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Survival of *Alcaligenes xylosoxidans* Degrading 2,2-Dichloropropionate and Horizontal Transfer of Its Halidohydrolase Gene in a Soil Microcosm

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Abstract. A soil microcosm, containing a mixture of sand and a well-characterized phaeozem soil from loess, was developed for biodegradative applications. It was inoculated with soil-borne *Alcaligenes xylosoxidans* AB IV, degrading 2,2-dichloropropionate (DCPA), by a plasmid-encoded haloalkanoic acid halidohydrolase. In long-term microcosm experiments, survival of *Alcaligenes xylosoxidans* ABIV in the presence and absence of selective pressure by the xenobiotic compound could be demonstrated as well as its capacity to maintain degradation of DCPA. At the same time its plasmid, pFL40, containing the degradative gene, *dhlC*, was horizontally transferred to *Pseudomonas fluorescens* in sterilized soil and also to different indigenous soil bacteria in nonsterilized soil.

The use of biodegradative bacteria could be an efficient tool to detoxify polluted soils. Little information is available about the ecological principles governing population dynamics and about biotic and abiotic factors affecting the survival and biodegradative activities of microorganisms in soil. Predictive information on the fate of microorganisms following their release into soils is nowadays an indispensable pre-release consideration. In particular, information on their survival and dispersal, as well as on transfer and stability of their DNA, is required.

For this purpose microcosms, simulating significant soil ecosystem parameters, can be used as tools to study ecological interactions of biodegradative organisms in soil communities. Thus we designed a particular microcosm, representing a reasonable approximation of the target soil ecosystem and useful as a reliable intermediate between test tube studies and field experiments for biodegradation.

Alcaligenes sp. AB IV was obtained from a garden soil plot and applied as a degradative model organism in these studies. It was found to utilize 2,2-dichloropropionate (DCPA) as sole carbon source. Up to now, many different bacteria had been isolated, utilizing short-chained haloalkanoic acids as sole source of carbon and energy [3, 6, 14]. The essential step in the degradation of these xenobiotic compounds was catalyzed by a halidohydrolase, known to be often encoded on mobile genetic elements [7, 15, 21]. Some of these biodegradative bacteria obviously possessed identical dehalogenases; others even harbored up to four different halidohydrolases [6, 18]. We present evidence for the successful removal of halogen substituents from pollutants by *A. xylosoxidans* AB IV in soil microcosms. Furthermore, data are presented not only indicating the survival of the biodegradative bacteria in soil, but also dissemination of its biodegradative genes in situ.

Materials and Methods

Bacterial strains and plasmids. Strain AB IV was isolated from a garden soil at Münster (Germany). A spontaneous rifampicin (Rif^r)-resistant mutant of *Pseudomonas fluorescens* R2f [24] was used as a recipient strain in mating experiments. Plasmid pME285 (Km^r) [11] was used as a cloning vector.

Construction of soil microcosms. Glass tubes, 30 cm in length and 3 cm internal diameter, were constructed as shown in Fig. 1 to obtain vertical soil microcosms. A phaeozem soil from loess from an agricultural plot near Braunschweig was used as the soil sample. Its properties and biomass content have been described [1, 20]. The columns were filled up to 25 cm in height with 250 g (wet weight) of a mixture of 40% sieved soil (mesh size 2 mm) and 60% seasand, to prevent anaerobic conditions by moisture. The bulk density was 1.4 g cm^{-3}, the moisture content was 18% ± 2%

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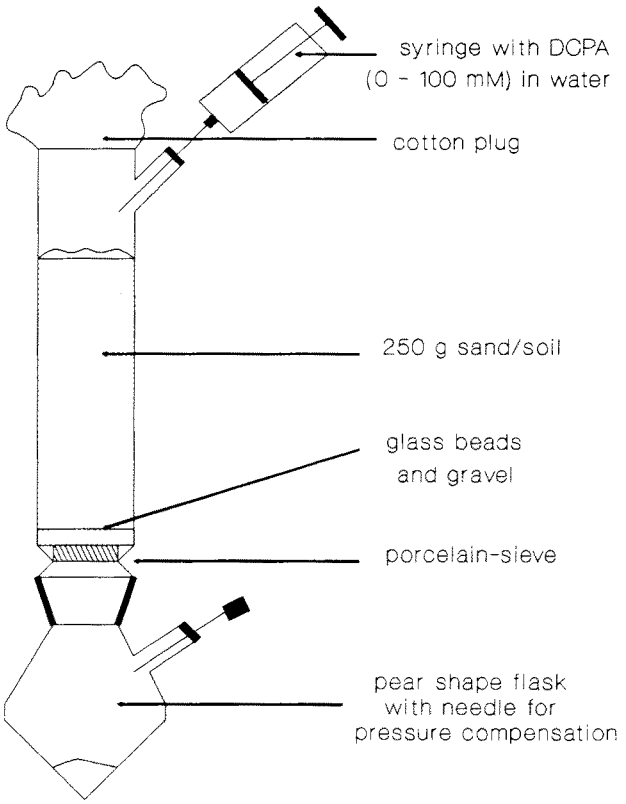

Fig. 1. Vertical soil microcosm used for studying survival, biodegradation and gene transfer of halidohydrolase-producing bacteria in soil. The 30 × 3 cm glass column contains a mixture of phaeozem soil from loess and sand. It was percolated with varying concentrations of DCPA. Gravel stones and a porcelain-sieve in the bottom of the funnel prevented the soil from escaping. Samples of effluent water that had been percolated through the microcosms were obtained from the collector flask and used for selective plating.

during the experiment, and the pH at the start was 7.5. The columns were stored in the dark at 16°–20°C.

Inoculation and analysis of microcosms. Column Bs was sterilized by autoclaving and then inoculated with 2×10^8 cells/g soil *P. fluorescens* R2f, to simulate the indigenous microflora. One day later the same quantity of cells of strain ABIV was introduced to the top of this column and also to a nonsterilized soil column B4. A nonsterilized soil column Bo without additional bacteria served as control. Every second day on the average, 5 mL sterilized tap water, containing 0–0.5 mmol DCPA, was added to the columns. Samples for determination of cell number and DCPA degradation were taken from the percolated liquid of the column, each time 2 h after the medium had been added to the soil. Samples were plated on nutrient agar or on King's B agar (KB) to estimate total colony forming units (CFU) after incubation at 30°C for 2 days. Degrading bacteria were enumerated on pH-indicator agar [22] with 20 m*M* chlorinated compound as sole carbon source after incubation for a maximum of 5 days.

Detection of transconjugant and transformant strains. Transconjugant *P. fluorescens* R2f containing the gene encoding haloalkanoic acid halidohydrolase (*dhlC*) were identified by plating on pH-indicator agar with 20 m*M* DCPA and rifampicin (100 μg/mL). Bacterial species, degrading DCPA and differing from the original strain ABIV, were detected by their morphological characteristics. Bacteria, capable of using halogenated compounds, were identified on API20NE (bioMerieux).

Soil analysis. The soil was cut into sections of 5 cm and analyzed for its bacterial content at the end of each experiment as previously described [8]. Bacteria were determined as described above.

Estimation of degradation of DCPA. Free chloride, which appears during the dehalogenation of DCPA, was measured by a modified [22] colorimetric method of Bergmann and Sanik [2]. Additionally, DCPA and pyruvate were measured by HPLC in a RP18-column, 50 m*M* KH_2PO_4-buffer, pH 2.5, flow rate 1.5 mL min^{-1}, retention time 4.0 min. respectively 1.8 min.

Enzyme activities and analysis of halidohydrolases. The halidohydrolases from different bacteria were compared in crude extracts [12] and in native polyacrylamide gel electrophoresis (PAGE), following activity staining [26]. A method was developed for rapid molecular weight estimations of dehalogenases. For this purpose active enzyme was electroeluted from a preparative native polyacrylamide gel in a biotrap chamber (Schleicher and Schuell). Estimation of molecular weight was made by SDS–PAGE.

DNA manipulations. Screening for plasmids in biodegradative bacteria, preparation and characterization of plasmid DNA, restriction enzyme analysis, molecular cloning, conjugal transfer, and transformation of biodegradative genes was carried out as described previously [7, 9, 10, 19]. Electroporation of DNA was performed in a Gene Pulser (BioRad) according to Fiedler [5].

Hybridization techniques. Plasmid DNA fragments were labeled with the Digoxigenin Labeling and Detection Kid (Boehringer) according to the manual of the producer. Colony hybridization was performed on Hybond-N-membranes (Amersham) and Southern blots were made with a VacuGene Blotting system (LKB-Pharmacia), following the manuals of the manufacturers.

Results and Discussion

Characterization of *Alcaligenes xylosoxidans* AB IV and its degradative enzyme. *Alcaligenes sp.* AB IV was obtained from an enrichment culture with chloroacetate (MCA). It utilized short-chained holalkanoic acids as sole carbon sources (Table 1), including DCPA, which is used as a herbicide (Dalapon). The dehalogenation of MCA and DCPA was found to be catalyzed by a halidohydrolase, yielding glycolate and pyruvate respectively. The enzyme was classified as a D,L-2-haloacid dehalogenase (E.C. 3.8.1.2), for acting on C-2 to C-4 halogenated acids except fluorinated compounds. In cell-free extracts a specific activity of 0.1 μkat/mg protein with MCA as dehalogenase-substrate was obtained. It was highly

Table 1. Growth of *A. xylosoxidans* AB IV on haloalkanoic acids

Growth substrates:[a]	NB	MCA	DCA	2-CPA	DCPA	CEO
Growth rate (h^{-1}):[b]	0.40	0.17	0.10	0.18	0.15	0

Sensitive to[c] Ampicillin (50), kanamycin (50), nalidixic acid (100), rifampicin (50), streptomycin (30), sulfonamide (500), $HgCl_2$ (20).

[a] NB, nutrient broth; MCA, chloroacetate; DCA, dichloroacetate; 2-CPA, D-,L-2-chloropropionate; DCPA, 2,2-dichloropropionate; CEO, chloroethanol.

[b] Growth rates were measured at 30°C with 20 m*M* haloalkanoic acid as substrate.

[c] Concentrations in μg/mL.

Table 2. Characterization of the halidohydrolase from *A. xylosoxidans* AB IV

Substrate		Relative activity (in % MCA activity)
Chloroacetic acid	(MCA)	100
Dichloroacetic acid	(DCA)	14 ± 2
Trichloroacetic acid	(TCA)	2 ± 0.5
D,L-2-Chloropropionic acid[a]	(CPA)	58 ± 3
L-2-Chloropropionic acid	(L-2-CPA)	72 ± 2
2,2-Dichloropropionic acid	(DCPA)	24 ± 2
2-Chlorobutyric acid	(MCBA)	17 ± 3
Bromoacetic acid	(MBA)	126 ± 4
Iodoacetic acid	(MJA)	61 ± 5

pH optimum, 9.0; temperature optimum, 42°C; molecular weight, 32 ± 1 kD.[b]

Measurements were performed with crude extracts at 30°C and pH 9.0. Given data were mean values of three to five estimations. No dehalogenation was observed from 3-chloropropionate and fluoroacetate (measured as glycolic acid).

[a] D- and L-CPA were both substrates of the halidohydrolase, determined by complete dechlorination of a given amount of D,L-2-CPA.

[b] Estimated by SDS-polyacrylamide gel electrophoresis.

induced during growth of strain AB IV on dichloroacetate. Table 2 summarizes the characteristics of the halidohydrolase. It is comparable to the fraction I dehalogenase from *P. putida PP3* [27].

Alcaligenes sp. AB IV was a coccoid rod (1 × 1.5 μm), occurring as single cells or in short chains, non- or only slowly motile and sensitive to common antibiotics (Table 1). Based upon morphological, biochemical, and serological characteristics, it was identified as *Alcaligenes xylosoxidans ssp.* according to Kiredjian et al. [16]).

Cloning of a plasmid-bound gene, *dhlC*, involved in 2,2-dichloropropionate metabolism. *Alcaligenes xylosoxidans* was found to harbor pFL40, a plasmid with a size of 60 kb which was transferred to *P. fluorescens* R2f with a frequency of 10^{-4} on filters. Analysis of transconjugants, utilizing DCPA, demonstrated the presence of a plasmid, identical with pFL40 in size and restriction fragment sizes, coupled with the expression of the halidohydrolase. Digestion of pFL40 with restriction endonucleases *EcoR*I- and *Sal*I, additional cloning of the fragments into pME285, and transfer into *P. fluorescens* R2f via electroporation resulted in a chimeric plasmid carrying a 6,9 kb fragment of pFL40. Because of the biochemical characteristics of the halidohydrolases, produced by the respective *P. fluorescens* transformants, this fragment comprised the halidohydrolase gene, *dhlC*.

Survival and biodegradation of *A. xylosoxidans* AB IV in a soil microcosm. As shown in Fig. 2, significant counts of *A. xylosoxidans* ABIV were detectable in the percolation water of microcosms with sterilized as well as in a nonsterilized soil for more than 5 months after inoculation. However, cell numbers were dependent on the concentration of DCPA. Subjected to an increasing concentration of 0.2–0.4 m*M* DCPA in an initial period (37 days), the number of biodegradative soil bacteria, estimated from the percolation water, stabilized at 10^6 in column B4 and 10^8 CFU/mL in column Bs, respectively. The release of free chloride and determination of DCPA from the percolation water indicated complete degradation of the DCPA, supplied during period 1 and 2 to column Bs, and 80% degradation of DCPA added to column B4, with a time delay of 2 weeks (Fig. 3).

In period 3 the substrate was replaced by sterile water, and the number of degrading bacteria decreased to 10^5 cells/mL (Fig. 2). When adjusting the substrate to 0.4 m*M* DCPA in period 4, progressively higher numbers of DCPA-degrading cells were detected in the percolation water, and DCPA degradation rates approximated the values of period 1, while the differences between total cell numbers and degrading bacteria decreased significantly (Figs. 2, 3). However, raising the substrate concentration to 0.1 *M* DCPA in period 5 resulted in a progressive decrease of cell numbers of the degradative population in the percolation water, probably owing to the toxic effect of high DCPA concentrations, confirmed by in vivo studies.

The final analysis of the vertical distribution of bacteria throughout the soil cores is shown in Fig. 4. It demonstrated cell numbers not significantly dif-

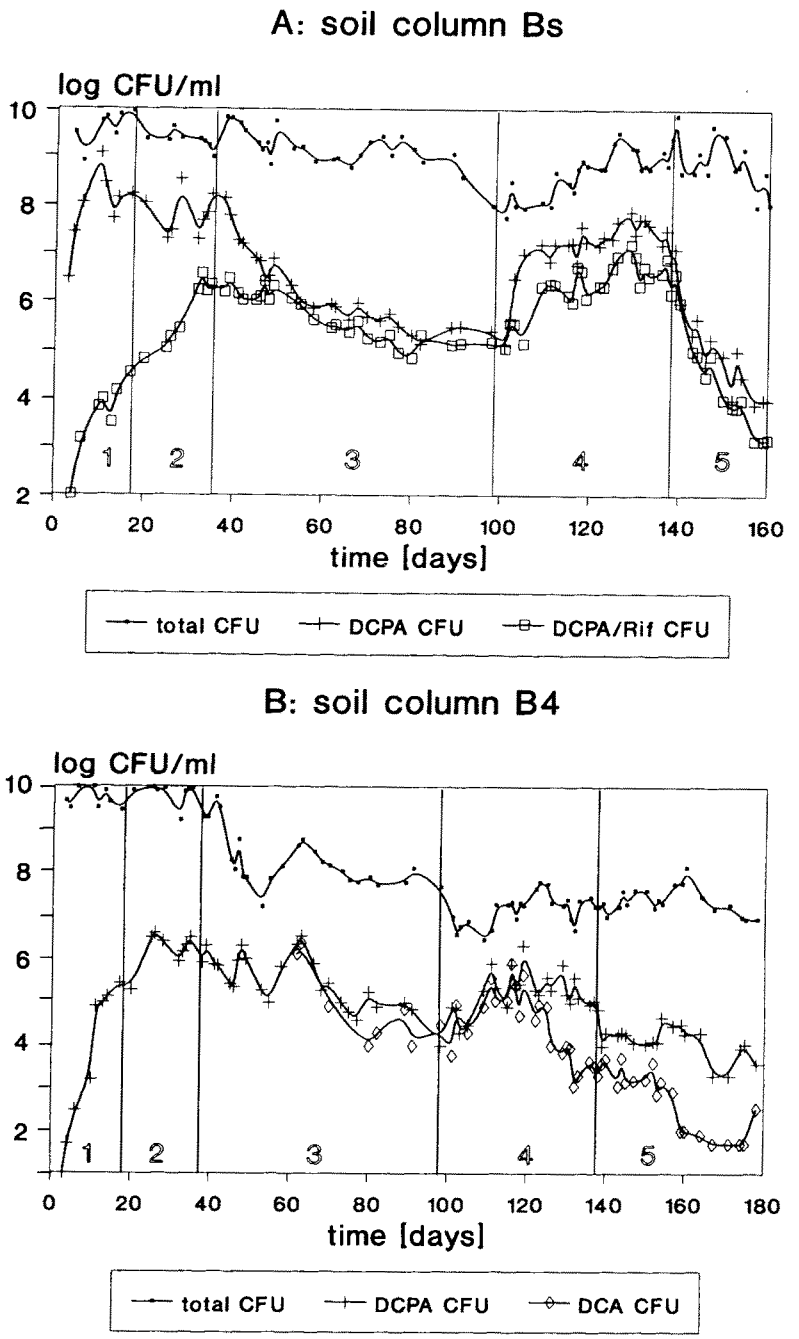

Fig. 2. Dynamics of long-term survival of *A. xylosoxidans* ABIV in soil in the presence and absence of DCPA. (A) The sterilized soil column Bs inoculated with the DCPA-degrading *A. xylosoxidans* ABIV and *P. fluorescens* R2f, and the occurrence of a DCPA-degrading, rifampicin-resistant transconjugant strain. (B) the nonsterilized column B4, inoculated with *A. xylosoxidans* ABIV. Phase 1, addition of 0.2 mmol DCPA; phases 2 and 4, 0.4 mmol DCPA; phase 5, 1.0 mmol DCPA/kg soil × day; phase 3, pure water was added. Samples were collected each time 2 h after the medium was added and were used for determination of CFU on nutrient or KB agar (total CFU), or on indicator agar, containing DCPA without (DCPA CFU) and with rifampicin (DCPA/Rif CFU), to differentiate between original Rif^S *A. xylosoxidans* AB IV and transconjugants.

ferent from those determined in the percolation water at this time (Fig. 2). These results suggested the long-term survival of *A. xylosoxidans* AB IV and its preserved capacity to utilize DCPA in soil independently from selective pressure by the xenobioticum.

A control microcosm, Bo, filled with nonsterilized soil devoid of *A. xylosoxidans* AB IV, revealed that within 6 months neither DCPA-utilizing bacteria nor any degradation of the xenobioticum could be detected. This result is in accordance with the findings of Burge [3]. However, two indigenous bacteria were isolated from microcosm Bo, naturally degrading DCA, and 2-chloroethanol (CEO) (Table 3).

Dispersal of halidohydrolase genes in soil communities. In column Bs a fluorescent Rif^r bacterium, capable of degrading DCPA, was isolated 5 days after inoculation with *A. xylosoxidans* ABIV (Fig. 2A). It

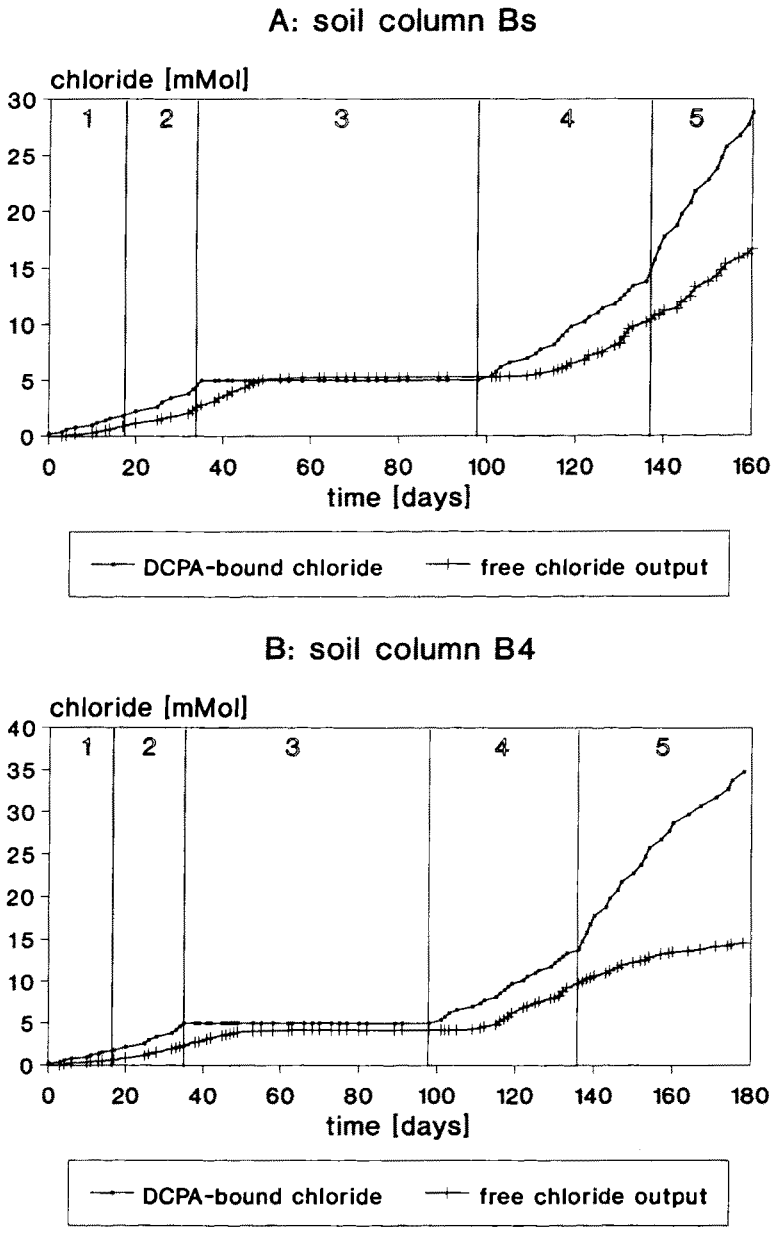

Fig. 3. Degradation of DCPA in soil microcosms. (A) Soil column Bs; (B) soil column B4. Degradation of DCPA was calculated on the basis of concentration of free chloride in the liquid effluence of the columns and set versus the DCPA-bound chloride input. Phases 1–5: refer to Fig. 2.

was identified as *P. fluorescens* R2f. Column Bs revealed that approximately every Rif^r cell was capable of degrading DCPA (Fig. 4A). However, DCPA-utilizing bacteria in column B4, differing from *A. xylosoxidans* AB IV, were first detected in period 4, as indicated by the difference in DCPA- and DCA-CFU (Fig. 2B). Such indigenous DCPA-utilizing soil bacteria were identified as members of the genera *Pseudomonas* and *Alcaligenes*, respectively (Table 3).

Comparative characterization of their dehalogenases demonstrated that all of these species produced an enzyme with corresponding electrophoretic mobility (R_F) in native PAGE (R_F = 0.35). The substrate specificity of these halidohydrolases was found to be identical with the enzyme, encoded by *dhlC* of pFL40 from *A. xylosoxidans* ABIV (Table 2), but differed in its substrate spectra and migration patterns (R.F. = 0.73) from the dehalogenase produced by *P. stutzeri* Bog (Table 3).

Restriction analysis and colony hybridization with probes from pFL40, constructed to screen either for *dhlC* or parts of the plasmid, indicated the presence of pFL40 in all of the indigenous transcon-

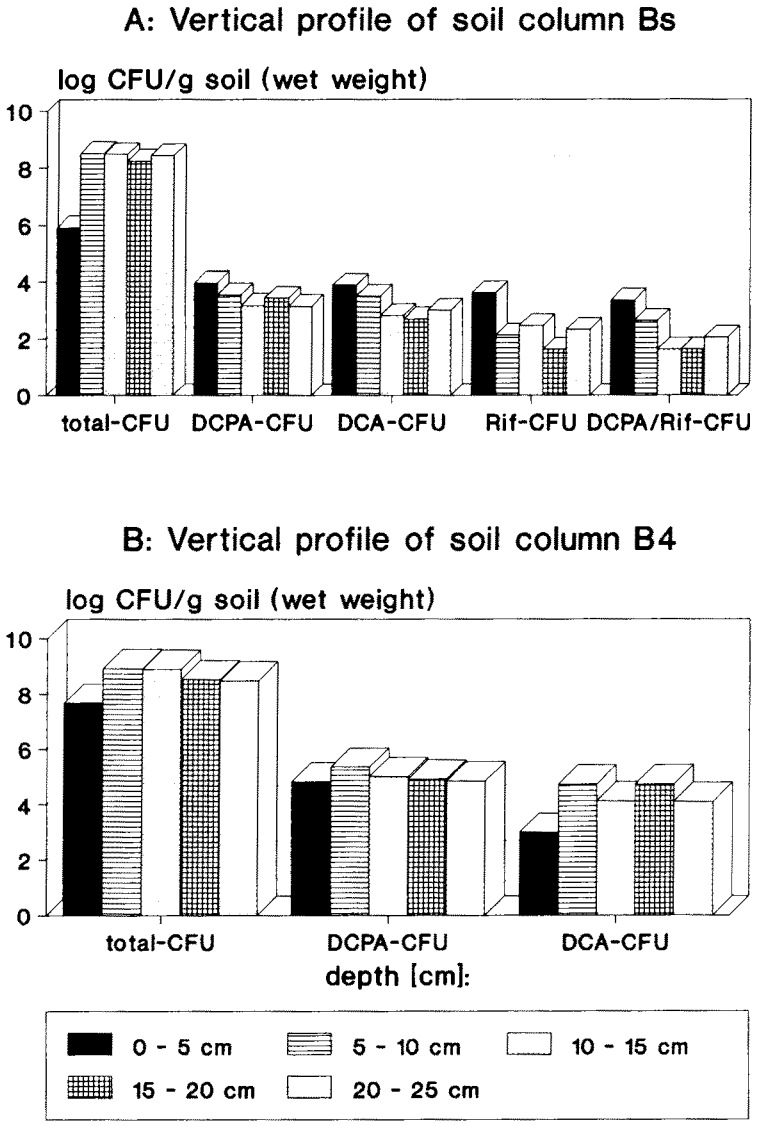

Fig. 4. Vertical distribution of bacteria from the soil columns. The soil core was removed from the glass columns and cut in 5-cm sections. Bacteria were detached from soil and enumerated on different media. Given data are mean values of three determinations.

jugants, metabolizing DCPA in microcosm B4, whereas DCPA$^-$ bacteria, capable of degrading other short-chained haloalkanoic acids, did not show homology with this probe (data not shown).

Kawasaki et al. [14] stated a great diversity of haloalkanoic acid dehalogenases. The isolation of indigenous soil bacteria, however, harboring plasmid pFL40, indicates that a horizontal gene transfer is more likely than adaptation to DCPA under the given conditions. Thus, horizontal dispersal of degradative functions may occur in natural environments and be one reason for the widespread appearance of bacteria encoding one or more of these dehalogenases. Comparative sequence analysis of dehalogenases from different sources [13, 14, 17] could provide first evidence for this assumption.

Thus, for effective enhancement of the detoxification of polluted soils, optimized laboratory strains could be preadapted in microcosms to gain ecologically fit biodegradative microorganisms, capable of transferring their detoxifying capacity to the autochthonous target microflora.

Our results indicate the potential of soil bacteria to pick up heterologous DNA and could recently be confirmed by horizontal transfer of an aminoglycoside resistance gene into indigenous soil bacteria of different species [9]. Such events favor the mobility and transfer potential of soil-borne genes and raise

Table 3. Characterization of DCPA-degrading strains, isolated from soil columns

Origin (microcosm)	Species	Growth/degradation[a]			Plasmid (kb)
		DCPA	DCA	CEO	
Bs	*A. xylosoxidans* ABIV	+	+	−	pFL40 (60)
	P. fluorescens R2f	−	−	−	n.d.
	P. fluorescens Bsf (Transconjugant *P. flu.* R2f)	+	−	−	pFL40-1 (60)
B4	*Pseudomonas sp.* B4r	+	(+)	−	pFL40-2 (60)
	P. fluorescens B4f	+	−	−	pFL40-3 (60)
	P. paucimobilis B4y	+	−	−	pFL40-4 (60)
	Alcaligenes sp. B4hr	+	+	−	pFL40-5 (60)
Bo	*P. stutzeri* Bog	−	+	+	(5.5)
	P. stutzeri Bor	−	+	+	n.d.

Bacteria were identified on API20NE.
[a] +, normal growth; (+), reduced growth; −, no growth and degradation; n.d., not detected.

unexpected implications for deliberate release of genetically engineered biodegradative microorganisms into the environment. Microcosm experiments with any organism designed to be released should be helpful to evaluate potential risks and to identify the roles of either abiotic soil variables like nutrient limitations, temperature, or biotic factors like survival, selection, stress factors, and barriers for gene mobility in situ [4, 8, 19, 23–25]. However, the increased need to warrant the detoxification of polluted environments could demand an effective expression adaptation and also interspecies transfer of the biodegradative capacity being released.

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