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Isolation and Characterization of Coumaphos-Metabolizing Bacteria from Cattle Dip

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Coumaphos, an organophosphate insecticide, is used for tick control in cattle dipping vats along the U.S.-Mexican border. Recently, several vats (problem vats) have experienced a loss of efficacy because of microbial degradation. Three morphologically distinct bacteria (designated B-1, B-2, and B-3) that metabolized coumaphos were isolated from enrichment cultures that were initiated from problem vat dip material. In general, amino acids, pyrimidines, and acetate supported growth; carbohydrates were not utilized. Only B-2 required growth factors. In resting cell experiments, coumaphos was hydrolyzed to diethylthiophosphoric acid and chlorferon by all three isolates. Chlorferon was subsequently metabolized by B-1 and B-2 to α-chloro-β-methyl-2,3,4-trihydroxy-*trans*-cinnamic acid. Only B-1 produced additional metabolites. Experiments with [benzo ring-labeled U-^{14}C]coumaphos or chlorferon demonstrated that B-1 was capable of both mineralizing and incorporating into biomass the aromatic portion of the molecule. The majority of label, however, was recovered in the form of soluble products, including α-chloro-β-methyl-2,3,4-trihydroxy-*trans*-cinnamic acid. Although B-1 had the capacity to use chlorferon as a carbon source at low concentrations (100 μg/ml), visible growth at higher concentrations (1,000 μg/ml) was not observed. The addition of 400 μg of chlorferon per ml to B-1 cells in the mid-log phase of growth resulted in complete inhibition of growth, while the addition of 100 to 200 μg of chlorferon per ml resulted in partial inhibition. The growth of B-2 and B-3 was inhibited by 100 μg of chlorferon per ml. These data suggest that, although B-1 and, to a lesser extent, B-2 and B-3 are responsible for the primary degradation of coumaphos, other organisms in the enrichment culture may play a secondary role in coumaphos degradation by removing inhibitory products of coumaphos metabolism.

Coumaphos [*O*,*O*-diethyl *O*-(3-chloro-4-methyl-2-oxo-2*H*-1-benzopyran-7-yl)phosphorothioate] is used as an acaricide for the control of the southern cattle tick (*Boophilus microplus*) and the cattle tick (*Boophilus annulatus*) by the Animal and Plant Health Inspection Service, U.S. Department of Agriculture, in its Tick Eradication Program. The Animal and Plant Health Inspection Service dips several hundred thousand cattle annually for tick control along the U.S.-Mexican border in 1 of 42 vats, each of which contains ca. 12,000 liters of a solution of flowable cattle insecticide (42% coumaphos, 58% inert ingredients; Co-Ral [Mobay Corp.]). Recently, a loss of efficacy has been observed in several vats (problem vats) because of accelerated rates of coumaphos degradation. We have previously reported on the degradation of coumaphos in cattle dipping solutions from both problem and nonproblem vats (8a). Under aerobic conditions, coumaphos was degraded in all dip vat solutions, although the rates were variable. Results of experiments with radiolabeled coumaphos demonstrate that the aromatic portion of the molecule is susceptible to mineralization. Enrichment cultures with coumaphos (as Co-Ral) were initiated, and stable consortia that were able to metabolize coumaphos were obtained. We report here on the isolation and characterization of three bacteria (B-1, B-2, and B-3) obtained from enrichment cultures that were capable of metabolizing coumaphos.

MATERIALS AND METHODS

Chemicals. Analytical grade coumaphos, chlorferon (3-chloro-4-methyl-2-oxo-2*H*-1-benzopyran-7-yl), formulation blank (inert ingredients), and [benzo ring-labeled U-^{14}C] coumaphos were gifts from the Animal Health Division, Mobay Corp. (Shawnee, Kans.). [Benzo ring-labeled U-^{14}C] chlorferon was synthesized by hydrolyzing 0.5 μCi of labeled coumaphos in 10 ml of deionized water with 0.9 IU of parathion hydrolase enzyme purified from a *Flavobacterium* sp. (courtesy of Jeffrey Karns, Pesticide Degradation Laboratory, Beltsville Agricultural Research Center, Beltsville, Md.) (8). After the reaction had gone to completion, chlorferon was extracted with ethyl acetate, concentrated, and purified (>99%) by thin-layer chromatography. Coumaphos (as Co-Ral) was a gift from the U.S. Tick Force Headquarters, Animal and Plant Health Inspection Service (Laredo, Tex.).

Diethylthiophosphoric acid (DETP) was synthesized by the following procedure. Equal amounts (0.106 mol) of triethylamine (10.7 g in 250 ml of H_2O; Aldrich Chemical Co., Inc., Milwaukee, Wis.) and chlorodiethylthiophosphate (20 g in 100 ml of H_2O; Aldrich) were combined and heated at 70°C for 6 h (pH 1.0). After the starting materials disappeared, the reaction was cooled, extracted 4 times with methylene chloride, dried over Na_2SO_4, filtered, and concentrated in vacuo. The reaction was quantitative. The infrared spectrum indicated the presence of P—O—H, P—O—C, and P=S bonds at 2,700 (broad), 1,000 (sharp), and 900 (sharp) cm^{-1}, respectively. The mass spectrum showed a parent peak at 170 *m*/*e*.

Isolation and characterization. Enrichment cultures were initiated from the Laredo City (Laredo, Tex.) dip vat solution by using coumaphos (as Co-Ral) as a carbon source. Substrate concentrations were monitored routinely, and when degradation was complete a 10% inoculum transfer was made into fresh mineral salts medium containing ca. 0.1% coumaphos. The mineral salts medium was essentially

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that described by Brunner et al. (2). It consisted of 20 mM potassium phosphate buffer (pH 7.0); $(NH_4)_2SO_4$, 0.5 g/liter; $MgSO_4 \cdot 7H_2O$, 0.2 g/liter; $CaCl_2$, 5.3 mg/liter; $FeSO_4 \cdot 7H_2O$, 2 mg/liter; $MnSO_4 \cdot 5H_2O$, 0.2 mg/liter; $CuSO_4 \cdot 5H_2O$, 0.4 mg/liter; $ZnSO_4 \cdot 7H_2O$, 0.2 mg/liter; H_3BO_3, 30 μg/liter; CuCl, 40 μg/liter; and $NaMoO_4 \cdot 2H_2O$, 40 μg/liter.

Pure cultures were obtained by performing appropriate serial dilutions of the enrichment culture and spread plating them onto diluted nutrient broth (1 g/liter) with 1.6% agar. Colonies were screened for organophosphate hydrolase activity by suspending colonies in microtiter wells containing 300 μl of 100 μg of parathion per ml in 50 mM Tris buffer (pH 8.0). The appearance of a bright yellow color caused by the accumulation of the hydrolysis product *p*-nitrophenol was indicative of the presence of organophosphate hydrolase activity. Organophosphate hydrolase-positive isolates were restreaked for purity.

For substrate range determinations, compounds were tested at 0.1% in mineral salts (plus 50 μg of yeast extract per ml for B-2); growth was assessed by examining cultures for visible turbidity within 10 days. Doubling times (T_2) were determined by using a meter (Klett-Summerson) with sidearm flasks shaken at 180 rpm at 27°C.

Metabolism studies. Resting cell experiments were conducted by growing isolates to the stationary phase in dilute nutrient broth (2 g/liter), centrifuging them at 6,000 rpm for 10 min, and suspending them in mineral salts to give a density of ca. 100 Klett units. Coumaphos (as Co-Ral) was added, and the culture was incubated on a shaker at 180 rpm at 27°C. Additional experiments with B-1 were performed by adding solid chlorferon directly to flasks that were cultured to a density of ca. 120 Klett units with dilute nutrient broth (2 g/liter), 0.1% arginine, or 0.1% tyrosine.

Radiolabeled experiments were conducted by culturing B-1 with 0.1% tyrosine or 0.1% arginine. During the late log phase of growth, 20 ml of culture was aseptically transferred to sterile biometer flasks (1) containing a foam rubber plug in the connection between the culture fluid and the CO_2 trapping solution to trap volatile metabolites. [Benzo ring-labeled U-^{14}C]coumaphos or -chlorferon (ca. 10^5 dpm) in methanol was added earlier to the flasks, and the methanol was allowed to evaporate. Unlabeled coumaphos or chlorferon was added in the solid form. The side arm contained 10 ml of 0.1 N KOH to trap the $^{14}CO_2$. After incubation at 120 rpm and 27°C, 1-ml fractions of KOH were mixed with 10 ml of aqueous scintillation cocktail (Ready-Solv HP; Beckman Instruments, Inc., Fullerton, Calif.) and counted in a scintillation counter (LS 6800; Beckman). Fractions (1.5 ml) of culture solution were transferred to Eppendorf tubes and centrifuged at a high speed in a microcentrifuge. The supernatant was collected and counted as described above. The pellet was suspended, washed three times with mineral salts, and counted as described above.

Inhibition experiments were conducted by culturing B-1 with 0.1% tyrosine or 0.1% arginine or by culturing B-2 and B-3 with 0.1% acetate in sidearm flasks. During the early to mid-log phases of growth, chlorferon was added to the cultures as a solid, and the incubations were continued as described above. Inhibition was monitored either by high-pressure liquid chromatography (HPLC) (substrate disappearance) or turbidity (Klett-Summerson meter).

Analytical methods. For determination of coumaphos, 1 ml of culture was diluted with 9 ml of methanol, shaken vigorously, and centrifuged for 10 min at 2,000 × *g*; and the supernatant was stored at 4°C until it was analyzed. For determination of DETP, chlorferon, α-chloro-β-methyl-2,3,4-trihydroxy-*trans*-cinnamic acid (CMTC), and tyrosine, culture solutions were either filtered through a 0.2-μm-pore-size disposable filter assembly (Acro LC13; Gelman Sciences, Inc., Ann Arbor, Mich.) or transferred to Eppendorf tubes and centrifuged at a high speed in a microcentrifuge; and the supernatant was stored at 4°C until it was analyzed. Coumaphos, DETP, chlorferon, CMTC, and tyrosine were quantified by using an HPLC system (Waters Associates, Inc., Milford, Mass.) consisting of two 6000A pumps, a 721 system controller, a 720 data module, a radial compression module, and a 712 WISP autosampler, with a UV-visible variable wavelength detector (LC-95; The Perkin-Elmer Corp., Norwalk, Conn.) set at 320 nm for the determination of coumaphos or 210 nm for the determination of DETP, chlorferon, CMTC, and tyrosine. Separations were achieved by using a radially compressed cartridge (C-18 Nova-Pak [4 μm]; Waters) with a mobile phase of 80% methanol–20% phosphoric acid (0.75 mM) for coumaphos or 50% methanol–50% phosphoric acid (0.75 mM) for DETP, chlorferon, CMTC, and tyrosine; the flow rate was 2.0 ml/min.

Radiolabeled metabolites were quantified by using an HPLC system (model 42; Gilson) equipped with a 116 programmable UV detector, a 231-401 autosampler, a 202-C fraction collector, and a system controller (PC-AT; IBM). A total of 440 μl of supernatant was injected onto a column (10 mm by 25 cm; 5 μm; octadecylsilane; Axxiom), and samples were collected at 15-s intervals. The mobile phase was 40% methanol–60% phosphoric acid (0.75 mM) at a flow rate of 4.5 ml/min. Samples were counted as described above.

Isolation and identification of CMTC. The B-1 culture solution (500 ml), after growth to the stationary phase in dilute nutruient broth (1 g/liter), was pelleted by centrifugation (6,000 × *g*), and the pellet was suspended in 100 ml of mineral salts with 500 μg of chlorferon per ml. After 3 days of incubation at 27°C, the solution was acidified (pH 2.0) and concentrated with a C-18 Sep Pak column (Waters). The metabolite was purified by semipreparative HPLC by using the HPLC system (Gilson) described above. Separations were achieved by using a gradient mobile phase of 60% methanol–40% phosphoric acid (0.75 mM) (0 to 5 min) that increased linearly over 2 min to 100% methanol; the flow rate was 4.5 ml/min. Fractions containing only the metabolite were pooled, extracted 3 times with methylene chloride, dried over sodium sulfate, and concentrated in vacuo. Mass spectra were obtained by the direct probe technique on a mass spectrometer (model MS25RFA; Kratos), and nuclear magnetic resonance (NMR) spectra were obtained by using a spectrometer (QE-300 NMR; General Electric Co., Palo Alto, Calif.). NMR samples were dissolved in $CDCl_3$, with trimethylsilane used as a internal reference. UV absorption spectra were obtained by using a photodiode array detector (990; Waters) with a controller (NEC APC-III) in line with the HPLC system (Waters) described above.

RESULTS

Isolation and characterization. After enrichment cultures were maintained for ca. 3 months, serial dilutions of the culture solution were performed to yield 20 to 80 colonies per plate. A distinctive colony type (resembling a fried egg) was present which gave a rapid (<5 min) positive organophosphate hydrolase response. This isolate (designated B-1) was restreaked to ensure purity. From a separate enrichment culture, two additional colonies were present which also gave a positive organophosphate hydrolase response (30 to 60 min). These isolates (designated B-2 and B-3) were also restreaked to ensure purity.

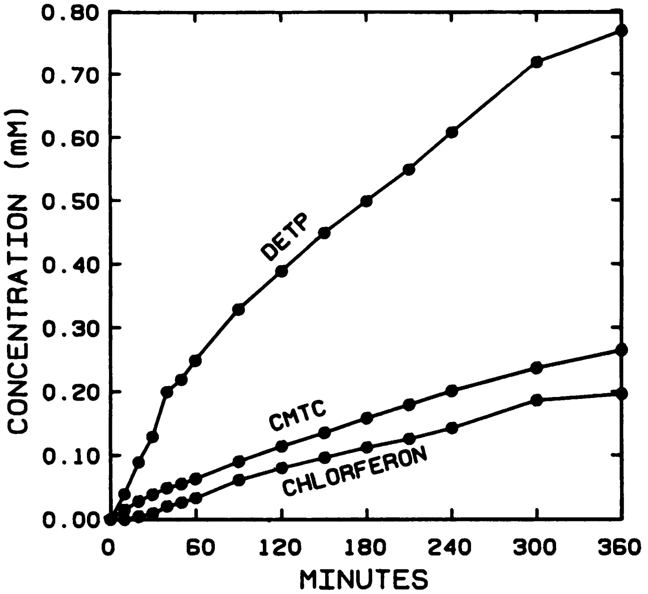

FIG. 1. Metabolism of coumaphos (as Co-Ral) to DETP, chlorferon, and an aromatic metabolite (CMTC) by B-1.

B-1 was a gram-negative, oxidase-negative, strictly aerobic, straight to slightly curved rod which exhibited motility during the mid-log phase of growth. Growth factors were not required. B-1 grew best with nutrient broth (T_2 = 2.3 h), arginine (T_2 = 2.3 h), and acetate (T_2 = 3.7 h). Tyrosine (T_2 = 5.1 h) and threonine supported growth at a moderate rate. Substrates that supported growth, but at a slower rate, were alanine, glutamate, histidine, isoleucine, leucine, β-alanine, lactate, succinate, cytosine, thymine, uracil, and DNA. Substrates that did not support visible growth included formulation blank, coumaphos, chlorferon, 7-hydroxy-4-methyl-coumarin, 7-hydroxycoumarin, the remaining 12 standard amino acids, carbohydrates, and purines.

B-2 was a gram-negative, oxidase-negative, strictly aerobic, nonmotile rod with a tendency to form clumps of cells. B-2 did not grow in the absence of 50 μg of yeast extract per ml. Substrates that supported growth were acetate, glutamate, aspartate, histidine, thymine, cytosine, and uracil. Substrates that did not support visible growth included formulation blank, coumaphos, chlorferon, 7-hydroxy-4-methylcoumarin, the remaining standard amino acids, carbohydrates, and purines.

B-3 was a gram-negative, oxidase-negative strictly aerobic, straight short rod that exhibited motility during the mid-log phase of growth. Growth factors were not required. Substrates that supported growth were acetate (T_2 = 8 h), butyrate, and glutamate. Substrates that did not support visible growth included formulation blank, coumaphos, chlorferon, 7-hydroxy-4-methylcoumarin, the remaining standard amino acids, and carbohydrates.

Metabolism of coumaphos. In resting cell experiments, all three isolates hydrolyzed coumaphos to DETP and chlorferon; B-1 exhibited the fastest rate (Fig. 1). Chlorferon was further metabolized to CMTC by B-1 and B-2, although the yield from B-1 was significantly greater than that observed from B-2. The addition of ca. 450 μg of chlorferon per ml to nutrient broth, arginine, or tyrosine-grown B-1 cells in the late log phase of growth resulted in the production of CMTC after 10 min of incubation. Although the rates of CMTC production varied with growth substrate (tyrosine > nutrient broth > arginine), almost all of the chlorferon was metabolized within 24 h (Table 1).

TABLE 1. Effect of growth substrate on transformation of chlorferon to CMTC by B-1

Time	μg of CMTC per ml (% yield) on the following growth substrate[a]:		
	Nutrient broth	Arginine	Tyrosine
10 min[b]	9 (2)	15 (3)	26 (6)
60 min	105 (23)	35 (8)	150 (33)
24 h[c]	240 (53)	300 (67)	250 (55)

[a] Cell density was normalized to 120 Klett units.

[b] Initial chlorferon concentration, 450 μg/ml.

[c] Final chlorferon concentration, ≤15 μg/ml.

CMTC was isolated and characterized by spectroscopic methods. The NMR spectrum showed a set of doublets at 6.58 and 6.48 ppm (J = 10 Hz) and a singlet at 1.96 ppm corresponding to the aromatic and methyl protons, respectively. Three absorption bands were observed in the UV spectrum at 210, 240, and 310 nm. Ion fragments in the mass spectrum were observed at 226 (228), 210 (212), 192, 184 (186), 182 (184), and 147 *m/e*, which refer to the loss of H_2O; the loss of O and H_2O; the loss of Cl and OH; the loss of CO_2H and CH_3; the loss of H_2O and CO_2; and the loss of H_2O, CO_2, and Cl, respectively (Fig. 2). The base peak was observed at 184 *m/e*.

The mass spectrum indicates the presence of a chlorine atom and the addition of at least one oxygen atom to the chlorferon skeleton. To determine which aromatic proton was oxidized, the aromatic region of the NMR spectrum of CMTC was compared with that of chlorferon (Fig. 3). The H_a of chlorferon was coupled to the proton in its *para* position, H_c, with a coupling constant of 2 Hz and was not coupled to the *meta* proton H_b. H_b, on the other hand, was coupled strongly to its *ortho* proton, H_c, with coupling observed at 9 Hz. Since coupling of the two aromatic protons in CMTC was observed at 10 Hz, one can unequivocably conclude that hydroxylation occurred at H_a.

Another structural question that arises in hydrolysis of the lactone. Unfortunately, no molecule ion peak was observed in the mass spectrum, but the base peak at 184 *m/e* can only be explained if the lactone was hydrolyzed. Further evidence for hydrolysis was found in the UV and NMR spectra. Hydrolysis of the lactone would allow free rotation around bond *a* (Fig. 3), giving rise to the least sterically hindered conformation of CMTC, in which the side chain is orthogonal to the aromatic ring. The methyl group would then be moved out of the plane of the aromatic ring and would not be subject to the anisotropic effects. The methyl protons of chlorferon which are in the plane of the aromatic ring are shifted downfield (2.57 ppm) relative to the methyl protons of CMTC (1.96 ppm). Furthermore, this conformation would force the α,β-unsaturated carbonyl moiety out of conjugation with the aromatic ring, causing a loss of resonance stabilization and a blue shift in the UV absorption of the $n \rightarrow \pi^*$ band. Indeed, this absorption band of 340 nm in chlorferon appears at 310 nm in the spectrum of CMTC.

Yields of chlorferon (B-3) or chlorferon and CMTC (B-2) from coumaphos were consistently stoichiometric. However, yields of CMTC from chlorferon (or coumaphos) by B-1 were consistently less than stoichiometric (29 to 78%), depending on the amount of chlorferon added and the growth substrate. This suggests that additional metabolites are produced by B-1. Experiments with tyrosine- or arginine-grown

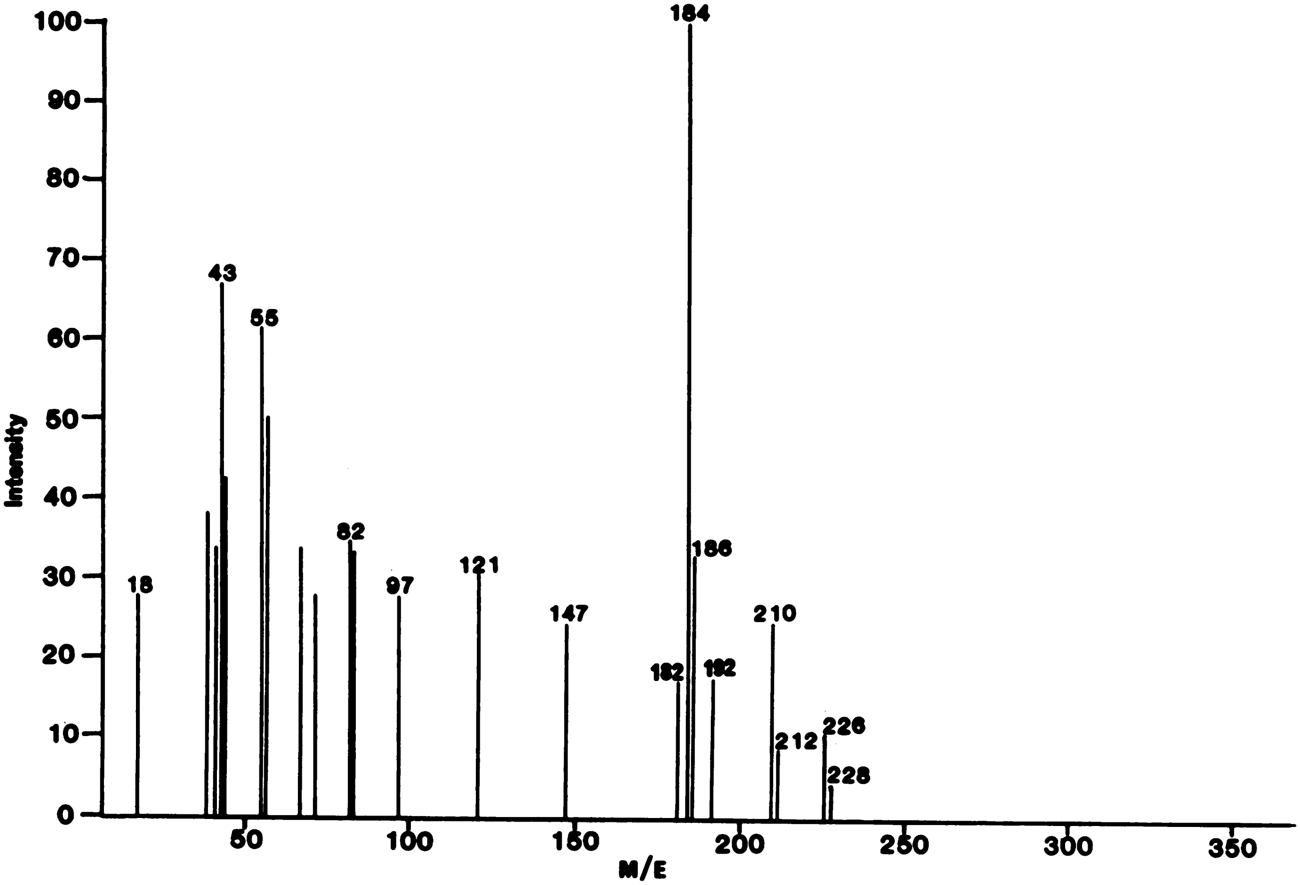

FIG. 2. Mass spectrum of CMTC.

cells in the late log phase of growth incubated with 100 μg of either [benzo ring-labeled U-^{14}C]coumaphos or -chlorferon per ml were conducted to determine the fate of the remaining material. Results were comparable in all four experiments, with a portion of the ring mineralized to CO_2, a portion incorporated into biomass, and the remaining label present in the supernatant (Table 2). The only exception was that a significantly greater proportion of carbon was mineralized in the tyrosine-grown, coumaphos-incubated cells. Quantitative recoveries of label indicated that little, if any, volatile metabolites were produced. The supernatant of the tyrosine-grown cells inoculated with chlorferon was analyzed by HPLC to determine the number and relative amounts of metabolites produced (Fig. 4). The chromatogram showed residual chlorferon, CMTC, and several additional metabolites which eluted at or near the void volume.

These data demonstrate that chlorferon can be used as a carbon source by B-1; however, repeated attempts to grow B-1 on 1,000 μg of chlorferon per ml were unsuccessful. The addition of 400 μg of chlorferon per ml to tyrosine-grown cells in the mid-log phase of growth resulted in the rapid inhibition of tyrosine consumption (Fig. 5). Chlorferon was completely metabolized, with the comcomitant production of CMTC and presumably other metabolites. Dose-response

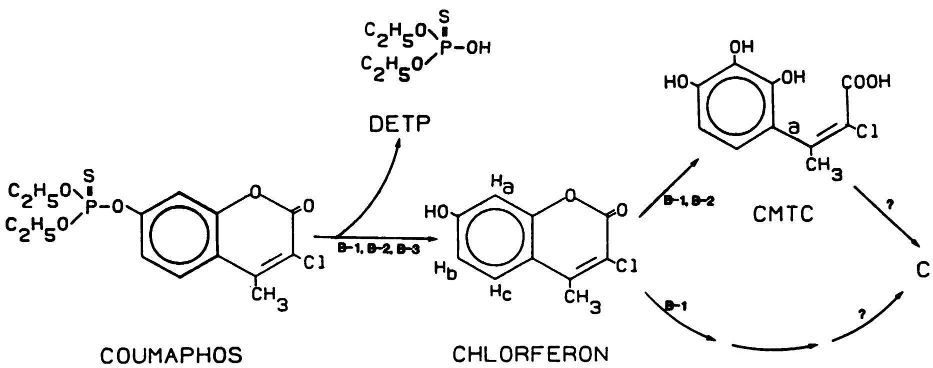

FIG. 3. Initial products of coumaphos metabolism.

TABLE 2. Distribution of radioactivity from metabolism of 100 μg of [benzo ring-labeled U-^{14}C]coumaphos or -chlorferon per ml by B-1

Insecticide and growth substrate	% Radioactivity in:			
	CO_2	Supernatant	Biomass	Total
Coumaphos[a]				
Tyrosine	62	20	12	94
Arginine	23	64	13	100
Chlorferon[b]				
Tyrosine	20	78	10	108
Arginine	25	72	11	108

[a] Incubation was for 72 h.

[b] Incubation was for 18 h.

experiments were conducted with tyrosine- or arginine-grown cells in the early to mid-log phase of growth, and inhibition was monitored turbidimetrically. Data were qualitative because of the tendency of the cells to flocculate with the addition of chlorferon. The addition of 50 or 100 μg of chlorferon per ml resulted in the partial inhibition of growth, although the effect was more severe with tyrosine-grown cells. The addition of 200 μg of chlorferon per ml resulted in the complete inhibition of tyrosine-grown cells and the severe inhibition of arginine-grown cells, while the addition of 400 μg/ml resulted in the complete inhibition of growth with both substrates. In all experiments chlorferon was metabolized to or below the limit of detection (1 μg/ml). Chlorferon also inhibited the growth of B-2 and B-3 on acetate. The addition of 50 μg/ml resulted in a partial inhibition of growth, while 100 μg/ml was completely inhibitory.

DISCUSSION

A variety of bacteria that are capable of degrading organophosphate pesticides have been described in the literature. Sethunathan and Yoshida (8) isolated from rice paddy water a *Flavobacterium* sp. that was able to use diazinon as a sole carbon and energy source and that was able to hydrolyze parathion. Siddaramappa et al. (9) isolated a *Pseudomonas* sp. that was able to hydrolyze parathion and utilize the hydrolysis product *p*-nitrophenol as a carbon and nitrogen source. Daughton and Hsieh (3) isolated a bacterium, *Pseudomonas stutzeri*, from a continuous culture which hydrolyzed parathion; *p*-nitrophenol was metabolized by a separate bacterium. Rosenberg and Alexander (7) described two *Pseudomonas* spp. that were able to hydrolyze a variety of organophosphate compounds and to use the ionic cleavage products as a sole phosphorous source. Nelson (5) isolated numerous *Bacillus* spp. and *Arthrobacter* spp. that were capable of hydrolyzing parathion; one of the *Arthrobacter* strains was also able to utilize *p*-nitrophenol as a sole carbon source. Recently, Racke and Coats (6) isolated from soil a *Pseudomonas* sp. that utilized isophenphos as a sole energy and carbon source.

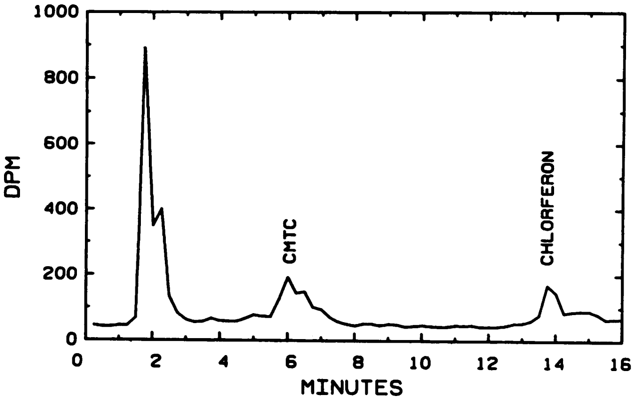

FIG. 4. Chromatogram of the supernatant of tyrosine-grown cells showing the metabolism of radiolabeled chlorferon to CMTC and polar products. Fractions were collected at 15-s intervals.

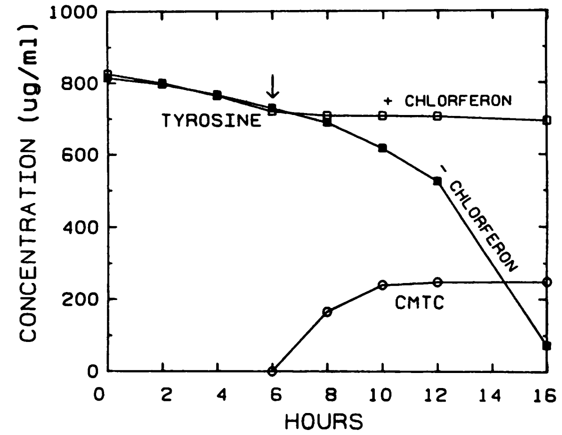

FIG. 5. Inhibition of tyrosine consumption by B-1 as a result of the addition of chlorferon. Chlorferon was added at the time indicated by the arrow, and CMTC appearance was monitored. Symbols: ■, tyrosine (without chlorferon); □, tyrosine (with 400 μg of chlorferon per ml); ○, CMTC.

B-1, B-2, and B-3 are similar to the isolates described above in that their initial mode of attack on coumaphos is hydrolysis of the organophosphate bond. Hydrolysis of coumaphos is not unique; the *Flavobacterium* sp. isolated by Sethunathan and Yoshida (8) has also been shown to hydrolyze coumaphos to DETP and chlorferon (4). The metabolism of chlorferon, however, is unique. Both B-1 and B-2 partially converted chlorferon to CMTC. There was no evidence for further metabolism of chlorferon or CMTC by B-2. B-1 partially mineralized chlorferon, incorporated a portion of the molecule into biomass, and produced several unidentified polar metabolites, in addition to CMTC.

It is not clear whether CMTC was an intermediate in chlorferon mineralization or an end product of a divergent metabolic pathway (Fig. 3). There was no evidence for consumption of CMTC by B-1 in any experiment, suggesting that CMTC may be an end product of chlorferon metabolism. However, the fact that chlorferon or its metabolites were inhibitory to growth of the culture could account for CMTC accumulation in the culture supernatant. Further studies with noninhibitory substrates analogous to chlorferon, as well as determination of the structures of the remaining metabolites, should aid in the elucidation of the chlorferon pathway.

Results of experiments with radiolabeled chlorferon or coumaphos demonstrated that B-1 is capable of using these compounds as a carbon source at low concentrations (100 μg/ml), although visible growth was not observed at higher concentrations (1,000 μg/ml). The addition of 400 μg of chlorferon per ml resulted in the complete inhibition of growth of B-1, while the addition of 100 to 200 μg/ml resulted in partial inhibition, depending on the growth substrate. These data indicate that B-1 has the potential to use coumaphos as a growth substrate but that the accumulation of toxic intermediates is inhibitory to the culture. Since chlorferon was consistently metabolized to below the detection limit (1 μg/ml), it is unlikely that chlorferon itself was responsible for inhibition of growth of B-1; rather, one or more products of chlorferon metabolism were inhibitory. By comparison, chlorferon appeared to be directly responsible for the inhibition of B-2 and B-3.

The observation that chlorferon or its metabolites were inhibitory is particularly interesting with respect to the ecology of the enrichment cultures. Enrichment cultures were maintained over several transfers, with initial coumaphos concentrations of ca. 1,000 μg/ml (equivalent to 580 μg of chlorferon per ml) with no apparent inhibition. Also, high-pressure liquid chromatograms of the enrichment culture did not show the accumulation of CMTC with time (data not shown). There are two possible explanations for this. One, the rate of consumption of coumaphos metabolites by B-1 was greater than or equal to the rate of production; or two, other organisms that were present in the enriched consortium were responsible for the removal of inhibitory metabolites. Our data are not consistent with the first hypothesis. With one exception, experiments with analytical chlorferon, analytical coumaphos, or coumaphos (as Co-Ral) resulted in significant accumulation of soluble products. However, conditions in pure culture experiments were sufficiently different from conditions in the enrichment culture experiments that extrapolations regarding rates of transformation may not be valid.

The more likely explanation for the lack of inhibition in the enriched consortium is that other organisms in the consortium were responsible for the removal of the inhibitory metabolites. The enriched consortium consisted of at least eight bacteria with distinct morphologies, which were present at greater than 10^6/ml, and several eucaryotic microorganisms. This is not surprising, considering that both the formulation blank and DETP are excellent carbon sources. We isolated several of the more dominant bacteria from the enrichment culture and are testing these isolates for their ability to degrade metabolites of coumaphos.

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