labelling of hybridization probes was performed with biotin-16-dUTP by using a nick translation kit (GIBCO/BRL, Eggenstein, Germany) by following the manufacturer's instructions. Southern blotting of target DNA to nylon membranes, hybridization conditions, and detection of labeled DNA were essentially as described by Oelmüller et al. (27).

Chemicals. Restriction enzymes were purchased from GIBCO/BRL. Agarose-type NA was obtained from Pharmacia, Freiburg, Germany. Antibiotics were obtained from Boehringer, Mannheim, Germany. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside and isopropyl-β-D-thiogalactopyranoside (IPTG) were obtained from Biomol, Ilvesheim, Germany. Heavy metal salts were purchased from E. Merck AG, Darmstadt, Germany.

RESULTS

Isolation and identification of strains. *Alcaligenes xylosoxydans* 31A was isolated in 1986 from a copper galvanization tank of a factory in Holzminden, Germany, 60 km from Göttingen (31). Strain 31A was able to grow in the presence of high concentrations of heavy metal salts (40 mM $NiCl_2$, 20 mM $CoCl_2$, 10 mM $ZnCl_2$, 1 mM $CdCl_2$, and 1.2 mM $CuCl_2$). The metal resistance genes were localized on megaplasmids pTOM8 (340 kb) and pTOM9 (200 kb) as reported previously (31). Strain 31A shared with strain CH34 the ability to grow

TABLE 3. Metal resistances conferred by megaplasmids and cloned fragments

Strain(s)	Plasmid (kb)	Resistance to (mM)[a]:				Strains mated
		Ni^{2+}	Co^{2+}	Zn^{2+}	Cd^{2+}	
AE104		0.5	0.1	0.3	0.2	
AE104	pVDZ'2 (20.5)	0.5	0.1	0.3	0.2	
H16, HF33, N9A, N9AF06, G29	—[b]	0.2	0.2	0.5	0.1	
N9AF06	pVDZ'2 (20.5)	0.2	0.2	0.5	0.1	
CT15	pTOM8 (340)	25	15	0.3	0.6	31A × AE104
CT16	pTOM9 (200)	25	15	0.3	0.6	31A × AE104
CHT18	pTOM8 (340), pTOM9 (200)	40	20	10	1.0	31A × AE104
FT20	pTOM8 (340)	15	0.2	0.5	0.1	31A × N9AF06
FT30	pTOM9 (200)	15	0.2	0.5	0.1	31A × N9AF06
HT20	pTOM8 (340)	15	0.2	0.5	0.1	31A × HF33
HT30	pTOM9 (200)	15	0.2	0.5	0.1	31A × HF33
FK2	pGOE2 (210)	25	15	0.3	0.6	KTO2(pULB113) × AE104
M255, M256	pGOE2 (210)	15	ND	ND	ND	KTO2(pULB113) × N9A
M257, M258	pGOE2 (210)	15	0.2	0.5	0.1	KTO2(pULB113) × N9AF06
M282	pGOE1 (250)	**	**	2.5	1.0	KTO2(pULB113) × N9AF06
M251, M252	pGOE2 (210)	15	***	***	***	KTO2(pULB113) × H16
M280	pGOE1 (250)	**	**	2.5	1.0	KTO2(pULB113) × H16
M253, M254	pGOE2 (210)	15	0.2	0.5	0.1	KTO2(pULB113) × HF33
M281	pGOE1 (250)	**	**	2.5	1.0	KTO2(pULB113) × HF33
M259, M262	pGOE2 (210)	20	***	***	***	KTO2(pULB113) × G29
M283	pGOE1 (250)	ND	ND	2.5	1.0	KTO2(pULB113) × G29
AE104	pVDZ'2::TBA8/9	30	15	0.5	0.8	
AE104	pVDZ'2::GBA	30	15	0.5	0.8	
N9AF06	pVDZ'2::TBA8/9	15	0.2	0.5	0.1	
N9AF06	pVDZ'2::GBA	15	0.2	0.5	0.1	

[a] **, does not grow in the presence of 3 mM $NiCl_2$ or 5 mM $CoCl_2$; ***, does not grow in the presence of 5 mM $CoCl_2$ or 2.5 mM $ZnCl_2$ or 1 mM $CdCl_2$. The maximally tolerable concentrations were not determined. ND, not determined.

[b] —, wild-type strains H16, N9A, and G29 carry only the autotrophy megaplasmid pHG (458 kb), which is not relevant to metal resistance and was therefore not listed in the column; derivatives HF33 and N9AF06 lack the autotrophy plasmids.

on gluconate and the inability to grow on glucose, fructose, lactose, and other sugars or on salicylate. In contrast to CH34, strain 31A does not grow lithoautotrophically on H_2 plus CO_2.

A. eutrophus KTO2 was isolated in 1984 from the wastewater treatment plant of the city of Göttingen (39). The patterns and degrees of resistances to heavy metals are similar to those of strain 31A. Strain KTO2 contains, however, three megaplasmids: pGOE1 (250 kb), determining zinc and cadmium resistance; pGOE2 (210 kb), determining nickel and cobalt resistance; and pGOE3 (170 kb), so far cryptic. The estimation of plasmid sizes (data not shown) was performed by agarose gel electrophoresis using reference plasmids pHG1, pMOL28, and pMOL30, whose sizes were determined by contour length measurements (13, 22). KTO2 shares with CH34 the ability to grow lithoautotrophically; it grows in mineral medium under an atmosphere of H_2 plus CO_2 plus O_2 at 30°C with a doubling time of about 5 h. It forms both a NAD-dependent cytoplasmic hydrogenase and a membrane-bound hydrogenase. KTO2 grows on gluconate but not on glucose, fructose, lactose, or other sugars tested and not on salicylate.

Transfer of megaplasmids by conjugation. Megaplasmids pTOM8, pTOM9, pGOE1, and pGOE2 were found to be self-transmissible by conjugation. In matings of *A. xylosoxydans* 31A and *A. eutrophus* KTO2 with strain AE104, which is a plasmid-free derivative of *A. eutrophus* CH34, and *A. eutrophus* N9AF06, which is a derivative of the wild-type strain N9A lacking the autotrophy (Aut) plasmid pHG3, nickel-resistant transconjugants occurred with a frequency of 10^{-8} per donor cell. In helper plasmid (pULB113)-assisted matings, the frequency of transfer rose up to 10^{-5} per donor cell. Attempts to establish those megaplasmids in *E. coli* strains (K-12, S17-1, and HB101) did not result in any success. With strain 31A as a donor, nickel-resistant transconjugants contained at least one of the donor megaplasmids (pTOM8 or pTOM9), whereas matings with *A. eutrophus* KTO2 as a donor resulted in transconjugants which contained only one megaplasmid, namely, pGOE2. When cadmium- or zinc-resistant transconjugants were selected after mating, they were found to contain pGOE1. The designation of transconjugants and the corresponding matings are listed in Table 3. Plasmid patterns of some representative strains and transconjugants are shown in Fig. 1.

Transconjugants of *A. eutrophus* AE104 with pTOM8 or pTOM9 were resistant to 25 mM $NiCl_2$, 15 mM $CoCl_2$, and 0.6 mM $CdCl_2$. If both megaplasmids were present, the three metal resistances were higher and, in addition, resistance to 10 mM $ZnCl_2$ was expressed. But transconjugants of *A. eutrophus* N9AF06 containing one of these megaplasmids showed exclusively nickel resistance (up to 15 mM $NiCl_2$).

Plasmid pGOE1 was transferred by helper (pULB113)-assisted conjugation from KTO2 to *A. eutrophus* H16, HF33, N9AF06, and G29 and was expressed to the level of about 2.5 mM Zn^{2+}, 1.0 mM Cd^{2+}, and 5 mM Co^{2+}. Thus, plasmid pGOE1 is, with respect to size and metal resistance, similar to pMOL30. Plasmid pGOE2 was transferred from the same donor strain, KTO2(pULB113), to *A. eutrophus*

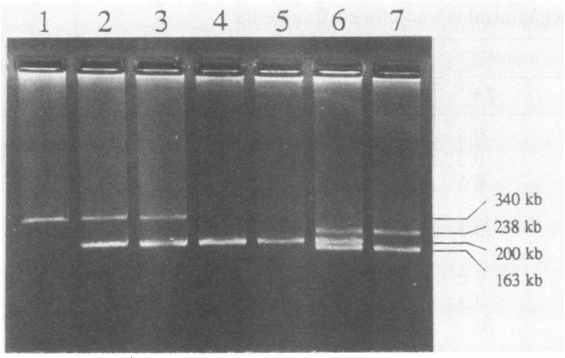

FIG. 1. Plasmid patterns of *A. xylosoxydans* 31A, *A. eutrophus* KTO2, and several transconjugants of *A. eutrophus* AE104. Agarose gel electrophoresis of crude lysates of the following strains: transconjugant CT15 with pTOM8 (lane 1); *A. xylosoxydans* 31A with pTOM8 and pTOM9 (lane 2); transconjugant CHT18 with pTOM8 and pTOM9 (lane 3); transconjugant CT16 with pTOM9 (lane 4); transconjugant FK2 with pGOE2 (lane 5); *A. eutrophus* KTO2 with pGOE1, pGOE2, and pGOE3 (lane 6); and *A. eutrophus* CH34 with pMOL28 and pMOL30 (lane 7). Electrophoresis proceeded for 12 h at 110 V (0.6% agarose). As there were no small plasmids present in the lower part of the figure, it was cut off.

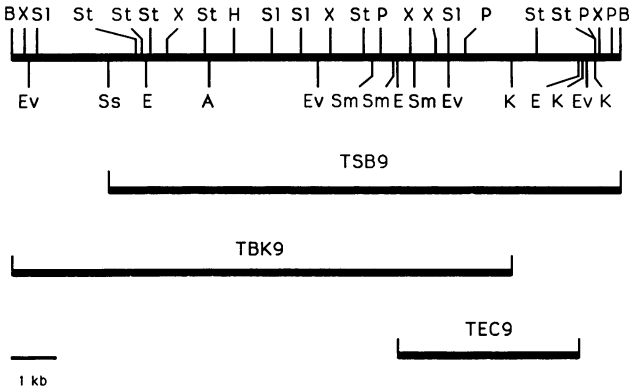

FIG. 2. Physical map of 14.5-kb *Bam*HI fragments TBA8, TBA9, and GBA. Subfragments TSB9, TBK9, and TEC9 are also shown. Restriction sites: A, *Apa*I; B, *Bam*HI, E, *Eco*RI; Ev, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; Sl, *Sal*I; Sm, *Sma*I; Ss, *Sst*I; St, *Sst*II; X, *Xho*I.

H16, HF33, N9A, N9AF06, G29, and AE104; in all transconjugants, expression of nickel resistance occurred to a degree higher than that reached with pMOL28. Plasmids pTOM9 and pGOE2 strongly resemble each other in size, degree of expression, and restriction patterns. Although the last are not shown here in extenso, the *Bam*HI restriction patterns of pTOM9 and pGOE2 are visible in Fig. 4A, lanes 7 and 3, respectively.

Cloning of nickel resistance genes. For cloning the nickel resistance genes, plasmid DNA was isolated from transconjugants harboring only one of the megaplasmids: plasmid pTOM8 was isolated from strain CT15, plasmid pTOM9 was isolated from strain CT16, and plasmid pGOE2 was isolated from strain M254. Plasmid DNA was digested with *Bam*HI and cloned into the *Bam*HI site of vector pSUP202. After ligation recombinant plasmids were transferred to *E. coli* S17-1 by transformation. Recombinant clones were screened on Tris mineral agar containing 1 mM $NiCl_2$, whereas *E. coli* S17-1 tolerates only 0.2 mM $NiCl_2$. One nickel-resistant S17-1 clone per 500 recombinants was found. The cloned nickel resistance genes of pTOM8, pTOM9, and pGOE2 were localized on 14.5-kb *Bam*HI fragments. Fragments originating from pTOM8, pTOM9, and pGOE2 were named TBA8, TBA9, and GBA, respectively.

Physical mapping of the 14.5-kb *Bam*HI-fragments TBA8, TBA9, and GBA. Physical maps of TBA8, TBA9, and GBA were constructed by using 12 restriction enzymes: *Apa*I, *Bam*HI, *Eco*RI, *Eco*RV, *Hin*dIII, *Kpn*I, *Pst*I, *Sal*I, *Sma*I, *Sst*I, *Sst*II, and *Xho*I. The maps were identical. Subfragments of equal enzyme digestions were compared directly on agarose gels. The sizes and the patterns of these subfragments were the same (data not shown, but partially presented in Fig. 5A). The physical map of TBA8, TBA9, and GBA is shown in Fig. 2. There are no similarities with the corresponding maps of pMOL28 and pMOL30 (25, 33).

Expression of the cloned fragments in *A. eutrophus*. Fragments TBA8, TBA9, and GBA were inserted into the mobilizable broad-host-range vector, pVDZ'2, and the hybrid vectors were transferred to *A. eutrophus* AE104 and *A. eutrophus* N9AF06 by conjugation. Transconjugants were grown in Tris-gluconate medium and spread on Tris-gluconate agar containing various concentrations of Ni^{2+}, Co^{2+}, Zn^{2+}, and Cd^{2+}. Strains, plasmids, and metal concentrations tolerated are presented in Table 3. The fragments caused the same metal resistances as the megaplasmids from which they were derived. In transconjugants of strain AE104, nickel, cobalt, and cadmium resistances were completely expressed, whereas in transconjugants of N9AF06, only nickel resistance was expressed. Nickel and cadmium resistances conferred by the cloned fragments were slightly higher than those resistances conferred by the original megaplasmids.

Expression of nickel resistance in *E. coli*. As mentioned above, the transfer of megaplasmids pTOM8, pTOM9, and pGOE2 to *E. coli* K-12 by conjugation or transformation was not successful. But fragments TBA8, TBA9, and GBA as hybrid plasmids in vector pVDZ'2 were readily transferred to *E. coli* K-12 by transformation and expressed, to our surprise. Nickel resistance of transformants was examined not only in Tris-buffered mineral medium but also in TES-buffered mineral medium. Cells were grown in the appropriate mineral medium and spread on mineral gluconate agar containing various nickel concentrations. The degree of nickel resistance in *E. coli* depended on the buffer system used; in TES-buffered medium the degree of resistance was about thrice as high as in Tris-buffered medium. Fragments TBA8, TBA9, and GBA as well as subfragments TSB9 and TEC9 conferred nickel resistance to 3 mM $NiCl_2$ (in Tris buffer) or 9 mM $NiCl_2$ (TES), whereas the corresponding sensitive *E. coli* strain tolerated only up to 0.2 mM $NiCl_2$ (Tris) or 0.5 mM $NiCl_2$ (TES).

Regulation of cloned nickel resistance genes. The regulation of nickel resistance genes located on fragments TBA8, TBA9, and GBA was examined by measuring growth curves. The experiments were performed with transconjugants derived from *A. eutrophus* AE104, *A. eutrophus* N9AF06, and *E. coli* K-12. Each of these transconjugants harbored hybrid vectors of one of the fragments in pVDZ'2. Proper controls with strains containing one of the original megaplasmids or vector pVDZ'2 were also examined. Each strain was pregrown either without added nickel or with an appropriate nickel concentration (1 to 3 mM $NiCl_2$), which might cause induction of nickel resistance. These precultures were used as inocula for growth experiments in the presence of high nickel concentrations (15 to 20 mM $NiCl_2$).

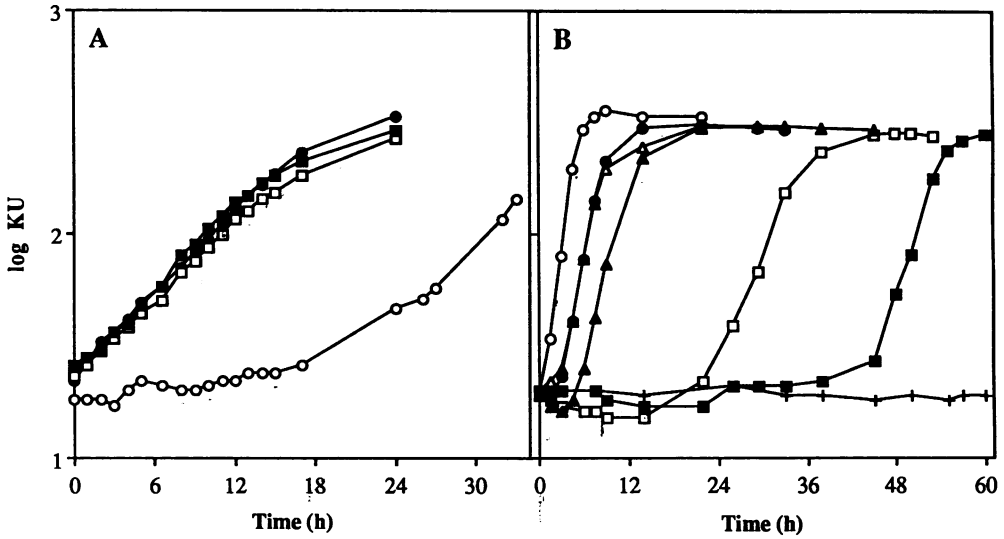

FIG. 3. (A) Growth of *A. eutrophus* AE104 harboring pTOM8 or the DNA fragment TBA8 cloned in vector pVDZ'2. The strains were grown in Tris-gluconate medium containing 20 mM $NiCl_2$ at 30°C. Precultures with 2 mM $NiCl_2$ and precultures without additional $NiCl_2$ were used as inocula (induced and uninduced cells). Symbols: ○, with pTOM8, uninduced; ●, with pTOM8, induced; □, with TBA8, uninduced; ■, with TBA8, induced. KU, Klett units. (B) Growth of *E. coli* K-12 harboring fragment GBA from pGOE2 in vector pVDZ'2. The cells were grown at 37°C in TES-gluconate medium containing 5 mM $NiCl_2$. The transformants harboring pVDZ'2::GBA were precultured in the presence of 0 mM (■), 1 mM (□), 3 mM (▲), 5 mM (●), and 7 mM (△) $NiCl_2$. K-12 carrying vector pVDZ'2 was precultured without added $NiCl_2$ and grown without nickel (○) or in presence of 5 mM $NiCl_2$ (+). Identical growth curves were obtained when fragment GBA was cloned in vector pVDZ'2 in the opposite orientation.

Exemplary growth of two AE104 strains is shown in Fig. 3. AE104 with megaplasmid pTOM8 exhibited an extended lag time (16 h) if the cells were pregrown without additional nickel salt. No lag time was observed if the cells were pregrown in the presence of 2 mM $NiCl_2$. So nickel resistance caused by pTOM8 was assumed to be inducible. But strain AE104 containing the corresponding DNA fragment TBA8 exhibited no significant lag time regardless of whether the cells were precultivated in the presence or absence of additional nickel salt. This difference between pTOM8 and TBA8 was observed in strain N9AF06, too (data not shown). Fragments TBA9 and GBA gave similar results. Thus, while the original megaplasmids were inducibly expressed in derivatives of *A. eutrophus*, the DNA fragments were expressed constitutively.

In contrast, transconjugants of *E. coli* K-12 harboring one of the fragments TBA8, TBA9, and GBA and pregrown in the presence of 3 to 7 mM $NiCl_2$ exhibited no or short lag times when transferred to high nickel concentrations (Tris medium 3 mM $NiCl_2$; TES medium, 5 and 7 mM $NiCl_2$). Cells precultivated without added nickel showed extended lag times, up to 90 h. (Fig. 3B). These results indicate that the hybrid plasmid-encoded nickel resistance is inducibly expressed in *E. coli* while it is constitutively expressed in *A. eutrophus* derivatives.

Nickel resistance caused by subfragments of TBA9. Several subfragments of TBA9 were cloned into vector pVDZ'2: the 12.2-kb *Sst*I-*Bam*HI fragment TSB9, the 11.9-kb *Bam*HI-*Kpn*I fragment TBK9, and the 4.3-kb *Eco*RI fragment TEC9 (Fig. 2). These hybrid plasmids were transferred to *A. eutrophus* AE104, *A. eutrophus* N9AF06, and *E. coli* K-12. The resulting clones, transformants, and transconjugants, respectively, were grown in Tris-gluconate medium and spread on Tris-gluconate agar containing various nickel concentrations. Subfragment TBK9 caused different levels of nickel resistance in *A. eutrophus* AE104. Depending on its orientation in vector pVDZ'2, we observed resistance to 30 or 10 mM $NiCl_2$. Obviously, the *lac* promoter located on pVDZ'2 affected the expression of the nickel resistance genes when the directions of transcription were different. TSB9 and TEC9 caused only low-level nickel resistance, up to 2 mM $NiCl_2$ in AE104 and up to 0.5 mM $NiCl_2$ in N9AF06. This result indicates that the genes for high-level nickel resistance are contained on a DNA fragment of about 12 kb.

In *E. coli* K-12, subfragments TSB9 and TEC9 caused nickel resistance as high level as that caused by fragment TBA9 (up to 9 mM $NiCl_2$ in TES buffer). But subfragment TBK9 conferred no nickel resistance. These results indicate that a comparatively small part of TBA9, namely, subfragment TEC9 (4.3 kb), is sufficient for the expression of nickel resistance in *E. coli* K-12.

DNA-DNA hybridization. DNA-DNA hybridization was performed with fragment GBA as a biotinylated DNA probe in order to investigate homologies between the nickel resistance genes of pTOM8 and pTOM9 from *A. xylosoxydans* 31A, pGOE2 from *A. eutrophus* KTO2, and pMOL28 from *A. eutrophus* CH34. Hybridization signals corresponding to the 14.5-kb *Bam*HI fragments of both pTOM8 (Fig. 4, lanes 5) and pTOM9 (Fig. 4, lanes 6 and 7) were detected. Thus, the hybridization experiments confirmed the presence of a significant homology between fragments GBA and TBA which was already deduced from the restriction maps. In addition, three signals corresponding to (i) a 9.6-kb fragment from *Bam*HI-digested plasmid DNA from pMOL28, (ii) a 12.2-kb fragment from the *Xba*I- and *Eco*RI-digested hybrid plasmid pVDZ'2::XEI, and (iii) a 13.5-kb fragment from the *Hin*dIII-digested hybrid plasmid pUC19::HKI were obtained (Fig. 4, lanes 9 to 11). Fragment HKI carries the structural genes of pMOL28 (33). Fragment XEI carries both the structural genes and the regulatory genes of pMOL28-encoded nickel resistance (18a).

In order to challenge these results and to determine the

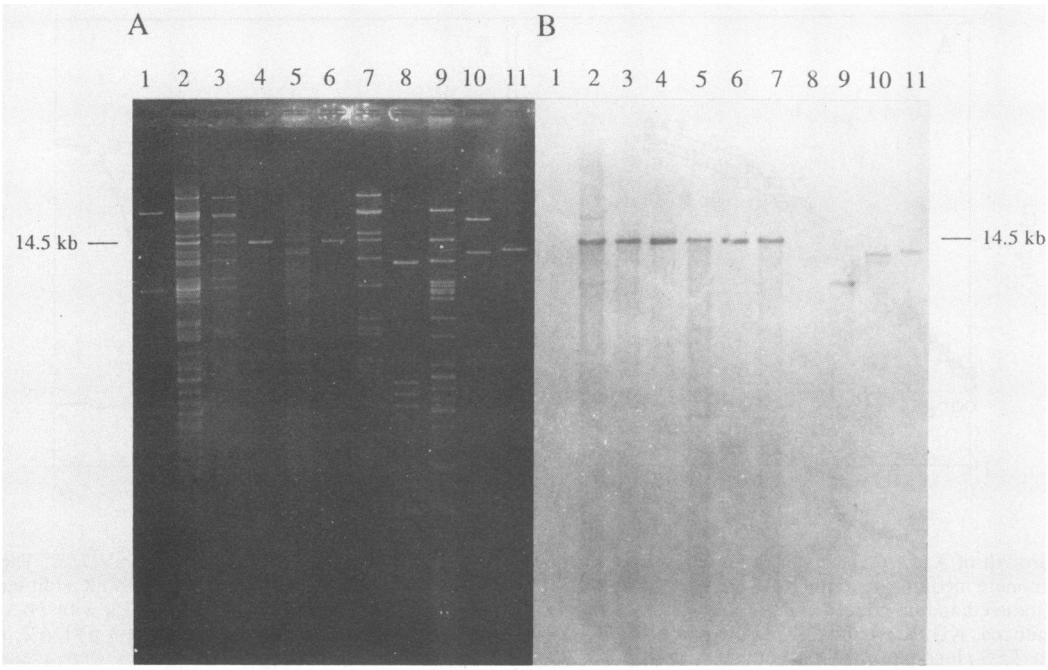

FIG. 4. DNA-DNA hybridization of *Bam*HI-digested plasmid DNA of *A. eutrophus* KTO2(pGOE1, pGOE2, pGOE3); transconjugants HF33(pGOE2), AE104(pTOM8), AE104(pTOM9), and AE126(pMOL28); fragments GBA and TBA; and cloned fragments from pMOL28, by using the biotinylated DNA fragment GBA as a DNA probe. Restriction fragments were separated by electrophoresis in 0.8% agarose (A), blotted onto a nylon filter, and probed with the biotinylated GBA probe as described in Materials and Methods (B). Hybridization signals were obtained with *Bam*HI-digested plasmid DNA from pTOM8, pTOM9, and pMOL28 (lanes 5, 7, and 9). Fragment TBA, *Xba*I- and *Eco*RI-digested hybrid plasmid pVDZ'2::XEI, and *Hin*dIII-digested hybrid plasmid pUC19::HKI showed hybridization signals, too (lanes 6, 10, and 11). *Bam*HI-digested plasmid DNA from the sum of pGOE1, pGOE2, and pGOE3, from pGOE2 alone, and from fragment GBA served as positive controls for hybridization (lanes 2 through 4). No hybridization signals were obtained with lambda DNA, which was *Hin*dIII digested (lane 1) or *Pst*I digested (lane 8).

subfragments of TBA and GBA, which share homology with the nickel resistance genes of pMOL28, the whole fragments TBA and GBA and their subfragments were hybridized with the biotinylated BS probe. Fragment BS is a 9.6-kb *Bam*HI fragment carrying nickel resistance genes of pMOL28 (27a). Hybridization signals were obtained with the whole fragments TBA and GBA (Fig. 5, lanes 9 and 10). Furthermore, hybridization signals which corresponded to the 6-kb fragment of the *Bam*HI- and *Eco*RI-digested hybrid plasmids Bluescript::TBA and Bluescript::GBA, respectively, were detected (Fig. 5, lanes 4 and 5). Two hybridization signals corresponding to the 2.7- and 0.9-kb fragments of the *Eco*RV-, *Sst*II-, and *Xho*I-digested hybrid plasmids Bluescript::TBA and Bluescript::GBA, respectively (Fig. 5, lanes 1 and 2), were also visible. These data indicate that the region of fragments TBA and GBA from the high-level nickel resistance plasmids pTOM9 and pGOE2, which showed homology with low-level nickel resistance genes from pMOL28, is only approximately 3.6 kb in size.

The hybridization experiments allow the following conclusions. (i) The hybridization signals obtained with the biotinylated probe of fragment GBA and the 14.5-kb *Bam*HI fragments from pTOM8 and pTOM9 confirm that fragments GBA and TBA (from pGOE2 of strain KTO2 and from pTOM9 of strain 31A, respectively) are identical as far as the restriction maps are concerned. (ii) The signals obtained with fragments derived from pMOL28 indicate relationships with plasmids pGOE2, pTOM8, and pTOM9. (iii) The signals obtained with the 9.6-kb *Bam*HI fragment BS (from pMOL28) as a biotinylated DNA probe confirm the relationship between both nickel resistance systems. The signals, which can be assigned to two *Eco*RV-, *Sst*II-, and *Xho*I-subfragments of TBA and GBA, indicate that the region homologous to pMOL28 is restricted to a maximally 3.6-kb region of the 6.0-kb *Eco*RI subfragments of TBA and GBA. (iv) This homologous region lies outside the 4.3-kb *Eco*RI subfragments of TBA and GBA which are specific and sufficient for expression of nickel resistance in *E. coli*. This result is in agreement with the lack of expression of pMOL28-encoded nickel resistance in *E. coli*.

DISCUSSION

From a physiological and ecological point of view, the main aim of research on heavy metal toxicity to microorganisms concerns the mechanisms of resistance. How do bacteria cope with toxic metals in the environment? Several model resistance mechanisms, such as those against mercury, cadmium, and arsenate, have been satisfactorily elucidated and adequately reviewed (28, 36).

We have chosen nickel resistance as a model system to study metal toxicity and selected *A. eutrophus* CH34 as the appropriate model organism (22). One reason for this choice concerns the metabolic type of this bacterium. It is an aerobic hydrogen-oxidizing bacterium; i.e., it requires nickel as a microelement for hydrogenase formation and autotrophic growth. Thus, it deals with the control of intracellular nickel concentration anyway (10, 12, 19). As some resistance mechanisms rely on energy-dependent extrusion of the toxic ions, a strict respiratory bacterium like CH34

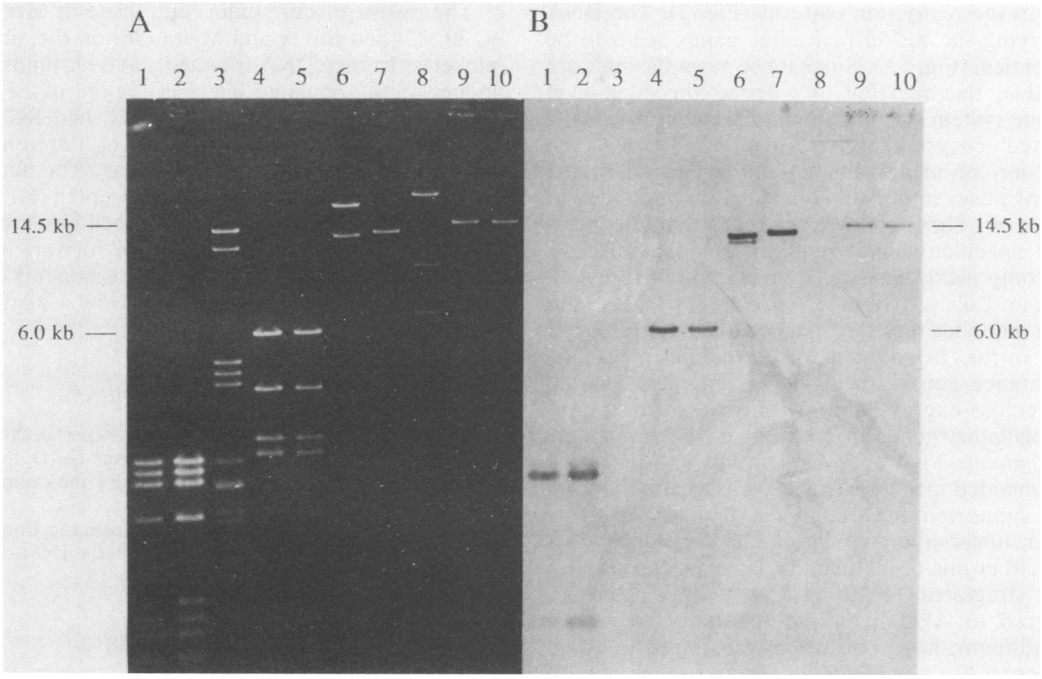

FIG. 5. DNA-DNA hybridization of fragments GBA and TBA derived from plasmids pGOE2 and pTOM9 as well as cloned fragments XEI and HKI from plasmid pMOL28 and subfragments of fragments GBA and TBA, by using the biotinylated fragment BS as a DNA probe. The restriction fragments were separated by electrophoresis in 0.8% agarose (A), blotted onto a nylon filter, and probed with the biotinylated BS probe as described in Materials and Methods (B). Hybridization signals were found with the whole fragments GBA and TBA (lanes 9 and 10), with one *Eco*RI subfragment of GBA and TBA (lanes 4 and 5), and with two *Eco*RV, *Sst*II, and *Xho*I subfragments of fragments GBA and TBA (lanes 1 and 2). *Xba*I- and *Eco*RI-digested hybrid plasmid pVDZ'2::XEI and *Hin*dIII-digested hybrid plasmid pUC19::HKI served as positive controls for hybridization (lanes 6 and 7). No hybridization signal was detected with *Pst*I-digested (lane 3) or *Hin*dIII-digested (lane 8) lambda DNA.

lends the advantage that metabolic energy can be generated only by respiration and can be drastically deprived just by removal of oxygen (4, 32). Furthermore, strain CH34 offers the convenience of carrying the resistance genes on a single plasmid, pMOL28.

Other characters of strain CH34 are of less advantage. This strain tolerates only 3 to 5 mM Ni^{2+}. Neither its plasmid pMOL28 nor the resistance gene-carrying DNA fragments are expressed in *E. coli*. Thus, various genetechnical methods as well as several procedures well established for *E. coli*, such as using membrane vesicles for studies on membrane transport, cannot be applied.

To come across further interesting nickel-resistant bacteria and to satisfy ecological interests, efforts were made to search for further nickel-resistant bacterial strains. When samples of forest and agricultural soils were used as inocula for enrichment cultures, the scarcity or absence of nickel-resistant bacteria from "clean" ecosystems became obvious. Only from industrially polluted areas, composts, wastewaters, and sediments could such bacteria be isolated (31, 39). And among 400 isolates were only four strains which tolerated nickel concentrations higher than 20 mM. These results led to the conclusion that bacteria with high-level nickel resistance can be isolated only from locations where resistance has survival value. The isolation of about a dozen nickel-resistant bacterial strains in industrial regions in Belgium and from mine refuse in Zaire (8) is in accordance with our experience. The studies discussed concern bacteria from anthropogenically polluted ecosystems, i.e., from places which have been exposed to nickel pollution for not more than a millenium.

Recently, we started to investigate the microflora in natural ecosystems which have evolved in environments enriched with heavy metals. Soils that developed over ore bodies or metalliferous minerals carry metallophyte plants and distinctive floras, and indicator species have been described for various metalliferous soils (1, 2, 5). Serpentine soils are frequently enriched with nickel, cobalt, and chromate. Many hundreds of plants grow in these extreme environments. Some plants growing on these nickel-rich soils even take up nickel and accumulate it in stems and leaves. More than 165 such nickel-hyperaccumulating plants are now known. The most outstanding example is the New Caledonian tree *Sebertia acuminata*. It can accumulate more than 1% (dry weight) nickel in the leaves and about 25% (dry weight) in its blue-green latex (30). It is imaginable that this kind of nickel-hyperaccumulating plants drives a nickel cycle resulting in the continuous percolation of the topsoil by nickel ions. These conclusions were drawn from a study on the abundance of nickel-resistant bacteria in soils of Western Australia and natural forests in New Caledonia (30). In the soils under the nickel-hyperaccumulating trees, the number of nickel-resistant bacteria almost equalled the total number of viable bacteria. About 200 bacterial isolates, many of which tolerate 30 mM Ni^{2+}, were collected, and we look forward to studying their properties.

The two strains of bacteria with high-level nickel resistances, *A. xylosoxydans* 31A and *A. eutrophus* KTO2, which are described in this paper, were isolated near Göttingen. The nickel resistance genes of both strains are located on megaplasmids. Plasmids pTOM9 of strain 31A and pGOE2 of strain KTO2 resemble each other strongly, not only in

size, but also in the restriction patterns (Fig. 2). The DNA fragments carrying the nickel resistance genes seem to be completely identical (Fig. 5A). Since these megaplasmids are self-transmissible, the question of whether this high-level nickel resistance system is abundant among bacteria growing in metal-polluted ecosystems arises.

The expression of nickel and cobalt resistance in the available strains poses interesting questions to be treated at the molecular level. These concern the regulation of expression, the host specificity of expression, and the selective expression of only nickel resistance or of combined nickel-cobalt resistance. Like pMOL28 (35), pTOM8, pTOM9, and pGOE2 confer inducible nickel resistance to all *A. eutrophus* strains tested so far. In contrast to the original megaplasmids, all resistance gene-carrying DNA fragments within their hybrid vectors expressed constitutive resistance. Apparently the regulatory genes are located on DNA fragments adjacent to fragments TBA8, TBA9, and GBA. Concerning the pMOL28-encoded nickel resistance, this assumption has already been supported by studying extended fragments (18a). Another observation concerns the host-dependent expression of either one or all metal resistances carried by a fragment. e.g., fragment TBA9 (14.5 kb, within pVDZ'2), when transferred to AE104, caused resistance to nickel, cobalt, and cadmium; however, when transferred to strain H16 or N9A, or to *E. coli*, TBA9 caused resistance only to nickel. A host-dependent expression of metal resistance was also described by Siddiqui et al. (33) and Nies et al. (23, 25). DNA fragment HKI of pMOL28 causes nickel and cobalt resistance in several *Alcaligenes* and *Pseudomonas* strains. DNA fragment aE8 of pMOL30 causes resistance to cobalt, zinc, and cadmium in *A. eutrophus* AE104 and *Alcaligenes hydrogenophilus*, whereas in *A. eutrophus* H16 this fragment only causes slight resistance to the last two metals. Neither the pMOL28-encoded nor the pMOL30-encoded metal resistances were expressed in *E. coli*. Although nickel resistance determined by pTOM8, pTOM9, and pGOE2 on the one hand and pMOL28 on the other hand show profound differences, a significant DNA homology has been detected. DNA-DNA hybridization experiments indicate that homology is restricted to a small area (3.6 kb) of TBA9. As was to be expected, it is not located on subfragment TEC9, which is responsible for nickel resistance in *E. coli*.

As mentioned above, the expression of fragment TBA9-encoded nickel resistance in *E. coli* shows a special feature. A 4.3-kb subfragment of TBA9, named TEC9, confers the same degree of nickel resistance to *E. coli* as the whole TBA9 fragment (14.5 kb). Consequently, several genes essential for complete nickel resistance in *Alcaligenes* strains exert no phenotypically recognizable effects in *E. coli*; this observation has not been analyzed so far. Possibly the high-level resistance systems of strains 31A and KTO2 comprise two groups of genes which are separately expressed.

There is another strain, isolated in 1984 (39), worth mentioning. This bacterium, *Alcaligenes denitrificans* 4a-2, is outstanding by three properties: (i) it is an aerobic hydrogen-oxidizing bacterium and forms only a single NAD-reducing hydrogenase, (ii) it tolerates more than 20 mM $NiCl_2$, and its nickel resistance is constitutively expressed, and (iii) the nickel resistance genes are chromosomally coded (17). The nickel resistance genes were transferred to *A. eutrophus* H16, G29, and AE104, the cured derivative of CH34, by helper-assisted conjugation. There is strong homology between the nickel resistance genes of 4a-2 and of pMOL28.

The results of our studies and those of Mergeay et al. (8, 9, 20, 22) and Silver and Misra (36) on the similarity of the nickel resistance DNA fragments and plasmids isolated from independently isolated bacteria remind us of the antibiotic resistance plasmids RP1, RP4, R68, and RK2 which were isolated from clinical specimens at different times from different bacteria and different areas. The almost complete identity of these plasmids is now accepted (6). With the wide distribution of the well-known broad-host-range antibiotic resistance plasmids in mind, we look forward to studying the nickel resistance plasmids and their fragments of the bacteria isolated in Belgium, Zaire, and New Caledonia in more detail.

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