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Degradation of 1,3-dichloropropene by a soil bacterial consortium and *Rhodococcus* sp. AS2C isolated from the consortium

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Abstract

A bacterial consortium capable of degrading the fumigant 1,3-D ((Z)- and (E)-1,3-dichloropropene) was enriched from an enhanced soil. This mixed culture degraded (Z)- and (E)-1,3-D only in the presence of a suitable biodegradable organic substrate, such as tryptone, tryptophan, or alanine. After 8 months of subculturing at 2- to 3-week intervals, a strain of Rhodococcus sp. (AS2C) that was capable of degrading 1,3-D cometabolically in the presence of a suitable second substrate was isolated. (Z)-3-chloroallyl alcohol (3-CAA) and (Z)-3-chloroacrylic acid (3-CAAC), and (E)-3-CAA and (E)-3-CAAC were the metabolites of (Z)- and (E)-1,3-D, respectively. (E)-1,3-D was degraded faster than (Z)-1,3-D by the strain AS2C and the consortium. AS2C also degraded (E)-3-CAA faster than (Z)-3-CAA. Isomerization of (E)-1,3-D to (Z)-1,3-D or the (Z) form to the (E) form did not occur.

Abbreviations: 1,3-D = 1,3-dichloropropene; 3-CAA = 3-chloroallyl alcohol; 3-CAAC = 3-chloroacrylic acid; GC = gas chromatography.

Introduction

1,3-Dichloropropene (1,3-D) is the main active ingredient of the commercial fumigants Telone II (94% 1,3-D), Telone C17 (83% 1,3-D and 17% chloropicrin), and Telone C35 (65% 1,3-D and 35% chloropicrin), and consists of two isomers, (Z)- and (E)-1,3-D in near equal ratio. This chemical is slightly more volatile than water, with vapor pressures of 34.3 and 23.0 mm Hg at 25 °C for (Z) and (E) isomers, respectively (DowElanco 1996), and is fairly water soluble, 2.18 g l^{-1} for cis isomer and 2.32 g l^{-1} for trans isomer. Telone C17 and Telone C35 are considered to be likely alternatives to the fumigant methyl bromide in the short term (Holman 1999). Use of methyl bromide in agriculture in the USA will be phased out in 2005.

It has been known since 1989 that repeated field treatments of 1,3-D may result in enhanced degradation of the chemical in the soils (Chung et al. 1999; Lebbink et al. 1989; Ou et al. 1995; Smelt et al. 1989; 1996; Verhagen et al. 1995; 1996). The majority of these studies did not determine (Z)- and (E)-1,3-D individually. Ou et al. (1995) found that (E)-1,3-D in an enhanced soil in Florida was degraded faster than (Z)-1,3-D. Microorganisms are responsible for enhanced degradation of pesticides in soils (Racke & Coats 1990).

Lebbink et al. (1989) isolated a strain of fluorescent *Pseudomonas* sp. from an enhanced soil that they reported had the capacity to utilize 1,3-D as a sole source of carbon for growth. This organism was maintained in a medium containing a number of organic substrates including glucose, meat extract, yeast extract, and soil extract. Fifteen bacterial isolates from five enhanced soils, when grown in a mineral medium containing 10 mg l^{-1} yeast extract, were found to have the capacity to degrade 1,3-D (Verhagen et al. 1995) and six Gram-negative strains each harbored a plasmid that carried a *dhlA* (haloalkane dehalogenase)-like

gene. The gene was suspected to be involved in 1,3-D degradation. All the isolates but one were *Pseudomonas* species. One isolate, *Pseudomonas pavonaceae* 170 (previously known as *Pseudomonas cichorii* 170), was found to have the capacity to utilize 3-chloroallyl alcohol (3-CAA) and 3-chloroacrylic acid (3-CAAC), the first two degradation products of 1,3-D, for growth (Poelarends et al. 1998). This organism produced at least three different dehalogenases: a hydrolytic 1,3-D dehalogenase that hydrolyses (Z)- and (E)-1,3-D to respective (Z)- and (E)-3-CAA, and two 3-CAAC dehalogenases, one specific for (Z)-3-CAAC and the other specific for (E)-3-CAAC.

In this study, we report enrichment of a mixed bacterial culture capable of cometabolically degrading (Z)- and (E)-1,3-D from an 1,3-D enhanced soil in Florida, and isolation of an axenic culture (*Rhodococcus* sp. strain AS2C) from the mixed culture. Degradation rates of (Z)- and (E)-1,3-D, and (Z)- and (E)-3-CAA by AS2C were determined. In addition, formation of (Z)- and (E)-CAA, and (Z)- and (E)-3-CAAC during degradation of (Z)- and (E)- 1,3-D in some experiments were also determined.

Materials and methods

Soil

A soil sample (0–15 cm depth) was collected in 1997 from an experimental site located 15 km northwest of the University of Florida campus in Gainesville, Florida. A part of this site, where the soil sample was collected, had been treated with 1,3-D annually between 1994 and 1997, and six times between 1982 and 1993. Since 1994, this soil had exhibited enhanced degradation toward (Z)- and (E)- 1,3-D (Chung et al. 1999; Ou et al. 1995). The soil sample was placed in a plastic bag and stored in the dark at 4 °C.

Chemicals and culture media

Analytical grade (Z)- and (E)- 1,3-D, and analytical grade (Z)- and (E)-3-CAA were gifts from Dow AgroSciences (Indianapolis, IN). Analytical grade (Z)- and (E)-3-CAAC were purchased from Aldrich (Milwaukee, WI). All other chemicals and organic solvents were either analytical grade or GC grade, and obtained from Fisher Scientific (Orlando, FL) or Aldrich.

A basal mineral medium previously used for enrichment and isolation of fenamiphos-degrading bacteria from soil (Ou & Thomas 1994) was used for this study. Trace minerals were supplemented to the medium (Ou & Thomas 1994). In addition, soil extract and soil leachate were used for initial screening of mixed bacterial cultures enriched from the enhanced soil to determine their capacity to degrade (Z)- and (E)-1,3-D. Soil extract was prepared by autoclaving equal weights of soil and deionized water as described previously (Ou 1991). Luria-Bertani (LB) agar plates were used for screening and purification of individual isolates from mixed bacterial cultures.

Enrichment and isolation of 1,3-D-degrading bacteria

For isolation of bacteria capable of utilizing 1,3-D as a sole source of carbon for growth, 1 g of the enhanced soil was added to a 250-ml glass Erlenmeyer flask with a Teflon lined screw cap containing 50 ml of basal mineral medium supplemented with trace minerals, and 50 mg l^{-1} of 1,3-D (equal ratio of analytical grade (Z)- and (E)-1,3-D). Control flasks contained no soil or autoclaved soil. To prevent leakage, the top of the flasks were wrapped with Teflon tape and after addition of 1,3-D, the flasks were immediately closed tightly with caps. The flasks were incubated in a rotary shaker at 28 °C. At two-week intervals, 5 ml of the suspension was transferred to fresh basal mineral medium. After four 1-to-10 transfers, the culture medium was essentially free of soil particles. Turbidity developed in the medium was considered to be microbial growth, a sign of successful enrichment of bacteria capable of utilizing 1,3-D for growth.

Several approaches were used to enrich bacteria capable of cometablizing 1,3-D from the enhanced soil. They included: (1) a small amount of the enhanced soil (0.01–0.05 g) was inoculated into soil extract; (2) a small amount of the enhanced soil (0.01–0.05 g) was inoculated into soil leachate; (3) during a series of 1-to-10 transfers of enhanced soil-basal mineral medium, a second biodegradable organic substrate was added; (4) during a series of 1-to-10 transfers of enhanced soil-basal mineral medium, autoclaved enhanced soil, or soil leachate, or a combination of the two was added. If 1,3-D was degraded microbially in some of the culture media, a small amount of the culture fluids was streaked on a LB agar plate. Individual colonies were tested for their capacity to degrade 1,3-D.

Degradation studies

Degradation of (Z)- and (E)-1,3-D by bacterial isolates was carried out in 250-ml glass Erlenmeyer flasks with Teflon-lined caps containing basal mineral medium (50 ml), 1,3-D, and a suitable biodegradable organic substrate (100 mg l^{-1}), usually DL-alanine or L-tryptone. The top of the flasks was wrapped with Teflon tape. To reduce volatilization, 1,3-D was added last and the flasks were immediately closed tightly with the caps. To avoid photodegradation, the flasks were wrapped with aluminum foil. Control flasks contained no live cells. The flasks were incubated in a rotary shaker at 28 °C. At appropriate time intervals, 0.5 ml of culture fluids was transferred to 28-ml glass tubes that had been wrapped with Teflon tape on the top of the tubes and immediately closed with Teflon-lined caps. Prior to carrying out solvent extraction, the tubes were kept in a −30 °C freezer for 1 hour.

Degradation of (Z)- and (E)-3-CAA and other chlorinated short-chained aliphatic hydrocarbons by a 1,3-D-degrading isolate (*Rhodococcus* sp. strain AS2C) was carried out in a similar manner as described above.

Extraction and GC analysis

To minimize volatilization loss, cold organic solvents (acetone, ethyl acetate, and hexane) were used for extraction. For extraction of (Z)- and (E)-1,3-D from culture fluids, 0.5 ml of subsamples in glass extraction tubes was extracted with 10 ml hexane by shaking on a reciprocal shaker at 500 strokes per min for 5 min. One ml of the hexane was transferred to GC vials and the vials were immediately capped. For extraction of (Z)- and (E)-3-CAA from culture fluids, 0.5 ml of subsamples in extraction tubes was extracted with 10 ml ethyl acetate by shaking on a reciprocal shaker for 5 min. One ml of the ethyl acetate was transferred to GC vials. Extractions were duplicated or triplicated.

1,3-Dichloropropene ((Z) and (E) isomers) and 3-CAA ((Z) and (E) isomers) were determined separately using a Perkin–Elmer Autosystem GC under the same conditions, with the exception of injection volumes. The GC was equipped with an autosampler, ^{63}Ni electron capture detector, split-splitless injector, Turbochrom software, and a 486 computer. The GC parameters and operational conditions were: column, 30 m × 0.25 mm, RTX-624 coated with 1.4 μm film; flow rates for carrier gas (He) and make-up gas (95% N_2 and 5% CH4), 0.5 ml min^{-1} and 30 ml min^{-1}, respectively; injection volumes, 1 μl for 1,3-D and 5 μl for 3-CAA; splitless, then split on after 1.5 min; injection temperature, 200 °C; detector temperature, 375 °C; and oven temperature, 50 °C for the first min, ramp at 40 °C min^{-1} to 120 °C, and held for 9 min. Under these conditions, the retention times (min) for (Z)- and (E)-1,3-D and (Z)- and (E)-3-CAA were 4.00, 4.37, 4.76, and 5.34, respectively.

For extraction of (Z)- and (E)-3-CAAC in culture fluids, 10 ml of subsamples was placed in glass extraction tubes, 5 ml of ethyl acetate-acetone (1:3) and 5 g of NaCl were added to the tubes. After pH of the samples was adjusted to 4.19 ± 0.05 with concentrated acetic acid, the tubes were shaken on a reciprocal shaker for 10 min. The solutions were transferred to glass separatory funnels. Each tube was rinsed 3 times with 1 ml of ethyl acetate-acetone (1:3), and the rinses were added to the funnel. The funnel was shaken by hand for 1 min, and then layers were allowed to separate. After discarding precipitate and the lower layer, the upper layer was transferred to a glass tube. The funnel was rinsed 3 times with 1 ml isooctane and the rinses were added to the tube. The solution in the tube was evaporated to <1 ml under a gentle stream of N_2. Isooctane was added the tube to make a volume of 2 ml. Approximately 0.1 g of anhydrous sodium sulfate was added to the solution to remove moisture. One ml of the solution was then transferred to a GC vial and then 10 μl of the devitalization agent chloromethyl-dimethylchlorosilane was added.

The Perkin–Elmer Autosystem GC was used for determination of (Z)- and (E)-CAAC. The GC parameters and operational conditions were: column, 60 m × 0.32 mm, RTX-1 coated with 5 μm film; flow rates for carrier gas (He) and make-up gas (95% N2 and 5% CH_4), 0.5 ml min^{-1} and 30 ml min^{-1}, respectively; injection volume, 2 μl; splitless, then split on after 1 min; injection temperature, 230 °C; detector temperature, 375 °C; and oven temperature, 45 °C for the first min, ramp at 10 °C min^{-1} to 220 °C, and hold for 30 min. Under these conditions, the retention times (min) for (Z)- and (E)-3-CAAC were 29.55 and 27.84, respectively.

16S ribosomal DNA sequencing

Genomic DNA from the strain AS2C was isolated by cetyltrimethylammonium bromide and isopropanol (Ausubel et al. 1996). The 16S ribosomal DNA (rDNA) was amplified by PCR using universal bacterial primers 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492r

(5′-TACGGYTACCTTGGTTACGACTT-3′) (Lane 1991). The PCR product was ligated into a TOPO TA cloning vector following the vendor's instructions (Invitrogen, San Diego, CA). The vector was transformed into *Escherichia coli* TOP10F′. Positive recombinants were verified using LB agar plates containing 50 μg ml^{-1} ampicillin. Plasmid DNA from a positive recombinant was purified by Wizard^{TM} Minipreps DNA Purification System (Promega, Madison, WI) for direct DNA sequencing. DNA sequencing was carried out by the ICBR (Interdisciplinary Center for Biotechnology Research) core sequencing facility at the University of Florida.

Results

(Z)- and (E)-1,3-D in aqueous media are subject to chemical hydrolysis (McCall 1987). The hydrolysis rate depends on temperature, but is independent of pH at each temperature. Chemical hydrolysis of 1,3-D is fairly rapid, and at 30 °C, half-life value was 3.1 days (McCall 1987). Therefore, in order to differentiate microbial degradation from chemical degradation, controls (without soil or live microorganisms) were accompanied with degradation experiments involving soil or live microorganisms.

Enrichment and screening

1,3-D ((Z)- and (E)-1,3-D, 24 mg l^{-1} each) was microbially degraded when applied to the basal mineral medium containing the enhanced soil at the amount of 0.1 kg l^{-1} (Table 1). After the second or third 1-to-10 transfer, (Z)- and (E)-1,3-D were not microbially degraded. If a small amount of the enhanced soil (0.02 kg l^{-1}) was added to the third transfer medium, microbial degradation resumed (Table 1). However, microbial degradation did not occur when autoclaved soil was added. Furthermore, when a small volume of the medium (0.05 l l^{-1}) from the third transfer was inoculated into soil extract, LB broth, or basal mineral medium containing glucose or yeast extract, good bacterial growth was observed, but microbial degradation of 1,3-D was absent (Table 1). This indicated that bacteria were present, but either 1,3-D degraders were not present or heat labile chemicals associated with the soil, which were responsible for inducing enzymes for 1,3-D degradation were destroyed by autoclaving. To test the last hypothesis, soil leachate (sterilized by filtration) was supplemented to the third transfer medium either containing autoclaved soil at an amount of 0.1 l l^{-1} or without autoclaved soil, and it was found that 1,3-D was microbially degraded. This indicated that 1,3-D degraders were present even after the third 1-to-10 transfer, and they required a heat labile component of the soil to have the capacity to degrade 1,3-D.

Although the bacterial consortium enriched from the enhanced soil when grown in LB broth did not microbially degrade 1,3-D, the consortium when grown in the basal mineral medium containing a small amount of LB broth (0.005–0.01 l l^{-1}) had the capacity to microbially degrade 1,3-D (Table 1). LB broth consists of two organic components, yeast extract and tryptone. Yeast extract had been previously shown not to stimulate microbial degradation of 1,3-D, so tryptone was the only likely candidate for testing. Tryptone (50–400 mg l^{-1}) was found to have the capacity to stimulate the microbial degradation (Table 1). Tryptone is rich in L-tryptophan, an aromatic substituted amino acid (Anonymous 1953). This amino acid at high temperatures, such as autoclaving (121 °C), will be hydrolyzed to indole and alanine. DL-alanine was found to have the capacity to stimulate the microbial degradation as well, but not indole (Table 1).

Degradation of 1,3-D by the mixed bacterial culture

When the complex organic co-substrate LB broth (10 ml l^{-1}), tryptone (100 mg l^{-1}), L-tryptophan (100 mg l^{-1}), or DL-alanine (100 mg l^{-1}) was supplemented to the basal mineral medium, trans-1,3-D (48 mg l^{-1}) was microbially degraded (Figure 1). Typically, rapid microbial degradation occurred after 1 day of incubation. Repeated applications of (Z)- and (E)-1,3-D to the mixed culture resulted in an increase in degradation rate. For example, in the presence of DL-alanine, (E)-1,3-D was completely degraded in 2 and 1 day after the second and third treatment, respectively.

Screening and evolvement of an axenic culture capable of degrading 1,3-D

The mixed culture was maintained in DL-alanine-basal mineral medium supplemented with 48 mg l^{-1} of 1,3-D (equal mixture of (Z)- and (E)-1,3-D). Every 2–3 weeks, the mixed culture was transferred to fresh medium, and at the same time, a small amount of the liquid culture was streaked on LB agar plates. Biomass from different colonies was inoculated into alanine-basal mineral medium containing (E)-1,3-D or

Table 1. Occurrence of microbial degradation of 1,3-D in basal mineral medium containing live enhanced soil, autoclaved soil, and various organic substrates as well as by a bacterial consortium enriched from the enhanced soil grown in basal mineral medium supplemented with various organic substrates

Basal mineral medium (BMM) + enhanced soil (0.1 kg l^{-1})	+[a]
1st 1-to-10 transfer	+
2nd 1-to-10 transfer	±[b]
3rd 1-to-10 transfer	−[c]
3rd 1-to-10 transfer + enhanced soil (0.02 kg l^{-1})	+
3rd 1-to-10 transfer + autoclaved soil (0.02 kg l^{-1})	−
3rd 1-to-10 transfer + autoclaved soil (0.02 kg l^{-1}) + enhanced soil (0.02 kg l^{-1})	+
3rd 1-to-10 transfer + autoclaved soil (0.02 kg l^{-1}) + soil leachate (0.1 l l^{-1})	+
3rd 1-to-10 transfer + glucose (200 mg l^{-1})	−
3rd 1-to-10 transfer + yeast extract (200 mg l^{-1})	−
3rd 1-to-10 transfer + soil leachate (200 mg l^{-1})	+
Soil extract + 3rd 1-to-10 transfer (0.05 l l^{-1})	−
LB broth + 3rd 1-to-10 transfer (0.05 l l^{-1})	−
BMM + LB broth (0.005–0.01 l l^{-1}) + culture grown in BMM + soil leachate (0.05 l l^{-1})	+
BMM + tryptone (50–400 mg l^{-1}) + culture grown in BMM + LB broth (0.05 l l^{-1})	+
BMM + L-tryptophan (50–400 mg l^{-1}) + culture grown in BMM + tryptone (100 mg l^{-1})	+
BMM + DL-alanine (50–400 mg l^{-1}) + culture grown in BMM + L-tryptophan (100 mg l^{-1})	+
BMM + indole (100 mg l^{-1}) + culture grown in BMM + L-tryptophan (100 mg l^{-1})	−

[a] Microbial degradation was observed.
[b] Microbial degradation may or may not be observed.
[c] Microbial degradation was not observed.

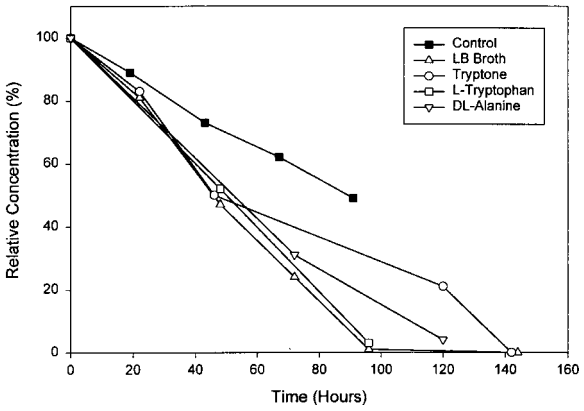

Figure 1. Degradation of (E)-1,3-D by the bacterial consortium grown in basal mineral medium containing LB broth, tryptone, L-tryptophan, or DL-alanine.

the mixture of (Z)- and (E)-1,3-D and checked for their capacity to degrade 1,3-D.

During the first 8 months of screening, no individual bacteria developed on LB plates had the capacity to degrade 1,3-D. Degradation was the same as chemical hydrolysis in the control medium. However, after 8 months, a type of colonies, which were not observed before appeared on LB plates. These colonies grew slowly, and they were small and light brownish color. This axenic culture (designated strain AS2C), when grown in the basal mineral medium containing DL-alanine, was found to have the capacity to degrade (Z)- and (E)-1,3-D.

Strain AS2C is Gram-positive, aerobic, nonmotile, small short rods or near cocci, and often club-shaped or v-shaped. According to the analysis of the 16S rDNA of AS2C, this organism belongs to the genus *Rhodococcus*.

Degradation of 1,3-D by AS2C

Alanine was not the only simple organic substrate that could serve as a substrate for strain AS2C to degrade 1,3-D. A number of other simple organic compounds including amino acids, short-chained aliphatic acids, and short-chained alcohols also had the capacity to stimulate 1,3-D degradation by this organism. They were L-tryptophan, L-aspartic acid, acetic acid, propionic acid, pyruvic acid, ethanol, propanol, and 3-CAA. AS2C could not degrade the two short chained-chlorinated aliphatic hydrocarbons, trichloroethylene and perchloroethylene, which are common groundwater contaminants. Nor could it cometabolically degrade these compounds in the presence of DL-alanine and 1,3-D. However, in the presence of DL-alanine and 1,3-D, AS2C had some capacity to

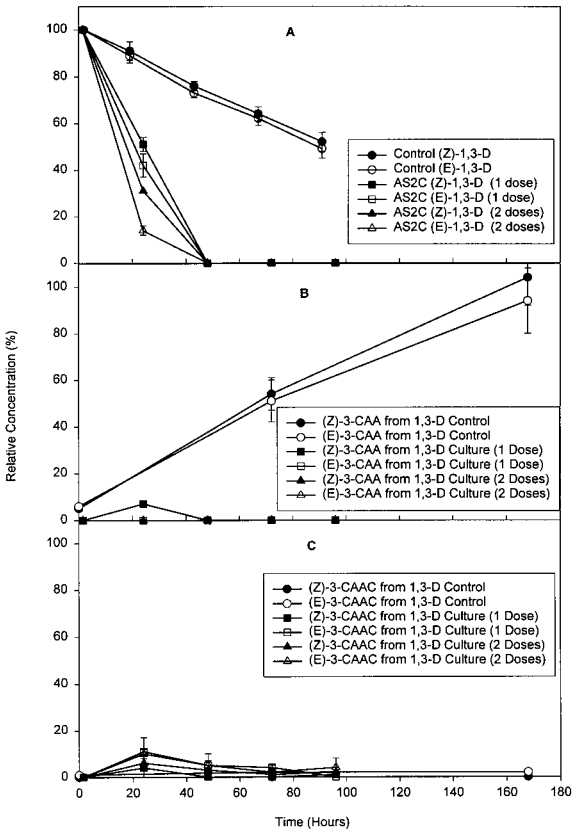

Figure 2. Degradation of (Z)- and (E)-1,3-D (A) and formation of metabolites, (Z)- and (E)-3- CAA (B) and (Z)- and (E)-3-CAAC (C), by the strain AS2C grown in basal mineral medium containing (Z)- and (E)-1,3-D (48 mg l^{-1} each) and DL-alanine (100 mg l^{-1}). One week after (Z)- and (E)-1,3-D were added to the culture medium, a second dose of 1,3-D (40 mg l^{-1}) was added to the medium.

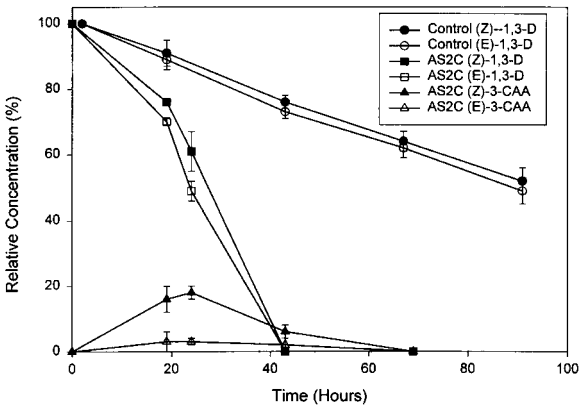

Figure 3. Degradation of (Z)- and (E)-1,3-D and formation of metabolites, (Z)- and (E)-3-CAA, by the strain AS2C grown in basal mineral medium containing (Z)- and (E)-1,3-D (24 mg l^{-1} each) and DL-alanine (100 mg l^{-1}).

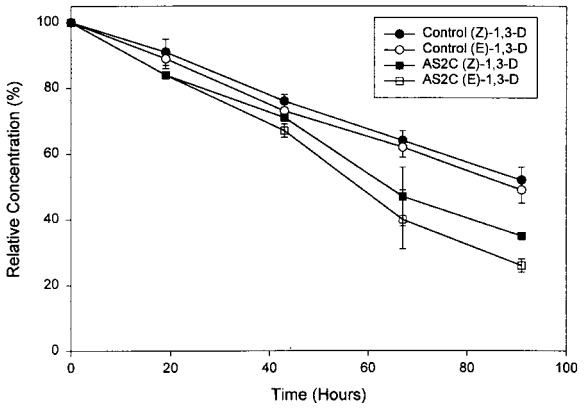

Figure 4. Degradation of (Z)- and (E)-1,3-D by the strain AS2C grown in basal mineral medium containing (Z)- and (E)-1,3-D (60 mg l^{-1} each) and DL-alanine (100 mg l^{-1}).

degrade 1,2-dichloropropane, a minor component of a commercial product of 1,3-D (Telone II).

We found that degradation rates of (Z)- and (E)-1,3-D in the alanine (and other co- substrates)-basal mineral medium containing no live AS2C (controls) were the same, and were independent of their concentrations, ranging from 24 to 72 mg l^{-1} for each isomer. Therefore, degradation curves based on average values were used to represent the chemical degradation (hydrolysis) of (Z)- and (E)-1,3-D with time in control media. The same two curves were attached to Figures 2, 3 and 4.

In the presence of DL-alanine (100 mg l^{-1}), (Z)- and (E)-1,3-D at a concentration of 48 mg l^{-1} each were rapidly degraded by AS2C (Figure 2A) and were not detected after 48 hours. Degradation of (E)-1,3-D was faster than (Z)-1,3-D. A small amount of (Z)-3-CAA appeared briefly (Figure 2B). (E)-3-CAA was not detected during the entire incubation period. (Z)- and (E)-3-CAA in the control medium were steadily formed during the entire incubation period, and after 7 days, nearly all (Z)- and (E)-1,3-D were chemically hydrolyzed to (Z)- and (E)-3- CAA, respectively. Small amounts of (Z)- and (E)-3-CAAC were formed, and after 24 hours they slowly declined (Figure 2C), with the concentration of (E)-3-CAAC being slightly larger than (Z)-3-CAAC. Reapplication of 1,3-D to the culture fluids resulted in more rapid degradation of the two isomers than the first application (Figure 2A), with (E)- 1,3-D still being more rapidly degraded than (Z)-1,3-D. Neither (Z)-3-CAA nor (E)-3- CAA was detected during the entire incubation period (Figure 2B). However, small amounts of (Z)- and (E)-3-CAAC were briefly detected (Figure 2C), but rapidly disappeared. Similar degradation patterns were observed for

(Z)- and (E)-1,3-D at a lower concentration (24 mg l^{-1} each) (Figure 3). Except that, in addition to (Z)-3-CAA, (E)-3-CAA in trace amounts were detected and the two alcohols persisted to near the end of incubation period.

At the concentration of 60 mg l^{-1} each, degradation of (Z)- and (E)-1,3-D was retarded and somewhat faster than chemical hydrolysis (Figure 4). At this concentration, growth of AS2C was not inhibited, however. At the concentration of 72 mg l^{-1} each, even though growth of AS2C was not inhibited, degradation for the two isomers was the same as chemical hydrolysis (data not shown). It appears that the concentration threshold for occurrence of microbial degradation was between 120 and 144 mg l^{-1} of 1,3-D in equal ratio of the two isomers. (Z)- and (E)-3-CAA were not determined.

Isomerization of cis-1,3-D or trans-1,3-D

No (Z)-1,3-D was found in AS2C culture media containing only (E)-1,3-D at 48 or 96 mg l^{-1}, and the (E) isomer was not detected when only the (Z) isomer was applied to AS2C media. This indicated that isomerization of the (E) isomer to the (Z) isomer or the (Z) isomer to the (E) isomer did not occur.

Degradation of 3-CAA by AS2C

The strain AS2C utilized 3-CAA (40 mg l^{-1} for each isomer) for growth and degraded the two isomers without the supplement of a second organic substrate such as alanine. Similar to (Z)- and (E)-1,3-D, AS2C degraded (E)-3-CAA faster than (Z)-3-CAA (Figure 5). There was a lag phase period of about 2 days for (Z)-3-CAA and then the alcohol was exponentially degraded. Biphasic degradation was observed for (E)-3-CAA. The isomer was initially slowly degraded, followed by rapid degradation. (Z)- and (E)-3-CAA were stable in control medium during the entire incubation period (Figure 5).

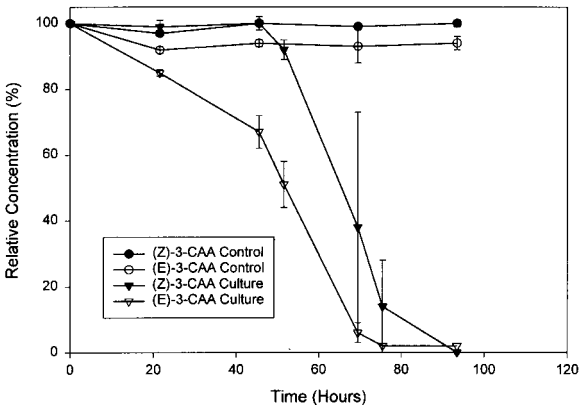

Figure 5. Degradation of (Z)- and (E)-3-CAA by the strain AS2C grown in basal mineral medium containing (Z)- and (E)-3-CAA (40 mg l^{-1}).

Discussion

Although microbial degradation, in addition to chemical hydrolysis, was involved in the enhanced degradation of 1,3-D in the 1,3-D treated soil (Chung et al. 1999; Ou et al. 1995), isolation of bacteria from the soil responsible for the degradation turned out to be a difficult task. Since we discovered in 1995 that enhanced degradation of (Z)- and (E)-1,3-D occurred in soil from the site that had a history of repeated applications of 1,3-D (Ou et al. 1995), repeated efforts to isolate axenic bacterial cultures from soil samples collected from the site failed (Ou, unpublished results). Thus, we came to the conclusion that cometabolism was likely involved in the degradation of (Z)- and (E)-1,3-D.

Free, short-chained aliphatic acids, such as formic acid, acetic acid, propionic acid, butyric acid and oxalic acid, and free amino acids are present in soils (Alexander 1977; Stevenson 1982). Only small amounts of amino acids, <2 mg kg^{-1} soil, are in free form. The majority of amino acids as well as alcohols are bound to soil humus. Therefore, it was likely that short-chained aliphatic acids, such as acetic acid and propionic acid, served as substrates for the degradation of 1,3-D by 1,3-D-degrading microorganisms in the enhanced soil. Although these aliphatic acids are thermally stable in water, during sterilization of soil by autoclaving, the aliphatic acids may be lost from soil by volatilization and, in addition, some of them may be bound to humus components to form bound residues. Free amino acids may be bound to soil humus during autoclaving as well. As a result, after autoclaving the concentrations of free aliphatic acids and amino acids in soil may be reduced to negligible amounts. The fact that replenishment of volatile and/or heat-sensitive chemicals, by adding soil leachate to autoclaved soil, resulted in occurrence of 1,3-D degradation confirmed that some volatile and/or heat-sensitive chemicals were required for the degradation by the mixed culture.

The strain AS2C was isolated from the mixed bacterial culture after 8 months of repeated subculturing.

An atrazine degrader, *Pseudomonas* sp. ADP, was isolated from a mixed bacterial culture capable of mineralizing atrazine after an extended period of subculturing (Mandelbaum et al. 1993; 1995). They attributed interspecies genetic transfer among the strains of the mixed culture that resulted in transferring all the genes responsible for mineralization of atrazine to one strain (ADP). A strain of *Pseudomonas* sp. isolated from soil utilized 3-CAA as a sole source of carbon for growth (Belser & Castro 1971) and a Gram positive coryneform bacterium isolated from a fresh water sediment utilized 3-CAA (van Hylckama Vlieg & Jansen 1992). If a strain of our mixed culture has the capacity to degrade (Z)- and (E)-3-CAA or (Z)- and (E)-CAAC, this strain needs to recruit dehalogenase (hydrolase) gene(s) or dehalogenase gene(s) and oxygenase genes to have the capacity to mineralize (Z)- and (E)- 1,3-D. Poelarends et al. (2000) suggested that bacteria acquire the capacity to degrade simple halogenated alkanes and alkenes via horizontal gene transfer. Therefore, it was possible that horizontal transfer of 1,3-D hydrolytic dehalogenase gene(s) may be the mechanism for strain AS2C, if AS2C was a (Z)- and (E)-3-CAA degrader, to acquire its capacity to degrade (Z)- and (E)-1,3-D.

The strain AS2C catabolized (Z)- and (E)-1,3-D to (Z)- and (E)-3-CAA, respectively, via hydrolysis, where the hydrolytic dehalogenase(s) had a higher activity toward the (E) isomer, resulting in more rapid degradation of (E)-1,3-D. Isomerization did not occur for either isomer and thus did not contribute to more rapid degradation of (E)-1,3-D. At present, it is not known whether one enzyme was responsible for hydrolysis of the two isomers, or two enzymes, one for each isomer. The Gram-negative 1,3-D degrader (*P. pavonaceae* 170) produced one hydrolytic dehalogenase responsible for the hydrolysis of the two isomers. The hydrolase gene was found to be likely plasmid borne (Verhagen et al. 1995), and the gene has been cloned (Poelarends et al. 1998). Our study is the first to demonstrate that (Z)- and (E)-3-CAAC were the degradation products of (Z)- and (E)-1,3-D by a microbial culture (in this case, AS2C). Our finding confirm the proposed pathway of 1,3-D degradation by Poelarends et al. (1998) that 1,3-D was first hydrolyzed to 3-CAA and then oxidized to 3-CAAC. Two strains of soil bacteria, a Gram-negative *Pseudomonas cepacia* and a Gram-positive coryneform bacterium, were found to have the capacity to utilize 3-CAAC as a sole source of carbon for growth (Hartmans et al. 1991). The Gram-negative organism utilized only the (Z) isomer, whereas the Gram-positive organism utilized both isomers. A Gram-positive coryneform bacterium, a 3-CAAC degrader, that was isolated from a fresh water sediment produced two dehalogenases, one specific for dechlorination of (Z)-3-CAAC and the other for the (E) isomer (van Hylckama Vlieg & Janssen 1992). Whether or not AS2C produces two dehalogenases for dechlorination of (Z)- and (E)-3-CAAC remains to be studied.

The strain AS2C and the mixed culture from which strain AS2C was isolated consistently degraded (E)-1,3-D faster than (Z)-1,3-D. The enhanced soil where the mixed culture was enriched also degraded the (E) isomer faster than the (Z) isomer (Chung et al. 1999; Ou et al. 1995). Thus, it was likely that bacteria in the mixed culture that were involved in degradation of (Z)- and (E)-1,3-D were also involved in the degradation of the two isomers in the enhanced soil. Microbial degradation contributed little to degradation of the two isomers in nonenhanced soil; chemical degradation was the main factor (Chung et al. 1999). AS2C also degraded (E)-3-CAA faster than (Z)-3-CAA, which had also occurred in the enhanced soil (Ou et al. 1995). In contrast, the Gram-negative *P. pavonaceae* 170 degraded (Z)-1,3-D faster than (E)-1,3-D. The 1,3-D dehalogenase produced by the organism had a specific activity 2 times larger toward (Z)-1,3-D than toward (E)-1,3-D (Poelarends et al. 1998). The strain 170 was isolated from an 1,3-D enhanced soil in the Netherlands (Verhagen et al. 1995), and individual degradation rates of the two isomers in the soil were not determined, however. Whether or not it is a general rule that Gram-positive organisms degrade (E)-1,3-D faster than (Z)-1,3-D and vice versa for Gram-negative organisms needs to be investigated.

Conclusions

A bacterial consortium enriched from a Florida sandy soil that exhibited enhanced degradation toward (Z)- and (E)-1,3-D, when in the presence of a suitable second organic substrate, differentially degraded (Z)- and (E)-1,3-D. An axenic bacterial culture capable of degrading (Z)- and (E)-1,3-D was isolated from the consortium after a long period of continuous subculturing. This axenic culture (*Rhodococcus* sp. AS2C) cometabolically degraded (Z)- and (E)-1,3-D in the presence of a suitable organic substrate, with the (E) isomer being degraded faster. More rapid degradation of (E)-1,3-D was not due to isomerization of the (E)

isomer to the (Z) isomer, rather was likely due to higher enzyme activity toward (E)-1,3-D. Initial appearance and subsequent disappearance of 3-CAA and 3-CAAC suggest that (Z)- and (E)-1,3-D are completely degraded by AS2C to CO_2, H_2O, and Cl^-. Since strain AS2C has a good capacity to degrade 1,3-D, this organism may be used to reduce or eliminate 1,3-D residues in soil after fumigation is completed to alleviate volatilization into the atmosphere and leaching into the groundwater.

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References

Alexander M (1977) Introduction to Soil Microbiology, 2nd Edition. John Wiley & Sons, New York, NY

Anonymous (1953) Difco Manual, 9th Edition. Difco, Detroit, MI

Ausubel F, Brent MR, Kingston, RE, Moore DD, Seidman JG, Smith JA & Struhl K (1996) Current Protocols in Molecular Biology. John Wiley & Sons, New York, NY

Belser NO & Castro CE (1971) Biodehalogenation – The metabolism of the nematocides *cis*- and *trans*-chloroallyl alcohol by a bacterium isolated from soil. J. Agric. Food Chem. 19: 23–26

Chung KY, Dickson DW & Ou LT (1999) Differential enhanced degradation of *cis*- and *trans*-1,3-D in soil with a history of repeated field applications of 1,3-D. J. Environ. Sci. Health B34: 749–768

DowElanco (1996) 1,3-Dichloropropene – a profile. DowElanco, Indianapolis, IN

Hartmans S, Janssen MW, van der Werf MJ & De Bont JAM (1991) Bacterial metabolism of 3-chloroacrylic acid. J. Gen. Microbiol. 137: 2025–2032

Holman D (1999) Finding an alternative cure. Ag Consultant 55(5): 45–46

Lane DJ (1991) 16S/23S rRNA sequencing. In Stackebrandt E & Goodfellow M (Eds.) Nucleic Acid Techniques in Bacterial Systematics (pp 115–175). John Wiley & Sons, New York, NY

Lebbink G, Proper B & Nipshagen A (1989) Accelerated degradation of 1,3-dichloropropene. Acta Hortic. 255: 361–371

Mandelbaum RT, Allan DL & Wackett LP (1995) Isolation and characterization of a *Pseudomonas* sp. that mineralizes the s-triazine herbicide atrazine. Appl. Environ. Microbiol. 61: 1451–1457

Mandelbaum RT, Wackett LP & Allan DL (1993) Mineralization of the s-triazine ring of atrazine by stable soil bacteria. Appl. Environ. Microbiol. 59: 1695–1701

McCall PJ (1987) Hydrolysis of 1,3-dichloropropene in diluted aqueous solution. Pestic. Sci. 19: 235–242

Ou LT (1991) Interactions of microorganisms and soil during fenamiphos degradation. Soil Sci. Soc. Am. J. 55: 716–722

Ou LT, Chung KY, Thomas JE, Obreza TA & Dickson DW (1995) Degradation of 1,3-dichloropropene (1,3-D) in soils with different histories of field applications of 1,3-D. J. Nematol. 27: 127–242

Ou LT & Thomas JE (1994) Influence of soil organic matter and soil surfaces on a bacterial consortium that mineralizes fenamiphos. Soil Sci. Soc. Am. J. 58: 1148–1153

Poelarends GJ, Kulakov LA, Larkin MJ, van Hylckama Vlieg JET & Janssen DB (2000) Roles of horizontal gene transfer and gene integration in evolution of 1,3-dichloropropene- and 1,2-dibromoethane-degradative pathways. J. Bacteriol. 182: 2191–2199

Poelarends GJ, Wilkene M, Larkin MJ, van Elsas JD & Janssen DB (1998) Degradation of 1,3-dichloropropene by Pseudomonas cichorii 170. Appl. Environ. Microbiol. 64: 2931–2936

Racke KD & Coats JR (1990) Enhanced Biodegradation of Pesticides in the Environment. ACS Symp. Ser. 426, American Chemical Society, Washington, DC

Smelt JH, Teunissen W, Crum SJH & Leistra M (1989) Accelerated transformation of 1,3-dichloropropene in soil. Pestic. Sci. 37: 173–183

Smelt JH, van der Peppel-Groen AE, van der Pas LJT & Dijksterhuis A (1996) Development and duration of accelerated degradation of nematicides in different soils. Soil Biol. Biochem. 28: 1757–1765

Stevenson FJ (1982) Humus Chemistry. John Wiley & Sons, New York, NY

van Hylckama Vlieg JET & Janssen DB (1992) Bacterial degradation of 3-chloroacrylic acid and the characterization of *cis*- and *trans*-specific dehalogenases. Biodegradation 2: 139–150

Verhagen C, Lebbink G & Bloem J (1996) Enhanced biodegradation of the nematicides dichloropropene and methylisothiocyanate in a variety of soils. Soil Biol. Biochem. 28: 1753–1756

Verhagen C, Smit E, Janssen DB & van Elsas JD (1995) Bacterial dichloropropene degradation in soil: screening of soils and involvement of plasmids carrying the *dhlA* gene. Soil Biol. Biochem. 27: 1547–1557