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BACTERIAL DICHLOROPROPENE DEGRADATION IN SOIL; SCREENING OF SOILS AND INVOLVEMENT OF PLASMIDS CARRYING THE *dhlA* GENE

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Summary—The nematocide *cis*-1,3-dichloropropene is widely used in intensive agricultural practice against root knot nematodes. As a result of the regular application of dichloropropene to six out of eight different soils, the compound was rapidly degraded to CO_2, H_2O and chloride ions in subsequent applications (adapted soils). Such degradation occurred at a lower rate in the same soil types without a history of dichloropropene use (unadapted soils). In two soils, the effect of repeated dichloropropene treatment was not found, since dichloropropene was already rapidly decomposed in the untreated controls. Since the dehalogenating enzyme haloalkane dehalogenase, encoded by the *dhlA* gene, is possibly involved in the degradation of dichloropropene, a specific PCR detection system combined with a *dhlA* probe was developed to detect *dhlA* in soil DNA extracts. Five of the eight adapted soils as well as three unadapted ones showed a response to this system, indicating the presence of DNA with sequence similarity to *dhlA*. Fifteen bacterial strains with dichloropropene-degradative capacity were isolated from enrichment cultures grown in the presence of this compound and inoculated with adapted soil (five soil types). Six selected isolates were identified as, respectively, *Alcaligenes paradoxus*, *Pseudomonas cichorii* I, *Pseudomonas corrugata* (2 ×), *Pseudomonas putida* and *Pseudomonas sp*. Each of the six strains harboured a plasmid of 50–60 kb in size and all but one carried resistance to $HgCl_2$. Filter matings were performed to investigate the possible cotransfer of biodegradative genes and Hg resistance to suitably-marked *P. fluorescens* R2f recipient strains. Three plasmid donor strains, *P. cichorii* I, *P. corrugata* and *Pseudomonas* sp., transferred the Hg resistance and the plasmid to the recipient used, suggesting that the Hg resistance marker was located on the plasmid. In addition, transconjugants produced with *P. cichorii* I and *P. corrugata* donor strains had also acquired 1,3-dichloropropene-degrading capability. PCR amplification with *dhlA* specific primers, of the plasmids obtained both from the original *P. cichorii* I and *P. corrugata* strains and from the respective transconjugant *P. fluorescens* R2f strains generated 400–500 bp amplification products. After cloning of the product of the *P. cichorii* I plasmid and sequencing of 180 bases, extensive homology with the *dhlA* gene was detected. These results suggest that a plasmid-located *dhlA*-like gene may be involved in dichloropropene degradation in soil by soil bacteria, but the extent to which the gene is involved in the process *in situ* is not clear.

INTRODUCTION

The repeated treatment of soils under continuous potato cropping with nematocides, such as *cis*-1,3-dichloropropene (DCPe) and methylisothiocyanate (MITC), which is common practice in The Netherlands (Smelt *et al.*, 1974), can result in soils with the capability of accelerated breakdown of these compounds. In such soils, which are called adapted (Kearney and Kellogg, 1985; Katan and Aharonson, 1989), ensuing applications of the same compounds are often poorly effective due to the greatly enhanced degradation rates. For agricultural practice, the irreversible decomposition of DCPe into 3-chloroallylalcohol is the most important detoxification step because chloroallylalcohol is only weakly toxic to nematodes.

Lebbink *et al.* (1989) suggested that the adaptation of soils to DCPe was due to the selection of a microbial population with DCPe degradative capacity. In particular fluorescent pseudomonads isolated from adapted soils were suggested to be involved in this process since they possessed degradative capacity. The occurrence of accelerated degradation of DCPe in soils was assessed by chromatographically determining the disappearance rate of DCPe in soil microplot systems by Verhagen *et al.* (1995).

The degradation of DCPe follows a pathway described by Roberts and Stoydin (1976). DCPe is decomposed into choroallylalcohol via the cleaving off of one chloride ion by nucleophilic substitution. This step is analogous to the degradation of

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Table 1. Properties of the soils used in field microplots. Fumigant treatment histories for these soils, indicated as (+), are given over a period before the microplot arrangement (1980–1989). The soils are listed here in order of increasing silt/clay content (the sum of the fractions <2 and 16 μm)

Soil	Soil type	Treatment history* MITC	Treatment history* DCPe	pH (KCl)	Organic matter	Particle size distribution (%) <2 μm	<16 μm	<50 μm	>200μm
Smilde SW1	Reclaimed cut-over peat soil	−	+	4.7	5.5	0	2.1	8.5	24.7
Odoorn AZ2	Sand	+	+	4.9	3.9	0	5.2	20.7	21.8
Ubbena	Sand	−	−	5.0	5.5	0	6.0	28.8	15.6
AB Haren	Sand	−	−	5.2	4.3	0	8.7	35.0	8.6
Wieringermeer	Sandy clay	−	+	7.3	3.8	4.9	7.2	17.6	6.2
Kloosterburen	Sandy loam	−	−	7.4	1.8	15.1	22.8	39.0	0.2
Lovinkhoeve	Loamy sand	−	−	7.4	2.7	16.9	29.8	79.7	1.4
Westmaas	Clay	−	−	7.5	2.6	20.1	31.2	63.8	0.5

*Treatment history: +, compound applied; −, no previous application of compound.

1,2-dichloroethane, in which a haloalkane dehalogenase (HAD) encoded by the *dhlA* gene, is involved (Janssen *et al.*, 1989). The active site of HAD was described by Franken *et al.* (1991). The active site showed homology with the dienelactone hydrolase molecule indicating a conserved region in the active site of both molecules.

Since it is likely that soil bacteria are involved in the enhanced biodegradative capacity observed in adapted soils, we initiated a study on the genetic background of DCPe degradation in bacteria obtained from DCPe-degrading soil communities. Chemical stress imposed on bacterial communities in soil may enhance plasmid incidence in the culturable population (Wickham and Atlas, 1988), and such plasmids may carry genes which confer the ability to degrade pesticides, as shown for example by 2,4-dichlorophenoxyacetic acid and 4-chloro-2-methylphenoxyacetic acid (Don and Pemberton, 1981). Transfer of biodegradative plasmids from the original hosts to other bacteria in soil may represent a mechanism by which bacterial populations evolve rapidly to cope with the imposed stress (Brokamp and Schmidt, 1991).

We have investigated the occurrence of adaptation in several soils, the enrichment of DCPe-degrading bacteria, their isolation and identification and the screening for plasmid localization of the biodegradative capacity. We used the active site of HAD to design primers for a PCR-based detection system of *dhlA*, since we surmised *dhlA* might be a key gene in DCPe degradation by soil bacteria. Evidence is presented for the occurrence of plasmid-borne and transferable active *dhlA* in the soil isolates *Pseudomonas cichorii* I and *Pseudomonas corrugata*.

MATERIALS AND METHODS

Soils and soil microplots

Top-layer soil from eight fields at different locations representing regions in Holland with an intensive use of nematocides were collected and transferred into microplots. Each soil was divided over 12 subplots. The soils differed in many aspects, including organic matter content, pH and particle size distribution (Table 1). The soils were treated with nematocides as outlined in the following, whereas for each soil a control was kept untreated. During 1992 and 1993, a number of subplots was treated once every 2 months with DCPe (6×), separately or alternately with MITC (3 × DCPe, 3 × MITC). MITC was included since it is an alternative nematocidal agent which can be used in conjunction with DCPe. Following these conditioning treatments, degradation of DCPe was examined in 1994. Soils from alternately-treated subplots showing enhanced degradation of DCPe (Table 2) were used for the

Table 2. Accelerated degradation of DCPe in soil presented as the 50 and 95% disappearance times (Dt_{50} and Dt_{95}, respectively) of the initial dose*

Soil	Fig.	Control Dt_{50} (day)	Control Dt_{95} (day)	Control Curve adaptation	Control *dhlA*	6 × treated in 1992 Dt_{50} (day)	6 × treated in 1992 Dt_{95} (day)	6 × treated in 1992 Curve adaptation	6 × treated in 1992 *dhlA*
Smilde SW1	1(a)	3.3	11.2	+/−	ND	4.7	9.2	+	ND
Odoorn AZ2	1(b)	8.6	21.5	+/−	0	5.8	12.5	+	+
Ubbena	1(c)	25.5	114.9	−	+	4.2	7.4	+	+
AB Haren	1(d)	5.0	17.7	−	0	2.7	5.7	+	0
Wieringermeer	1(e)	4.9	5.5	−	0	2.7	4.5	+	0
Kloosterburen	1(f)	11.7	50.3	−	+	3.4	7.6	+	+
Lovinkhoeve	1(g)	3.4	7.8	+	0	3.3	6.1	+	+
Westmaas	1(h)	2.2	4.2	+	+	1.7	3.8	+	+

*These results are derived from the polynomal interpolation and extrapolation of the DCPe dissipation values in soil. The column "Fig." indicates the corresponding figures. Curve adaptation is defined as positive (+) when a deviation from the first-order model is observed. Detection of *dhlA* was by qualitative PCR in combination with the *dhlA* gene fragment as a probe. +, Signal detected (*dhlA*) or curve adapted; +/−, curve adaptation unclear; −, no curve adaptation; 0, no signal detected; ND, not determined.

enrichment and isolation of DCPe-degrading bacteria described in this paper.

Application of nematocides to soil microplots

DCPe and MITC were injected into the top 18 cm of the field microplots as fumigants, by using an injector (doses 10 ml DCPe and 30 ml Metham-sodium m^{-2}; 16 injections m^{-2}). The fumigants used were AAMonam (Schering AAgrunol, The Netherlands), which contained 50% Metham-sodium (in water), the source of MITC, and Telone-*cis* (gift from Dow Elanco, The Netherlands) which contained 98.4% DCPe. After injection into soil, Metham-sodium is largely converted to MITC (Smelt *et al.*, 1974). Telone-*cis* was used in the field microplots as well as for all laboratory enrichment and DCPe degradation experiments.

Soil sampling and incubation

Soil samples were taken from the top 20 cm layers of each soil microplot using a bore (25 mm dia). Eight to ten cores randomly taken over a 1 m^2 area were mixed to produce a composite sample. The composite samples were homogenized and stored at 4°C in closed plastic bags. For each, 18 subsamples (5 g, corresponding to 4–4.3 g dry soil) were weighed into 15-ml glass vials (Schott, Germany) equipped with Teflon inlayed gas-proof screw caps. Subsequently, the closed vials were stored for 48 h at 20°C. DCPe was added to each series of flasks (Fig. 2) using a syringe, to an initial content of 47 mg DCPe kg^{-1}. Some vials received MITC (from an aqueous solution) alternated with DCPe, at 71 mg kg^{-1}. All vials were kept in the dark at 20°C. On days 1, 2, 5, 7, 10 and 14, three flasks of each series were sampled for extraction of DCPe and chloroallylalcohol, and analysed as described below.

Analytical methods

DCPe in soil was determined by first extracting a 5-g soil sample with 2.5 ml demineralized water and 5 ml ethylacetate containing 5 mg 1,1,1-trichloroethane l^{-1} (internal standard in analysis) for 40 min. The concentrations of DCPe and chloroallylalcohol (CAA) in the enrichment and pure cultures were determined by extracting a 2.5 ml aliquot of culture with 2.5 ml ethylacetate containing 1,1,1-trichloroethane for 20 min.

A 1 μl aliquot of the ethylacetate solution obtained was injected into a gas chromatograph (GC; Carlo Erba, Vega 6130) with a 25 m Chrompack CP-Wax58 CB column and a ^{63}Ni electron capture detector. Quantitation was carried out on a 286 personal computer running the Baseline integration programme.

Chloride production was determined by a colorimetric assay according to Bergmann and Sanik (1957).

Assessment of soil adaptation to DCPe

A first-order kinetic reaction model has been commonly used to describe the dissipation kinetics of several pesticides in soil (Nash, 1988). As indicated, deviation from this first-order kinetics can be regarded as a sign of accelerated degradation and adaptation of the soil to DCPe. Polynomial interpolation and extrapolation (Press *et al.*, 1987) was applied to the DCPe dissipation values, in order to derive the dissipation time (Dt, in days) values for 50 and 95% of the original doses (Dt_{50} and Dt_{95}, respectively).

DNA extraction from soil for PCR amplification

Soils treated with DCPe as well as untreated ones were collected from the microplots and analysed for DCPe degradation, as described below. These soil samples were also used for extraction of total microbial community DNA according to the direct extraction and purification procedure described by Smalla *et al.* (1993). Using this flexible procedure, DNA was purified to the minimum degree of purification required for successful PCR amplification, as assessed by checking the amplification of added target DNA. Using selected primers, PCR amplification of a *dhlA*-specific region (see below), was then applied on the soil DNA extract.

Enrichment, isolation and identification of DCPe degrading bacteria

Flasks containing mineral medium with dichloropropene, modified by replacing the vitamins by 10 mg yeast extract l^{-1} (MMY; Janssen *et al.*, 1987), were inoculated with soil obtained from microplots receiving alternate DCPe–MITC applications. Periodically following inoculation, dilutions of the cultures were plated onto solid modified mineral medium (MMY agar) with dichloropropene as the sole C source (Loos, 1975). The plates were kept for 7 days at 24°C in closed glass jars stoppered with Viton rubber to prevent DCPe evaporating.

Separate colonies growing on MMY agar were regrown after streaking on MMY agar, tested for dichloropropene degrading capacity in MMY medium supplemented with DCPe, and if positive, identified using Gram staining and the BIOLOG identification system (MicroLog, La Jolla, CA, U.S.A.). Selected strains were assayed for the occurrence of antibiotic (kanamycin—Km; chloramphenicol—Cm; rifampicin—Rp; streptomycin—Sm; tetracycline—Tc; nalidixic acid—Nx; gentamycin—Gm) or mercury ($HgCl_2$) resistance markers, by scoring single colony development on Luria–Bertani (LB) agar (Bacto peptone 10 g, yeast extract 5 g, NaCl 5 g, demineralized water 1 l., agar 15 g; pH 7.2) plates containing these inhibitory agents (50 μg of each antibiotic ml^{-1}, 20 μg of $HgCl_2$ ml^{-1}).

Table 3. Characteristics of strains with DCPe-degradative ability isolated from adapted soils, and possible transfer of the biodegradation genes to *P. fluorescens* R2f recipient strains

Soil	Identification	Code	Resistance markers+	P_D +	*dhlA* D(PCR)*	Transfer**	P_R +	Degr***	*dhlA* T(PCR)+ + +
Smilde	*A. paradoxus*	164	Sm Km	+	+	0	ND	ND	ND
Odoorn	*P. corrugata*	172	Hg Nx	+	+	+	+	+	+
Kloosterburen	*P. putida* B	159	Hg Nx	+	+	+	+	0	0
Kloosterburen	*Pseudomonas* sp.	158	Hg Nx	+	+	+	0	0	0
Lovinkhoeve	*P. corrugata*	168	Hg Nx Cm	+	+	+	0	0	0
Westmaas	*P. chichorii* I	170	Hg	+	+	+	+	+	+

+Hg, mercury; Gm, gentamycin; Km, kanamycin; Rp, rifamipicin; Sm, streptomycin; Cm, chloramphenicol; Nx, nalidixic acid.
**dhlA* D(PCR): *dhlA*-specific PCR amplification using the plasmid of the isolated biodegradative strain.
** Transfer: selectable marker was transferred to either one of the *P. fluorescens* R2f recipient strains.
+ + P_D: plasmid present in (donor) strain (50–70 kb in size). P_R: plasmid of similar size to that in the donor detected in *P. fluorescens* R2f transconjugants.
*** Degr: transconjugant showed DCPe degradation capacity.
+ + + *dhlA* T(PCR): *dhlA*-specific amplification using plasmids isolated from transconjugant strains.
ND, not determined; +, result positive; 0, result negative.
Recipient strain characteristics: *P. fluorescens*, isolated from grassland, denoted R2f (*chr*::KTG), resistance markers Gm^R Km^R Rp^R, and denoted R2f Sm^R Rp^R.

Bacterial strains, plasmids and cultivation conditions

Two derivatives of *Pseudomonas fluorescens* strain R2f, a spontaneous rifampicin (Rp) and streptomycin (Sm) resistant mutant (R2f Rp^r Sm^r) and a chromosomally-marked strain containing the KTG marker gene cassette (R2f *chr*::KTG; Smit and van Elsas, 1992), which confers resistance to Km and Gm, were used as recipient strains. Strain R2f was originally isolated from a grass rhizosphere (van Elsas *et al.*, 1988). The R2f derivatives were cultured overnight at 27°C, with shaking, in 20-ml aliquots of LB broth containing the appropriate antibiotics (50 μg ml^{-1} of both Rp and Sm for R2f Rp^r Sm^r and 50 μg ml^{-1} of Km for R2f *chr*::KTG).

Escherichia coli HB101 (pPJ123), used as the source of the *dhlA* gene, was grown overnight, with shaking, at 37°C in LB broth containing 12 μg tetracycline ml^{-1}. Plasmid pPJ123 is pLAFR1 with a 1.15 kb *Bam*H1–*Eco*R1 *dhlA* specific insert (Janssen *et al.*, 1989).

Strains with dichloropropene-degrading capacity isolated from soil and their plasmids can be found in Table 3. These strains were cultured at 27°C in LB broth or on LB agar plates.

Plasmid extraction

A modified Kado and Liu (1981) method was used for plasmid extraction. Briefly, 1.5 ml of overnight culture was centrifuged in an Eppendorf centrifuge (1 min; 14,000 rev min^{-1}) and resuspended in 100 μl TEA buffer (40 mM Tris acetate, 1 mM EDTA; pH 7.9). Then, 200 μl of freshly-prepared solution A (50 mM Tris, 3% SDS, 120 mM NaOH; pH 12.5) was added, and the resulting solution was quickly mixed and kept at 57°C for 70 min. The solution was then extracted with phenol–chloroform and the aqueous phase was diluted with 200 μl sterile demineralized water. DNA was precipitated by the addition of 50 μl 3 M Na-acetate (pH 5.2) and 1 ml 100% ethanol at −20°C (at least 1 h, usually overnight). Plasmid DNA was pelleted by centrifugation (10 min; 14,000 rev min^{-1}). Pellets were washed once with 70% ethanol and dried under vacuum. The resulting plasmid DNA was resuspended in 25 μl TE buffer (Tris–HCl 10 mM, EDTA 10 mM, pH 8.0), and analysed via electrophoresis in a 0.7% agarose gel (Sambrook *et al.*, 1989).

To quickly obtain plasmid DNA free of chromosomal DNA, the QIAGEN plasmid mini kit (QIAGEN Inc., Chatsworth, CA, U.S.A.) was used. The manufacturer's protocol was followed, except that 20 ml of overnight culture was used and the elution buffer was heated to 50°C to facilitate elution of the plasmid DNA from the column.

Filter mating experiments

Matings between strains with dichloropropene-degradative capacity and selectable markers (Table 3) and the marked *P. fluorescens* R2f derivatives used as recipients were done with equal cell densities of early stationary-phase cells grown in LB broth. Aliquots (0.1 ml) of washed (twice in 0.85% NaCl) suspensions of the respective donor and recipient strains were pipetted onto sterile membrane filters (Millipore, 0.45 μm) placed on LB agar plates. The plates were kept for 16–20 h at 30°C, after which cells were resuspended by transferring filters to small volumes (1–2 ml) of LB medium. Aliquots (0.1 ml) of undiluted or diluted cell suspensions were plated on LB agar plates containing appropriate selective agents (Table 3). Plates were kept at 28°C for 3–5 days. Resulting colonies were picked and purified, via streaking onto the same selective medium. Subsequently, single colonies were picked and examined for the presence of plasmids and DCPe degradation capacity.

PCR

A PCR system was developed based on the active site of HAD encoded by *dhlA* (Franken *et al.*, 1991) so as to obtain products characteristic for this functional site. Primer sequences selected were for the forward primer 5′-TGG GGC GGA TTT TTG GGG CT-3′ and for the reverse one 5′-GTA CGA AAT

GGC CAG CGT CC-3′. Primers spanned a 461-bp *dhlA*-specific region, the total product size being 501 bp. PCR was performed in 50-ml reaction volumes using standard concentrations of primers, dNTP's, $MgCl_2$, and Taq polymerase, Stoffel fragment (Perkin–Elmer/Cetus). Either whole bacterial cells carrying DCPe degradative genes, or purified DNA obtained from these isolates or directly from soil, were used as targets. Negative controls contained no added target DNA. The cycling scheme used was 35 times 94°C (1 min), 55°C (1 min), 72°C (2 min), followed by 10 min final extension at 72°C. The PCR reaction mixture was then kept at 4°C until analysis.

Molecular analysis of PCR products and plasmids

PCR products and plasmids were analysed via gel electrophoresis and Southern blotting according to standard procedures as described in Sambrook *et al.* (1989). Plasmids were restricted with *Eco*RI, *Pst*I and *Bam*HI. A 1.15 kb *Eco*R1–*Bam*H1 fragment of *dhlA* (Janssen *et al.*, 1989), as well as the *dhlA* specific PCR product were used as molecular probes. For plasmid comparisons, whole plasmid pPC170 was used as a probe. Probes were labelled with [^{32}P]ATP using the Megaprime random primer labelling kit (Amersham). Hybridization was carried out overnight at high stringency (Sambrook *et al.*, 1989) and washes were performed at low, medium and high stringencies. Hybridization signals were scored as positive when these were clearly visible on blots, with positive controls (target DNA) also showing clear signals and negative controls (non-target DNA) showing negligible signals.

Cloning and sequence analysis of dhlA-specific PCR product

For cloning, the TA PCR cloning vector (Invitrogen, Leek, The Netherlands) was used following the instructions of the manufacturer. Sequencing via the dideoxymethod was performed by R. Pomp (Laboratory for Monoclonal Antibodies, IPO-DLO, Wageningen, The Netherlands). The sequence obtained from the sequenced PCR product was compared to the published sequence of *dhlA*, as well as to sequences in the EMBL database, accessed via the Nijmegen CAOS/CAMMSA system, using a FASTA homology search.

RESULTS

Detection of biodegradative capacity and dhlA-specific PCR products in soils

Most soils of varying characteristics, including clayey, sandy and peaty soils (Table 1), showed a pattern of accelerated degradation of DCPe when they had been repeatedly treated with DCPe [Table 2, Fig. 1(a–h)] or alternately with DCPe and MITC (exemplified in Fig. 2). The effect was in particular strong in four soils, i.e. Ubbena sand, AB Haren sand, Wieringermeer sandy clay and Kloosterburen sandy loam, and was detected to a much lesser extent in Smilde peaty soil and Odoorn AZ2 sand. The criteria applied to assess soil adaptation to DCPe (as outlined in Materials and Methods) led to the conclusion that each of these six soils adapted following repeated DCPe application, whereas accelerated degradation of DCPe was not found in the controls not treated with DCPe (Table 2). The remaining two (polder) soils, Lovinkhoeve loamy sand and Westmaas clay, also showed rapid degradation of DCPe, however this adaptation was apparently not related to previous treatment with this compound since the controls were also shown to rapidly decompose DCPe.

Using the *dhlA*-specific primers and probe on total community soil DNA extracts, a positive signal was found in five of the eight DCPe-treated soils, but also in three of eight untreated (control) soils. This result was qualitative and no further attempt was made to quantify the number of targets involved, in particular since quantitative PCR applied to soil DNA is still a difficult task.

Enrichment cultures and isolation and characterization of DCPe-degradative strains

Five soils with DCPe treatment history (Smilde peat, Odoorn sand, Kloosterburen sandy loam, Lovinkhoeve silt loam and Westmaas clay) were used to enrich bacteria with DCPe-degradative capacity. Four of these soils showed a signal with the *dhlA* detection system. The DCPe degradative capacity developing in these enrichment cultures was similar for all cultures, and is exemplified for Westmaas clay soil (Fig. 3).

Plating of dilutions of the enrichment cultures obtained from the five soils on selective medium (Loos, 1975), gave rise to numerous morphologically-different colonies. A total of 32 colonies was randomly picked from different isolation plates and purified. Following purification, approximately 50% of these (15 out of 32) were able to unequivocally degrade DCPe when GC tested in a shake culture set-up [see Fig. 4(A)]. Six Gram-negative strains were selected for further genetic work based on the presence of plasmids (see further). Their identification according to the BioLog system can be found in Table 3. An analysis of antibiotic and heavy metal resistance patterns revealed that several strains were resistant to Hg^{2+}, Nx, Km, Sm or Cm. The Hg and Km resistances were used as selectable markers to assess plasmid localization and possible (co-)transfer of the DCPe-degradative genes.

Plasmid profiles of isolated strains

All six selected DCPe-degradative strains were shown to contain at least one plasmid (Table 3), as evidenced by plasmid extraction followed by analysis of the extract by gel electrophoresis. All plasmids visualized in their CCC form were estimated to range

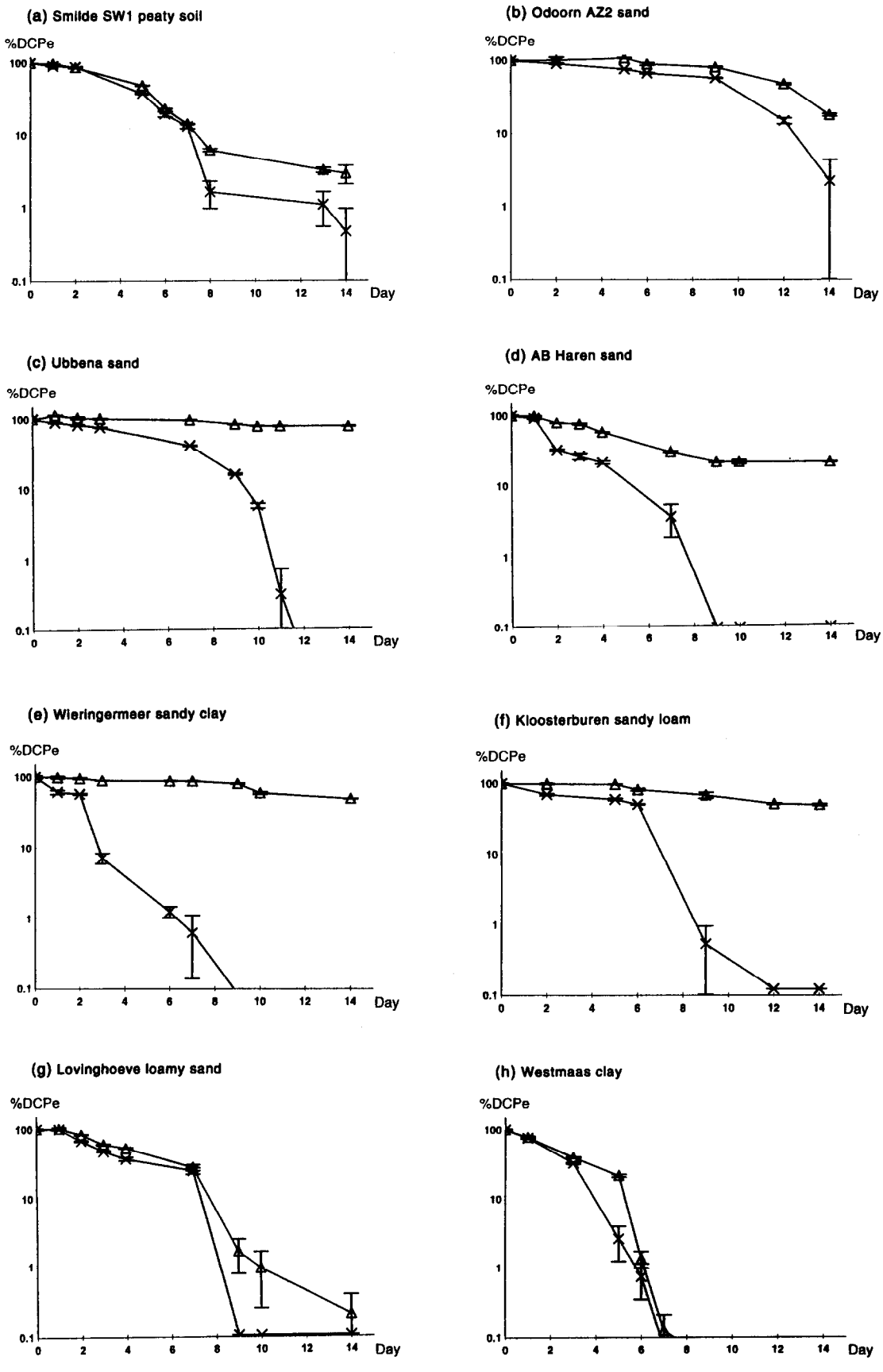

Fig. 1. Effect of repeated application of DCPe on its degradation rate in eight soils. The DCPe concentration as a percentage of that at zero time is presented (% DCPe): △, untreated; ×, six DCPe treatments given.

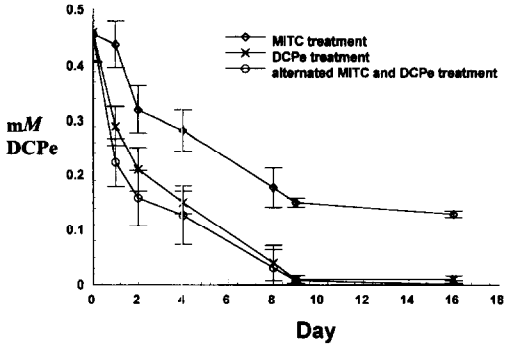

Fig. 2. Degradation of DCPe in MITC, DCPe- and alternately-treated Westmaas clay soils. Per data point, the standard deviation between replicates is indicated with vertical bars. Since it revealed extremely fast degradation, the alternately-treated soil was used for enrichment of DCPe degraders. mM DCPe = mM DCPe g^{-1} soil.

in size from about 50 to 60 kb, by comparison to standard plasmids of known sizes (not shown). To analyse their structure and relatedness, three of the transferable plasmids (see further), i.e. pPC170 (of *P. cichorii* 170), pCO172 (of *P. corrugata* 172) and pP159 (of *Pseudomonas* sp. 158), were restricted using *Eco*RI, *Pst*I and *Bam*HI, and blotted to a membrane, after which they were hybridized to plasmid pPC170 as a probe. Restriction patterns of the three plasmids were similar and about eight bands appeared on the blots of both the *Eco*RI and *Pst*I digests of pPC170, pCO172 and pP159 DNA. From the blots, the sizes of these plasmids were estimated to be about 50–55 kb. *Bam*HI digestion of the three plasmids was clearly different in that only two restriction fragments were generated.

Plasmid transfer experiments

Filter matings between the 6 DCPe-degradative strains and the *P. fluorescens* R2f recipients (both marked strains used in order to enhance the chance of selection of the transconjugants) revealed that in five cases the selectable marker used (Hg resistance)

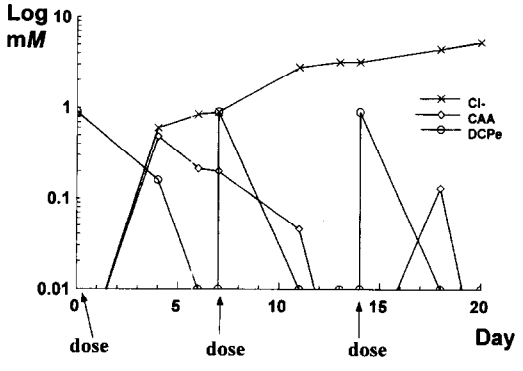

Fig. 3. Degradation of DCPe in enrichment culture (third transfer) of Westmaas clay soil. A 0.9 mM dose of DCPe was given on days 0, 7 and 14 (indicated with "dose"). Cl^- = free chlorine ion concentration; CAA = chloroallylalcohol concentration; DCPe = *cis*-1,3-dichloropropene concentration.

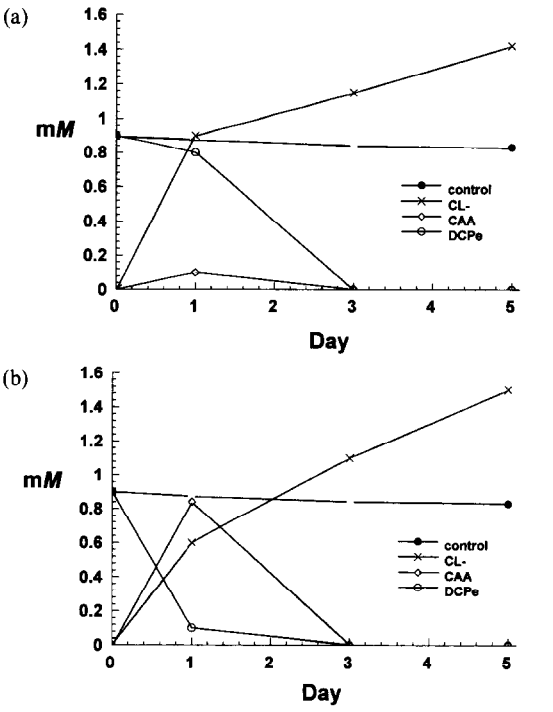

Fig. 4. Degradation of DCPe and production of CAA and free chlorine (Cl^-) in MMY medium by: (A) *P. cichorii I* strain 170 [during the experiment, the culture remained at optical density (OD_{660}) 0.24]; (B) *P. fluorescens* R2f transconjugant containing the *P. cichorii* I pPC170 plasmid (during the incubation, the OD_{660} of the culture remained at 0.28). As a control on possible adsorption effects of bacterial cells, the rate of disappearance of DCPe in the presence of a non-degrading organism, *Enterobacter cloaceae*, is shown (indicated with "control").

was transferred to the recipient, with frequencies per donor ranging from about 10^{-4} (*P. cichorii* 170 donor) to 10^{-7}–10^{-8} (*Pseudomonas* sp. 158 and *P. putida* subgroup B 159 as donors). Results are summarized in Table 3. The remaining mating, with *Alcaligenes paradoxus* 164 as the putative donor strain, did not result in detectable transfer of the Km resistance marker. Ten colonies of potentially plasmid-containing transconjugants (two colonies per successful mating) were purified by streaking on selective agar, and the resulting strains were tested for the presence of plasmids. Plasmids with sizes similar to those in the respective donor strains were found in transconjugants from three matings, with respectively *P. corrugata* 172, *P. putida* B strain 159 and *P. cichorii* I strain 170 as donors (Table 3). We did not detect plasmids in transconjugants originating from matings with *P. corrugata* strain 168 or *Pseudomonas* sp. 158 as donors.

Degradation of DCPe by transconjugant strains

Transconjugants obtained from matings with *P. cichorii* I strain 170 and *P. corrugata* strain 172 were capable of degrading DCPe, as shown by GC analysis of cultures in MMY supplemented with DCPe. A representative pattern of degradation is shown for

the *P. fluorescens* R2f transconjugant strain originating from the mating with *P. cichorii* I strain 170 [Fig. 4(B)]. The rate of DCPe degradation in this strain was at least as high as that in the culture of the plasmid donor *P. cichorii* strain 170, which suggested optimal expression of the DCPe-degradative gene in the new host. The Hg-resistant transconjugants obtained in matings of *P. fluorescens* R2f with *Pseudomonas* sp. 158, *P. putida* subgroup B strain 159 and *P. corrugata* strain 168 did not show DCPe-degradative capacity (Table 3).

PCR amplification of dhlA in DCPe-degradative donor and transconjugant strains

PCR products of about 400–500 bp were generated with all six isolated DCPe-degradative strains listed in Table 3, as well as with the *P. fluorescens* R2f transconjugants generated with *P. cichorii* 170 and *P. corrugata* 172 as donors, whereas no products were found in the plasmidless *P. fluorescens* R2f recipients. The PCR products showed strong hybridization signals with the *dhlA*-specific probe used (Fig. 5), suggesting substantial sequence homology. *DhlA*-specific products could not be generated on the transconjugants obtained with *Pseudomonas* sp 158, *P. putida* subgroup B strain 159 and *P. corrugata* strain 168. The PCR products obtained were all similar in size and in the range of the product generated with cloned *dhlA* as a target, suggesting similarity between all these products (Fig. 5).

Sequence analysis of dhlA-specific PCR amplification product generated on the P. cichorii plasmid pPC170

About 180 bases of the 3′ end of the *dhlA* fragment were sequenced, and the sequence information was compared by a FASTA homology search run on the CAOS/CAMMSA system (Nijmegen, The Netherlands) to that of *dhlA* as well as to the entire EMBL data base. Results showed high (>95%) similarity between the cloned fragment and the *dhlA* published sequence. The mismatches found included several base substitutions as well as single-base insertions, which are currently under investigation. There was no substantial homology to any other sequence of the data base, suggesting high similarity of the 180 bp sequence only to *dhlA*.

DISCUSSION

Six of the eight soils tested (Tables 1 and 2; Fig. 1) showed a pattern of accelerated degradation of DCPe following repeated treatment with DCPe or with DCPe and MITC (alternated), whereas two (clayey, polder) soils did not show a response to the conditioning treatment. This lack of response was related to the high DCPe degradation rate already present in these two soils. The occurrence of adaptation in soils resulting in accelerated degradation of pesticides is often attributed to the selection of a novel microbial population with degradative capacity (Kearney and Kellogg, 1985; Katan and Aharonson, 1989). Adaptation to DCPe has also been observed before in some of the soils tested here (Lebbink *et al.*, 1989). However, unfortunately the bacteria (under which pseudomonads) able to decompose DCPe implicated in the adaptation, have not been further characterized with respect to the genetic background and molecular mechanisms of DCPe decomposition (Lebbink *et al.*, 1989).

Repeated treatment with DCPe is possibly not the only cause for soil adaptation to DCPe to occur, since the two polder soils, Lovinkhoeve silt loam

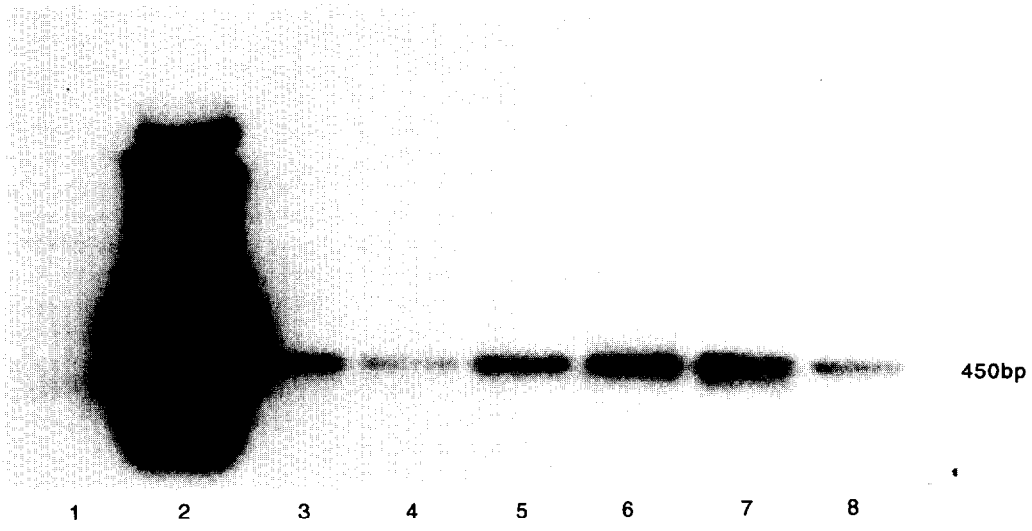

Fig. 5. Southern blot hybridization of PCR products obtained with the *dhlA* specific amplification system, with the *dhlA* probe. Lanes: **1**, negative (water) control; **2**, positive control (*dhlA* product generated on the cloned *Xanthobacter autotrophicus* GJ10 *dhlA* gene; **3**, product of *Alcaligenes paradoxus* 164; **4**, product of *P. putida* B 159; **5**, product of *P. fluorescens* R2f transconjugant obtained with *P. cichorii* 170 donor; **6**, product of *P. cichorii* strain 170; **7**, *idem* lane 3; **8**, *idem* lane 5 (different transconjugant).

and Westmaas clay (Table 2), were found to be conditioned before application of DCPe in the microplots. A possible reason for the apparent preadaptation might be that polders are former marine environments, in which algae are known to produce halogenated hydrocarbons which may select for microorganisms degrading these compounds. However, alternatively we cannot rule out the possibility that these soils have been in contact with DCPe in the past, since these soils have been used agriculturally. An important feature of these polder soils is also the relatively high pH, which is regarded as a prime factor favouring microbial degradative activity (N. Mulder, pers. commun.; Suett and Walker, 1988; Suett *et al.*, 1996). Soils with no known history of application of pesticides which naturally show accelerated degradation have been described before (Kaufman and Edwards, 1983). This naturally-occurring degradative activity has been related to cropping history, to natural soil characteristics, or to previous treatment with a structurally related compound.

It has further been shown that treatment of soil with two agents alternatedly may protect either of the two chemicals from accelerated microbial degradation (Avidov *et al.*, 1985), possibly by not allowing a large and active degrading microbial population to develop. Hence, we tested whether the alternation of the two nematocidal agents, DCPe and MITC, could affect the persistance in soil of either one or both of these fumigants. No evidence was obtained for such a mechanism (Verhagen *et al.*, 1995), but as a corollary, we obtained soils with pronounced accelerated DCPe degradation, of which five were selected for enrichment and isolation of bacteria with DCPe-degrading activity.

Although a direct pathway for DCPe degradation as described by Roberts and Stoydin (1976) is likely, DCPe can also be degraded cometabolically, with enzymes like ammonia monooxygenase (Rasche *et al.*, 1990) and alkene monooxygenase (Ensign *et al.*, 1992). Previous field observations have revealed that nitrification is strongly suppressed after fumigation with DCPe, due to loss of most of the nitrifying bacteria (Lebbink and Kolenbrander, 1974). The high DCPe concentrations in a fumigated field (0.43 mM) probably also inhibit the cometabolic pathways. We therefore surmised that direct dehalogenation via nucleophilic substitution was possibly a key mechanism, and we screened for the occurrence of the HAD encoding *dhlA* gene using a specific PCR detection system based on the active site of the enzyme. The *dhlA*-specific PCR detection applied to the soil microplot experiment showed that five out of seven soils with DCPe treatment history in these plots (one not assessed) contained *dhlA*-homologous sequences, indicating their potential for adaptation. However, three of the control soils without DCPe conditioning treatment history also revealed a *dhlA*-homologous amplification product. Hence, there was no strong correlation between adaptation to DCPe and qualitative detection of *dhlA* via PCR amplification. The qualitative results obtained with PCR have to be viewed with caution, though, since they merely indicate the presence of the target gene without providing any indication of the number of targets. PCR is known to be extremely sensitive. Using other target DNA, the soil DNA extraction and PCR amplification scheme used here has been shown to permit the detection of about 10^3 copies of target DNA g^{-1} soil (Smalla *et al.*, 1993), and this might be a rough indication of the minimal copy number of *dhlA*-like genes present in case of a positive PCR. The presence of such gene copy numbers in soils not conditioned with DCPe might suggest a small population of microorganisms carrying *dhlA*-like sequences is commonly present in these. Quantification of the *dhlA*-specific PCR applied to soil DNA extracts is clearly needed to assess the potential for enhancement of *dhlA* genes under DCPe selection. Quantification might be achieved using, for example, most probable number (MPN) PCR (Nesme *et al.*, 1995) or PCR with competitor DNA.

Since five out of six selected strains with DCPe-degradative capacity were identified as pseudomonads, this taxon can be pinpointed as a source of DCPe degradation genes in soil. This corroborated the results of Lebbink *et al.* (1989). The six strains were shown to contain at least one plasmid as well as a Hg or Km resistance determinant, and therefore genetic transfer of these selectable markers to *P. fluorescens* R2f recipient strains was used as a strategy to assess the genomic localization of the DCPe-degradative genes. In Hg-resistant transconjugants from matings with three putative donor strains, plasmids with sizes similar to those in the respective Hg-resistant donor strains were detected (Table 3), indicating that the Hg resistance marker was localized on the self-transmissible plasmids. These plasmids, pPC170, pCO172 and pP159, were closely related, as evidenced by restriction and Southern hybridization with pPC170 DNA. Transconjugants of two matings, with *P. cichorii* 170 as well as with *P. corrugata* 172 as donors, were capable of degrading DCPe, suggesting that DCPe-degradative genes were associated with plasmids pPC170 and pCO172. We did not investigate whether a possibly truncated inactive DCPe degradative gene was present on the related plasmid pP159.

The generation of *dhlA*-specific PCR products of 400–500 bp with the six selected strains (Table 3) suggested *dhlA*-homologous sequences were present in all these strains. *DhlA*-homologous sequences were also found in Hg-resistant and DCPe-degrading *P. fluorescens* R2f transconjugants containing either the *P. cichorii* I 170 plasmid pPC170 or the *P. corrugata* 172 plasmid pCO172. The absence of such sequences from the remainder of the Hg-resistant transconjugants, including the plasmid pP159 containing one,

was consistent with the absence of DCPe degradative capacity and indicates functional *dhlA* was not transferable and plasmid-associated in these strains. Furthermore, the high similarity of the sequence of the PCR amplification product generated with pPC170 to the *X. autotrophicus dhlA* gene indicated the active site of *dhlA* was present in plasmid pPC170. Together, these data suggested that functional *dhlA*-like genes present on the self-transmissible plasmids pPC170 and pCO172 were responsable for the DCPe-degradative capacity in *P. cichorii* I and *P. corrugata* 172. They were also functional in the *P. fluorescens* R2f transconjugants. The relevance of the *dhlA*-homologous PCR product in the remainder of the isolated strains for the nature and localization of their DCPe biodegradative capacity is currently uncertain, but it is possible that a chromosomal *dhlA*-like gene is involved.

The involvement of plasmids as carriers of dehalogenation genes has been found before. For instance, Slater and Bull (1982) described plasmid-located genes coding for degradation (dehalogenation) of 2-chloropropionic acid and monochloro-acetic acid in pseudomonads. Furthermore, plasmid-borne haloalkanoic acid dehalogenase genes have been found in soil-isolated *Moraxella* sp. (Kawasaki *et al.*, 1981) and in *Alcaligenes xylosoxidans* (Brokamp and Schmidt, 1991). The latter plasmid was found to be transferable between bacteria in soil, and horizontal transfer of such plasmids was invoked as a key mechanism in the rapid adaptation of soil microbial populations to the degradable compounds. The fact that in our study genes responsible for bacterial DCPe degradation were located on self-transmissible plasmids also points at a role for self-transmissible elements in spreading these genes through soil bacterial populations, thereby conferring enhanced adaptability to these.

Since it is our aim to develop a rapid detection method indicative of enhanced degradation of DCPe in field soils, we will pursue *dhlA* quantification via PCR as a means to characterize the adaptation process. However, it is clear that more research in this area is needed, in particular since other DCPe decomposition mechanisms may also play a role in soil microbial communities.

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