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Construction and Characterization of Heavy Metal-Resistant Haloaromatic-Degrading *Alcaligenes eutrophus* Strains

DIRK SPRINGAEL,* LUDO DIELS, LILIANE HOOYBERGHS, SABINE KREPS, AND MAX MERGEAY

Laboratory of Genetics and Biotechnology, Center of Studies for Nuclear Energy and Flemish Institute for Technological Research, SCK/CEN-VITO, Boeretang 200, B-2400 Mol, Belgium

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***Alcaligenes eutrophus* strains exhibiting both plasmid-borne heavy metal resistance and haloaromatic-degrading functions were obtained by intraspecific conjugation. The strains which we constructed expressed catabolic and resistance markers together. Degradation of various polychlorinated biphenyl isomers and 2,4-D (2,4-dichlorophenoxyacetic acid) was observed in the presence of 1 mM nickel or 2 mM zinc, provided that the metal resistance determinant was present in the catabolizing strain. Such strains may be useful for decontamination of sites that are polluted with both organic compounds and heavy metals.**

The use of specialized bacterial strains that are able to degrade organic pollutants, such as polychlorinated biphenyls (PCBs) and pesticides, is becoming increasingly important for decontaminating polluted soils, sludges, and groundwaters (16). The use of these microorganisms may face various obstacles, including poor survival and functioning outside laboratory conditions because of the presence of toxins or other inhibitory and/or competing compounds, physical inaccessibility, competition, predation etc. (12).

In the United States, 37% of sites polluted with organic compounds were found to also contain inorganic pollutants, such as heavy metals (20). Heavy metals are known to influence organic matter decomposition by the natural bacterial flora and are considered important inhibitors of activated sludge processes (1, 2, 18, 29, 35). Accordingly, bioremediation of sites polluted with organic compounds might be impaired by the presence of heavy metals. Indeed, we tested the responses to heavy metals of different bacterial strains selected for their capacity to degrade organic xenobiotic compounds (Table 1). The strains were tested on Tris minimal medium agar plates containing an appropriate carbon source and a heavy metal salt at different concentrations. Tris minimal medium is peculiarly convenient for testing resistance to heavy metals, as described previously (24). The plates were examined for growth after 1 week of incubation at 30°C. In most cases, growth of the xenocatabolic strains was totally inhibited at low heavy metal concentrations (0.1 mM Co^{2+}, less than 0.5 mM CrO_4^{2-}, 0.2 mM Ni^{2+}, 0.2 mM Cd^{2+}, 0.1 mM merbromine, and 0.5 mM Zn^{2+}). Thus, the availability of bacteria that are able to degrade organic pollutants and at the same time are able to resist contamination with multiple heavy metals is important.

Alcaligenes eutrophus is a suitable choice for such constructions because strains belonging to the genus *Alcaligenes* have been isolated as both multiple-heavy-metal-resistant (7–9, 22) and chloroaromatic compound-degrading (3, 10, 14, 30, 31) bacteria. Moreover, these strains have been isolated as important colonizers of severely polluted sites and, therefore, seem to be well-adapted to the constraints imposed by such environments (8).

A. eutrophus A5 harboring IncP1 plasmid pSS50, which is involved in biphenyl (BP)–4-chlorobiphenyl degradation (Bph^+/Cbp^+) (31), and *A. eutrophus* JMP134 harboring IncP1 plasmid pJP4, which is involved in 2,4-dichlorophenoxyacetic acid (2,4-D) degradation (Tfd^+) (10), were mated with *A. eutrophus* CH34 or SV661 carrying megaplasmids conferring multiple resistance to heavy metals (9). The characteristics of the strains are shown in Table 2. The heavy metal-resistant strains were kept on selective agar plates containing Tris minimal medium supplemented with gluconate as a carbon source and the appropriate heavy metal, as described previously (24). Strains A5 and JMP134 were kept on Tris minimal medium containing BP and 2,4-D, respectively, as sole sources of carbon and energy. BP (purchased from Janssen Chimica, Beerse, Belgium) was supplied on the lid of the petri dish. 2,4-D was added at a concentration of 5 mM. Plate mating occurred as described previously (21). The selective agar media contained Tris medium, BP or 2,4-D (5 mM) as the sole source of carbon and energy, and 1 mM $NiCl_2 \cdot H_2O$ or 2 mM $ZnSO_4 \cdot 7H_2O$. The large metal-resistant plasmids of strains CH34 and SV661 are poorly self-transferable (frequency, 10^{-7} to 10^{-8} per donor cell) (8, 24) in intraspecific matings. To allow selection for catabolic strains receiving heavy metal resistance markers, the catabolic strains were mated with a multiple auxotrophic derivative of *A. eutrophus* CH34 (strain AE81) (24), and the amino acids required by strain AE81 were omitted from the selection medium. In matings in which transfer of catabolic and resistance markers could occur in either direction, the transconjugants were analyzed for chromosomal markers to differentiate between donor and recipient strains.

Strain JMP134 and A5 transconjugants which had received Nic^+ or Zin^+ markers from strain AE81 were identified, and strain CH34 and SV661 transconjugants which had received the catabolic marker from strain JMP134 or A5 were also found. Representative strains from each mating are described in Table 3. The plasmid profiles of the transconjugants were examined by using the crude plasmid extraction method of Kado and Liu (17). As described previously, pJP4 was transferred unchanged into strain CH34 at a high frequency (10^{-1}) (13). Transfer of pMOL28 from strain AE81 to strain JMP134 happened at a frequency of 6×10^{-6} and was enhanced by IncP1 catabolic plasmid pJP4, which was present in the recipient strain (i.e., by retromobilization) (23). Transconjugant strains contained a very large plasmid

* Corresponding author.

TABLE 1. Maximum tolerated concentrations of heavy metals in minimal medium for various xenobiotic compound-catabolic strains and *A. eutrophus* CH34

Strain	Xenobiotic compound catabolic property (reference)[a]	Maximum tolerated concn (mM) of[b]:						
		Co^{2+}	Zn^{2+}	CrO_4^{2-}[c]	Ni^{2+}	Cu^{2+}	Cd^{2+}	Hg^{2+}[d]
A. eutrophus A5	Cbp^+ (31)	<0.1	1	<0.5	0.2	0.3	0.2	0.5
Alcaligenes sp. strain BR60	Clc^+ (14)	1.0	>2	<0.5	1.0	0.5	0.2	>0.5
A. eutrophus JMP134	Tfd^+ (10)	0.1	0.5	<0.5	0.1	0.3	0.1	0.3
Pseudomonas putida PaW1	Xyl^+ (15)	<0.1	2.0	<0.5	0.5	0.3	0.1	0.1
P. putida PRS2015	Clc^+ (6)	0.1	1.0	<0.5	0.2	0.3	0.2	0.1
P. putida RE204	Ipb^+ (11)	0.1	0.2	<0.5	1.0	ND[e]	0.1	<0.1
P. putida PgG1901	Nah^+ (36)	<0.1	0.5	<0.5	0.2	<0.1	0.1	<0.1
A. eutrophus CH34	(24)	10	5.0	0.5	2.0	1.0	1.0	>0.5
Arthrobacter sp. strain SK15	Cbp^+ (34)	<0.1	<0.2	<0.5	0.2	<0.1	<0.1	<0.1
Moraxella sp. strain SK12	Cbp^+ (34)	<0.1	<0.2	<0.5	0.2	<0.1	<0.1	<0.1
Pseudomonas fluorescens GS15	Cbp^+ (34)	<0.1	0.2	<0.5	0.2	0.3	0.2	<0.1
Pseudomonas stutzeri GS3	Bph^+ (34)	<0.1	0.2	<0.5	0.2	0.3	0.2	0.1
Alcaligenes denitrificans JB1	Cbp^+ (28)	0.1	0.2	<0.5	0.2	<0.1	0.2	<0.1

[a] Abbreviations: Cbp^+, Clc^+, Tfd^+, Xyl^+, Ipb^+, Nah^+, and Bph^+, ability to utilize 4-chlorobiphenyl, 3-chlorobenzoate, 2,4-D, xylene, isopropylbenzene, naphthalene, and BP as a carbon sources, respectively.

[b] The corresponding MIC may be estimated by multiplying the maximum tolerated concentration by a factor 2 to 2.5. Cultures were grown for 4 days at 30°C on minimal medium agar containing metal salts.

[c] All strains except strain CH34 were susceptible to 0.5 mM CrO_4^{2-}.

[d] Resistance to mercury (Hg^{2+}) was monitored as resistance to merbromin in Luria-Bertani rich medium (24).

[e] ND, not determined.

which might have been a pJP4::pMOL28 cointegrated plasmid (Fig. 1A, lane 3), and no transfer of Nic^+ was observed when a plasmid-free derivative of strain JMP134 was used as the recipient strain. Transfer of the Bph^+ marker from strain A5 to strains CH34 and SV661 happened at a rather low frequency (10^{-6}) considering that pSS50 is an IncP1 plasmid (5) and was accompanied by a 60-kb enlarged version of 51-kb plasmid pSS50 (Fig. 1B, lanes 4, 7, and 8). These rearrangements will be described elsewhere (34). Transfer of Nic^+ and Zin^+ from strain AE81 to strain A5 occurred at a frequency of 10^{-7} to 10^{-8}, which was comparable to the frequency reported previously when a plasmid-free derivative of strain CH34 was used as the recipient strain (24). Although plasmid-free strain A5 was not available to check whether pSS50-mediated retrotransfer was involved in the transfer of pMOL28 and pMOL30 from strain AE81 to strain A5, the fact that some transconjugants harbored a slightly enlarged plasmid pSS50 containing a Tn*4378* insertion, as shown by restriction enzyme digestion (Fig. 1B and C) and DNA-DNA hybridization (data not shown), which was derived from pMOL28, indicated that pMOL28 can be transferred to strain A5 by means of pSS50.

As observed previously for other *Alcaligenes* strains (26, 27, 32, 33), all unselected metal determinants of pMOL28 and pMOL30 were also expressed in the *A. eutrophus* A5 and JMP134 hosts (Table 3). The strain CH34 and SV661

TABLE 2. Bacterial strains and plasmids used for construction of heavy metal-resistant haloaromatic-degrading strains

Strain	Plasmid		Relevant plasmid marker(s)[a]	Relevant chromosomal marker(s)[a]	Reference
	Designation	Size (kb)			
A. eutrophus A5	pSS50	51	Involved in 4-chlorobiphenyl degradation		31
Heavy metal-resistant *A. eutrophus* strains					
CH34	pMOL28	165	Nic^+ Chr^+ $CobA^+$ Mer^+ (Tn*4378*)		24
	pMOL30	240	Cad^+ $CobB^+$ Zin^+ Plu^+ Cup^+ Mer^+ (Tn*4380*)		
AE81	pMOL28	165	Nic^+ Chr^+ $CobA^+$ Mer^+ (Tn*4378*)	*trpE50 leu-27 met-81*	24
	pMOL30	240	Zin^+ $CobB^+$ Cad^+ Plu^+ Cup^+ Mer^+ (Tn*4380*)		
SV661	pMOL284	165	Nic^+		8
	pMOL304	240	Zin^+		
A. eutrophus strains					
JMP134	pJP4	80	Tfd^+ Clc^+ Mer^+		10
JMP222	Plasmid-free derivative of JMP134			Sm^r	10

[a] Abbreviations: Clc^+, Tfd^+, Bph^+, and Cbp^+, ability to utilize 3-chlorobenzoate; 2,4-D, BP, and 4-chlorobiphenyl as carbon sources, respectively; Nic^+, Chr^+, Cob^+, Mer^+, Cad^+, Zin^+, Plu^+, and Cup^+, resistance to nickel (1 mM), chromate (0.5 mM), cobalt (10 mM), mercury compounds (0.5 mM), cadmium (0.8 mM), zinc (0.2 mM), lead (0.5 mM, and copper (0.8 mM), respectively; Sm^r, resistance to streptomycin.

TABLE 3. Characteristics of representative heavy metal-resistant haloaromatic-degrading *A. eutrophus* constructions

Strain	Origin	Selected markers[a]	Relevant plasmid(s)	Heavy metal resistance phenotype[a]	Xenobiotic compound-degrading phenotype[a]
AE1200	CH34 × JMP134	Tfd^+ Nic^+	pJP4, pMOL28, pMOL30	Nic^+ Chr^+ Cob^+ Mer^+ Cad^+ Zin^+ Plu^+	Tfd^+ Clc^+
AE300	JMP134 × AE81	Tfd^+ Nic^+	pJP4::pMOL28	Nic^+ Mer^+	Tfd^+ Clc^+
AE707	CH34 × A5	Bph^+ Zin^+	pSS50Ω, pMOL28, pMOL30[b]	Nic^+ Chr^+ Cob^+ Mer^+ Cad^+ Zin^+ Plu^+ Cup^+	Bph^+ Cbp^+
AE860	SV661 × A5	Bph^+ Zin^+	pSS50Ω, pMOL284, pMOL304[b]	Zin^+ Mer^+ Nic^+	Bph^+ Cbp^+
A5.4	A5 × AE81	Bph^+ Nic^+	pSS50::Tn*4378*, pMOL28	Nic^+ Chr^+ Cob^+ Mer^{+c}	Bph^+ Cbp^+

[a] Abbreviations: Tfd^+, Clc^+, Bph^+, and Cbp^+, ability to degrade 2,4-D, 3-chlorobenzoate, BP, and 4-chlorobiphenyl, respectively. The following concentrations of heavy metals were tested: Ni^{2+}, 1 mM; CrO_4^{2-}, 0.5 mM; Co^{2+}, 0.5 mM; Hg^{2+}, 0.5 mM; Cd^{2+}, 0.8 mM; Zn^{2+}, 2 mM; Pb^{2+}, 0.5 mM; and Cu^{2+}, 0.8 mM.

[b] Strains AE707 and AE860 carry an enlarged plasmid pSS50 (110 kb), designated pSS50Ω.

[c] Expression of cobalt resistance is lower than in *A. eutrophus* CH34 but greater than in parental strain A5.

transconjugants which received the Bph^+ phenotype from strain A5 were also able to grow in the presence of 4-chlorobiphenyl (Cbp^+).

The influence of heavy metals on the PCB-catabolic activity of heavy metal-resistant PCB-degrading bacterial strains A5.4 and AE707 (Table 3) was examined by monitoring the growth of these organisms in the presence of BP and the degradation of PCB congeners in resting cell assays in the absence and in the presence of heavy metals (1 mM Ni and 2 mM Zn). Cells were grown in 20 ml of Tris minimal medium containing BP as the carbon source without or with heavy metals (1 mM Ni or 2 mM Zn) in 125-ml Wiame shake flasks at 30°C, and cell growth was followed by measuring as optical density at 660 nm with a Spectronic 21 spectrophotometer (Milton Ray Company/Analis). In the resting cell assay, the cells were harvested from late exponential growth on BP by centrifugation, washed twice, and resuspended in the same mineral medium without and with heavy metals (1 mM Ni or 2 mM Zn) to a final optical density of 0.8. Portions (2 ml) of the cell suspensions were added to 24-ml screw-cap vials which were lined with aluminum foil and contained 20 μg of Aroclor 1242 or 20 μg of a defined PCB congener mixture. Aroclor 1242 was added as a concentrated methanol solution, and the PCB mixture was added as a concentrated *n*-hexane solution. The methanol or *n*-hexane was allowed to evaporate prior to addition of cells. Then the cultures were incubated at 30°C on a rotary platform shaker. As a control, *A. eutrophus* CH34, which does not contain a

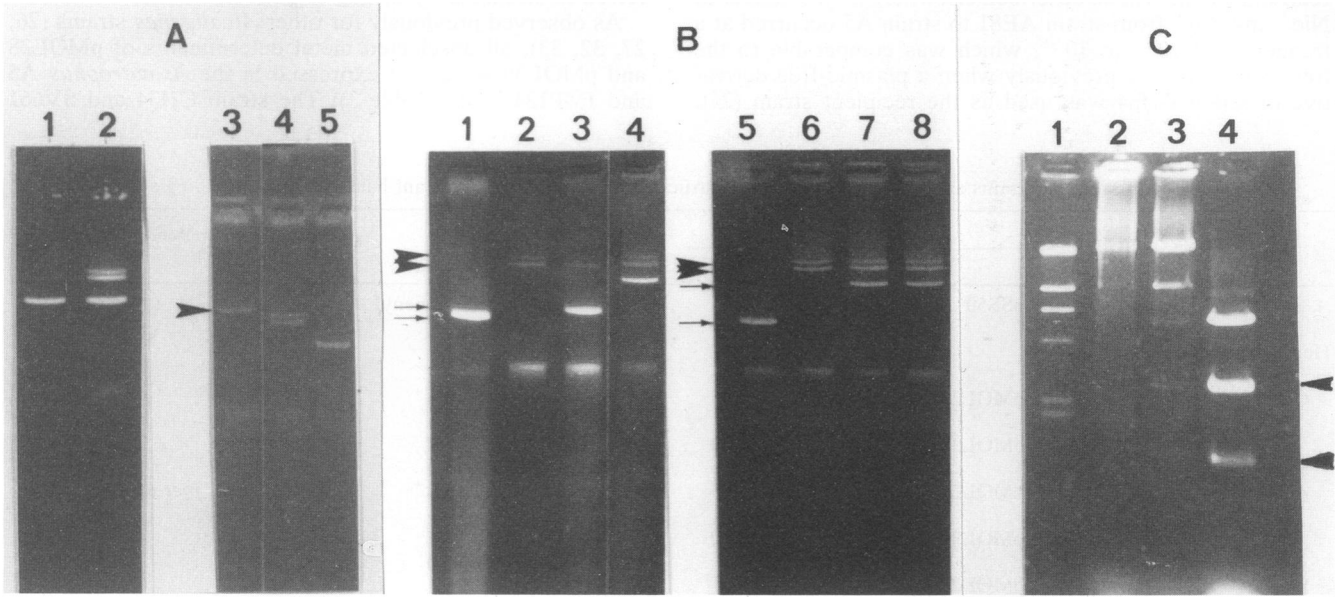

FIG. 1. (A) Plasmid profiles of representative Nic^+ Tfd^+ transconjugants. Lane 1, strain JMP134(pJP4); lane 2, strain AE1200 (pMOL28,pMOL30,pJP4); lane 3, strain AE300(pJP4::pMOL28) (arrowhead); lane 4, strain AE81(pMOL28,pMOL30); lane 5, strain JMP134 (pJP4). (B) Plasmid patterns of representative heavy metal-resistant PCB-degrading *A. eutrophus* strains. The arrows indicate the positions of pSS50 and pSS50 derivatives. The arrowheads indicate the positions of plasmids bearing heavy metal resistance markers (pMOL30, pMOL28, pMOL304, and pMOL284). Lane 1, strain A5(pSS50); lane 2, strain CH34(pMOL30, pMOL28); lane 3, strain A5.4; lane 4, strain AE707; lane 5, strain A5(pSS50); lane 6, strain SV661(pMOL304, pMOL384); lane 7, strain AE860; lane 8, strain AE707. (C) Lane 1, λ *Hin*dIII; lane 2, *Eco*RI digest of pSS50; lane 3, *Eco*RI digest of pSS50::Tn*4378* of strain A5.4; lane 4, *Eco*RI digest of plasmid R1Cm::Tn*4378* (13 kb) (7). Preparations were digested according to the recommendations of the restriction enzyme manufacturer. The arrowheads indicate the positions of internal fragments of Tn*4378*.

TABLE 4. Cometabolization of PCB isomers in a defined PCB-congener mixture by *A. eutrophus* A5 and constructed strains in the presence of heavy metals[a]

Strain	Plasmid(s)	Incubation medium	% Degradation of:						
			4-Chloro-biphenyl	2,4'-Dichloro-biphenyl	3,3'-Dichloro-biphenyl	4,4'-Dichloro-biphenyl	2,4,4'-Trichloro-biphenyl	2,2',5,5'-Tetra-chlorobiphenyl	2,4,5,2',3'-Penta-chlorobiphenyl	2,4,5,2',3',5'-Hexa-chlorobiphenyl
A5	pSS50	Tris	100	100	100	100	95	0	0	0
A5	pSS50	Tris + Zn^{2+}	100	80	85	50	50	0	0	0
A5	pSS50	Tris + Ni^{2+}	100	100	100	100	65	0	0	0
A5.4	pSS50::Tn*4378*, pMOL28	Tris + Ni^{2+}	100	100	100	100	75	0	0	0
AE707	pSSD51, pMOL28, pMOL30	Tris + Zn^{2+}	100	100	100	100	80	0	0	0
AE707	pSSD51, pMOL28, pMOL30	Tris + Ni^{2+}	100	100	100	100	80	0	0	0
CH34	pMOL28, pMOL30	Tris	0	0	0	0	0	0	0	0

[a] Strain A5.4 was incubated for 72 h at 30°C, and strains A5 and AE707 were incubated for 48 h at 30°C. All experiments were done in duplicate.

TABLE 5. Cometabolization of di- and trichlorobiphenyl isomers in Aroclor 1242 by *A. eutrophus* A5 and constructed strains in the presence and absence of heavy metals[a]

Strain	Plasmid(s)	Incubation medium	% Degradation of:				
			Dichlorobiphenyl isomers			Trichlorobiphenyl isomers	
			1	2	3	1	2
A5	pSS50	Tris	92.3	91.5	92.3	28.6	60.3
A5	pSS50	Tris + Zn^{2+}	0	12.2	16.5	4.8	1.1
A5	pSS50	Tris + Ni^{2+}	40.0	14.7	17.3	0	0
A5.4	pSS50::Tn*4378*, pMOL28	Tris + Ni^{2+}	72.5	100	99.4	88.6	89.8
AE707	pSSD51, pMOL28, pMOL30	Tris + Zn^{2+}	95	91.3	93.2	56.7	63.9
AE707	pSSD51, pMOL28, pMOL30	Tris + Ni^{2+}	50	26.9	31.2	4.8	10.3
CH34	pMOL28, pMOL30	Tris	0	4	0	0	0

[a] Cultures were incubated for 72 h at 30°C. PCBs with more than three chlorine substitutions were not degraded. All experiments were done in duplicate.

BP-degrading pathway, was included in the assay. After a defined time, the vials were removed and treated with perchloric acid (final concentration, 0.7%) to stop metabolism. The PCBs were extracted twice with 1 volume of *n*-hexane, and the phases were separated by centrifugation when necessary. The PCB analysis was done with a model QDM1000 gas chromatograph-mass spectrometer by using a column containing type WCOT fused silica. [3,3',4,4'-^{14}C] tetrachlorobiphenyl (final concentration, 50 ppm) was added to the incubation vials prior to extraction and was included as an internal standard. Pure PCB congeners and Aroclor 1242 were obtained from Ventron, Karlsruhe, Germany.

Strain A5.4 had the same growth rate (doubling time, 4 h) in the presence and in the absence of 1 mM Ni^{2+}. Strain AE707 had a doubling time of 2 h in the presence and in the absence of 2 mM Zn^{2+}, but an increase in doubling time (to 4 h) was observed in the presence of 1 mM Ni^{2+} (data not shown). Furthermore, strain A5 degraded higher chlorinated PCBs only to a minor extent, degrading only PCBs with up to three chlorine substitutions (Tables 4 and 5). However, a slight inhibition of PCB degradation was observed with strain A5 when heavy metals were present in the medium; this was also observed for the degradation of PCB isomers in the presence of Aroclor 1242. On the other hand, the PCB-degrading activities of Bph^+ Nic^+ or Bph^+ Zin^+ strains A5.4 and AE707 were not impaired in the presence of nickel or zinc (Tables 4 and 5).

Table 6 shows the responses of *A. eutrophus* JMP134 and

TABLE 6. Growth in the presence of 3 mM 2,4-D in batch cultures and production of free chloride ions from 2,4-D by strains JMP134 and AE1200 in the presence and absence of heavy metals (1 mM Ni and 2 mM Zn) in batch cultures

Medium	Strain JMP134(pJP4)		Strain AE1200(pJP4, pMOL28, pMOL30)	
	Optical density	Cl^- concn (mM)	Optical density	Cl^- concn (mM)
Tris + 2,4-D	0.2	3.2	0.18	3.1
Tris + 2,4-D + Ni	0.02	0.0	0.145	2.7
Tris + 2,4-D + Zn	0.02	0.0	0.15	2.5

Nic$^+$ Zin$^+$ Tfd$^+$ *A. eutrophus* AE1200 on 2,4-D degradation in the presence and in the absence of zinc and nickel. Cells were grown at 30°C in a chloride-free minimal medium which contained 1 g of $(NH_4)_2SO_4$ per liter, 0.05 g of $MgSO_4 \cdot 7H_2O$ per liter, 0.01 g of $FeSO_4 \cdot 7H_2O$ per liter, 0.294 g of β-glycerophosphate per liter, 3 mM 2,4-D, and 1 mM $NiCl_2 \cdot 6H_2O$ or 2 mM $Zn(SO_4)_2$. The β-glycerophosphate was added after sterilization. In contrast to parental strain JMP134, strain AE1200 did grow in the presence of nickel and zinc, and free chloride ions were released, demonstrating that 2,4-D was completely degraded. Chloride levels were assayed by the colorimetric method of Bergmann and Sanik (4).

The strains which we constructed were able to grow in the presence of nickel and zinc and exhibited unimpaired biodegradation activities. Both of these properties are important considering the use of microorganisms for decontamination of environments polluted by organic xenobiotics compounds and heavy metals. Indeed, growing strains have been shown to be superior for degradation of a xenobiotic compound compared with resting cells (19, 25). Accordingly, supplying nutrients or carbon sources together with the introduced bacterium can promote biodegradation. Introduction of bacteria that are sensitive to heavy metals but still retain activity against the organic contaminant in heavy metal-contaminated environments could lead to decreased degradation in time and may require subsequent inoculation of the organisms.

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REFERENCES

1. **Abbas, A. S., and C. Edwards.** 1990. Effects of metals on *Streptomyces coelicolor* growth and actinorhodin production. Appl. Environ. Microbiol. **56:**675–680.
2. **Babich, H., and G. Stotzky.** 1983. Toxicity of nickel to microbes: environmental aspects. Adv. Appl. Microbiol. **29:**6782–6790.
3. **Bedard, D. L., M. L. Haberl, R. J. May, and M. J. Brennan.** 1987. Evidence for novel mechanisms of polychlorinated biphenyl metabolism in *Alcaligenes eutrophus* H850. Appl. Environ. Microbiol. **53:**1103–1112.
4. **Bergmann, J. G., and J. Sanik.** 1957. Determination of trace amounts of chlorine in naphtha. Anal. Chem. **29:**241–243.
5. **Burlage, R. S., L. A. Bemis, A. C. Layton, G. S. Sayler, and F. Larimer.** 1990. Comparative genetic organization of incompatibility group P degradative plasmids. J. Bacteriol. **172:**6818–6825.
6. **Chatterjee, D. K., S. T. Kellogg, S. Hamada, and A. M. Chakrabarty.** 1981. Plasmid specifying total degradation of 3-chlorobenzoate by a modified *ortho* pathway. J. Bacteriol. **146:**639–646.
7. **Diels, L., M. Faelen, M. Mergeay, and D. Nies.** 1985. Mercury transposons from plasmids governing multiple resistance to heavy metals in *Alcaligenes eutrophus* CH34. Arch. Int. Physiol. Biochim. **93:**B27–B28.
8. **Diels, L., and M. Mergeay.** 1990. DNA probe-mediated detection of resistant bacteria from soils highly polluted by heavy metals. Appl. Environ. Microbiol. **56:**1485–1491.
9. **Diels, L., A. Sadouk, and M. Mergeay.** 1988. Large plasmids governing multiple resistances to heavy metals: a genetic approach. Toxicol. Environ. Chem. **23:**79–89.
10. **Don, R. H., A. J. Weightman, H. J. Knackmuss, and K. N. Timmis.** 1985. Transposon mutagenesis and cloning analysis of the pathways for degradation of 2,4-dichlorophenoxyacetic acid and 3-chlorobenzoate in *Alcaligenes eutrophus* JMP134(pJP4). J. Bacteriol. **161:**85–90.
11. **Eaton, R. W., and K. N. Timmis.** 1986. Characterization of a plasmid-specified pathway for catabolism of isopropylbenzene in *Pseudomonas putida* RE204. J. Bacteriol. **168:**123–131.
12. **Fewson, C. A.** 1991. Factors affecting the degradation of hazardous recalcitrant materials, p. 173–183. Abstr. Int. Symp. Environ. Biotechnol., 22 to 25 April 1991, Oostende, Belgium.
13. **Friedrich, B., M. Meyer, and H. G. Schlegel.** 1983. Transfer and expression of the herbicide-degrading plasmid pJP4 in aerobic autotrophic bacteria. Arch. Microbiol. **134:**92–97.
14. **Fulthorpe, R. A., and R. Campbell Wyndham.** 1989. Survival and activity of a 3-chlorobenzoate-catabolic genotype in a natural system. Appl. Environ. Microbiol. **55:**1584–1590.
15. **Harayama, S., and M. Rekik.** 1990. The meta cleavage operon of TOL degradative plasmid pWWO comprises 13 genes. Mol. Gen. Genet. **221:**113–120.
16. **Jain, R. K., and G. S. Sayler.** 1987. Problems and potential for *in situ* treatment of environmental pollutants by engineered microorganisms. Microbiol. Sci. **4:**59–63.
17. **Kado, C. I., and S. T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145:**1365–1373.
18. **Klein, T. M., and M. Alexander.** 1986. Bacterial inhibitors in lake water. Appl. Environ. Microbiol. **52:**114–118.
19. **Kohler, H. P. E., D. Kohler-Staub, and D. D. Focht.** 1988. Cometabolism of polychlorinated biphenyls: enhanced transformation of aroclor 1254 by growing bacterial cells. Appl. Environ. Microbiol. **54:**1940–1945.
20. **Kovalick, W.** 1991. Perspectives on health and environmental risks of soil pollution and experiences with innovative remediation technologies, abstr. 3.3-1. Abstr. 4th World Congr. Chem. Eng., Karlsruhe, Germany, 16 to 21 June 1991.
21. **Lejeune, P., M. Mergeay, F. Van Gijsegem, M. Faelen, J. Gerits, and A. Toussaint.** 1983. Chromosome transfer and R-prime plasmid formation mediated by plasmid pULB113 (RP4:: Mini-Mu) in *Alcaligenes eutrophus* CH34 and *Pseudomonas fluorescens* 6.2. J. Bacteriol. **155:**1015–1026.
22. **Mergeay, M.** 1991. Towards an understanding of the genetics of bacterial metal resistance. Trends Biotechnol. **9:**17–24.
23. **Mergeay, M., P. Lejeune, A. Sadouk, J. Gerits, and L. Fabry.** 1987. Shuttle transfer (or retrotransfer) of chromosomal markers mediated by plasmid pULB113. Mol. Gen. Genet. **209:**61–70.
24. **Mergeay, M., D. Nies, H. G. Schlegel, J. Gerits, P. Charles, and F. Van Gijsegem.** 1985. *Alcaligenes eutrophus* CH34 is a facultative chemolithotroph with plasmid-bound resistance to heavy metals. J. Bacteriol. **162:**328–334.
25. **Mondello, F. J.** 1989. Cloning and expression in *Escherichia coli* of *Pseudomonas* sp. strain LB400 genes encoding polychlorinated biphenyl degradation. J. Bacteriol. **171:**1725–1732.
26. **Nies, A., D. H. Nies, and S. Silver.** 1989. Cloning and expression of plasmid genes encoding resistances to chromate and cobalt in *Alcaligenes eutrophus*. J. Bacteriol. **171:**5065–5070.
27. **Nies, D., M. Mergeay, B. Friedrich, and H. G. Schlegel.** 1987. Cloning of plasmid genes encoding resistance to cadmium, zinc, and cobalt in *Alcaligenes eutrophus* CH34. J. Bacteriol. **169:**4865–4868.
28. **Parsons, J. R., D. T. H. M. Sijm, A. van Laar, and O. Hutzinger.** 1988. Biodegradation of chlorinated biphenyls and benzoic acids by a *Pseudomonas* strain. Appl. Microbiol. Biotechnol. **29:**81–84.
29. **Said, W. A., and D. L. Lewis.** 1991. Quantitative assessment of the effects of metals on microbial degradation of organic chemicals. Appl. Environ. Microbiol. **57:**1498–1503.
30. **Schlömann, M., D. H. Pieper, and H. J. Knackmuss.** 1990. Enzymes of haloaromatic degradation: variations by *Alcaligenes* on a theme by *Pseudomonas*, p. 185–196. *In* S. Silver, A. M. Chakrabarty, B. Iglewsk, and S. Kaplan (ed.), *Pseudomonas*: biotransformations, pathogenesis, and evolving biotechnology. American Society for Microbiology, Washington, D.C.

31. **Shields, M. S., S. W. Hooper, and G. S. Sayler.** 1985. Plasmid-mediated mineralization of 4-chlorobiphenyl. J. Bacteriol. **163:** 882–889.
32. **Siddiqui, R. A., K. Benthin, and H. G. Schlegel.** 1989. Cloning of pMOL28-encoded nickel resistance genes and expression of the genes in *Alcaligenes eutrophus* and *Pseudomonas* spp. J. Bacteriol. **171:**5071–5078.
33. **Siddiqui, R. A., H. G. Schlegel, and M. Meyer.** 1988. Inducible and constitutive expression of pMOL28-encoded nickel resistance in *Alcaligenes eutrophus* N9A. J. Bacteriol. **170:**4188–4193.
34. **Springael, D.** 1992. Ph.D. thesis. Vrije Universiteit Brussel, Brussels, Belgium.
35. **Tyagi, R. D., D. Couillard, and J. P. Villeneuve.** 1986. Functional design of activated sludge processes with heavy metal inhibition. Can. J. Chem. Eng. **64:**632–638.
36. **Yen, K. M., and I. C. Gunsalus.** 1982. Plasmid gene organization: naphthalene/salicylate oxidation. Proc. Natl. Acad. Sci. USA **79:**874–878.