APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1990, p. 1485–1491
0099-2240/90/051485-07$02.00/0

Vol. 56, No. 5

DNA Probe-Mediated Detection of Resistant Bacteria from Soils Highly Polluted by Heavy Metals

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Received 2 October 1989/Accepted 15 February 1990

Alcaligenes eutrophus **CH34 DNA fragments encoding resistance to Cd^{2+}, Co^{2+}, Zn^{2+} (*czc*), or Hg^{2+} (*merA*) were cloned and used as probes in colony hybridization procedures with bacteria isolated from polluted environments such as a zinc factory area (desertified because of the toxic effects of zinc contamination) and from sediments from factories of nonferrous metallurgy in Belgium and mine areas in Zaire. From the different soil samples, strains could be isolated and hybridized with the *czc* probe (resistance to Cd^{2+}, Co^{2+}, and Zn^{2+} from plasmid pMOL30). Percentages of CFU isolated on nonselective plates which hybridized with the *czc* and the mercury resistance probes were, respectively, 25 and 0% for the zinc desert, 15 to 20 and 10 to 20% for the two Belgian factories, and 40 and 40% for the Likasi mine area. Most of these strains also carried two large plasmids of about the same size as those of *A. eutrophus* CH34 and shared many phenotypic traits with this strain. These findings indicated a certain correlation between the heavy-metal content in contaminated soils and the presence of heavy-metal-resistant megaplasmid-bearing *A. eutrophus* strains.**

Plasmid-bound multiple resistance to heavy metals in *Alcaligenes eutrophus* CH34 isolated from sediments of a decantation basin of a zinc factory has been described elsewhere (10, 11). These sediments contained up to 10,000 ppm (10,000 μg/g) of Zn^{2+}. *A. eutrophus* CH34 carries two large plasmids: pMOL28 (165 kilobases [kb]; resistance to nickel, cobalt [CobA], chromate, and mercury [associated with Tn*4378*]) and pMOL30 (238 kb; resistance to cadmium, cobalt [CobB], zinc, copper, lead, and mercury [Tn*4380*]) (5, 11). Resistance to cadmium, cobalt, and zinc in pMOL30 can be inactivated by a single Tn*5* insertion and was associated with an *Eco*RI fragment (*czc*) cloned in various vectors, including the broad-host-range vector pRK290 (13). The resulting plasmid, pDN2, and apparent derivatives such as pDN7 or pDN705 can be mobilized to a variety of bacteria, but the expression of resistance has up to now been found only in *A. eutrophus* strains and related strains (13). The resistance genes code for efflux mechanisms (15, 19). The nucleotide sequence of the *czc* region of pMOL30 (13) is now available and contains four open reading frames (14).

The presence of plasmid-bound multiple resistance to heavy metals leads to the question of the dispersion of such plasmids in the environment. Therefore, we looked at other environments strongly polluted by heavy metals, and we used the 9.1-kb *czc* fragment (13) isolated from plasmid pMOL30 of *A. eutrophus* CH34 as a detecting probe. The selected samples came from a zinc-desertified area in Limburg (Belgium), from areas around factories involved in nonferrous metallurgy in Belgium, and from mine areas in Zaire. In the different contaminated soils, heavy-metal-resistant *A. eutrophus* strains could be isolated. These strains also bear large plasmids that hybridize with the *czc* and *mer* probes.

Strains. Table 1 reports the strains used in this work and their relevant characteristics. Luria agar with or without 10 mM Zn^{2+} ($ZnSO_4$) was used to count the aerobic CFUs from the soil samples. As a minimal medium, Tris gluconate supplemented with heavy metals was used (11). Chemolithotrophy was tested on Schatz plates (18) incubated in GasPak jars with H_2-CO_2 without a catalyzer. Phenotypic tests with carbon sources were carried out as described before (11).

Analysis of soil samples. Soil samples (from the surface to a depth of 20 cm) of approximately 1 kg were taken five times with a clean steel spade sterilized with alcohol and stored in sterile plastic bags at 18°C. Investigations were started 3 days after sampling. Samples were first examined for their content of CFUs and then for their content of heavy metals. To count aerobic, culturable, heterotrophic bacteria, samples were suspended in 10 mM $MgSO_4$ and agitated briskly for 2 h. After appropriate dilutions, suspensions were spread on rich medium. Heavy-metal content was determined with an atomic emission spectroscopy-induced coupled plasma torch (100 Kontron). Each sample was divided into two parts; one was subjected to boiling water extraction for 2 h and the other was subjected to total acid extraction. Acid extraction was done by agitating 10 g of dry soil in 10 ml of acid (HCl-HNO_3, 3:1) for 1 h. Afterwards, 80 ml of water was added and the mixture was boiled for 2 h.

Plasmid isolation. Large plasmids were isolated by the method of Kado and Liu (7). Plasmid isolation for restriction endonuclease analysis was performed by a modification of the same procedure (6).

DNA hybridization techniques. DNA transfer onto nitrocellulose filters and DNA hybridization were done according to standard procedures (9, 20). Replica plating of different colonies on nitrocellulose filters was done by the method of Sayler et al. (17) with some modifications. The membranes were treated for 7 min on 0.5 M NaOH–1.5 M NaCl and washed for 1 min with 1 M Tris hydrochloride (pH 7.5) and for 5 min with 1.5 M Tris hydrochloride (pH 7.5)–3 M NaCl. After being baked at 80°C under vacuum, the membranes were hybridized with DNA probes at 65°C in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (2). The *czc* probe was a 9-kb *Eco*RI restriction enzyme fragment of pMOL30 cloned in pBR325 (CM485) (5, 13). *merA* was a *Nar*I-*Kpn*I DNA fragment from R100 in recombinant plasmid pDG60 (A. Summers, personal communication). Every pos-

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TABLE 1. Strains and plasmids

Strain	Origin	Plasmid	Relevant markers	Reference or source
Alcaligenes eutrophus				
CH34	Liège, zinc factory	pMOL28 (165 kb)	Nic^+, Chr^+, $CobA^+$, Mer^+ (Tn*4378*)	10, 11
		pMOL30 (240 kb)	Cad^+, $CobB^+$, Zin^+, Mer^+ (Tn*4380*), Plu^+, Cup^+	10, 11
AE104	Cured CH34	None		Plasmid-free derivative of CH34 (11)
AE126	Cured CH34	pMOL28	Nic^+, Chr^+, $CobA^+$, Mer^+ (Tn*4378*)	11
AE128	Cured CH34	pMOL30	Cad^+, $CobB^+$, Zin^+, Mer^+ (Tn*4380*), Plu^+, Cup^+	11
AE578	Mating between AE104 and DS185	pMOL85 (250 kb)	Zin^+	This study
DS185	Lommel, zinc desert	pMOL90 (260 kb)	None	Same as AE579
		pMOL85 (250 kb)	Zin^+	This study
		pMOL80 (4 kb)	None	
DS309	Lommel, zinc desert	As in DS185		This study
DS308	Cured DS185	pMOL90 (260 kb)	None	
		pMOL86 (4 kb)	None	
SV513	Beerse, nonferrous metallurgy factory	pMOL281		This study
		pMOL301	Zin^+	
SV572	Beerse, nonferrous metallurgy factory	pMOL282		This study
		pMOL302	Zin^+	
SV620	Beerse, nonferrous metallurgy factory	pMOL283		This study
		pMOL303	Zin^+	
SV661	Beerse, nonferrous metallurgy factory	pMOL284		Same as SV662 and SV663
		pMOL304	Zin^+	
SV820	Beerse, nonferrous metallurgy factory	pMOL285		This study
		pMOL305	Zin^+	
AS2	Likasi, mine area	pMOL286	Nic^+	This study
		pMOL306	Zin^+, Cad^+	
AS39	Likasi, mine area	pMOL287	Nic^+	This study
		pMOL307	Zin^+, Cad^+	
AS60	Likasi, mine area	pMOL288		This study
		pMOL308	Zin^+	
AS82	Likasi, mine area	pMOL289		This study
		pMOL309	Zin^+	
AS130	Likasi, mine area	pMOL290		This study
		pMOL310	Zin^+	
AS168	Likasi, mine area	pMOL291		This study
		pMOL311	Zin^+	
SH1	Shituru, mine area	pMOL292		This study
		pMOL312	Zin^+	
ER8	Overpelt, non-iron-producing factory	pMOL61	None	This study
		pMOL62	Cad^+, Zin^+	
ER107		pMOL66	None	This study
		pMOL67	Cad^+, Zin^+	
ER121		pMOL63	None	This study
		pMOL64	None	
		pMOL65	Cad^+, Zin^+	
ER122		pMOL68	None	This study
		pMOL69	None	
		pMOL70	Cad^+, Zin^+	
H16		pHG1	Chemolithotrophy	H. G. Schlegel
ATCC 17707			Chemolithotrophy	H. G. Schlegel
N9A			Chemolithotrophy	H. G. Schlegel
Escherichia coli				
CM485	HB101	pMOL149	Tc Ap	Cloning of *czc* region of pMOL30 in pBR325 (5, 13)
CM537		pDG60	Ap	*Nar*I-*Kpn*I fragment of *merA* (1,051 base pairs) (R100) in pBR325 (A. O. Summers)

TABLE 2. Metal content and bacterial counts in metal-polluted soil

Sample origin[a]	% Moisture	Metal concn[b] in soil (ppm)							Bacteria (CFU[c]/g of soil) on:	
		Al	Cd	Co	Cu	Ni	Pb	Zn	Luria agar	Luria agar plus 10 mM Zn^+
Zinc desert (Lommel, Belgium)	5.2	6,000 (10)	<10 (1)	(<2)	1,000 (2)	90 (<5)	2,000 (<15)	7,000 (150)	2.8×10^3	6×10^2
Zinc desert (Lommel, Belgium)[d]	14.0	39,000 (<5)	<10 (<1)	(<2)	800 (2)	160 (<5)	1,650 (<15)	11,000 (35)	5.5×10^5	6×10^5
Beerse, Belgium	6.6	5,270 (124)	<5 (<1)	<5 (<1)	501 (1)	13 (2)	260 (6)	241 (47)	7.9×10^2	2×10^2
Overpelt, Belgium	12.6		36 (1)	<5 (<1)	97 (<1)	7 (0)	548 (<1)	1,307 (62)	6.0×10^3	7×10^2
Likasi, Zaire	31.7	29,700 (1)	92 (4)	2,650 (166)	31,700 (73)	58 (5)	370 (<1)	1,850 (35)	4.8×10^8	5.6×10^7
Shituru, Zaire	15.0	15,800 (<1)	61 (8)	283 (14)	6,400 (3)	28 (<4)	850 (<1)	13,700 (605)	7.0×10^3	1.3×10^2

[a] The pH of the different soils was between 5 and 6.
[b] In all tested samples, Hg was lower than 0.1 ppm. The Beerse sample also contained 44 ppm of Sb (3 ppm in the water-extractable fraction). Numbers in parentheses are concentrations of metal extracted by hot water.
[c] Estimated after 72 h of incubation at 28°C
[d] Sample taken under a tree.

itive hybridization was confirmed in dot blots by using purified plasmid DNA. Positive and negative controls were included on all membranes with CH34 and AE104 colonies or their DNA in colony or dot blot hybridizations, respectively.

Genetic manipulations. Matings (AE104 × DS185) were done by growing donor and recipient strains at 30°C in Luria broth, and agar mating was done as described by Lejeune et al. (8). The selective agar medium contained Tris medium supplemented with 2 mM $ZnSO_4$ and was incubated in a GasPak jar with air, H_2, and CO_2. Electroporation was done with a gene pulser (Bio-Rad Laboratories) at 25 μF and 2.5 kV with resistance of the pulse controller set at 200 Ω. Cells were prepared by the Bio-Rad protocol. DNA of pMOL286 was electroporated into AE104 (plasmidless derivative of CH34), and selection was done on minimal medium supplemented with 2 mM $NiCl_2$. DS185 was cured by growing the strain at a sublethal concentration (100 μg/ml) of novobiocin and selecting for zinc-sensitive strains.

RESULTS

Description of soil samples. Table 2 reports the heavy-metal contents and bacterial counts for various soil samples taken in industrial regions in Belgium (at a site involved in the manipulation and storage of Zn ores [Overpelt] and in the environment of copper, zinc, and antimony purification plants [Beerse]), mining areas from Zaire (Likasi and Shituru), and one zinc-desertified area in Limburg (Lommel in Belgium).

The zinc desert (approximately 90 ha) (Fig. 1) is in an area that was occupied by a zinc factory that released large amounts of zinc into the air from the first years of this century until 1973, when the factory was dismantled. The area is covered with a porous, sandy soil inhabited only by some liverwort and very few trees (*Acer* or *Pinus* spp.) with impaired or dwarflike growth. Neighboring areas were originally sandy dunes, now often covered with heaths that have been invaded by *Pinus silvestris*, introduced to the region as a wood for mine carpentering, or converted to pastures or crop fields.

Every tested sample had a high content of heavy metals; there was a predominance of zinc in the Belgian samples and of copper, cobalt, and zinc in the Zairian samples (Table 2). From the data on water-soluble fractions, it seems that zinc became available to the aerobic microflora as a soluble form in the Belgian samples, and copper, zinc, and cobalt were available in the Likasi samples. In every tested sample, the CFUs found on the selective plates were not very different from those found on the nonselective plates or were at least within the same order of magnitude. Thus, in most of the tested samples, representative aerobic populations were specifically adapted to grow or to survive in high concentrations of Zn^{2+} ions. From both selective and nonselective plates, colonies were picked and hybridized with two probes: the *czc* fragment of plasmid pMOL30 and the *Nar*I-*Kpn*I *merA* fragment of R100. (A strongly homologous *Nar*I-*Kpn*I fragment is also present in mercury resistance transposons found in both plasmids pMOL28 and pMOL30 of *A. eutrophus* CH34 [4]). Table 3 shows the percentage of positive hybridizations among colonies picked from selective and nonselective plates. A positive response to the *czc* probe was found with almost all colonies on selective plates and a notable fraction of colonies on nonselective plates. Except in the samples from Lommel, a strong positive relationship was found between the isolates responding to the *czc* probe and those hybridizing with the *merA* probe (in the Lommel samples, no *merA* hybridization was found). Confirmation of colony hybridization was first made by dot blots (Fig. 2); this technique was found to be much more replicable than colony hybridization. On the other hand, all colonies found to hybridize with the *czc* probe were able to grow on plates with 10 mM Zn^{2+}. Furthermore, they were shown to be *A. eutrophus* on the basis of their phenotypic properties and polyacrylamide gel electrophoresis protein fingerprints. The reverse situation was also true, suggesting that growth on Luria agar with 10 mM zinc was almost diagnostic for the

TABLE 3. Colony hybridization with *czc* and *merA* probes

Origin	% of colonies hybridized with probes from[a]:			
	Nonselective plates		Zn^{2+} selective plates	
	czc	*merA*	*czc*	*merA*
Zinc desert (Lommel)	25	<1	100	<1
Likasi	40	40	100	100
Overpelt	15	10	100	NT[b]
Beerse	20	20	100	100

[a] For each sample, 50 to 100 colonies were tested.
[b] NT, Not tested.

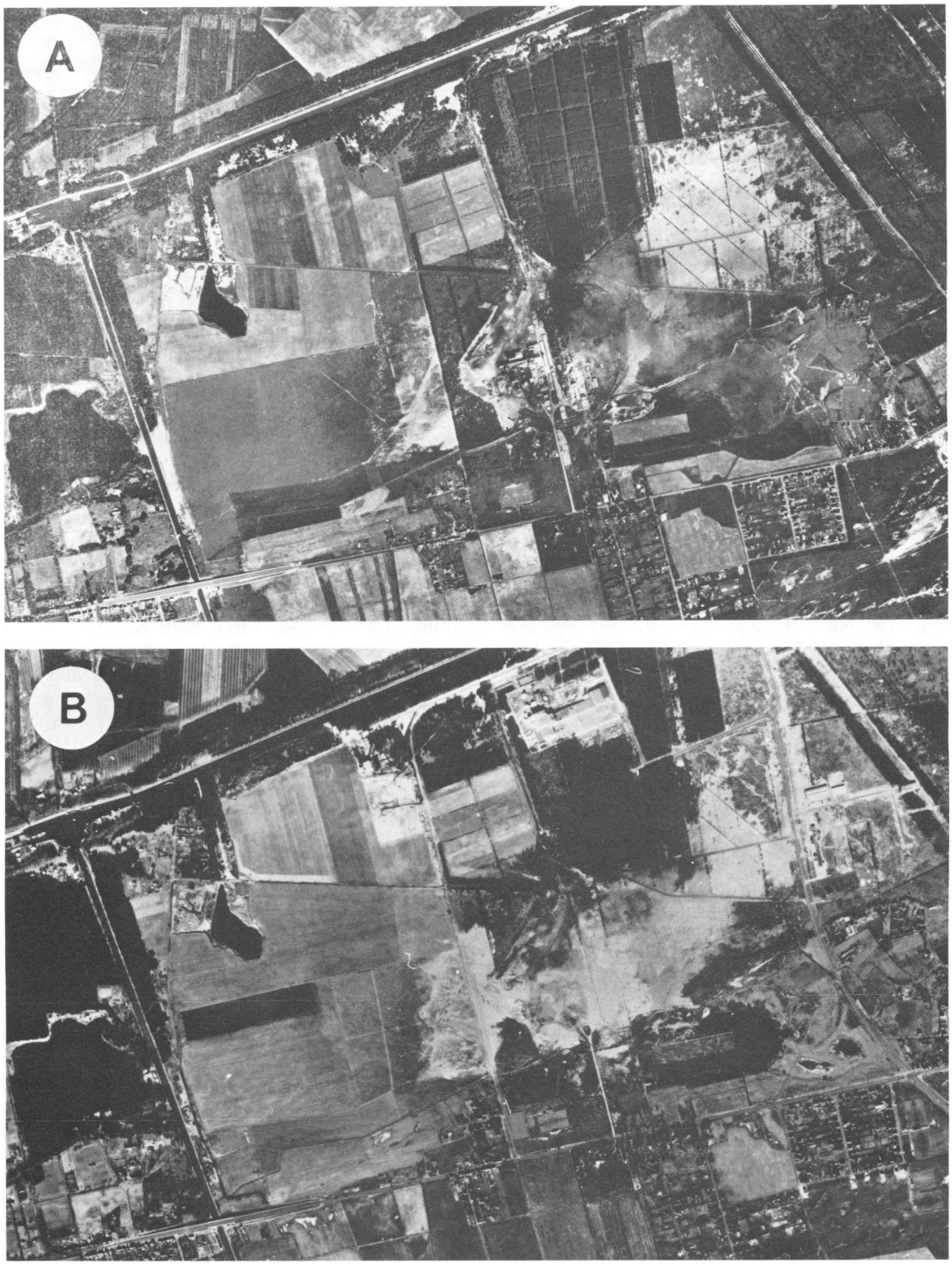

FIG. 1. Aerial view of desertified zone (zinc desert). Pictures were taken after the zinc factory stopped activities. (A) 1970 (factory still present); (B) 1985 (11 years after the zinc factory was dismantled). The progression of desertification is seen in the center right part of the pictures.

presence of a fragment strongly homologous by hybridization with the *czc* fragment, especially if the growth was supported by further growth at 20 mM Zn^{2+}.

Phenotypic and taxonomic analyses of zinc-resistant bacteria hybridizing with the *czc* probe. Table 4 reports the characteristics of representative strains found in the various soil samples and compares *A. eutrophus* CH34, from which the *czc* probe was isolated, with the type strain *A. eutrophus* H16 (ATCC 17699, NCIB 10442), which is representative of this chemolithotrophic genus. Carbon sources used to support bacterial growth, resistances to heavy metals tested on minimal media, and growth at different temperatures are shown in Table 4. In addition, Fig. 3 shows the plasmid content for the same strains.

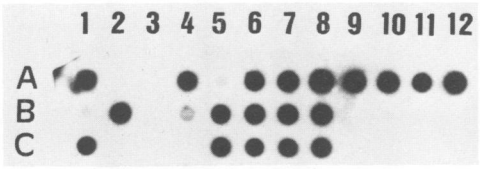

FIG. 2. Plasmids in bacteria hybridizing with the *czc* probe. Plasmid DNA was isolated from different strains resistant to heavy metals, dot blotted with a Hybridot-BRL apparatus, and hybridized with the ^{32}P-labeled *czc* DNA fragment from pMOL30. Dot blots in row A: 1, AE578; 2, DS308; 3, AE104; 4, AE579; 5, ER201; 6, SV661; 7, SV513; 8, SV663; 9, AS39; 10, AS2; 11, AS168; and 12, AS130. Dot blots in row B: 1, DS308; 2, AE150; 3, SV662; 5, SV620; 6, SV820; 7, CH34; 8, DS185; 9, AE104; 10, AE3; 11, AE126; and 12, AE296. Dot blots in row C: 1, AE128; 3, AE453; 4, ER8; 5, ER121; 6, ER122; 7, ER124; and 8, ER107.

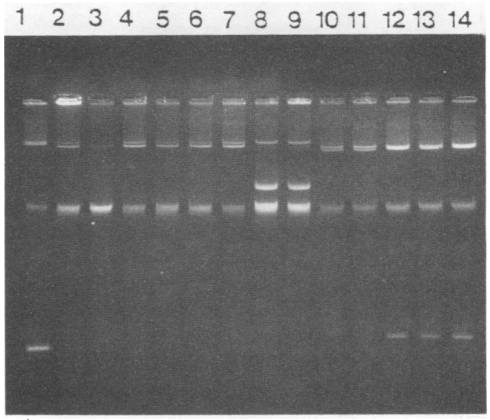

FIG. 3. Plasmid DNA patterns of different *A. eutrophus* strains. Agarose (0.8%) gel electrophoresis was done on the large plasmids from DS185 (lane 1), CH34 (lane 2), SV513 (lane 3), SV620 (lane 4), SV661 (lane 5), AS39 (lane 6), AS168 (lane 7), AS130 (lane 8), AS2 (lane 9), ER8 (lane 10), ER107 (lane 11), ER121 (lane 12), ER122 (lane 13), and ER124 (lane 14).

Data from Table 4 and Fig. 3 show a convergence of phenotypic traits in all the tested strains. This convergence was extended thanks to the presence of large plasmids banding in the same region, multiple resistance to heavy metals, and in different cases, the ability to grow in chemolithotrophic conditions. In addition, all strains grew on gluconate, lactate, azelate, citrate, ethanol, propanol, butanol, benzoate, *p*-hydroxybenzoate, threonine, histidine, tyrosine, and proline and did not grow on glucose, mannitol, *n*-valerate, geraniol, chlorobenzoate, methionine, toluate, and salicylate. This convergence would allow identification of all these strains as *A. eutrophus*. It may be relevant to recall that strain CH34 was assigned to *A. eutrophus* because of the presence of two hydrogenases (one cytoplasmic and one membrane bound) (11). Also, a few very specific differences could be found; for DS185, Cd resistance was expressed only at 37°C, and in ER121, Cd resistance was expressed after induction by Zn^{2+}.

A substantial difference between strains H16 and CH34 is that chemolithotrophy is encoded by the megaplasmid pHG1 in the former and by chromosomal determinants in the latter. Another difference is growth on fructose (Table 4). The electrophoretic pattern of proteins (data not shown) also suggests a divergence between H16, ATCC 17707, and N9A on the one hand and multiply resistant strains on the other hand.

From the data on temperature, nickel resistance, and chemolithotrophy, two subgroups within the metal-resistant strains are indicated (Table 4): one strongly clustered around strain CH34, and the other encompassed the strains isolated in Lommel and Overpelt (DS185 and ER121). The strains clustered around *A. eutrophus* CH34 share a remarkable array of characteristics: hybridization with both *czc* and the *merA* probes, multiple resistance to heavy metals associated with two plasmids, growth in chemolithotrophic conditions, and a high level of mutagenesis induced at 37°C. However, Zairian samples include a notable fraction of thermoresistant nonchemolithotrophic strains, of which strain AS2 is representative. In the first plasmid preparations, AS2 showed two megaplasmids (250 and 165 kb) that were very similar to pMOL30 (*czc*) and pMOL28 (*chr cnr*). The smaller plasmid was replaced by a band migrating much lower in the gel (Fig. 3). This band was eluted and used to transform the plasmid-free derivative AE104 by electroporation with selection for nickel resistance. All the transformants harbored a plasmid migrating just as pMOL28 did (Fig. 4). These Nic^+ transformants inherited resistance to cobalt, chromate, and mercury as well.

Zinc resistance was also assigned to pMOL62, pMOL67,

TABLE 4. Phenotypic analysis

Strain[a]	Origin	Use of carbon sources					Resistance to heavy metals					Temp (°C)		
		Chemolitho-trophy	Glucose	Fructose	Lactate	Dicarboxylic acids[b]	Zn^{2+}	Cd^{2+}	Co^{2+}	Ni^{2+}	Cu^{2+}	30	37	41
CH34	Liège	+	−	−	+	+	+	+	+	+	+	+	(+)[c]	−
H16		+	−	+	+	+	−	−	−	−	−	+	(+)	−
DS185	Lommel, zinc desert	−[d]	−	−	+	+	+	(−)[e]	(−)[e]	−	+	+	+	(+)
ER121	Overpelt	−	−	−	+	+	+	(−)[f]	+	−	+	+	+	(±)
SV661	Beerse	+	−	−	+	+	+	+	+	+	+	+	(+)[c]	−
AS39	Likasi	+	−	−	+	+	+	+	+	+	+	+	(+)[c]	−
AS2	Likasi	−	−	−	+	+	+	+	+	+	+	+	+	+
SH1	Shituru	+	−	−	+	+	+	+	+	+	+	+	(+)[c]	−

[a] Each phenotype of every strain represents at least 10 isolates found in the same sample or in different samples from the same area. Plates with carbon sources and heavy metals were incubated at 30°C.
[b] Azelaic and sebacic acids.
[c] Cultures of these strains give rise to survivors exhibiting a variety of mutant phenotypes at a frequency of 10^{-5} (12; Sadouk et al., unpublished data).
[d] Spontaneous mutants able to grow chemolithotrophically were found.
[e] Strains could grow on Cd and Co at 37°C.
[f] Cd resistance after induction with Zn.

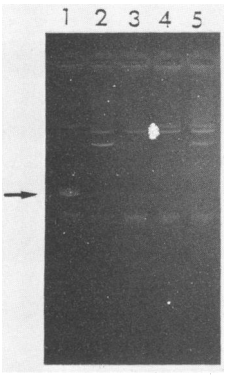

FIG. 4. Original AS2 plasmids. Lane 1, DNA band (indicated by arrow) of pMOL286 was used for electroporation; lanes 2 to 5, after electroporation and selection on Ni plates, different Ni-resistant transformants were obtained. They all bear pMOL286 at the site where pMOL28 migrates.

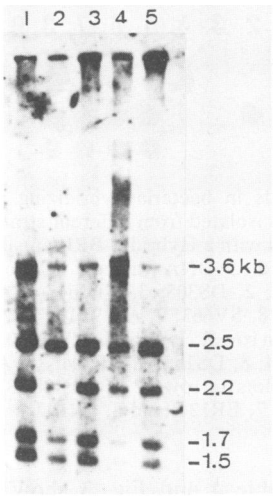

FIG. 5. *Pst*I digestions of pMOL85 and pMOL30 plasmid DNA. Lane 1, CH34; lanes 2 and 3, zinc-resistant transconjugants from the mating DS185 × AE104; lane 4, DS309; land 5, DS185. As a probe, the 9.1-kb *czc* fragment of plasmid pMOL30 was used.

pMOL65, and pMOL70 (Table 1) in strains ER8, ER107, ER121, and ER122, respectively, by IncP1-mediated mobilization of the plasmids to *A. eutrophus* recipients. A deleted form of pMOL304 (strain SV661) yielded zinc-sensitive strains.

Strain AS2 is also of interest for studying the genetic basis of temperature-induced mutagenesis; for example, this could be studied through intraspecies matings with strain CH34. The strains found in the zinc desert and their relatives from Lommel will be discussed in the next section.

Bacteria of the zinc desert and their plasmids. Apart from the zinc-resistant *Alcaligenes* spp. (of which isolate DS185 is the representative), samples from the zinc desert contained *Pseudomonas aeruginosa*, especially in a sample taken at the base of a tree. Strain DS185 is considered an important aerobic colonizer of the zinc desert (more than 25% of the total detected CFU). DS185 carries three plasmids (Fig. 3), i.e., two megaplasmids (pMOL90, 260 kb; pMOL85, 240 kb) and a very small plasmid (pMOL80, 4 kb). The relationships between plasmids and resistance to heavy metals were revealed by curing attempts and by transfer of the plasmids to a sensitive strain. Cured DS185 bacteria sensitive to Zn^{2+} were obtained by the action of novobiocin and were shown to have lost pMOL85 (data not shown).

Transfer was made by mating DS185 and AE104, a plasmid-free derivative of strain *A. eutrophus* CH34. The transfer of resistance to Zn^{2+} from DS185 to AE104 occurred at rather high frequencies (up to 10^{-4}). Transconjugants carried a plasmid which again would be identified as pMOL85. Interestingly, these transconjugants obtained at 30°C exhibited resistance not only to Zn^{2+} but also to Cd^{2+}, Co^{2+}, and Cu^{2+}, although resistance to Cd^{2+} and Co^{2+} was not expressed in the original host at 30°C (but was expressed at 37°C). Thus, pMOL85 seems to carry these four resistance elements, as pMOL30 does.

Now the question of homology between the DNA of strains found in the desertified zone and the fragment *czc* from pMOL30 can be addressed. Figure 5 shows hybridizations with *Pst*I digests. *Pst*I endonuclease cleaves the *czc* fragment of pMOL30 at five sites (13). Hybridization of *Pst*I digests with the 9-kb probe yielded a remarkably similar pattern in *A. eutrophus* CH34, DS185, DS309 (another isolate from the same zone), and two Zn^R transconjugants from the mating of AE104 × DS185 that contain pMOL85.

DISCUSSION

We have applied DNA probes to detect specific genes in the environment; probes have previously helped to detect catabolic genes (17) or mercury resistance genes (1, 2). The 9-kb *czc* probe encoded resistance to cadmium, cobalt, and zinc. pMOL30 is a 240-kb plasmid in CH34 isolated in a zinc factory (province of Liège, Belgium). The *czc* probe (now sequenced [15]) allowed the detection of new resistant isolates in soils with a high content of heavy metals. The soil samples came from Belgium and Zaire. In Zaire, samples from two mining areas in the Shaba copper belt were analyzed. The Belgian samples came from two factories other than iron factories and from a zinc-desertified area that had been the site of a zinc factory. The samples tested contained up to 40,000 ppm of the following heavy metals: Zn^{2+}, Cd^{2+}, Pb^{2+}, Co^{2+}, Cu^{2+}, and Ni^{2+}. If the soluble fraction is considered, it seems that Zn^{2+} is the ion most available to bacteria and thus the ion most likely to exert a selection pressure, although in the Zairian samples, Cu^{2+} and Co^{2+} were apparently also effective in this way, because many strains resistant to copper and cobalt were isolated from the Zairian soil samples.

Zinc-resistant bacteria were thus found in all the tested samples, and in most cases, the number of CFU on selective plates and nonselective plates and hybridizing with the *czc* probe had a maximum difference of 1 order of magnitude. The correlation was especially strong between the colonies detected on rich medium plus 10 mM Zn^{2+} and those hybridizing with the *czc* probe. Luria agar plus 10 mM (or 20 mM) Zn^{2+} thus seems very useful in detecting or enriching bacteria related to *A. eutrophus* CH34. All the isolates seemed to share many phenotypic properties with *A. eutrophus* CH34. Although the classification as *A. eutrophus* is not questioned, strains showing multiple resistance to heavy metals seem to be similar to *A. eutrophus* CH34 and to diverge somewhat from type strain H16 in a series of characteristics. Therefore, the name *A. eutrophus* "var. *metallotolerans*" would be useful. As far as aerobic CFU are concerned, resistant *A. eutrophus* seemed to be an important and often the main colonizer of the tested samples. However, some samples, such as those from the zinc desert,

seemed to contain appreciable numbers of anaerobic bacteria, including sulfate-reducing bacteria (data not shown). In order to have a more general view of the total numbers of living bacteria, use of the direct viable count method (21) may be necessary. Nevertheless, the tested samples seemed to host a microbial flora specifically adapted to the constraints exerted by heavy metals. Apart from zinc-resistant bacteria hybridizing with the *czc* probe, some fluorescent bacteria and some flavobacteria were encountered (on nonselective plates or in mixed colonies with *A. eutrophus*). Gram-positive bacteria were never isolated, with the exception of some streptomycetes; this result agrees with observations on the influence of chemical stresses on the aerobic population from various soil samples (3).

Thus, in soils with high levels (up to 5%) of heavy metals, heterotrophic resistant bacteria closely related to *A. eutrophus* CH34 constitute a substantial fraction of the aerobic microflora. This is probably relevant to the environmental transfer of genes in contaminated soils (for example, from released genetically manipulated microorganisms); in this respect, knowledge of *A. eutrophus* CH34 genetics could lead to some predictions. The strain has indeed been found to be a good recipient of foreign genes introduced through R-prime plasmids (8).

Other interesting questions or possible research areas arise from the microbiological study of heavy-metal-contaminated soils: for example, the influence of such bacteria on the biological availability of heavy metals to other soil microorganisms or to higher plants, possible participation by these bacteria in geochemical processes, or possible correlation between facultative chemolithotrophic growth of bacteria and resistance to heavy metals. It might indeed be speculated that oxidoreduction processes would occur in sulfide soils exposed to air and deliver some of the H_2 required for chemolithotrophic growth of *A. eutrophus* bacteria.

For most of these questions, further studies of plasmid-borne resistance mechanisms (essentially based on metal efflux [15, 19]), of metal uptake mechanisms (16), and of growth physiology (6) will be needed, with cooperation between workers in molecular biology, physiology, and microbial ecology.

ACKNOWLEDGMENTS

We thank P. Dejonghe and F. Van Assche for turning our attention to industrial sites in Limburg and their potential in environmental microbiology, Gecamines (Zaire) for her gracious collaboration, L. Gypen and N. Vermaut for taking soil samples, and A. O. Summers and H. G. Schlegel for the gift of the strains. The participation of D. Severens, S. Vets, A. Schildermans, and E. Renders was appreciated. Expert technical assistance of L. Hooyberghs and A. Ryngaert is acknowledged. We thank S. Silver, B. Powell, and H. G. Schlegel for their critical review of the manuscript.

This work was partially supported by an EEC grant (BAP O366 Risk Assessment Programme).

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