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Aerobic Degradation of 1,1,1-Trichloro-2,2-Bis(4-Chlorophenyl)Ethane (DDT) by *Alcaligenes eutrophus* A5

LLOYD J. NADEAU,[1]† FU-MIN MENN,[1] ALEC BREEN,[1]‡ AND GARY S. SAYLER[1,2,3]*

Center for Environmental Biotechnology,[1] the Department of Microbiology,[2] and the Graduate Program in Ecology,[3] The University of Tennessee, Knoxville, Tennessee 37932

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Biotransformation of 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT) by *Alcaligenes eutrophus* A5 was demonstrated by analysis of ethyl acetate-extracted products from resting cell cultures. Gas chromatography-mass spectrometry characterization of the neutral extracts revealed two hydroxy-DDT intermediates (m/z = 370) with retention times at 19.55 and 19.80 min that shared identical mass spectra. This result suggested that the hydroxylations occurred at the *ortho* and *meta* positions on the aromatic ring. UV-visible spectrum spectrophotometric analysis of a yellow metabolite in the culture supernatant showed a maximum A_{402} with, under acidic and basic conditions, spectrophotometric characteristics similar to those of the aromatic ring *meta*-cleavage products. 4-Chlorobenzoic acid was detected by thin-layer chromatography radiochemical scanning in samples from mineralization experiments by comparison of R_f values of [^{14}C]DDT intermediates with that of an authentic standard. These results were further confirmed by gas chromatography-mass spectrometry analysis. This study indicates that DDT appears to be oxidized by a dioxygenase in *A. eutrophus* A5 and that the products of this oxidation are subsequently subjected to ring fission to eventually yield 4-chlorobenzoic acid as a major stable intermediate.

Polychlorinated biphenyls (PCBs) and 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT) are widely distributed and persistent xenobiotic contaminants in the environment (14, 25, 26, 28). These contaminants share similar chemical structures and physical properties, such as low solubility and high lipid partitioning coefficients, and are relatively resistant to microbial degradation. Substantial information has accumulated that a variety of PCB congeners can be oxidatively degraded by a dioxygenase system in a few microbial species (2, 3, 6, 11, 16, 30, 31, 37). Recently, it has also been demonstrated that PCBs can be anaerobically dechlorinated by mixed microbial populations in sediments (24). Conversely, DDT degradation has only been documented as an anaerobic dechlorination pathway (35, 36), although degradative intermediates such as dichlorodiphenylmethane have been shown to be susceptible to aerobic metabolism (10).

Recent data suggest that a chlorobiphenyl (CB)-degrading strain (B-206) can mediate hydroxylation of DDT with phenyl ring dechlorination (20). However, no DDT ring-cleavage products were observed in these experiments. Because of these findings and structural similarities between some PCBs and DDT, it was of interest to determine whether other bacteria capable of CB or biphenyl aerobic biodegradation were competent for aerobic degradation of DDT.

Of primary interest in these studies was an evaluation of the ability of *Alcaligenes eutrophus* A5 (15, 19, 31) to carry out the degradation of DDT. *A. eutrophus* A5 was originally isolated by enrichment cultivation of PCB-contaminated sediments and was found to mediate the mineralization of 4-CB through a 4-chlorobenzoic acid (4-CBA) intermediate (31). This strain was found to have catabolic gene homology with *Pseudomonas testosteroni* B-356 (1) but over time lost the ability to mineralize 4-CB. The results of these current studies indicate that strain A5 is also capable of the aerobic degradation of DDT to 4-CBA.

MATERIALS AND METHODS

Bacterial cultures and cultivation methods. *A. eutrophus* A5 was identified and characterized previously as a PCB-degrading bacterium (22, 31). In order to obtain high cell densities for the resting cell assays, this organism was grown overnight at 28°C in yeast extract-peptone-glucose medium (29), which consisted of 0.2 g of yeast extract, 2.0 g of peptone, 0.2 g of ammonium nitrate, and 1.0 g of glucose in 1 liter of distilled water (pH 7.0).

Minimal salts medium supplemented with 0.005% yeast extract was used in the experiment for measuring [^{14}C]DDT mineralization and for characterizing the [^{14}C]DDT intermediates by thin-layer chromatography (TLC). The minimal salts (basal salts) medium consisted of 4.0 g of $NaNO_3$, 1.5 g of KH_2PO_4, 0.005 g of $FeCl_3$, 0.2 g of $MgSO_4$, 0.01 g of $CaCl_2$, and 0.5 g of Na_2HPO_4 in 1 liter of distilled water (pH 7.0).

Strain A5 was screened for DDT-degradative ability by the ether spray plate method (34). The organisms, including *Escherichia coli* controls, were plated onto yeast extract-peptone-glucose agar, sprayed with 1% *o,p'*-DDT and *p,p'*-DDT, and incubated at 30°C, and the colonies were periodically examined for formation of a yellow color.

[^{14}C]DDT mineralization experiments were performed in duplicate as described previously (22). [Ring-U-^{14}C]DDT (specific activity, 291 μCi/mg) was added at a final concentration of 1 ppm to 10^6 to 5×10^7 cells in 1 ml of minimal salts medium supplemented with 0.005% yeast extract. The cul-

* Corresponding author. Mailing address: Center for Environmental Biotechnology, the Department of Microbiology, and the Graduate Program in Ecology, The University of Tennessee, 10515 Research Dr., Suite 100, Knoxville, TN 37932. Phone: (615) 974-8080. Fax: (615) 974-8086.

† Present address: Air Force Engineering Service Center, Bldg. 1117, Armstrong Laboratory for Environmental Quality, Tyndall Air Force Base, FL 32403.

‡ Present address: Risk Reduction Engineering Laboratory, U.S. Environmental Protection Agency, Cincinnati, OH 45268.

tures were incubated at room temperature and aerated by shaking at 100 rpm. Duplicate cultures were acidified with 0.5 ml of 2 N H_2SO_4 at different intervals. Control cultures were autoclaved and treated as described above. NaOH (0.4 N) was used to trap $^{14}CO_2$ in mineralization assays by adding 0.5 ml to 1 ml of H_2O and 10 ml of Readysafe solution, and $^{14}CO_2$ was quantitated with a Beckman liquid scintillation counter (Fullerton, Calif.). Analysis of [^{14}C]DDT-degradative products was conducted with the TLC technique.

Batch cultivation experiments were used to characterize the DDT intermediates by UV-visible spectrum spectrophotometry and gas chromatography-mass spectrometry (GC-MS). Overnight cultures of *A. eutrophus* A5 grown in yeast extract-peptone-glucose medium at room temperature were washed three times and resuspended in 100 ml of minimal salts medium to a final cell density of 10^8 to 10^9 cells per ml in 500-ml flasks. DDT dissolved in *N',N*-dimethylformamide (0.5%, vol/vol) was added to the cells at a concentration of 5 mM. Abiotic controls consisted of minimal salts medium with 5 mM DDT, and biotic controls consisted of *A. eutrophus* A5 with *N',N*-dimethylformamide. Incubations were carried out at 30°C in a rotary shaker set at 225 rpm.

Analytical methods. TLC separation and quantitation of the metabolites was performed by spotting a 50-μl aliquot of cell suspensions from live and killed control cultures incubated with [^{14}C]DDT onto a TLC plate (silica gel GF; Alltech). [^{14}C]DDT and metabolites analyzed by TLC were separated by a hexane-ethanol (75:25) solvent system and were detected and quantitated with a Bioscan Imaging Scanner System 2000 with an AutoChanger 1000 (Bioscan, Inc., Washington, D.C.).

The UV-visible spectrum absorbance measurements of the supernatants from *A. eutrophus* A5 cultures supplemented with DDT were performed with a Beckman DU20 spectrophotometer. The supernatants were acidified to pH 3 with 1 N HCl and were made basic to pH 12 with 10 N NaOH. Samples were scanned over a wavelength of 220 to 550 nm at a scanning rate of 1,200 nm/min.

MS characterization of metabolites was performed with cultures incubated with and without 5 mM DDT and with abiotic controls. The cultures were centrifuged at 15,300 × *g* for 20 min at 4°C, and the supernatants were extracted three times with ethyl acetate. After the neutral extraction, the supernatants were acidified to pH 3 with 1 N HCl and extracted as described above. The extracts were dehydrated with anhydrous sodium sulfate and evaporated to approximately 2 ml under a vacuum with a rotary evaporator at 28°C. The flasks were washed three times with 1 ml of ethyl acetate and were dried to 0.5 ml under N_2 gas.

The metabolites were identified with a Hewlett-Packard GC-MS (Palo Alto, Calif. [model 5995A]) with a 12-m BP5 capillary column (film thickness, 0.25 μm) consisting of 5% diphenyl dimethyl siloxane (SGE, Austin, Tex.). The column temperature parameters were set at an initial temperature of 50°C for 1 min followed by a 10°C/min increase to 250°C. The ionization voltage was −70 eV. The 4-CBA metabolite was confirmed with an authentic standard.

Chemicals. The *o,p'*- and *p,p'*-DDT isomers (Ultrascientific, North Kingston, R.I.) solubilized in acetone at 1% (wt/vol) were used to screen for DDT degraders. [^{14}C]DDT at 99.9% radiolabeled purity (specific activity, 291 μCi/mg [Amersham, Arlington Heights, Ill.]) was used in the mineralization and metabolite experiments. The radiolabeled purity was further confirmed by a high-performance liquid chromatography system equipped with a radioactive flow detector (FLO-ONEβeta; Radiomatic Instruments and Chemical Co., Inc, Tampa, Fla.). Reagent-grade 4-CBA was used in the TLC and GC-MS metabolite characterization (Fisher Scientific, Atlanta, Ga.). Pesticide–GC-MS-grade hexane, ethyl acetate, and absolute ethanol were used for TLC and GC-MS analyses.

TABLE 1. TLC quantitation of [^{14}C]DDT metabolites produced from [^{14}C]DDT by *A. eutrophus* A5 and detected by TLC scanning radioisotope detector

Incubation time (days)	Detection (% of initial [^{14}C]DDT) of metabolite[a]:	
	I	II
0[b]	0	0
5	7.0	0
35	1.3	0.6
44	8.0	0.3
70	10.6	2.1
100	0	18.8

[a] Metabolite I has a TLC R_f value of 0.6, and metabolite II has a TLC R_f value of 0.1.
[b] Zero hour.

RESULTS

Screening of DDT-degrading organism. With DDT (1%) spray plate analysis, *A. eutrophus* A5 produced a yellow product from both the *o,p'*- and *p,p'*-DDT isomers after 30 days, persisting to over 45 days of incubation. The DDT film became clarified around the primary streaks after the detection of the yellow intermediate, indicating that the organism has the ability to degrade DDT.

The supernatants from *A. eutrophus* A5 supplemented with 5 mM DDT produced a yellow intermediate with an A_{402} peak. The intensity of the peak increased when the supernatant was made basic to pH 12. At pH 3, the absorbance peak disappeared and the supernatant became colorless. The phenomena described above are typical characteristics of the *meta*-ring-cleavage product formed during aromatic hydrocarbon oxidation (3, 5, 8, 9, 17). The yellow intermediate in the culture increased in intensity to an optical density of 0.65 at 402 nm until the 6th day of incubation.

Metabolite production from [^{14}C]DDT. *A. eutrophus* A5 was incubated with 1 ppm [^{14}C]DDT for distinct incubation times, and the aqueous fraction was analyzed by TLC radiochemical scanning. Two metabolites were separated (R_f, 0.6 and 0.1) from [^{14}C]DDT (R_f, 0.80). Metabolite I (R_f, 0.6) appeared on the 5th day of incubation and in subsequent experiments was detectable within 3 days. This metabolite increased in concentration to 10.6% of the original [^{14}C]DDT on the 70th day of incubation and was not detected by the 100th day of incubation (Table 1). Metabolite II appeared within 35 days and increased to 18.8% by the last day of incubation. During the course of the experiment, up to the 70th day of incubation, there was an increase in the amount of radiolabeled compound at the origin. No metabolites were detected in the killed control cultures.

To further characterize these intermediates, 1,1'-bis(*p*-chlorophenyl)-2,2,2-trichloroethane (DDD), 1,1'-bis(*p*-chlorophenyl)-2,2,2-trichloroethylene (DDE), and 4-CBA were spotted onto TLC plates with 50-μl aliquots of cell suspensions incubated for 5, 70, and 100 days. The 4-CBA comi-

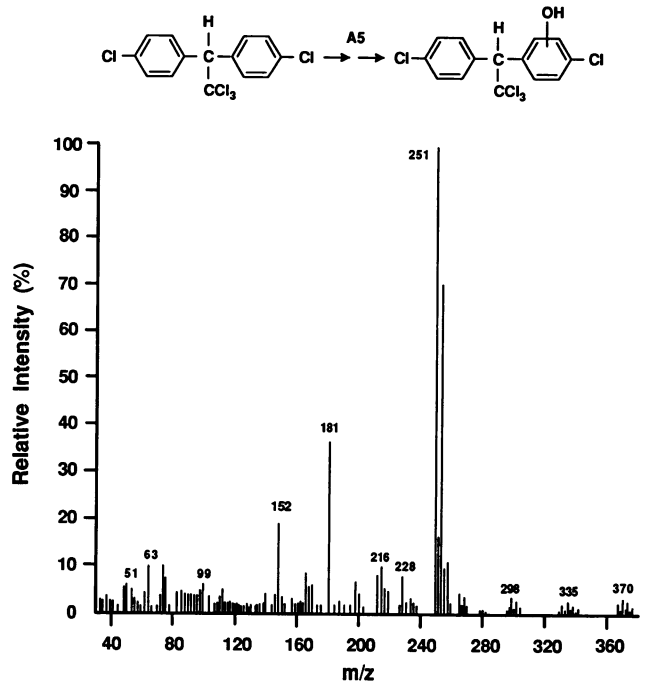

FIG. 1. Mass spectra of the hydroxy-DDT intermediate produced from the degradation of DDT by *A. eutrophus* A5.

grated with metabolite II. Authentic DDD and DDE did not comigrate with either metabolite I or II.

GC-MS characterization of metabolites. The GC-MS analysis was performed with sample extracts following the 6th day of incubation, typically when the yellow metabolite reached an optical density at 402 nm of 0.65. The GC-MS analysis of the neutral extracts from the cultures revealed a metabolite with retention times at 19.80 and 19.55 min, which showed identical mass spectra. The mass spectra of the intermediate at 19.80 min are shown in Fig. 1. This metabolite had a parent peak (M^+) of m/z = 370 and a base peak of m/z = 251 and was tentatively determined to be a hydroxy-DDT because it had a molecular weight of 370, which is 16 more than that of DDT (molecular weight, 354). The ionization fragment of m/z = 251 indicated the loss of $-CCl_3$. This ionization pattern consisted of three sequential ionization peaks, after the parent peak (m/z = 370), at m/z = 335, 298, and 262, which correspond to the loss of three chloride ions. The remaining ion fragment (m/z = 251) could be $C_{13}H_9OCl_2$ (molecular weight, 251). These metabolites are considered to be hydroxyl substituents at *meta* and *ortho* positions on the phenyl ring resulting from dehydration of the dihydrodiol metabolite (10, 11). Metabolites were not detected in extractions performed with the biotic and abiotic controls.

In the acid extract, one intermediate with a GC retention time of 8.0 min, a molecular ion peak of m/z = 156, and a base peak of m/z = 139 (M^+ − OH) was detected. This product was identified as 4-CBA by comparison with an authentic standard, which suggested that 4-CBA is a ring-cleavage product formed from DDT by strain A5.

DISCUSSION

This is the first report describing the aerobic bacterial degradation of DDT via 4-CBA. Earlier studies have demonstrated that DDT is decomposed under anaerobic circumstances (18, 21). Anaerobic degradation by mixed and defined bacterial cultures occurred by reductive dechlorination of the ethane group to DDD or by dehalogenation to DDE (4, 21, 35). While DDE was a dead-end intermediate, DDD was further degraded by sequential steps involving reductive dechlorination and hydroxylation of the ethane group, resulting in the accumulation of dichlorobenzophenone (23, 36). One of the anaerobic intermediates, *p,p'*-dichlorophenylmethane, was converted to a ring-fission product, *p*-chlorophenylacetic acid, by *Hydrogenomonas* sp. under aerobic conditions (10). These DDT intermediates were not detected in any experiments with *A. eutrophus* A5.

The initial aerobic degradative step by *A. eutrophus* A5 is oxidation on the phenyl ring at adjacent *ortho* and *meta* positions to form hydroxy-DDTs, essentially a dihydrodiol compound formed from insertion of two molecules of oxygen. This is in contrast to previous observations with strain B-206 (20) in which *ortho* or *para* hydroxylation of DDT occurred to produce a phenolic metabolite with concomitant phenyl ring dechlorination. There was no further degradation reported for the latter hydroxy-DDTs (20). In addition, the initial oxidation at the carbon bridge (C-1) to form kelthane [1,1-bis(*p*-chlorophenyl)-2,2,2-trichloroethanol] was not observed because the mass spectrum of kelthane does not match the spectra of the DDT metabolites observed here. In the present study, a yellow ring-cleavage compound was detected spectrophotometrically in DDT culture supernatants of *A. eutrophus* A5, suggesting a *meta*-cleavage DDT pathway.

On the basis of the information obtained from this study, an aerobic catabolic pathway for DDT is proposed (Fig. 2). *A. eutrophus* A5 initially oxidizes DDT (A) at the *ortho* and *meta* positions to form a 2,3-dihydrodiol–DDT intermediate (B). It is proposed that this is a dioxygenase type of attack resulting in the transient production of a DDT dihydrodiol. On the basis of previous work by Gibson et al. (12, 13), it is suggested that the dihydrodiol compound is unstable and easily dehydrates into two hydroxylated compounds (Fig. 1) under weak acidic conditions (pH < 7.0). Since there were two different retention times observed from GC analysis for the hydroxy-DDT metabolites, it is suggested that a dihydrodiol-DDT (B) may be formed in the catabolic pathway. However, the assumption about the formation of dihydrodiol-DDT needs further investigation. On the basis of the known degradation pathways of aromatic hydrocarbons and PCBs (27), the dihydrodiol-DDT would be further degraded to 2,3-dihydroxy-DDT (compound C) by a dehydrogenase. Compound C would be further metabolized through *meta* cleavage to form the yellow ring-fission product (compound D) which would then be catabolized to 4-CBA (compound E). The ring-cleavage product would be further degraded to either a C-6- or C-5-chlorinated acid, depending on where the hydrolytic cleavage took place. A C-6-chlorinated acid would be formed if the hydrolytic cleavage took place between C-1 and an adjunct carbon on the cleaved phenyl ring. A C-5-chlorinated acid would be formed if the hydrolytic cleavage took place in a way similar to that in the biphenyl pathway (8, 9), between C-5 and C-6 on the cleaved phenyl ring. The proposed pathway requires identification of compounds B, C, and D for further confirmation. At the present time, whether the dechlorination on C-1 takes place before (7, 33) or after ring cleavage by strain A5 is not known.

4-CBA appears to be a terminal product formed from DDT by *A. eutrophus* A5 because it has lost the ability to further

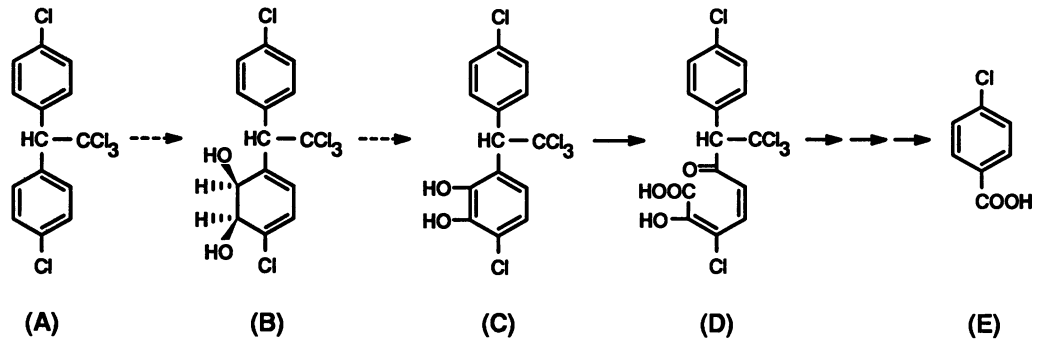

FIG. 2. The proposed DDT aerobic degradative pathway by *A. eutrophus* A5. Compounds B and C were not identified in this study. Compound D, the yellow ring-cleavage product, was determined spectrophotometrically. The dashed lines denote that these compounds have not been identified in this study.

degrade 4-CBA (22). It has recently been shown that metabolism of 4-CB to 4-CBA in strain A5 is associated with a large (59-kb) biphenyl transposon (Tn*4371* [32]). Layton et al. have shown that a pSS50-related plasmid (pSS70) appears to contain a unique 10-kb fragment responsible for dechlorination of 4-CBA to 4-hydroxybenzoate (19). It is not yet known whether individual isolates similar to strain A5 containing pSS50-related plasmids can mineralize DDT aerobically to completion either as a primary substrate or as a cometabolic substrate in a biphenyl pathway. In addition, the ability of consortia to mineralize DDT completely should also be taken into account as a direction for future investigation. It is also important to understand the capacity and evolution of microbial strains in the concomitant degradation of PCBs, DDT, and other related chloroaromatic pesticides such as DDD, DDE, and methoxychlor.

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