

## Isolation of *Terrabacter* sp. Strain DDE-1, Which Metabolizes 1,1-Dichloro-2,2-Bis(4-Chlorophenyl)Ethylene when Induced with Biphenyl

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***Terrabacter* sp. strain DDE-1, able to metabolize 1,1-dichloro-2,2-bis(4-chlorophenyl)ethylene (DDE) in pure culture when induced with biphenyl, was enriched from a 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane residue-contaminated agricultural soil. Gas chromatography-mass spectrometry analysis of culture extracts revealed a number of DDE catabolites, including 2-(4'-chlorophenyl)-3,3-dichloropropenoic acid, 2-(4'-chlorophenyl)-2-hydroxy acetic acid, 2-(4'-chlorophenyl) acetic acid, and 4-chlorobenzoic acid.**

The insecticide 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane, commonly known as DDT, was once used extensively to control both agricultural pests and disease vectors. Although many countries ceased using DDT over 20 years ago, its residues (DDTr) still persist in the environment, predominantly as 1,1-dichloro-2,2-bis(4-chlorophenyl)ethylene (DDE), 1,1-dichloro-2,2-bis(4-chlorophenyl)ethane (DDD), and 1,1,1-trichloro-2-(2-chlorophenyl)-2-(4-chlorophenyl)ethane (2,4'-DDT), as well as unchanged DDT (Fig. 1) (1). Among these residues, DDE is the most toxicologically significant residue, as it blocks the action of androgens in rats (12) and has been implicated in male reproductive abnormalities in alligators (9). In topsoils, the presence of DDTr, often as DDE, limits land use options and may have an impact on trade in agricultural products. Cost-effective remediation methods for widespread low-level contamination are therefore required. While bacterial degradation of DDE has recently been reported in a recombinant strain (10), it is not known how widespread this capacity is in nature. The presence of organisms with this degradative capacity in contaminated soil may be beneficial in developing treatment options.

DDE has been considered to be a dead-end metabolite of DDT formed under oxidizing conditions (1, 8). Although little is known about the microbial metabolism of DDE (1, 8, 16), recent reports indicate that it can be metabolized by chlorobiphenyl-degrading bacteria under aerobic conditions (10, 13). Bacterium strain B-206, for example, produced a number of phenolic metabolites from DDE (13), and subsequently, *Pseudomonas acidovorans* M3GY was shown to mediate ring cleavage of DDE proceeding via *meta*-fission to 4-chlorobenzoic acid (10).

To investigate whether aerobic bacteria from DDTr-contaminated agricultural soils have the potential to metabolize DDE, we established enrichment cultures to select for microbes that transform DDE when provided with biphenyl as a carbon source. An aerobic gram-positive bacterium, *Terra-*

*bacter* sp. strain DDE-1, which metabolizes DDE when grown with biphenyl, was isolated.

**Chemicals.** DDE was purchased from Aldrich Chemical Company, 2,4'-DDT was purchased from Riedel de Haen, and biphenyl was purchased from BDH. [<sup>14</sup>C]DDE, prepared from [<sup>14</sup>C]DDT, was obtained from NEN Research Products, Boston, Mass. (4). All chemicals were at least 98% pure. Diazomethane was generated from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide with the Diazald kit (Aldrich Chemical Company) according to the manufacturer's instructions.

**Enrichment cultures.** Enrichment cultures for the selection of microbes metabolizing DDE were established in 100 ml of minimal medium (18) with the addition of vitamins (17) and biphenyl (1 mg ml<sup>-1</sup>) and in the presence of DDE (0.25 mg ml<sup>-1</sup>). Biphenyl and DDE dissolved in a minimal amount of acetone were added to sterile flasks, the solvent was evaporated, and then sterile medium was added. The source of inocula (10 g per flask) was agricultural soil from the AgResearch Winchmore Research Station, Canterbury, New Zealand, in which levels of DDE may reach 2 mg kg<sup>-1</sup>. Details of these soils are described by Boul et al. (3). All enrichment cultures were incubated at 28°C on a rotary shaker at 200 rpm. Aliquots (10 ml) were subcultured in fresh medium every month. Growth was indicated by the increase in turbidity in enrichment cultures with biphenyl as a carbon source in the presence of DDE. After 3 months, a persistent yellow water-soluble product accumulated in one of the cultures. Removal of DDE from the culture accumulating the yellow product was revealed by high-pressure liquid chromatography analysis of solvent extracts from entire cultures after 1 month of incubation (4). There was no loss of DDE or accumulation of a yellow product in the abiotic control flasks.

**Isolation and identification of a DDE-metabolizing bacterium.** A bacterium which metabolizes DDE was isolated from the enrichment culture by an overlay technique (19). The DDE precipitated in the overlay medium formed an opaque layer, and biphenyl crystals were placed in the lid of the petri dishes. Presumptive DDE-metabolizing microbes were detected as colonies surrounded by a clear zone after at least 6 weeks of incubation at 28°C. Isolates were removed from the clear zones and purified by streaking them onto plate count agar (PCA) (Difco). A bacterium, designated strain DDE-1, was selected for further study. Within 48 h of incubation, it produced

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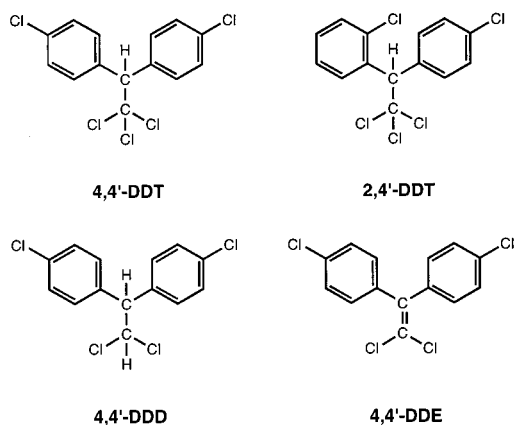


FIG. 1. 4,4'-DDT and its residues DDD, DDE, and 2,4'-DDT, which predominate in soil.

a yellow water-soluble product on PCA which had previously been spread with 0.1 ml of 1% (wt/vol) DDE in ether.

Strain DDE-1 is a gram-positive, coccobacillus-shaped bacterium which is catalase positive, oxidase negative, and non-motile. 16S rRNA sequence analysis (2, 6) showed that strain DDE-1 belonged within *Terrabacter*, a genus of the high-G+C gram-positive bacterial clade. Strain DDE-1 was 98.6% similar to *Terrabacter* sp. strain DPO1361 and 95.9% similar to *Terrabacter tumescens*. A phylogenetic tree was constructed (7, 20), and the position of strain DDE-1 is shown in Fig. 2. *Terrabacter* sp. strain DDE-1 has been deposited in the International Collection of Micro-organisms from Plants (ICMP; Auckland, New Zealand) as strain ICMP 13121.

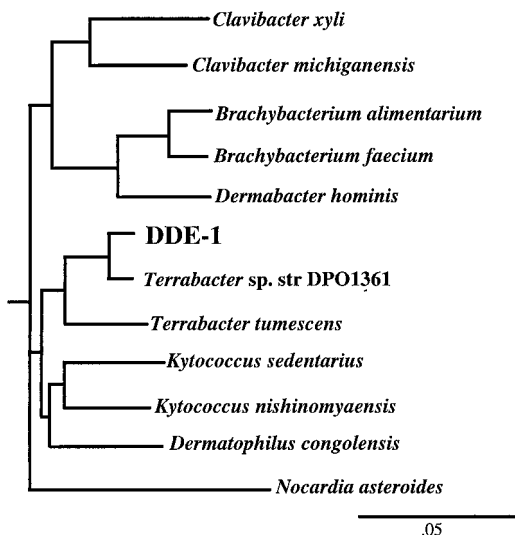


FIG. 2. 16S rRNA phylogenetic tree showing the position of *Terrabacter* sp. strain DDE-1. The GenBank accession numbers of the organisms used were as follows: *Terrabacter* sp. strain DPO1361, Y08853; *T. tumescens* NCIB 8914, X53215; *Dermatophilus congolensis* ATCC 14637, M59057; *Dermabacter hominis* DSM 7083, X91034; *Clavibacter xyli* clone pCG803, M60935; *Clavibacter michiganensis* DSM 7483, X77434; *Brachybacterium faecium* DSM 4810, X91032; *Brachybacterium alimentarium* CNR2 925, X91031; *Kytococcus sedentarius* DSM 20547, X87755; *Kytococcus nishinomyaensis* DSM 20448, X87757; and *Nocardia asteroides* ATCC 19247, Z36934. The bar represents a Jukes-Cantor distance of 0.05.

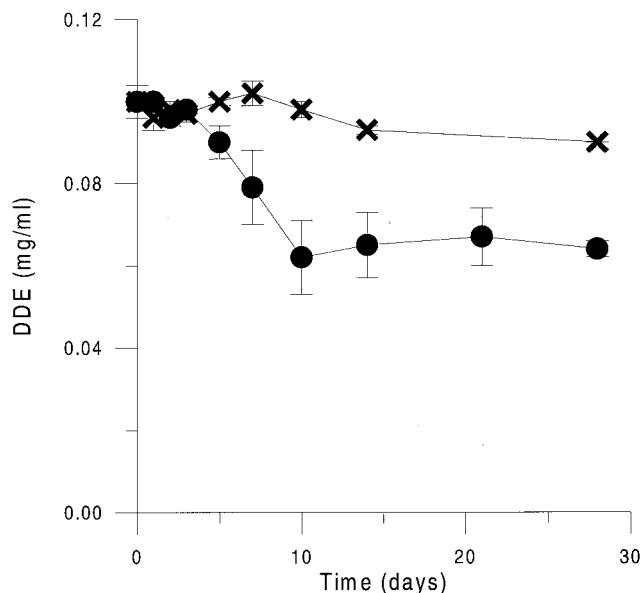


FIG. 3. Rate of transformation of DDE by *Terrabacter* sp. strain DDE-1 when incubated with biphenyl (●). Control flasks containing dead cells are also shown (×). The data are the means of values from three replicates; the bars indicate standard errors.

**Evidence for the metabolism of DDE by *Terrabacter* sp. strain DDE-1.** Metabolism of DDE by *Terrabacter* sp. strain DDE-1 in pure culture was confirmed by the loss of DDE from culture flasks containing minimal medium and biphenyl. Cells grown on PCA with biphenyl in the lid were removed from the agar, washed twice, and resuspended in phosphate buffer, and  $2.5 \times 10^9$  cells were inoculated into a series of flasks containing 50 ml of minimal medium supplied with biphenyl ( $0.5 \text{ mg ml}^{-1}$ ) and DDE ( $0.1 \text{ mg ml}^{-1}$ ). Biotic control flasks were inoculated with cells killed by autoclaving. All cultures were incubated at  $28^\circ\text{C}$  in the dark with shaking at 200 rpm. Entire flasks were sacrificed at regular intervals. The numbers of viable cells were monitored by plating culture dilutions on PCA. Triplicate cultures were acidified with 0.5 ml of concentrated HCl, 2,4' DDT (5 mg per flask) was added as an internal standard, and DDE residues were extracted with hexane. The extracts were diluted 100-fold in hexane and analyzed with an Econo-Cap EC-1 nonpolar silica column coated with polydimethylsiloxane (30 m by 0.32 mm [internal diameter]; 0.25- $\mu\text{m}$  film thickness; Alltech) and a Shimadzu model GC17AV2 gas chromatograph with a wide-range flame ionization detector. The operating conditions were as follows: injector port,  $250^\circ\text{C}$ ; detector,  $270^\circ\text{C}$ ; column temperature program,  $50^\circ\text{C}$  isothermal for 1 min,  $10^\circ\text{C}/\text{min}$  to  $200^\circ\text{C}$ ,  $20^\circ\text{C}/\text{min}$  to  $300^\circ\text{C}$ , and isothermal at  $300^\circ\text{C}$  for 5 min. The carrier gas was helium at 1 ml/min with a pressure program, and injections were 1  $\mu\text{l}$  splitless.

DDE was lost from the flasks containing live *Terrabacter* sp. strain DDE-1 but not from the abiotic control flasks after 28 days of incubation (Fig. 3). The concentration of DDE fell from 0.1 to  $0.062 \text{ mg ml}^{-1}$  after 10 days of incubation, after which no further loss occurred. While no metabolites of DDE were detected in the extracts by using a wide-range flame ionization detector, the accumulation of a yellow product in the medium, indicative of ring cleavage, was observed, as noted previously. No concomitant increase in viable cell numbers was detected by plating culture dilutions onto PCA (results not shown).

TABLE 1. Mass balance determination for *Terrabacter* sp. strain DDE-1 after 28 days of incubation with [ $^{14}\text{C}$ ]DDE

Culture flask	% Recovery of $^{14}\text{C}$ radiolabel <sup>a</sup>					Total
	Neutral extract	Acid extract	Aqueous extract	Cell-associated extract	CO <sub>2</sub>	
Sterile control	89.4 ± 12.7	1.5 ± 0.2	0.14 ± 0.05	ND <sup>c</sup>	<1.0	92.09 ± 12.4
Biotic control <sup>b</sup>	85.1 ± 2.1	1.2 ± 0.1	1.02 ± 0.15	<0.5	<1.0	87.96 ± 2.0
Strain DDE-1	57.6 ± 4.8	26.9 ± 1.5	2.78 ± 0.69	<0.5	<1.0	88.7 ± 3.4

<sup>a</sup> Values are averages ± standard deviation of three replicate flasks.

<sup>b</sup> Inoculated with  $2.5 \times 10^9$  cells of strain DDE-1 killed by autoclaving.

<sup>c</sup> ND, not determined.

Additional evidence for the metabolism of DDE by *Terrabacter* sp. strain DDE-1 was obtained with [ $^{14}\text{C}$ ]DDE. Biometer flasks containing 30 ml of minimal medium supplemented with DDE (0.1 mg ml<sup>-1</sup>) spiked with 0.225  $\mu\text{Ci}$  of [phenyl ring- $^{14}\text{C}$ (U)]DDE were inoculated with cells induced with biphenyl as described above. Potassium hydroxide (1 M) was used as a CO<sub>2</sub> trap, and the flasks were incubated at room temperature in the dark with shaking at 125 rpm. The experiment was carried out in triplicate with biotic and abiotic controls. At regular intervals, 0.5-ml samples of the CO<sub>2</sub> trap were removed and mixed with 11 ml of the scintillation cocktail (10 ml of PCS plus 1 ml of water), and the radioactivity was determined by liquid scintillation counting with a Beckman model 3800 liquid scintillation counter. The  $^{14}\text{C}$  residues in the neutral and acid organic phases, the cell biomass, and the aqueous phase of the cultures were quantified as follows. The cultures were adjusted to pH 7.5 with 5 M NaOH and extracted three times with an equal volume of ethyl acetate. The remaining culture was adjusted to pH 2.5 to 3.5 with 5 M HCl and reextracted with ethyl acetate as before. The culture was then centrifuged, and the pellet of cells was washed and then resuspended in 10 ml of buffer, from which a 1-ml aliquot was removed for scintillation counting. The radioactivity present in 1-ml aliquots of the supernatant combined with cell washings and in 1-ml aliquots of the solvent extracts was also measured.

A mass balance calculated for *Terrabacter* sp. strain DDE-1 (Table 1) reveals conclusively the ability of this bacterium to metabolize DDE. In comparison with the control flasks, those containing live cells transformed a significant percentage of the [ $^{14}\text{C}$ ]DDE to acid-extractable products. At the same time, the percentage of radioactivity recoverable from the neutral extract decreased in flasks with live cells. No mineralization of DDE to carbon dioxide was detected. Similar levels of conversion of DDE and the absence of mineralization activity were reported for *P. acidovorans* M3GY (10).

The spectral characteristics of the yellow product were obtained by inoculating washed cells of *Terrabacter* sp. strain DDE-1, induced with biphenyl as above, into minimal medium containing DDE (0.1 mg ml<sup>-1</sup>) only. The flasks were incubated at room temperature in the dark with shaking at 125 rpm for 10 days. An aliquot of 3 ml was taken from the flask and centrifuged, and the spectrum of the supernatant was determined over the range from 350 to 550 nm in a Beckman DU-640 spectrophotometer. The yellow compound had an absorbance maximum at 401 nm at neutral pH; acidification with orthophosphoric acid caused a shift in the spectrum and a concomitant decrease in the peak. These features are characteristic of *meta*-ring cleavage products of aromatic compounds that absorb over the 375 to 430-nm region (10, 14). A proposed structure for this product (catabolite II) is given in Fig. 4.

**Isolation and characterization of DDE catabolites.** A number of DDE catabolites were isolated from cultures of *Terrabacter* sp. strain DDE-1 in minimal medium with DDE (0.5 mg

ml<sup>-1</sup>) and induced with biphenyl (Fig. 4). To this end, the cultures were incubated in the dark at 25°C with shaking at 180 rpm for 7 or 21 days, acidified to pH 2 to 3 with orthophosphoric acid, and centrifuged (10,000  $\times$  g; 30 min; 4°C) to remove cells and debris. The supernatant was extracted with approximately 1.5 volumes of ethyl acetate. The extract was dried over anhydrous sodium sulfate and concentrated to approximately 500  $\mu\text{l}$  in vacuo with gentle heat (30°C). The concentrated sample was further reduced under a stream of nitrogen and methylated with diazomethane before gas chromatography-mass spectrometry (GC-MS) analysis (models GC 8000 and MD 800; Fison's Instrument). The gas chromatograph was fitted with a BPX5 column coated with polysilphenylene-siloxane (length, 25 m; internal diameter, 0.22 mm; 0.25- $\mu\text{m}$  film thickness). Injection (2  $\mu\text{l}$ ) was performed in split mode at 40°C. The column temperature was held at 40°C for 2 min and then raised by 5°C per min up to 280°C, where it was held for 5 min. The injector temperature was maintained at 250°C. The carrier gas was helium at a pressure of 80 kPa.

Mass spectra were obtained (electron ionization at 70 eV; trap current, 100  $\mu\text{A}$ ; mass range, 40 to 500; source temperature, 200°C), and possible structures were deduced by comparing the mass spectra recorded with the mass spectrum database of the National Institute of Standards and Technology and reports in the literature (10, 14).

GC-MS analyses of the culture extracts revealed many catabolites (Fig. 4) that were not detected in abiotic controls. The presence of chlorine in the catabolites was confirmed by analysis of the molecular ion. Compounds containing one chlorine atom typically show a molecular ion with an  $m/z$  of  $x + 2$  in a ratio of 3 to 1 that corresponds to the two naturally occurring chlorine isotopes (35 and 37). This feature helps confirm DDE as the parent compound from which the catabolite is derived (5).

Two compounds with different retention times (44.5 and 45.3 min, respectively) but identical mass spectra and molecular ions with  $m/z$  of 332 (16 mass units more than the parent molecule) were detected (Fig. 5). The major fragments of the ionization pattern were indicative of the loss of one chlorine ( $m/z$ , 297), two chlorines ( $m/z$ , 262), and HCl ( $m/z$ , 296) (Fig. 5). These catabolites were identified as isomers of the monohydroxylated derivatives (catabolite I [Fig. 4]) of DDE at the *meta* and *ortho* positions, based on comparison with the database reference spectra and similarity to the spectrum presented by Hay and Focht (10). We propose that the isomers are derived from dehydration of an initial dihydroxy compound, formed through dioxygenase attack on DDE, which is unstable in acidic extracts (10, 14).

A number of catabolites downstream of the proposed ring cleavage product (catabolite II) were isolated. Catabolite III (Fig. 6), identified as 2-(4'-chlorophenyl)-3,3-dichloropropenoic acid (methyl ester), had a molecular ion at an  $m/z$  of 264. This compound was also reported as a metabolite of DDE when degraded by *P. acidovorans* M3GY (10). No catabolites

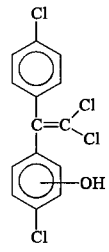
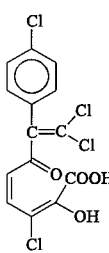
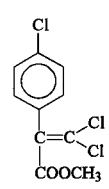
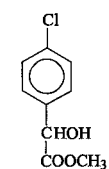
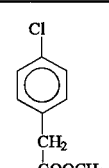
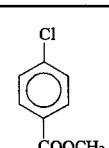
Catabolite	Structure	Molecular ion ( $M^+$ )	Major ions ( $m/z$ )	Presence	
				7d	21d
I Monohydroxy-DDE		332 $C_{14}H_8Cl_4O$	297(-Cl) 296 (-HCl) 262 (-2Cl)	+	+
II DDE-muonic semialdehyde  Inferred from spectrophotometric evidence; maximum absorbance at 401 nm		ND	ND	ND	ND
III 2-(4'-chlorophenyl)-3,3-dichloropropenoic acid		264 $C_{10}H_7Cl_3O_2$	229(-Cl) 205(-COOHCH <sub>3</sub> ) 170 (-Cl and -COOCH <sub>3</sub> )	+	+
IV 2-(4'-chlorophenyl)-2-hydroxy acetic acid		200 $C_9H_9ClO_3$	141(-COOCH <sub>3</sub> )	+	+
V 2-(4'-chlorophenyl) acetic acid		184 $C_9H_9ClO_2$	125(-COOCH <sub>3</sub> )	+	+
VI 4-chlorobenzoic acid		170 $C_8H_7ClO_2$	139(-CH <sub>3</sub> O) 111(-COOCH <sub>3</sub> )	+	+

FIG. 4. Catabolites identified in the degradation of DDE by *Terrabacter* sp. strain DDE-1 by GC-MS (methyl ester derivatives) and UV-visible-light spectrophotometry. ND, not visualized by GC-MS; +, present.

that were intermediate between compounds II and III were seen in the GC-MS analysis. Catabolite IV, 2-(4'-chlorophenyl)-2-hydroxy acetic acid (methyl ester), with a molecular ion with an  $m/z$  of 200, represents a loss of carbon dioxide and HCl from catabolite III, indicating sequential degradation. This compound could then be further degraded to 4-chlorobenzoic acid (catabolite VI [methyl ester]). Catabolite V ( $m/z$ , 184;

methyl ester) is also likely to be formed from catabolite IV, possibly through dehydration, but may be a dead-end product in this pathway. Catabolites V and VI have also been reported previously as degradation products of DDT (14, 15) and DDE (10).

In conclusion, a gram-positive bacterium, *Terrabacter* sp. strain DDE-1, was isolated that is able to metabolize DDE to

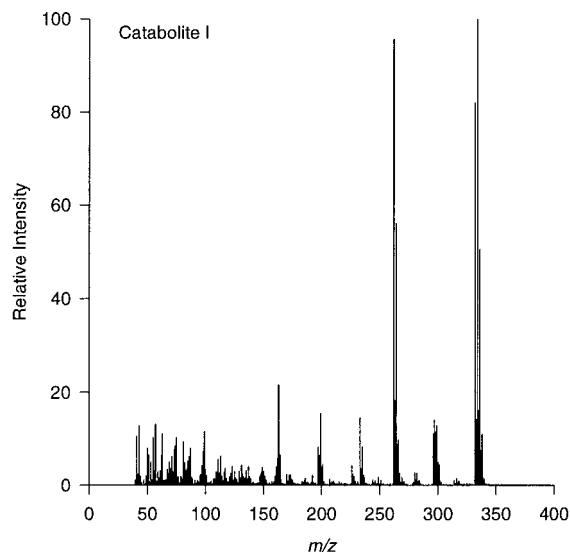


FIG. 5. Mass spectrum for catabolite I, monohydroxy-DDE.

4-chlorobenzoate when induced with biphenyl. The evidence points to the involvement of a dioxygenase and subsequent *meta*-ring cleavage similar to the degradation pathway proposed for *P. acidovorans* M3GY (10). This is only the second report of extensive metabolism of DDE and the first report of this metabolism in a gram-positive organism. As *Terrabacter* sp. strain DDE-1 was isolated from an agricultural soil contaminated with DDE, this study provides evidence for the usefulness of biphenyl-metabolizing bacteria for in situ remediation of DDE-contaminated agricultural soils. To enhance degradation of DDE in soils, information is required on the in situ abundance and distribution of bacteria with this type of metabolism and the substrates that will induce the expression of the catabolic pathway for DDE degradation. While biphenyl has proved a useful substrate for isolating *Terrabacter* sp. strain

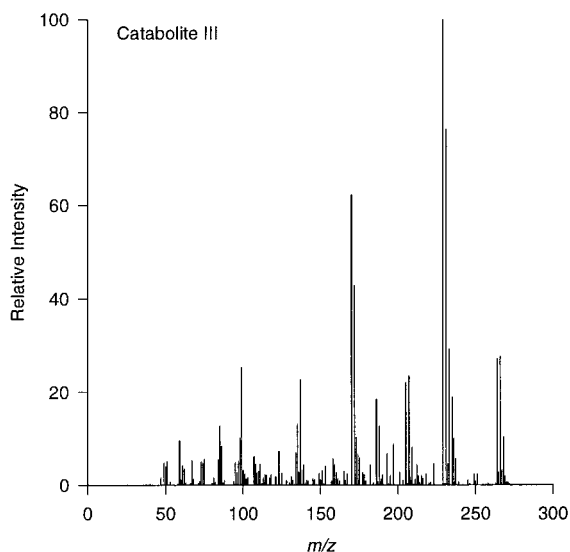


FIG. 6. Mass spectrum for catabolite III, 2-(4'-chlorophenyl)-3,3-dichloropropenoic acid (methyl ester).

DDE-1, the application of biphenyl to soils is not desirable, as it is on the Environmental Protection Agency's priority pollutant list. Fortunately, a number of plant terpenes, including cymene and limonene, have been shown to stimulate degradation of polychlorinated biphenyls (11). Whether these compounds also induce DDE degradation in *Terrabacter* sp. DDE-1 has yet to be determined.

Although remediation of DDTr-contaminated soils is difficult, the isolation of a bacterium with the ability to degrade DDE from such a site indicates that microbially mediated processes for cleanup of DDTr-contaminated soils are worthy of further investigation.

**Nucleotide sequence accession number.** The 16S rRNA sequence of strain DDE-1 has been submitted to GenBank and assigned accession no. U96645.

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