Nickel-Resistant Bacteria from Anthropogenically Nickel-Polluted and Naturally Nickel-Percolated Ecosystems

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DNA fragments harboring the nickel resistance determinants from bacteria isolated from anthropogenically polluted ecosystems in Europe and Zaïre were compared with those harboring the nickel resistance determinants from bacteria isolated from naturally nickel-percolated soils from New Caledonia by DNA-DNA hybridization. The biotinylated DNA probes were derived from the previously described *Alcaligenes eutrophus* CH34, *Alcaligenes xylosoxidans* 31A, *Alcaligenes denitrificans* 4a-2, and *Klebsiella oxytoca* CCUG 15788 and four new nickel resistance-determining fragments cloned from strains isolated from soils under nickel-hyperaccumulating trees. Nine probes were hybridized with endonuclease-cleaved plasmid and total DNA samples from 56 nickel-resistant strains. Some of the New Caledonian strains were tentatively identified as *Acinetobacter*, *Pseudomonas mendocina*, *Comamonas*, *Hafnia alvei*, *Burkholderia*, *Arthrobacter aureus*, and *Arthrobacter ramosus* strains. The DNA of most strains showed homologies to one or several of the following nickel resistance determinants: the *cnr* and *ncc* operons of the strains *A. eutrophus* CH34 and *A. xylosoxidans* 31A, respectively, the *nre* operon of strain 31A, and the nickel resistance determinants of *K. oxytoca*. On the basis of their hybridization reactions the nickel resistance determinants of the strains could be assigned to four groups: (i) *cnr/ncc* type, (ii) *cnr/ncc/nre* type, (iii) *K. oxytoca* type, and (iv) others. The majority of the strains were assigned to the known groups. Among the strains from Belgium and Zaïre, exclusively the *cnr/ncc* and the *cnr/ncc/nre* types were found. Among the New Caledonian strains all four types were represented. Homologies to the *nre* operon were found only in combination with the *cnr/ncc* operon. The homologies to the *cnr/ncc* operon were the most abundant and were detected alone or together with homologies to the *nre* operon. Only the DNA of the strains isolated from soil in Scotland and the United States and that of five of the New Caledonian strains did not show any detectable homologies to any of our probes. The nickel resistance fragment isolated from *Burkholderia* strain 32W-2 was studied in some detail. This 15-kb *Bam*HI fragment conferred resistance to 1 to 5 mM NiCl₂ to *Escherichia coli* and resistance to up to 25 mM NiCl₂ to *A. eutrophus*. It showed strong homologies to both the *cnr/ncc* operon and the *nre* operon and conferred strictly regulated (inducible) nickel resistance to *A. eutrophus*.

Among the heavy metal-resistant organisms, the nickel-resistant bacteria offer the unique opportunity to compare bacteria isolated from anthropogenically nickel-polluted ecosystems with those isolated from naturally nickel-percolated ecosystems. In our experience nickel-resistant bacteria could not be isolated from normal arable soils in Germany and New Caledonia (34, 37, 39, 49).

Nickel-resistant bacteria isolated from anthropogenically polluted ecosystems have been well studied, starting with a strain which was isolated from a zinc decantation tank in Liège, Belgium (19). This bacterium, characterized as *Alcaligenes eutrophus* CH34, harbors two large plasmids (8), pMOL28 (163 kbp), determining resistance to nickel, cobalt, mercury, and chromate, and pMOL30 (238 kbp), determining resistance to cadmium, zinc, cobalt, mercury, and copper (6, 20, 25, 27). From low-grade ore deposits in Belgium and Zaïre, metal-resistant bacteria which have many properties in common with strain CH34 were isolated. Some strains selected by enrichment culture on nickel-containing media turned out to grow lithoautotrophically on hydrogen and carbon dioxide just as CH34 does (6, 18). All these strains carry two plasmids and exhibit similar resistance patterns.

A. eutrophus KT02 was isolated from the wastewater treat-

ment plant of Göttingen (49); it is a lithoautotrophic bacterium and harbors the following three plasmids: plasmid pGOE1 (250 kbp), which determines cadmium and zinc resistance, plasmid pGOE2 (210 kbp), which encodes nickel and cobalt resistance, and plasmid pGOE3 (170 kbp), for which so far no function is known (39). *Alcaligenes xylosoxidans* 31A was isolated from the metalworking industry in Holzminden, Germany. It is an organotrophic bacterium and harbors two large plasmids, pTOM8 (340 kbp) and pTOM9 (200 kbp), both of which determine resistance to nickel, cobalt, zinc, cadmium, and copper ions (37). *Alcaligenes denitrificans* 4a-2, isolated from the wastewater treatment plant in Dransfeld, Germany (49), and *Klebsiella oxytoca* CCUG 15788, isolated from the metalworking industry in Göteborg, Sweden (17), are so far the only strains which have been shown to carry nickel resistance genes on the chromosome (11, 48).

Some of these bacteria have been studied in detail, and some of the nickel-cobalt-resistance determinants have been cloned and sequenced. The pMOL28-encoded cobalt and nickel resistance (*cnr*) is inducible (43) and is due to an energy-dependent efflux system (41, 51). In some mutants nickel and cobalt resistance is expressed constitutively (15, 44). Plasmid pMOL28 has been transferred to other wild-type strains of *A. eutrophus*, and it confers the ability to tolerate 3 mM NiCl₂ and 5 mM CoCl₂ to the transconjugants when they are grown on solid media or in liquid media (20). The plasmid-encoded *cnr* determinant was cloned and inserted in broad-host-range vectors (42). The hybrid plasmids complemented a nickel-sensi-

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tive Tn5 mutant of *A. eutrophus* to full nickel and cobalt resistance. From the same plasmid, pMOL28, the genes coding for chromate and cobalt-nickel resistance were cloned (22), and the nucleotide sequence of the chromate resistance gene was determined (23).

Detailed analysis of the nickel-cobalt-cadmium resistance determinants (*ncc*) of strains KT02 (plasmid pGOE2) and 31A (plasmid pTOM9) revealed high levels of similarity between both strains as well as similarities to and pronounced differences from the *cnr* genes of pMOL28. The plasmids pGOE2 and pTOM9 confer high degrees of nickel resistance (15 to 40 mM NiCl₂) to transconjugants. The restriction nuclease fragment patterns of the *ncc* regions from pGOE2 and pTOM9 (14.5-kbp *Bam*HI fragments) were identical but different from that of the *cnr* region from pMOL28 (39). However, alignment of the nucleotide sequences of the *cnr* cluster of pMOL28 and the *ncc* cluster of pTOM9 revealed high levels of homology (38). Surprisingly, the nickel resistance conferred to transconjugants by hybrid plasmids containing the 14.5-kbp *Bam*HI fragment of pGOE2 or pTOM9 was expressed not only in derivatives of *A. eutrophus* but also in *Escherichia coli*. The expression in *E. coli* was found to be due to a second nickel resistance determinant on the 14.5-kbp *Bam*HI fragment, named *nre* (38). Altogether, the present results indicate that the resistance to nickel and cobalt of the bacterial strains isolated from anthropogenically polluted ecosystems in Germany, Belgium, and Zaïre is due to determinants which resemble each other on the molecular level to a high degree. A further nickel-resistant bacterium was found among the bacteria which regularly grow in an oil emulsion used as lubrication fluid (17). This strain, a *K. oxytoca* strain, tolerates 10 mM NiCl₂. The nickel resistance determinants showed weak homologies to the *nre* operon and were transferable by conjugation to strains of *E. coli* and *Citrobacter freundii* (48).

Bacteria were isolated from naturally nickel-percolated soils when we counted the numbers of viable nickel-resistant bacteria in soils which developed over ore bodies containing nickel (34). The serpentine soils originating by weathering of serpentine rocks are frequently enriched with nickel, chromium, and cobalt in addition to very high iron and manganese concentrations (3). The flora on these soils is characterized by many endemic plant species. Most of these plants are resistant to nickel, and some are in addition nickel hyperaccumulating. More than 165 nickel-hyperaccumulating plants are known (2). The New Caledonian tree *Sebertia acuminata* is the most outstanding example; it has more than 25% nickel (weight/dry weight) in its blue-green latex and more than 1% nickel in its leaves. Thus, these leaves drive a "nickel cycle" (35) which involves the transportation of nickel from deep soil and rock layers to the leaves, where the nickel accumulated is transiently stored in the vacuoles. After the leaves fall the nickel is liberated from the decaying leaves and is percolated by the rain water through the top soil layer, resulting in contents of about 10 mg of nickel per g of soil. The soil under the canopy of nickel-hyperaccumulating trees has probably been percolated by nickel-containing leaf extracts for millions of years. In samples taken from the humus and mineral soil layers underneath the canopy of nickel-hyperaccumulating trees, the number of bacteria able to grow at 3, 10, and 20 mM NiCl₂ was almost as large as the number of bacteria growing on nutrient broth in the absence of nickel ions (34).

The aim of the present study is the comparison of various bacterial strains isolated (i) from anthropogenically polluted nickel-containing ecosystems and (ii) from naturally nickel-containing or nickel-percolated soils. Comparison of the mechanisms of nickel-cobalt resistance, the locations of the resis-

tance determinants, and the host specificities of determinants and plasmids will contribute to ecologically oriented understanding. We started these studies by applying DNA-DNA hybridization methods. Two efforts were made. (i) Four DNA fragments coding for nickel resistance were cloned from four strains isolated from naturally nickel-percolated soil (34). An additional nickel resistance fragment was cloned from *K. oxytoca*. These studies provided five new nickel resistance sequences in addition to the four nickel resistance-coding DNA fragments already described (11, 15, 39). Homologies of these nine nickel resistance DNA fragments were investigated by cross hybridization experiments. (ii) Plasmid and total DNA of 56 nickel-resistant strains from different ecosystems in Belgium, Germany, New Caledonia, Sweden, Scotland, the United States, and Zaïre was digested with the enzyme *Eco*RI and blotted onto nylon membranes. In hybridization experiments with these nylon filters the nine nickel resistance DNA fragments served as biotinylated DNA probes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacteria studied are listed in Table 1. Some of the nickel-resistant bacteria were gifts provided by M. Mergeay and I. Mattsby-Baltzer; others were isolated from soils in the course of viable count measurements (34, 35). Isolation procedures, habitats, concentrations of nickel in the soils, and ecological data were described in the publications mentioned and by Schmidt et al. (39). All bacterial strains listed in Table 1 were deposited in the culture collection of the Institut für Mikrobiologie, Göttingen, Germany.

In addition, some nickel-resistant strains were isolated after enrichment culture. *Hafnia alvei* 5-5, strains 5-1 and 6-1, and strains of *Acinetobacter*, *Comamonas*, *Alcaligenes*, *Pseudomonas denitrificans*, and *Pseudomonas stutzeri* were isolated from a soil sample from New Caledonia. The soil was provided by J.-P. Cosson. It was taken from the rhizosphere of seedlings of *S. acuminata* below the canopy of an adult tree of *S. acuminata* and mailed to us in tightly closed plastic bottles. For maintenance of the natural moisture content root cuttings were enclosed. Transportation took a week.

H. alvei 5-5 and strains 5-1 and 6-1 were isolated from aerobic enrichment cultures designed for nickel-resistant enterobacteria. The liquid medium contained 0.1% (wt/vol) nutrient broth, 1% lactose, and 10 mM NiCl₂ in Tris-buffered mineral medium (18). After about five transfers, the suspensions were streaked on solid medium containing 1% (wt/vol) peptone, 1% lactose, 10 mM NiCl₂, 2% bile salts (Difco), and 0.0013% brilliant green. Although enriched under aerobic conditions, the pure cultures were able to grow anaerobically on lactose. On eosin-methylene blue agar, metallic sheen did not appear, and β-D-glucuronidase assayed on Fluorocult agar (art. no. 4038; Merck, Darmstadt, Germany) was not present. Further properties were tested by using the API 20NE system (BioMérieux, Marcy l'Etoile, France). Strain 6-1 and an *Acinetobacter* strain were obtained by aerobic enrichment culture in Tris-buffered mineral media containing 0.2 or 0.1% (wt/vol) sodium acetate and 10 mM NiCl₂ and adjusted to pH 6.0. The solid medium contained 0.4% sodium gluconate. The *Alcaligenes* and *Pseudomonas* strains were isolated from anaerobic enrichment cultures designed for denitrifying bacteria. The Tris-buffered medium contained 0.5% potassium nitrate with either sodium-potassium tartrate, glucose, or sodium gluconate, each 0.2%, as a substrate. Incubation occurred in screw-cap test tubes containing Durham tubes. The incubation temperature was 30°C throughout. *E. coli* was grown at 37°C. Strains of *E. coli* and plasmids used in laboratory analysis are listed in Table 2.

Growth experiments. Growth curves for recognizing the regulatory properties of the nickel resistance system were prepared from growth experiments in 300-ml Klett sidearm flasks and by using a Klett-Summerson photometer for turbidity measurements. The flasks contained 30 ml of Tris-gluconate medium supplemented with nickel chloride at the desired concentration. Inocula (of induced cells) were pregrown in the presence of 1, 5, 10, or 20 mM NiCl₂. Uninduced cells had been grown for about 50 generations without added nickel. For inoculation, cells from the end of the exponential growth phase were used.

Estimation of highest tolerable metal concentrations. The estimation of the highest tolerable metal concentrations (MTCs) was done as described by Stoppel et al. (48).

Conjugation, transformation, and isolation of DNA. Conjugation was done as described previously (39). For transformation *E. coli* S17-1 and JM109 were incubated at 37°C in Luria-Bertani medium containing 20 mM MgCl₂. Cells were grown on a rotary shaker to approximately 40 Klett units of turbidity. Competence of cells was obtained by the calcium chloride procedure (9, 33). Genomic DNA was isolated by the method described in reference 1. Plasmid DNA of *Alcaligenes* strains and the soil isolates was isolated as described in reference 10

TABLE 1. Strains from various habitats, their heavy metal resistances, the presence of plasmids, and hybridization signals obtained with nine DNA probes

Place of isolation	Organism and/or strain	Reference(s)	Relevant resistance(s) and/or other characteristic(s) ^a	No. of plasmids ^b	Hybridization signals with DNA probe ^c :								
					CH34 8.5-kbp <i>Stu</i> I- <i>Sca</i> I	4a-2 11.5-kbp <i>Eco</i> RI	31A 8.8-kbp <i>Pst</i> I	31A 3.1-kbp <i>Pst</i> I	Kco 4.3-kbp <i>Hind</i> III	32W-2 15-kbp <i>Bam</i> HI	22-1 7.0-kbp <i>Eco</i> RI	12-1 7.0-kbp <i>Eco</i> RI	18-1 7.0-kbp <i>Eco</i> RI
Belgium	<i>A. eutrophus</i>												
	CH34	17	Ni (3), Co, Zn, Cd	2	+	+	+	(+)	-	+	(+)	(+)	(+)
	DS185	6	Co, Zn, Cd, Cu	4	-	(+)	-	-	-	B	+	+	+
	SV620	6	Ni (3), Co, Zn, Cd, Cu, Aut ⁺	2	+	+	+	+	-	+	+	+	+
	SV661	6	Ni (3), Co, Zn, Cd, Cu, Aut ⁺	2	+	+	+	+	-	+	+	+	+
	SV820	6	Ni (3), Co, Zn, (Cd), Cu	2	(+)	(+)	+	-	-	(+)	-	-	-
	ER8	6	Ni (3), Co, Zn, Cd, Cu	3	(+)	(+)	-	-	-	B	-	-	-
	ER107	6	Ni (1), Co, Zn, (Cd), Cu	2	(+)	-	-	-	-	B	-	-	-
	ER121	6	Ni (1), Co, Zn, Cd, Cu, Aut ⁺	3	(+)	-	-	-	-	B	-	-	-
	ER122	6	Ni (1), Co, Zn, Cd, Cu	3	(+)	(+)	-	-	-	B	-	-	-
Zaire	<i>A. eutrophus</i>												
	AS2	6	Ni (3), Co, Zn, Cd, Cu	2	+	+	B	-	-	B	-	-	-
	AS39	6	Ni (3), Co, Zn, Cd, Cu, Aut ⁺	2	+	+	+	(+)	-	+	(+)	(+)	(+)
	AS60	6	Ni (3), Co, Zn, Cd, Cu	2	+	+	B	-	-	B	-	-	-
	AS82	6	Ni (3), Co, Zn, Cd, Cu	2	+	+	B	-	-	B	-	-	-
	AS130	6	Ni (3), Co, Zn, Cd, Cu	2	+	+	+	-	-	B	-	-	-
	AS168	6	Ni (3), Co, Zn, Cd, Cu	2	+	+	+	(+)	-	+	+	+	+
	SH1	6	Ni (3), Co, Zn, Cd, Cu, Aut ⁺	2	+	+	+	(+)	-	+	+	+	+
Germany	<i>A. denitrificans</i> 4a-2	49	Ni (20)	1	+	+	(+)	(+)	-	(+)	(+)	(+)	(+)
	<i>A. xylosoxidans</i> 31A	37	Ni (40), Co, Zn, Cd	2	+	+	+	+	-	+	+	+	+
	<i>A. eutrophus</i> KT02	49	Ni (40), Co, Zn, Cd	3	+	+	+	+	-	+	+	+	+
New Caledonia	<i>Burkholderia</i> strain 12-1	34	Ni (20), Co, (Zn), (Cu)	0	+	+	+	+	-	+	+	+	+
	16	34	Ni (10), Co, Zn, Cu	0	+	+	+	+	-	+	+	+	+
	17.20	34	Ni (20), Co, Zn, Cu	0	+	+	+	+	-	+	+	+	+
	<i>Burkholderia</i> strain 18-1	34	Ni (10), Co, Zn	0	+	+	+	+	-	+	+	+	+
	<i>Burkholderia</i> strain 22-1	34	Ni (20), Co, Zn, Aut ⁻	2	+	+	+	+	-	+	+	+	+
	27	34	Ni (10), Co, Zn	0	+	+	+	+	-	+	+	+	+
	28	34	Ni (10), Co, Zn, Cu, Aut ⁻	1	+	+	+	+	-	+	-	-	-
	29	34	Ni (20), Co, Zn, Cu	0	+	+	+	-	-	+	-	-	-
	30-1	34	Ni (10)	2	-	-	(+)	-	-	-	-	-	-
	30-3	34	Ni (10)	1	-	-	-	-	-	-	-	-	-
	<i>Burkholderia</i> strain 32W-2	34	Ni (20), Co, Zn, Cu, Aut ⁻	1	+	+	+	+	-	+	+	+	+
	35-1	34	Ni (30), Co, Zn, Cu	0	-	(+)	(+)	-	-	-	-	-	-
	5-1	34	Ni (10), Co, Zn, Cu, Aut ⁻	0	-	-	-	-	+	-	-	-	-
	<i>H. alvei</i> 5-5	34	Ni (10), (Co)	2	-	-	-	(+)	+	(+)	-	-	-
	6-1	34	Ni (10), Co, Zn, Cu, Aut ⁻	1	+	+	+	+	-	(+)	(+)	(+)	(+)
	<i>Acinetobacter</i> sp.	This study	Ni (20), Co, Cu	0	-	-	(+)	-	-	(+)	(+)	(+)	(+)
	<i>Comamonas</i> sp.	This study	Ni (10), Zn, Cu	0	(+)	+	(+)	-	-	(+)	+	+	+
	<i>Alcaligenes</i> strain II	This study	Ni (10), (Zn)	1	-	-	-	-	-	-	-	-	-
	<i>Alcaligenes</i> strain IV	This study	Ni (10), (Zn)	1	-	-	-	-	-	-	-	-	-
	<i>Pseudomonas denitrificans</i>	This study	Ni (10), Zn, Cu	0	-	-	-	-	-	-	-	-	-
	<i>P. denitrificans</i> III	This study	Ni (20), Co, Zn, (Cu)	0	-	-	-	-	-	-	-	-	-
	<i>P. stutzeri</i>	This study	Ni (3), Co, Zn, Cu	1	+	(+)	(+)	-	-	(+)	-	-	-
Sweden	<i>K. oxytoca</i> 15788	17, 48	Ni (10)	2	-	-	-	-	+	-	-	-	-
Scotland	61-5	34	Ni (10), Co	1	-	-	-	-	-	-	-	-	-
	62-5	34	Ni (10), Co	0	-	-	-	-	-	-	-	-	-
	63-1	34	Ni (20), Co	1	-	-	-	-	-	-	-	-	-
	63-5	34	Ni (10), Co, Zn, Cu	2	-	-	-	-	-	-	-	-	-
United States	<i>Arthrobacter aureescens</i> 59-6	34	Ni (10), Co	0	-	-	-	-	-	-	-	-	-
	<i>A. ramosus</i> 60-6	34	Ni (10), Co	ND ^d	-	-	-	-	-	-	-	-	-

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TABLE 1—*Continued*

Place of isolation	Organism and/or strain	Reference(s)	Relevant resistance(s) and/or other characteristic(s) ^a	No. of plasmids ^b	Hybridization signals with DNA probe ^c :								
					CH34 8.5-kbp <i>Stu</i> I- <i>Sca</i> I	4a-2 11.5-kbp <i>Eco</i> RI	31A 8.8-kbp <i>Pst</i> I	31A 3.1-kbp <i>Pst</i> I	<i>K.o.</i> 4.3-kbp <i>Hind</i> III	32W-2 15-kbp <i>Bam</i> HI	22-1 7.0-kbp <i>Eco</i> RI	12-1 7.0-kbp <i>Eco</i> RI	18-1 7.0-kbp <i>Eco</i> RI
	Nickel-sensitive strains												
	<i>A. eutrophus</i> AE104	20	Cured mutant of CH34	0	—	—	—	—	—	—	—	—	—
	<i>A. eutrophus</i> N9A	40	Wild-type strain, Aut ⁺	1	—	+	—	—	—	—	—	—	—
	<i>A. eutrophus</i> H16	53	Wild-type strain, Aut ⁺	1	—	+	—	—	—	—	—	—	—
	<i>E. coli</i> CM214	13	Carrying plasmid pULB113	1	—	—	—	—	—	—	—	—	—
	<i>K. oxytoca</i> JCCM 1665	48	Type strain	0	—	—	—	—	—	—	—	—	—

^a Numbers in parentheses denote the maximum tolerable concentrations of Ni²⁺ (millimolar). The predominating concentration is 10, which means that at least 10 mM NiCl₂ is tolerated. For some strains the actual MTC (>10) was determined. Co, Zn, Cd, or Cu means that the strain is able to grow at 5 mM CoCl₂, 2.5 mM ZnCl₂, 1 mM CdCl₂, or 0.5 mM CuCl₂, respectively. An element symbol in parentheses means that the metal resistance is significant but rather low.

^b Plasmids were detectable by the method of Kado and Liu (10).

^c Hybridization was carried out under stringent conditions (29) expected to result in signals only with more than 95% DNA sequence identity. +, strong signal; —, no signal; (+), faint signal; B, background reaction.

^d ND, not determined.

and modified in reference 25. The alkaline lysis method (33) was used for isolation of vector DNA. Plasmid DNA was purified as described previously (39).

Hybridization experiments. Isolation of probe fragments from agarose gels, labelling of the designated probe with biotin-16-dUTP, and detection of the biotinylated probe were done as described previously (48). Restriction nuclease fragments of the target DNA were separated by electrophoresis in Tris-acetate-buffered 0.7% agarose. Four gels at a time were blotted to nylon membranes (12, 30). Hybridization conditions were essentially those described by Oelmüller et al. (29); for incubation a hybridization roll-incubator (Biometra, Göttingen, Germany) was used.

RESULTS

Characterization of bacterial strains isolated from naturally nickel-containing soils. All the bacterial strains which were isolated from soil samples collected in New Caledonia, Scotland, and the United States (Table 1) were originally grown on counting plates containing Tris-mineral-gluconate medium (34). For these strains, the ability to grow on gluconate and in the presence of 10 or 20 mM NiCl₂ was maintained after frequently repeated subculture. The purity of the cultures was verified by growth in liquid media and on solid media containing nutrient broth or 1 of about 20 organic compounds, such as glucose, fructose, galactose, sucrose, lactose, maltose, xylose, arabinose, starch, cellulose, acetate, propionate, lactate, citrate, benzoate, 4-hydroxy-benzoate, mandelate, capronate, and adipate, as a substrate. The detailed results are documented in the doctoral thesis of Stoppel (47). All strains were able to grow on gluconate or nutrient broth. Only about half of the strains grew on monosaccharides or disaccharides. Most strains grew on organic acids. Growth on cellulose or starch was rarely encountered. Only a few strains were tested for the ability to grow lithoautotrophically with hydrogen gas as an energy source. Only some strains isolated from soil samples collected in Belgium and Zaïre grew as hydrogen autotrophs (Aut⁺ in Table 1). Selected strains characteristic for other ecosystems failed to grow lithoautotrophically (Aut[−]). Therefore, we assume that hydrogen autotrophy is not coupled with nickel resistance. Most of the strains were not identified on the genus or species level.

In addition we used the soil from the rhizosphere of the New Caledonian tree *S. acuminata* to isolate some representatives of a few metabolic types after enrichment culture. The enrich-

ment conditions were described in Materials and Methods. They led to the isolation of three enterobacterial strains (*H. alvei* 5-5 and strains 5-1 and 6-1) and *Acinetobacter*, *Comamonas*, denitrifying *Alcaligenes*, and *Pseudomonas* strains. All of the strains maintained the ability to grow in the presence of heavy metals. All strains were able to grow in the presence of 3 mM NiCl₂, and the majority of the strains tolerated higher concentrations of nickel. The concentrations of nickel tolerated on Tris-gluconate agar medium are indicated in parentheses in Table 1. With the exception of a few strains, all nickel-resistant bacteria tolerated cobalt, zinc, and copper also (at least 5 mM CoCl₂, 2.5 mM ZnCl₂, and 0.5 mM CuCl₂, respectively). Some strains stood out by showing especially high nickel resistance which amounted to MTCs of 20 and 30 mM NiCl₂. These strains were, therefore, studied in more detail, and their nickel resistance determinants were expressed in *A. eutrophus* AE104 and cloned.

Examination for the presence of plasmids and the possible localization of nickel resistance determinants on plasmids proved to be difficult. Application of the technique of Kado and Liu (10) and related methods, which provided reproducible results when various strains of *E. coli*, *A. eutrophus*, *Pseudomonas*, and phototrophic bacteria were used, resulted in failures to either lyse the cells or make the plasmid bands clearly visible on electropherograms. The strains were resistant to lysozyme treatment, and the use of other peptidoglycan-digesting enzymes, such as mutanolysin from *Streptomyces globosporus*, lyticase from *Arthrobacter luteus*, lysing enzymes from *Aspergillus* and *Cytophaga* species, and achromopeptidase from *Achromobacter lyticus*, was not successful (31). Osmotic shock, freezing and thawing, or sonication did not lead to reproducible results, either. Thus, the data concerning the presence and number of plasmids presented in Table 1 have to be considered preliminary.

Transfer of nickel resistance determinants by conjugation. The failure to isolate the nickel resistance-encoding DNA directly from the wild-type strains prompted us to transfer the nickel resistance determinants from the wild-type strains to *A. eutrophus* AE104 as a recipient by conjugation. Although in some cases helper-unassisted transfer was successful, for further studies we used only transconjugants obtained by

TABLE 2. List of *E. coli* strains and plasmids used in laboratory analysis

Strain or plasmid	Relevant characteristic(s) ^a	Reference or source
Strains		
S17-1	Metal sensitive; <i>pro thi recA2 tra⁺</i>	45
JM109	Metal sensitive; <i>thi recA Δ(lac-proAB) F'(traD36 proAB lacI^qΔlacZM15)</i>	54
Plasmids		
pSUP202	Ap ^r Cm ^r Tc ^r	46
pVDZ'2	Tc ^r	5
pULB113	Ap ^r Km ^r Tc ^r	50
pBluescript	Ap ^r	Stratagene, Heidelberg, Germany

^a Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Tc, tetracycline

pULB113-assisted conjugation (Table 3). Not all transconjugants expressed nickel resistance at degrees as high as those observed for the wild-type donors, but they all were resistant to at least 20 or 25 mM NiCl₂.

Cloning of nickel resistance genes. Plasmid DNA of transconjugants M396, M401, M404, and M415 (Table 3) was isolated and digested with nuclease *EcoRI* or *BamHI*. Restriction DNA fragments were ligated into the *EcoRI* or *BamHI* site of vector pSUP202. The recombinant plasmids were transformed in *E. coli* S17-1. The resultant recombinant clones were screened with respect to nickel resistance. All four plasmid preparations resulted in isolation of nickel-resistant clones with a frequency of 1 per 400 to 600 antibiotic-resistant transformants. *EcoRI* digestion of hybrid plasmid DNA from the clones containing DNA fragments derived from strains M396, M401, and M404 revealed three fragments, approximately 7 kbp in size. A nickel resistance-mediating *BamHI*-fragment cloned from plasmid DNA of M415 was determined to be about 15 kbp. The fragments were designated 12-1 7-kbp *EcoRI*, 18-1 7-kbp *EcoRI*, 22-1 7-kbp *EcoRI*, and 32W-2 15-kbp *BamHI* (see Table 4).

Expression of the cloned fragments in *E. coli* and *A. eutrophus*. For investigating the expression of nickel resistance in *E. coli*, the four transformants of *E. coli* S17-1 were used. Each of the strains harbored hybrid vectors consisting of one of the four isolated fragments in vector pSUP202. In Tris-buffered mineral medium all fragments conferred resistance to at least 1 mM NiCl₂ (sensitive control, 0.2 mM NiCl₂). Since Tris is known to inhibit growth of *E. coli* (21), a second buffer system was used for this experiment. Among various buffers, such as HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), MOPS (morpholinepropanesulfonic acid), PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)], TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid], and Tris, TES buffer turned out to be the appropriate medium with respect to the expression of nickel resistance (36). In TES-buffered mineral medium the four fragments encoded resistance in *E. coli* to up to 5 to 7 mM NiCl₂ (sensitive control, 0.5 mM NiCl₂). To examine the expression in *A. eutrophus* strains, the fragments were cloned into the mobilizable broad-host-range vector pVDZ'2 (5). Hybrid plasmids were transformed in *E. coli* S17-1 and transferred to *A. eutrophus* AE104 by conjugation. In Tris-buffered medium hybrid plasmids consisting of fragment 12-1 7-kbp *EcoRI*, 18-1 7-kbp *EcoRI*, or 22-1 7-kbp *EcoRI* in pVDZ'2 encoded resistance to 3 to 5 mM

TABLE 3. Transconjugant *A. eutrophus* AE104 strains harboring nickel resistance determinants from wild-type donor strains

Transconjugant ^a	Wild-type donor	MTC of Ni (mM)	No. of plasmids ^b	Hybridization signals with DNA probe ^c :								
				CH34 8.5-kbp <i>StuI</i> - <i>ScaI</i>	4a-2 11.5-kbp <i>EcoRI</i>	31A 8.8-kbp <i>PstI</i>	31A 3.1-kbp <i>PstI</i>	<i>Kco</i> 4.3-kbp <i>HindIII</i>	32W-2 15-kbp <i>BamHI</i>	22-1 7.0-kbp <i>EcoRI</i>	12-1 7.0-kbp <i>EcoRI</i>	18-1 7.0-kbp <i>EcoRI</i>
M396	12-1	20	1 or 2	+	+	+	+	-	+	+	+	+
M399	16	20	1	+	+	+	+	-	+	+	+	+
M401	18-1	20	2	+	+	+	+	-	+	+	+	+
M404	22-1	20	1 or 2	+	+	+	+	-	+	+	+	+
M407	27	20	1	+	+	+	+	-	+	+	+	+
M409	28	20	1	+	+	+	+	-	+	-	-	-
M412	29	20	1	+	+	+	-	-	+	-	-	-
M415	32W-2	25	1	+	+	+	+	-	+	+	+	+

^a Transfer occurred by plasmid pULB113-assisted conjugation with *A. eutrophus* AE104 as the recipient.^b Plasmids were detectable by the method of Kado and Liu (10).^c Hybridization was carried out under stringent conditions expected to result in signals only with more than 95% DNA sequence identity.

NiCl₂, whereas fragment 32W-2 15-kbp *BamHI* conferred resistance to up to 25 mM NiCl₂ (Table 4).

Regulation of fragment 32W-2 15-kbp *BamHI* in *A. eutrophus*. As a representative of the New Caledonian bacteria, *Burkholderia* strain 32W-2 was selected for further physiological studies. The wild-type strain tolerated 30 mM Ni²⁺, 5 mM Co²⁺, 0.5 mM Cu²⁺, and 2.5 mM Zn²⁺, but not cadmium, when grown on solid medium. The transconjugant M415 (AE104 × 32W-2) tolerated 25 mM Ni²⁺. Finally, the hybrid plasmid pVDZ'2::32W-2 15-kbp *BamHI*, with the fragment ligated in one or the opposite orientation into the *BamHI* site of the broad-host-range vector pVDZ'2, was tested. The resulting two hybrid vectors were transformed into *E. coli* S17-1 and transferred to strain AE104 by conjugation. Nickel resistance was expressed in both orientations, in one orientation resistance to 30 mM Ni²⁺ and in the other orientation resistance to 25 mM Ni²⁺.

Growth experiments in liquid media showed that nickel resistance in the wild-type *Burkholderia* strain 32W-2 is an inducible property. When cells grown in nickel-free Tris-glucuronate medium were inoculated into the medium containing a challenge concentration of 10 mM Ni²⁺, they started to grow exponentially after 18 h, and at a challenge concentration of 20 mM Ni²⁺ they started to grow exponentially after 30 h. Cells pregrown at 1, 10, or 20 mM Ni²⁺ grew without lag time when exposed to a challenge concentration of 20 mM Ni²⁺. The results (Fig. 1A and additional data) mean that a low nickel concentration (1.0 mM) is sufficient for full induction. These data indicate that in 32W-2 the formation of the nickel resistance system is very strictly controlled.

To examine whether the nickel resistance determinants of the 32W-2 15-kbp *BamHI* fragment conferred inducible nickel resistance, the fragment was cloned and tested in both orientations, as shown in Fig. 1B. When cells of strain AE104 harboring the hybrid plasmid pVDZ'2::32W-2 15-kbp *BamHI*, in both orientations, were pregrown without nickel and transferred to a challenge concentration of 10 mM Ni²⁺, exponential growth started after an approximately 16-h lag time. Cells pregrown at 1, 5, or 10 mM Ni²⁺ started to grow as early as

TABLE 4. Expression of nickel resistance determinants from representative nickel-resistant bacteria in *E. coli* and in *A. eutrophus* AE104

Source of determinant (organism and strain)	Place or substance of isolation	Fragment	Reference	Expression (MTC [mM NiCl ₂]) ^a in:	
				<i>E. coli</i>	AE104
<i>A. eutrophus</i> CH34	Zinc factory, Liège, Belgium	8.5-kbp <i>StuI-ScaI</i> ^b	16	None	3
KT02	Sewage plant, Göttingen, Germany	14.5-kbp <i>Bam</i> HI	39	3 (9)	30
<i>A. xylooxidans</i> 31A	Galvanization tank, Holzminden, Germany	14.5-kbp <i>Bam</i> HI	39	3 (9)	30
		8.8-kbp <i>PstI</i> ^b	38	None	15
		3.1-kbp <i>PstI</i> ^b	38	(9)	1
<i>A. denitrificans</i> 4a-2	Sewage plant, Dransfeld, Germany	11.5-kbp <i>Eco</i> RI ^b	11	None	3
<i>K. oxytoca</i> 15788	Metal factory, Göteborg, Sweden	4.3-kbp <i>Hind</i> III ^b	48	3 (10)	3
New soil isolates 32W-2	Garden soil, Mont de Koghis, New Caledonia	15-kbp <i>Bam</i> HI ^b	This study	1 (5)	25
22-1	Under <i>S. acuminata</i> , Rivière Bleue, New Caledonia	7-kbp <i>Eco</i> RI ^b	This study	1 (5)	3 to 5
12-1	Under <i>S. acuminata</i> , Rivière Bleue, New Caledonia	7-kbp <i>Eco</i> RI ^b	This study	1 (5)	3 to 5
18-1	Under <i>S. acuminata</i> , Rivière Bleue, New Caledonia	7-kbp <i>Eco</i> RI ^b	This study	1 (5)	3 to 5

^a MTCs for Tris- and/or TES (in parentheses)-buffered mineral medium are given. The degree of expression of nickel resistance fragments in *E. coli* depended on the host strain and the vector used.

^b DNA fragment from which one of the nine DNA probes was prepared.

those of the nickel-free control. Thus, the 32W-2 15-kbp *Bam*HI fragment conferred inducible nickel resistance to AE104. This nickel resistance is characterized by its inducibility by already low concentrations of nickel and by a relatively high efficiency (MTCs of 25 to 30 mM Ni²⁺).

Physical mapping of the 15-kbp *Bam*HI fragment from 32W-2. Strain 32W-2 was one of the few strains the nickel resistance of which was expressed in *A. eutrophus* AE104 as well as in *E. coli*. The 15-kbp *Bam*HI fragment of strain 32W-2 was cloned into the *Bam*HI site of the vector pBluescript SK⁻ in one and the other orientation. Hybrid plasmids were transferred to *E. coli* JM109 by transformation. Plasmid DNA was isolated, and the physical map of the fragment (not shown here) was constructed by using 12 restriction enzymes: *Apa*I,

*Bam*HI, *Eco*RI, *Eco*RV, *Hind*III, *Kpn*I, *Nde*I, *Sfi*I, *Sma*I, *Sst*I, *Sst*II, and *Xba*I. Comparison of the corresponding maps of nickel resistance fragments of *A. eutrophus* CH34 (15, 42), *A. eutrophus* KT02, and *A. xylooxidans* 31A (39) revealed no similarities.

DNA-DNA hybridization with nine nickel resistance fragments as DNA probes. At the beginning of the present study five fragments determining nickel resistance in host bacteria were available. These were the nickel resistance determinants from *A. eutrophus* CH34, *A. denitrificans* 4a-2, *A. xylooxidans* 31A, *A. eutrophus* KT02, and *K. oxytoca* 15788.

In the present study we prepared a further four fragments from the New Caledonian strains. The nine total nickel resistance fragments listed in Table 4 served both as biotinylated

FIG. 1. Growth of the wild-type *Burkholderia* strain 32W-2 (A) and of *A. eutrophus* AE104 (pVDZ/2::32W-2 15-kbp *Bam*HI) (B) at a challenge concentration of 10 mM NiCl₂. The cells were grown as described in Materials and Methods. Precultures grown without NiCl₂ (■ and □) or with 1 (▼ and ▽) or 10 (▲ and △) mM NiCl₂ (uninduced and induced cells) were used as inocula of the Klett sidearm flasks containing Tris-gluconate medium supplemented with 10 mM NiCl₂. Solid and empty symbols designate the two possible orientations of fragment 32W-2 15-kbp *Bam*HI in vector pVDZ/2. Controls were as follows: ● and ○, strains grown without NiCl₂; and +, strain AE104 harboring pVDZ/2 without an insert at 5 mM NiCl₂.

FIG. 2. Restriction patterns (A) and DNA-DNA hybridization of nine *SalI*-digested nickel resistance fragments with the biotinylated fragment 31A 8.8-kbp *PstI* (B) or the fragment 31A 3.1-kbp *PstI* (C) as a DNA probe. The *SalI*-digested fragments were separated by electrophoresis in Tris-acetate-buffered 0.8% agarose and stained with ethidium bromide (A). The DNA was blotted onto a nylon filter and probed with one of the biotinylated fragments as described in Materials and Methods.

DNA probes and—digested with *SalI*—as target DNA. Five fragments were cloned from strains which were isolated from anthropogenically nickel-percolated sites: (i) fragment CH34 8.5-kbp *StuI-ScaI*, isolated from *A. eutrophus* CH34, harboring the *cnr* operon; (ii) fragment 4a-2 11.5-kbp *EcoRI*, isolated from *A. denitrificans* 4a-2, carrying chromosomally coded nickel resistance determinants; (iii) and (iv) fragments 31A 8.8-kbp *PstI* and 31A 3.1-kbp *PstI*, both subfragments of the 31A 14.5-kbp *BamHI* fragment (designated TBA by Schmidt et al. [39]), isolated from *A. xylosoxidans* 31A and carrying the *ncc* operon and the *nre* operon, respectively; and (v) fragment *K.o.* 4.3-kbp *HindIII* isolated from *K. oxytoca*, carrying chromosomally coded nickel resistance determinants. Fragments vi to ix were the four new nickel resistance fragments originating from the New Caledonian wild-type strains 32W-2, 12-1, 18-1, and 22-1 described in this study. Figure 2A shows the restriction patterns resulting from *SalI* digestion of the nine nickel resistance fragments. Figure 2B and C shows two of nine corresponding hybridized nylon filters presenting the hybridization signals obtained with biotinylated fragment 31A 8.8-kbp *PstI* (Fig. 2B) and 31A 3.1-kbp *PstI* (Fig. 2C) as DNA probes. By using the fragment 31A 8.8-kbp *PstI* as a biotinylated probe, hybridization signals could be detected with several *SalI* fragments of fragments CH34 8.5-kbp *StuI-ScaI*, 4a-2 11.5-kbp *EcoRI*, 31A 8.8-kbp *PstI*, and 32W-2 15-kbp *BamHI* (panel B, lanes 1 to 3 and 6). No signal was detected with *SalI*-digested fragments 31A 3.1-kbp *PstI*, *K.o.* 4.3-kbp *HindIII*, 22-1 7-kbp *EcoRI*, 12-1 7-kbp *EcoRI*, and 18-1 7-kbp *EcoRI* or with *HindIII*- or *PstI*-digested lambda DNA (lanes λ^H , 4, 5, 7 to 9, and λ^P). When fragment 31A 3.1-kbp *PstI* was used as a DNA probe (panel C), hybridization signals appeared with one *SalI* fragment of all fragments except fragment CH34 8.5-kbp *StuI-ScaI* (lane 1). The hybridization reaction with fragment *K.o.* 4.3-kbp *HindIII* (lane 5) was very faint. No hybridization reaction was obtained with *HindIII*- or *PstI*-digested lambda DNA (lanes λ^H and λ^P). Table 5 summarizes the results of the cross-hybridization experiments with the isolated resistance fragments. Fragments CH34 8.5-kbp *StuI-ScaI* and 31A 8.8-kbp *PstI* showed patterns of hybridization reactions which were identical. Both fragments had strong homologies to each other as well as to fragments 4a-2 11.5-kbp *EcoRI* and 32W-2 15-kbp *BamHI*. No signals or only very faint signals could be detected after the hybridization reactions of these two fragments with the fragments 31A 3.1-kbp *PstI*, 22-1 7-kbp *EcoRI*, 12-1 7-kbp *EcoRI*, 18-1 7-kbp *EcoRI*, and *K.o.* 4.3-kbp *HindIII*. Fragments 4a-2 11.5-kbp *EcoRI* and 32W-2 15-kbp *BamHI* showed almost the same hybridization reactions. They shared strong homologies with all of the other resistance fragments except fragment *K.o.* 4.3-kbp *HindIII*. For this fragment only weak signals could

be detected with fragments 31A 3.1-kbp *PstI*, 32W-2 15-kbp *BamHI*, and 22-1 7-kbp *EcoRI*. The remaining fragments did not show any homologies with fragment *K.o.* 4.3-kbp *HindIII*. Hybridization reactions of 31A 3.1-kbp *PstI*, 22-1 7-kbp *EcoRI*, 12-1 7-kbp *EcoRI*, and 18-1 7-kbp *EcoRI* can be combined in one group. These fragments shared strong homologies to each other and to fragment 4a-2 11.5-kbp *EcoRI*, but they did not show any hybridization reaction with fragments CH34 8.5-kbp *StuI-ScaI*, 31A 8.8-kbp *PstI*, and *K.o.* 4.3-kbp *HindIII*. Only fragments 31A 3.1-kbp *PstI* and 22-1 7-kbp *EcoRI* showed faint signals after the hybridization reaction with fragment *K.o.* 4.3-kbp *HindIII*.

Plasmid and total DNA of 56 nickel-resistant strains from different ecosystems in Belgium, Zaire, Germany, New Caledonia, Sweden, Scotland, and the United States was isolated, digested with *EcoRI*, separated on 63 agarose gels, and blotted onto nylon membranes. In hybridization experiments with these nylon filters the nine nickel resistance fragments listed in Table 4 served as biotinylated DNA probes. Table 1 summarizes the *EcoRI* fragments which gave positive signals. The results of the hybridization reactions with the plasmid DNA of the strains were not presented separately for the following reason: if isolation of plasmid DNA was feasible and if there was any signal with one of the DNA probes, the numbers of detectable signals and the sizes of the corresponding DNA

TABLE 5. Cross-hybridization signals originating from probing of nine nickel resistance-determining fragments

<i>SalI</i> -digested fragment	Cross-hybridization signal with DNA probe ^a :							
	CH34 8.5-kbp <i>StuI-ScaI</i>	4a-2 11.5-kbp <i>EcoRI</i>	31A 8.8-kbp <i>PstI</i>	31A 3.1-kbp <i>PstI</i>	<i>K.o.</i> 4.3-kbp <i>HindIII</i>	32W-2 15-kbp <i>BamHI</i>	22-1 7.0-kbp <i>EcoRI</i>	18-1 7.0-kbp <i>EcoRI</i>
CH34 8.5-kbp <i>StuI-ScaI</i>	+	+	+	+	+	+	+	+
4a-2 11.5-kbp <i>EcoRI</i>	+	+	+	+	+	+	+	+
31A 8.8-kbp <i>PstI</i>	+	+	+	(-)	-	+	(-)	(-)
31A 3.1-kbp <i>PstI</i>	-	+	-	+	(+)	+	+	+
<i>K.o.</i> 4.3-kbp <i>HindIII</i>	-	-	+	(+)	+	(-)	+	-
32W-2 15-kbp <i>BamHI</i>	+	+	+	+	(-)	+	+	+
22-1 7.0-kbp <i>EcoRI</i>	-	+	-	+	(-)	+	+	+
12-1 7.0-kbp <i>EcoRI</i>	-	+	-	+	(-)	+	+	+
18-1 7.0-kbp <i>EcoRI</i>	-	+	-	+	(-)	+	+	+

^a +, strong; (+), faint; (-), very faint; -, no signal.

fragments were the same in the lane with plasmid DNA and the lane with the genomic DNA. Thus, nickel resistance of approximately all investigated strains was deduced to be plasmid coded. Only in the case of strain 4a-2 did the signal appear solely in the genomic DNA lane. This observation confirms the previous result (11) indicating that in this strain nickel resistance is exclusively coded by the chromosome.

DISCUSSION

The resistance properties of bacteria towards antibiotics as discovered in the 1950s (52) directed the attention of microbiologists to ecological aspects, to the distribution and dispersal of resistance factors, and to their origin. The antibiotic resistance plasmids RP1, RP4, R68, and RK2, which were isolated from clinical specimens of different bacteria taken from different areas at different times, turned out to be almost identical (4). We encounter an analogous question regarding heavy metal resistance. In our studies we concentrated on nickel resistance. We posed the question of whether the nickel resistance determinants of the bacteria isolated from ecosystems in Belgium and Germany are similar, and in the present study we started to compare these determinants with those of bacteria isolated from naturally nickel-containing soil in New Caledonia, Scotland, and California.

The most advanced studies of the nickel resistance determinants on the molecular level are those performed on pMOL28 of *A. eutrophus* CH34. The regulatory and structural genes were isolated as an 8.5-kbp *EcoRI*-*PstI* fragment, and its sequencing revealed six potential genes, *cnrYXHCBA*, on a 7.1-kbp sequence. The genes *cnrYXH* encode proteins involved in the regulation of the operon (15). *CnrH* was shown to belong to a subfamily of alternative sigma factors (designated the extracytoplasmic function [ECF] subfamily) of σ^{70} factors (16). It apparently controls the transcription of the structural genes *cnrCBA*. Except for the regulatory genes, the sequence of the *cnr* operon is very similar to that of the cobalt-zinc-cadmium resistance operon *czc* located on pMOL30, also originating from strain CH34 (25, 27). It was cloned with a 9.1-kbp *EcoRI* fragment (25) and sequenced (26). Four open reading frames were assigned to polypeptides *CzcD*, *CzcC*, *CzcB*, and *CzcA*. *CzcA* was recognized as the potential transmembrane protein. The hypothetical functions of the proteins were presented as a model (24), and the relationships between the *czc*, *cnr*, and *ncc* systems and their relationships to other ion efflux systems were discussed (24, 28). The *Czc/Cnr* efflux system was characterized as a three-component chemiosmotic antiport pathway with a 12-transmembrane-helix protein as the center piece (7, 32). *CnrA/CzcA* was shown to be homologous to bacterial proteins involved in functions such as cell division, nodulation, and protein secretion (32). *CnrB/CzcB* and *CnrC/CzcC* are proteins associated with *CnrA/CzcA* which apparently influence the degree and specificity of metal ion efflux (7).

The detailed analysis of the nickel resistance determinant located on plasmid pTOM9 of *A. xylosoxidans* 31A, which was isolated as a 14.5-kbp *Bam*HI fragment, demonstrated a very homologous structure, but one that carries two operons. The *ncc* operon comprises structural genes (*nccCBA*) and regulatory genes (*nccYXH*), and the corresponding proteins are highly similar to the proteins encoded by the *cnrHCBA* genes (38). Studies of the expression of the 14.5-kbp *Bam*HI fragment in AE104 as well as in *E. coli* resulted in the detection of a second determinant, the *nre* operon, which is expressed in *E. coli*, too. It was cloned and sequenced also and was found to show no homologies to the *cnr*, *czc*, and *ncc* sequences (14). The detection of the *nre* operon is indicative of a second nickel

resistance determinant which is expressed not only in *Alcaligenes* strains but in *E. coli*, too, and which perhaps specifies a resistance mechanism quite different from that of the *cnr* or *ncc* operon.

The availability and construction of DNA fragments carrying the nickel resistance determinants of previously studied bacterial strains such as *A. eutrophus* CH34, *A. xylosoxidans* 31A, *A. denitrificans* 4a-2, and *K. oxytoca* 15788 prompted us to compare them with nickel resistance determinants from bacterial strains isolated from naturally nickel-percolated ecosystems. Thus, we were able to prepare altogether nine DNA probes which were used to examine about 60 nickel-resistant bacterial strains.

In drawing conclusions from the results of the DNA-DNA hybridization study, it has to be emphasized that each of the four nickel resistance fragments isolated from bacteria from naturally nickel-laden soil (32W-2, 12-1, 18-1, and 22-1) could be related to one or two of the well-characterized nickel resistance determinants from strains which were isolated from anthropogenically nickel-polluted ecosystems. Three of the four nickel resistance fragments, those from strains 12-1, 18-1, and 22-1, can be grouped together. The strains were isolated from the same spot and are physiologically alike. The resistance fragments isolated from these strains are *EcoRI* fragments, 7 kbp in size, and they confer resistance to 5 mM NiCl_2 only. The restriction patterns differ only slightly, e.g., fragment 22-1 7-kbp *EcoRI* has *SalI* cleavage sites which differ from those of the other two fragments. Hybridization experiments with strains 12-1, 18-1, and 22-1 and the nine biotinylated DNA probes resulted in identical hybridization signals and indicated resistance determinants which are homologous to the *ncc* operon and apparently to the *nre* operon as well. However, the three 7-kbp *EcoRI* fragments isolated from these strains harbored resistance determinants which showed homologies to the *nre* operon only. In contrast, the nickel resistance determinants of strain 32W-2 differ from those of the three strains mentioned. Strain 32W-2 was isolated from a different habitat and is physiologically different. The resistance determinants were isolated on a *Bam*HI fragment which is 15 kbp in size and confers resistance to 25 mM NiCl_2 . Hybridization indicated homologies to both the *ncc* and *nre* operons. Thus, the fragment resembles the 14.5-kbp *Bam*HI fragments from *A. xylosoxidans* 31A and *A. eutrophus* KT02. The 32W-2 15-kbp *Bam*HI fragment has, however, a completely different restriction map and differs from the 14.5-kbp *Bam*HI fragments of strains 31A and KT02 in a further outstanding property. While these fragments confer to strains of *A. eutrophus* only constitutive nickel resistance, the 32W-2 15-kbp *Bam*HI fragment confers inducible nickel resistance to *A. eutrophus* (Fig. 1), indicating the presence of the regulatory region.

A comparison of homologies and the distribution of nickel resistance determinants in the bacteria studied are presented in the right-hand columns of Table 1. On the basis of the hybridization patterns the strains examined can be divided into several groups. The strains isolated from desertified soil and waste ore deposits in Belgium and Zaïre form a homogeneous group; they belong to the species *A. eutrophus* (6). In contrast, the strains isolated from soils in New Caledonia comprise several groups. Strains 12-1, 16, 17.20, 18-1, 22-1, 27, 28, 29, 30-1, 30-3, 32W-2, and 35-1 were isolated from the Tris-glucanate agar plates which served to estimate numbers of CFU at varied nickel concentrations (34). The other New Caledonian strains were isolated from soil enrichment cultures designed either for the selection of enterobacterial strains (5-1, 5-5, and 6-1) or for the selection of *Acinetobacter* strains or denitrifying bacteria (*Alcaligenes* and *Pseudomonas* strains). *K.*

oxytoca was isolated from metalworking fluid (17) and studied in detail (48). Strains 61-5, 62-5, 63-1, and 63-5 were isolated from soil from Scotland, and strains 59-6 and 60-6 originated from soil samples from California (34).

With respect to their hybridization signals, the nickel resistance determinants of the strains could be assigned to four classes. (i) *cnr/ncc*-type determinants had strong homologies with the *cnr* operon and the *ncc* operon. The *cnr* operon and the *ncc* operon as well as the *czc* operon have been shown to be very homologous on the basis of DNA sequences (15, 22, 27, 38). (ii) *cnr/ncc/nre*-type determinants had strong homologies with both the *ncc/cnr* operon and the *nre* operon. (iii) *K. oxytoca*-type determinants had strong homologies with nickel resistance determinants of *K. oxytoca* and weak hybridization signals with the *nre* operon. (iv) Others had no hybridization reaction with any of the DNA probes used in this study. The majority of the strains could be assigned to group i, ii, or iii. Only the DNA of the strains isolated in Scotland and the United States and that of six of the New Caledonian strains showed no detectable homologies to any of our probes. Among the strains isolated in Belgium and Zaïre, exclusively the *cnr/ncc* type and the *cnr/ncc/nre* type were found. Among both groups the predominating type was the *cnr/ncc* type. Among the New Caledonian strains all four classes were represented. A remarkable result is that the *nre* operon was found only in combination with the *cnr/ncc* operon but never alone. In contrast, the *cnr/ncc* operon was found to occur not only in combination with the *nre* operon but also alone. Nickel resistance genes of *K. oxytoca* were not found to occur in combination with any of the other known nickel resistance determinants. Thus, the nickel resistance genes of *K. oxytoca* may encode a second type of nickel resistance which appears independently from other nickel resistance determinants.

Our studies allow the following conclusions. For nickel resistance determinants isolated from anthropogenically nickel-percolated ecosystems, strong homologies with nickel resistance determinants isolated from naturally nickel-rich ecosystems could be found. Under the experimental conditions used in our study, the nickel resistance operons *cnr* and *ncc* occurred alone or combined with the *nre* operon and were found to be the most abundant types of nickel resistance determinants.

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