APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1988, p. 1414–1419
0099-2240/88/061414-06$02.00/0

Vol. 54, No. 6

Bacterial Metabolism of Carbofuran†

G. RASUL CHAUDHRY* AND A. N. ALI

Institute of Food and Agricultural Sciences, University of Florida, Gainesville, Florida 32611

Received 6 November 1987/Accepted 7 March 1988

Fifteen bacteria capable of degrading carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate) were isolated from soil samples with a history of pesticide application. All isolates were gram negative and were oxidase- and catalase-positive rods; they occurred singly or as short chains. All of the identified isolates belonged to one of two genera, *Pseudomonas* and *Flavobacterium*. They were separated into three groups based on their mode of utilization of carbofuran. Six isolates were placed in group I; these isolates utilized carbofuran as a sole source of nitrogen. Seven isolates were placed in group II; these isolates utilized the pesticide as a sole source of carbon. Isolates of both groups I and II hydrolyzed carbofuran to carbofuran phenol. Two isolates, designated group III, also utilized carbofuran as a sole source of carbon. They degraded the pesticide more rapidly, however, so up to 40% of [^{14}C]carbofuran was lost as $^{14}CO_2$ in 1 h. The results suggest that these isolates degrade carbofuran by utilizing an oxidative pathway.

Carbofuran (2, 3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate) is a pesticide that is widely used in agriculture. It is a potent inhibitor of cholinesterase and, as a result, is highly toxic to mammals, having an oral 50% lethal dose of 2 mg/kg in mice (6). Therefore, it can be potentially hazardous as a result of accidental spills and runoff from areas of application. Also of concern is the general lack of disposal methods for excess carbofuran and other pesticides and for pesticide rinse solution washed off of farm equipment. In addition, several pesticides, including carbofuran, fail to control insects after they are used continuously for a number of years (7, 10, 11, 19, 20), resulting in economic loss from failed crops. This reduction in efficacy could result from the fact that (i) the target insect(s) may develop pesticide resistance analogous to the evolution of antibiotic resistance genes in microorganisms, and thus become insensitive to the pesticide; and (ii) soil microorganisms that repeatedly or continuously encounter synthetic toxic chemicals may develop new capabilities to degrade such chemicals. Many of these toxic chemicals are known mutagens, and some are even carcinogens (15). As such, they may cause changes in the vast and diverse gene pool that is present in soil, influencing gene expression, regulation, and genetic recombination in and among the soil microorganisms. Hence, they may play an important role in the evolution of new traits. Microorganisms with newly evolved traits have been implicated in the rapid inactivation of pesticides in problem soils (7).

It has become increasingly possible to isolate microorganisms that are capable of degrading xenobiotic and recalcitrant compounds from environments polluted with toxic chemicals. Several microorganisms responsible for the metabolism of toluene, xylene, naphthalene, and 2,4-dichlorophenoxyacetate have been isolated and investigated extensively (1, 2, 5, 14, 21, 22). The degradative pathways, their respective genetic determinants, and their regulation in these microorganisms have also been studied (3, 8, 9, 16, 18, 23, 24). Similar knowledge about the microorganisms that are capable of degrading other toxic chemicals, particularly those used as pesticides such as carbofuran, should help to mitigate the problems resulting from their agricultural and nonagricultural use. Although several soil microorganisms have been reported to degrade carbofuran (7, 10, 19, 20), their biochemical pathways for degradation of the pesticide are not well-known.

We isolated and partially characterized several bacteria that were capable of degrading carbofuran. These microorganisms were isolated from various fields in Florida. The isolates utilized carbofuran as a sole source of carbon or nitrogen.

MATERIALS AND METHODS

Chemicals. Analytical grade pesticides and their metabolites (purity, >99.5%) were obtained from the U.S. Environmental Protection Agency, Research Triangle Park, N.C.; FMC Corp., Princeton, N.J.; and Chem Service, Westchester, Pa. [^{14}C]carbofuran (ring-labeled; specific activity, 5 mCi/mmol) was a gift from the International Atomic Energy Agency, Vienna. [^{14}C]carbofuran phenol was obtained by hydrolyzing [^{14}C]carbofuran with NaOH, followed by purification by thin-layer chromatography (TLC). Catechol, sodium gentisate, and sodium benzoate were purchased from Fisher Scientific Co., Orlando, Fla. Methylamine and *o*-nitrophenyl dimethyl carbamate were obtained from Sigma Chemical Co., St. Louis, Mo.

Media and growth conditions. Microbial isolates were maintained on agar plates containing minimal medium (MM). MM contained the following constituents, in grams per liter: K_2HPO_4, 4.8; KH_2PO_4, 1.2; NH_4NO_3, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; $Ca(NO_3)_2 \cdot 4H_2O$, 0.4, and $Fe_2(SO_4)_3$, 0.001. The pH was adjusted to 7.0, and the medium was autoclaved and supplemented with carbofuran or other compounds as the sole carbon source just before inoculation. Cultures were grown at 28°C with shaking in a rotary shaker at 150 rpm. MM containing glucose (MMG) had the same composition as MM except that NH_4NO_3 was excluded, $Ca(NO_3)_2$ was replaced with $CaCl_2$, and 1% glucose was added. This medium was supplemented with carbofuran or other compounds as the only nitrogen source. Luria-Bertoli medium (LB) contained the following, in grams per liter: tryptone, 10; yeast extract, 5; and NaCl, 10. The pH was adjusted to 7.0. Plates were prepared by adding 15 g of agar to 1 liter of the LB medium. To determine the MICs, antibiotics and

* Corresponding author.

† This is a contribution from the Institute of Food and Agricultural Sciences, University of Florida, Gainesville, Fla., Agricultural Experiment Station Journal Series no. 8945.

metals (used as sulfate or chloride salts) were added to the desired growth medium.

Isolation of microorganisms. Soil samples were collected from various locations in Florida that had a previous history of treatment with carbofuran. Another source of these samples was an abandoned site previously used for the disposal of pesticides that were washed off of farm equipment.

To isolate mixed cultures, 5-g soil samples were suspended in 20 ml of MM containing 2 mg of carbofuran in 125-ml culture flasks. The flasks were incubated for 2 to 4 weeks at 20°C. They were then subcultured into fresh MM or MMG containing carbofuran. Subculturing was performed periodically, and the concentrations of carbofuran were gradually increased to 500 and 200 μg/ml in MM and MMG, respectively, during subsequent transfers. The cultures were analyzed for the loss of carbofuran by TLC, as described below. Individual microorganisms were then isolated from these cultures. Freshly grown mixed cultures were serially diluted in the respective MM, and 0.1-ml amounts were spread plated onto MM and MMG agar plates containing carbofuran as the sole source of carbon and nitrogen, respectively. The plates were then incubated at 20, 25, and 30°C and monitored for the appearance of colonies. The viable colonies were patched onto fresh plates containing the same medium and subsequently streaked for the isolation of single colonies. The isolated colonies were then tested for their ability to grow in liquid media containing 1,000 and 200 μg of carbofuran per ml as the only source of carbon and nitrogen, respectively. Two sets of control flasks were incubated simultaneously. One contained the medium and carbofuran and the other contained the medium and the isolate (no carbofuran). The cultures were shaken at 150 rpm for 1 week and checked for an increase in the optical density at 550 nm (OD_{550}) with a spectrophotometer (320; The Perkin-Elmer Corp., Norwalk, Conn.). The isolates that demonstrated growth were selected for further investigation.

Identification and characterization of bacterial isolates. Selected isolates were tested for Gram staining, motility by using the Simms motility test medium, growth on Simmons citrate agar slants, carbohydrate fermentation, and oxidase (1% tetramethyl *p*-phenylenediamine) and catalase (3% hydrogen peroxide). Subsequently, each culture was screened with a test kit (API 20E; Analytical Products, Inc., Plainview, N.Y.). For identification of *Pseudomonas* sp., the method of Stanier et al. (17) was used. Identification of *Flavobacterium* sp. was based on the scheme outlined in *Bergey's Manual of Systematic Bacteriology* (12).

To confirm the degradation of carbofuran, the isolates were grown in MM containing unlabeled carbofuran and [^{14}C]carbofuran (ring-labeled) (0.05 μCi/ml) under normal growth conditions, as described above. The experiments were performed by using sets of 12 culture tubes in duplicate; each tube contained 2 ml of medium. The tubes were inoculated with the microorganisms to an OD_{550} of 0.05 and were incubated at 28°C. Control culture tubes without labeled carbofuran and without inoculum were treated in the same way. At each interval (2 to 4 h), one tube was removed from the incubator; and after the OD of the culture was measured, the contents of the tube were extracted 3 times with chloroform. The extracts were pooled and evaporated to near dryness and dissolved in 100 μl of methanol. A portion of the dissolved extract (1 to 5 μl) was used for scintillation counting. The extracts and the labeled standards (5 to 20 μl), in methanol, were spotted onto TLC plates (Eastman Kodak Co., Rochester, N.Y.) and developed in an ether-hexane (3:1; vol/vol) mixture. ^{14}C-labeled compounds were detected on the thin-layer plates by autoradiography with X-ray film (X-Omat AR; Eastman Kodak). The disappearance of [^{14}C]carbofuran from extract samples was regarded as evidence of pesticide degradation by the isolated microorganisms. In extracts from controls, [^{14}C]carbofuran exhibited identical behavior as the standard on TLC plates, whereas its concentration in the culture supernatant decreased with an increase in the period of incubation. To quantify the transformation of [^{14}C]carbofuran into its metabolites, the spots on the thin-layer plate identified by autoradiography were scraped, eluted with chloroform, and subjected to scintillation counting. Unlabeled carbofuran was also quantified by the same method, except that carbofuran was located by spraying the plate with 1% potassium permanganate (4).

Estimation of $^{14}CO_2$ and identification of metabolites. Selected isolates were grown to the exponential growth phase in their respective media containing carbofuran and were harvested by centrifugation (15,000 × *g* for 5 min). Each cell pellet was washed twice with fresh medium and suspended in 1.5 ml of the same medium at a 20-fold concentration. The cell suspension was then used to inoculate 2.5 ml of MM or MMG containing unlabeled carbofuran (500 or 100 μg/ml as the carbon or nitrogen source, respectively) and labeled carbofuran (0.2 μCi/ml). This was done in a sealable small culture flask (50 ml) which had a replaceable small glass vial (2.5 ml) containing filter paper strips soaked in 1 N NaOH that were used to trap the $^{14}CO_2$. Control flasks, one without cells and one without labeled carbofuran, were maintained. The purity of labeled carbofuran was tested by TLC and autoradiography before carbofuran was used in the experiments. The flasks were then incubated at 28°C in a water bath shaker. At predetermined intervals, 200-μl samples were withdrawn and centrifuged (15,000 × *g* for 10 min). The supernatant and the cell pellet (washed twice with MM) were saved for analysis. At each sampling interval, the vials containing NaOH-soaked filter paper strips in the culture flask were replaced with fresh vials. The filter paper strips were transferred to a scintillation vial, and the glass vial was rinsed twice with scintillation fluid. The rinse solution that was washed off was transferred into a scintillation vial, and after the volume of scintillation fluid was made up to 10 ml, the samples were counted for estimation of $^{14}CO_2$ evolved during the period of incubation. The cell pellet was extracted 3 times with chloroform. The extracts were pooled, evaporated to near dryness, and dissolved in 50 μl of methanol and used for the determination of the internal metabolite pool. The metabolites were identified by TLC and autoradiography as described above. After chloroform extraction the cell pellet was dissolved in 100 μl of 0.1 N NaOH and subjected to scintillation counting for estimation of radioactivity (from the labeled substrate) incorporated into the biomass.

Hydrolase assay. The bacterial isolates were grown to the late logarithmic phase in 100 ml of selective medium (with carbofuran as the sole source of carbon or nitrogen). The cells were harvested by centrifugation (10,000 × *g* for 10 min at 5°C), washed, and suspended in 5 ml of 50 mM potassium phosphate buffer (pH 7.0)–1 mM dithiothreitol–1 mM $MnCl_2$–5% glycerol. The cooled cells were disrupted by sonication (Sonifier cell disruptor; model W140; Heat Systems-Ultrasonics, Inc., Plainview, N.Y.) for 10 min in 30-s bursts, allowing a 15-s cooling time between each burst. The disrupted cell suspension was then centrifuged at 30,000 × *g* at 5°C for 1 h, and the supernatant was used as the cell extract for the hydrolase assay. Assays were carried out by

TABLE 1. Carbofuran-metabolizing bacteria isolated from Florida soils[a]

Group and isolate	Genus	History	Location in Florida
I[b]			
50075	*Pseudomonas*	?[c]	Gainesville
50076	?	[d]	Port St. Lucie
50077	*Flavobacterium*	[e]	Gainesville
50078	*Pseudomonas*	?	Tallahassee
50117	*Flavobacterium*	[d]	Gainesville
50118	*Pseudomonas*	?	Belle Glade
II[f]			
50083	*Pseudomonas*	[e]	Gainesville
50084	?	?	Gainesville
50085	*Flavobacterium*	[e]	Belle Glade
50201	*Pseudomonas*	?	Newberry
50202	*Pseudomonas*	?	Newberry
50454	?	[e]	Gainesville
50456	*Flavobacterium*	[g]	Sanford
II[h]			
50432	*Pseudomonas*	[g]	Sanford
50453	?	[e]	Gainesville

[a] Soil samples were collected from farm sites with or without a known history of carbofuran application.
[b] Carbofuran was utilized as the sole source of nitrogen.
[c] ?, Unknown.
[d] Soil with no known exposure to carbofuran in the past.
[e] Soil with repeated exposure to carbofuran.
[f] Carbofuran was utilized as the sole source of carbon.
[g] Abandoned pesticide waste disposal site.
[h] Carbofuran was utilized as the sole source of carbon and degraded to CO_2.

using cuvettes with a path length of 1 cm at 37°C, and the results are expressed as the means of duplicates. The assay mixture contained 50 mM Tris hydrochloride (pH 8.5), 1 mM $MnCl_2$, 1 mg of *O*-nitrophenyl dimethyl carbamate (substrate) per ml, and the enzyme. The reaction was started by adding the substrate. Controls that lacked either the substrate or the enzyme were used in each set of experiments. Enzyme activity was measured in units, with 1 U being equal to 1 nmol of the substrate hydrolyzed per mg per min. Protein was estimated by the method of Lowry et al. (13).

RESULTS

Sixteen bacterial isolates capable of degrading carbofuran were originally isolated from 11 enrichment cultures. One isolate lost its ability to degrade carbofuran during subculturing and was not investigated further. Six of the isolates utilized carbofuran as a sole source of nitrogen and 9 used the pesticide as a sole source of carbon and energy (Table 1). Soil samples with a history of exposure to carbofuran were consistently positive for the presence of carbofuran-degrading bacteria. Four of five samples obtained from areas with no known history of carbofuran application were found negative for the presence of the pesticide-degrading bacteria by our procedure. These results are in agreement with the hypothesis that the application of pesticides promotes the evolution of microorganisms that are capable of degrading these xenobiotic compounds in the soil.

All isolates were gram-negative, motile rods, occurred singly or as short chains, and were oxidase and catalase positive. A total of 12 isolates hydrolyzed gelatin, and 11 were positive for starch hydrolysis. One of the isolates (50117) produced pink pigments even in rich (LB) medium. This isolate could not be lysed completely, even in Tris

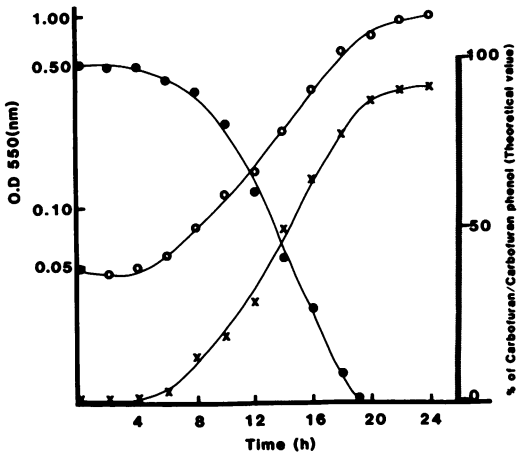

FIG. 1. Growth of isolate 50118 in MMG containing 200 μg of carbofuran per ml as the sole source of nitrogen. The OD_{550} of the culture (○) was measured with a spectrophotometer. Carbofuran (●) and carbofuran phenol (×) concentrations were determined by TLC, as described in the text. The carbofuran concentration is expressed as a percentage of that present at the start of the experiment. The amount of carbofuran phenol was calculated as the percentage of the theoretical amount of carbofuran that would be derived from 200 μg of carbofuran per ml.

buffer (pH 12.5), with heating at 37°C for 30 min. Eight other isolates had pigments ranging in color from yellow to orange. Three isolates (50076, 50078, and 50118) synthesized a large amount of cell wall-associated mucilagenous material. Selected isolates of the three groups were studied for their resistance to antibiotics. They were all found to be resistant to several antibiotics to variable extents. Many microorganisms are known to have developed such resistances in order to survive in such hostile environments as soil. Most of the isolates were tentatively identified as species of either *Pseudomonas* or *Flavobacterium*. Bacteria of these genera are commonly found in sewage, soil, and water and are often responsible for the degradation of xenobiotic compounds in the environment.

The isolated bacteria were sorted into three groups based on their ability to utilize carbofuran. Six isolates were placed in group I; these isolates utilized carbofuran as the sole source of nitrogen and they converted carbofuran into a metabolite similar to carbofuran phenol, as judged by TLC by using authentic compounds as standards. The growth of one such isolate (50118) and its ability to degrade carbofuran are shown in Fig. 1. This isolate slowly hydrolyzed carbofuran up to a concentration of 200 μg/ml and grew optimally at 28 to 30°C. Higher concentrations of carbofuran did not influence the growth rate of the microorganisms, but a considerable amount of the pesticide remained in the spent medium (unpublished observations). Nine other isolates utilized carbofuran as the sole source of carbon (Table 1) and were placed in groups II and III. Seven of these isolates grew slowly and degraded carbofuran to carbofuran phenol, as did members of group I. No additional metabolite could be detected from the culture medium of these isolates, as judged by TLC when labeled carbofuran was used as a substrate. Carbofuran phenol accumulated in the culture medium concurrently with the loss of carbofuran, as shown in Fig. 2 for one of these isolates (50085). Two isolates (50432 and 50453) that degraded carbofuran extensively were placed in group III. The growth of isolate 50432 in MM in the

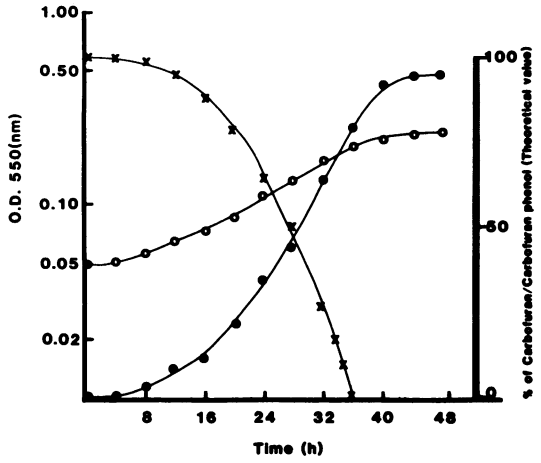

FIG. 2. Growth of isolate 50085 in MM containing 1,000 μg of carbofuran per ml as the sole source of carbon. The OD of the culture (○) and the carbofuran (×) and carbofuran phenol (●) concentrations were measured as described in the legend to Fig. 1.

presence of carbofuran is shown in Fig. 3. Isolate 50432 also utilized carbofuran as the sole carbon source, and no metabolic products were detected. The rate of degradation for this isolate was much faster than those for any of the other isolated bacteria, and it grew poorly in LB medium. The isolates belonging to group I also had a slow growth rate, but the isolates of group II grew rapidly in LB medium.

In order to obtain additional insight into the mechanism of carbofuran degradation by the isolated bacteria, logarithmically growing cultures were concentrated by centrifugation and incubated under controlled conditions in the medium containing labeled carbofuran; the fate of the ^{14}C-labeled pesticide was followed in the various fractions. In the case of isolate 50118, all of the radioactivity was found in the culture medium in the form of carbofuran phenol, as judged by TLC, and no loss of ^{14}C as CO_2 was detected. Similar results were obtained with isolates 50083 and 50085, although some ^{14}C was found in cells that were extracted with chloroform (Table 2). However, the results for isolate 50432 were quite

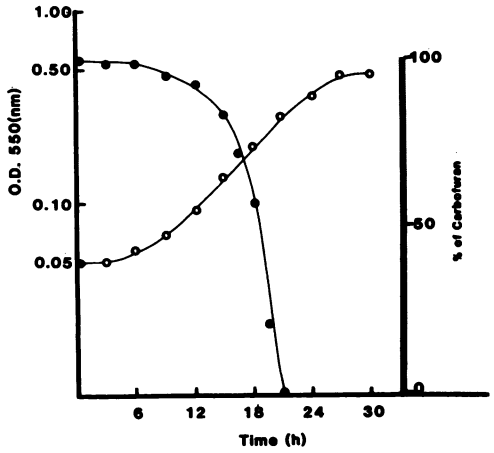

FIG. 3. Growth of isolate 50432 in MM containing 1,000 μg of carbofuran per ml as the sole source of carbon. The OD of the culture (○) and the carbofuran concentration (●) were measured as described in the legend to Fig. 1.

TABLE 2. Distribution of ^{14}C after incubation in isolated bacterial cultures with ring-labeled carbofuran[a]

Isolate	% ^{14}C recovered in the following fractions:				
	Chloroform extractable		Chloroform nonextractable		CO_2
	Medium	Cell	Medium	Cell	
50117	96	<1	<1	<1	<1
50118	95	<1	<1	<1	<1
50083	87	<1	<1	5	<1
50085	85	<1	<1	6	<1
50432	<1	<1	26	32	40

[a] Cultures were grown to the late logarithmic phase in the respective minimal media containing carbofuran, harvested, and suspended in the same minimal media to the desired cell density (OD_{550}, 2). Unlabeled and labeled carbofuran was added to these media. The mixture was incubated in a water bath shaker at 28°C for 2 h. The amount of ^{14}C in CO_2 was trapped in 1 N NaOH. Controls were incubated similarly but contained no cells or labeled carbofuran. At the end of each experiment, cultures were centrifuged and cell pellets were washed twice with MM; this fraction is referred to as cell. The culture supernatant and washings were pooled; this fraction is referred to as medium. These fractions were extracted three times with chloroform; and extracts were pooled, dried, and suspended in 100 μl of methanol. The radioactivity in the various fractions was determined by liquid scintillation counting with a counter (model LS 5801; Beckman Instruments, Inc., Fullerton, Calif.). Results are averages of duplicate experiments.

different. Approximately 40% of the radioactivity was evolved as $^{14}CO_2$ during the course of the experiments (Table 2), and it often occurred over a period of 1 h (Fig. 4). An additional 32% radioactivity was incorporated into the cell biomass, whereas 26% of the ^{14}C remained in the culture medium. No metabolite could be detected by TLC or autoradiography.

Selected isolates grown in LB medium were unable to degrade the pesticide as such. Cells grown in LB medium were washed twice with the respective minimal media and inoculated into the carbofuran-containing medium. They

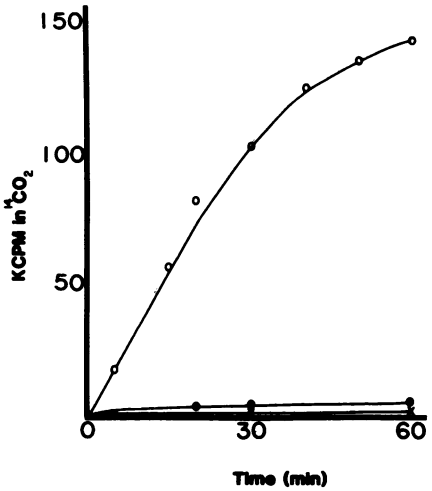

FIG. 4. Release of $^{14}CO_2$ from ^{14}C-labeled carbofuran. Selected strains were grown to the late-logarithmic phase in the respective minimal media containing carbofuran. The cultures were concentrated to the desired OD by centrifugation in the same media containing labeled and unlabeled carbofuran in a sealable culture flask, with provision for trapping the CO_2 in NaOH, as described in the text. The amounts of $^{14}CO_2$ released at various intervals from suspensions of isolates 50432 (○), 50085 (●), and 50118 (×) were determined by scintillation counting.

TABLE 3. Growth of bacterial isolates in minimal medium containing various organic compounds[a]

Compound	Growth of the following bacterial isolates[b]:				
	50117	50118	50083	50085	50432
Carbofuran	+	+	+	+	+
Carbofuran phenol	−	−	−	−	−
3-Ketocarbofuran	+	−	+	+	ND
Carbaryl	+	+	+	+	−
Aldicarb	+	+	+	+	ND
Aldicarb sulfoxide	−	+	ND	ND	ND
Aldicarb sulfone	−	+	ND	ND	ND
Methylamine	+	+	+	+	−
Sodium salicylate	ND	ND	ND	ND	+

[a] The minimal media containing appropriate compounds were inoculated with selected isolates and incubated at 28°C. The OD_{550} of the culture was monitored for 1 week. A set of controls, one without inoculum and the other without either a carbon or a nitrogen source, was treated in the same way.

[b] Abbreviations: +, growth in the presence of the appropriate compound used as the sole source of carbon or nitrogen; −, no growth; ND, not determined.

required several hours of adaptation before they could utilize the pesticide. The lag period for each isolate varied, depending on the length of time for which the cells were grown in LB medium, the number of washings performed, the amount of inoculum, and the concentration of carbofuran used. None of the tested isolates degraded carbofuran when it was included in rich medium (data not shown). This suggests that the degradative enzyme(s) are not constitutive but are induced only in the presence of carbofuran.

The ability of the selected isolates to utilize other carbamates, organic compounds, and known metabolites of carbofuran for growth was also tested, and the results are shown in Table 3. The isolates from groups I and II that were tested utilized aldicarb and its metabolites carbaryl and 3-ketocarbofuran for growth. Isolate 50432 grew in the presence of sodium salicylate (0.1%) as the only carbon source (Table 3). This isolate could not utilize carbofuran phenol for growth, suggesting that carbofuran phenol may not be a metabolite of the degradative pathway in this isolate. If carbofuran is degraded via hydrolysis to carbofuran phenol, as evidenced by the results of the experiments described above, methylamine is the second metabolite in the hydrolytic pathway that could be used as a carbon or a nitrogen source. When carbofuran was replaced with methylamine in the growth media, the members of groups I and II grew equally well. Isolates 50432 and 50453, however, were not able to utilize methylamine as either a carbon or a nitrogen source.

An *Achromobacter* sp. that was capable of utilizing carbofuran as the sole source of nitrogen has been reported to exhibit hydrolase activity (11). Crude cell extracts from the selected isolates were tested for the presence of such enzyme activity. Cell extracts of the tested isolates, except for that of isolate 50432, hydrolyzed *o*-nitrophenyl dimethyl carbamate, an analog of carbofuran (Table 4). This compound was used for the hydrolase assays, as the resulting product *o*-nitrophenyl can be conveniently detected spectrophotometrically. These results indicate that hydrolase activity is greater in isolates that utilize carbofuran as the sole source of carbon than in those that utilize it as the sole source of nitrogen. The hydrolase activity was increased with an increase in pH (maximum at pH 9) and temperature (maximum at 45°C).

TABLE 4. Hydrolase activity of crude extracts from the isolated carbofuran-degrading microorganisms[a]

Isolate	Sp act (U) of enzyme
50083	155
50085	185
50118	94
50432	ND

[a] Enzyme assays were performed by measuring the hydrolysis of *o*-nitrophenyl dimethyl carbamate. The assay mixture contained 20 mM Tris hydrochloride buffer (pH 8.5), 1 mM $MgCl_2$, and 1 mg of the substrate per ml. The reaction was started by adding the enzyme, and the increase in the A_{413} was measured. A blank sample contained the reaction mixture without enzyme. One unit of activity is defined as the hydrolysis of 1 nmol of the substrate in 1 min at 37°C. ND, Not determined.

DISCUSSION

On the basis of the results presented here, we propose that members of groups I and II degrade carbofuran via hydrolysis, whereas those of group III metabolize it via an oxidative pathway (Fig. 5). An *Achromobacter* sp. that is capable of hydroloyzing carbofuran has been reported (11). It would be of interest to elucidate further the proposed oxidative degradative pathway of isolate 50432, which completely and efficiently degrades carbofuran.

As expected, the group I isolates that were tested exhibited hydrolase enzyme activity (Table 4). Likewise, similar enzyme activity was present in group II isolates, which also converted carbofuran to carbofuran phenol but utilized it as a carbon source. It is yet to be determined whether the enzyme(s) and genetic determinant(s) in all these isolates (members of groups I and II) are similar. The isolates belonging to the first two groups were capable of utilizing other carbamate compounds as well (Table 3). This suggests that the degradative enzymes of these isolates are rather nonspecific, as far as the basic molecular structure of the target compound is concerned, and are specific to the carbamate moiety only.

Although carbofuran-degrading microorganisms were found in almost all of the samples collected from soils with a history of carbofuran application, most isolates degraded the pesticide slowly and only to carbofuran phenol. However, we were able to isolate more than one bacterium from some enrichment cultures with differences in morphology, mode of carbofuran utilization, or both. This suggests that a variety of soil microorganisms have developed the capability to degrade carbofuran. More potent isolates (50432 and 50453) that degraded carbofuran rapidly were obtained only

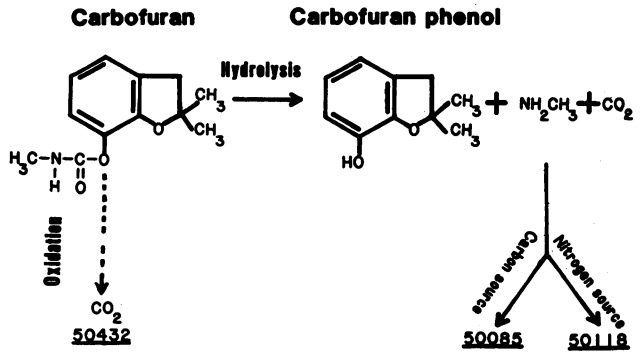

FIG. 5. Proposed pathways for the degradation of the carbofuran used by the microorganisms isolated in this study.

from a pesticide waste disposal site and from a soil sample to which carbofuran had been applied for a number of years (Table 1). This indicates that repeated applications or exposure of soil to xenobiotic compounds for a long period of time can result in the evolution of microorganisms capable of degrading these compounds rapidly and more extensively. Attempts to isolate and characterize microorganisms from problem soils in which carbofuran is rapidly inactivated and to compare them with the microorganisms from normal soils in which carbofuran is effective for the control of pests should be useful. The difference between isolates from normal soils in which carbofuran is effective and problem soils in which the pesticide is rapidly inactivated may be analogous to the difference between the members of groups I and II and group III. Such studies should further our understanding of the enhanced degradation of pesticides in problem soils, as well as further the development of strategies for improving the efficacy of pesticides.

Finally, the group III isolates extensively degraded carbofuran and utilized the pesticide more rapidly than did isolates of groups I and II. Such microorganisms potentially could be used to decontaminate pesticide-polluted sites.

ACKNOWLEDGMENTS

We thank V. Carlisle, V. Waddill, and W. Scudder for help in collecting some of the samples and W. Wheeler, D. Hubbell, and G. Bitton for critically reviewing the manuscript.

This study was supported by grant WM150 from the Florida Department of Environmental Regulation.

LITERATURE CITED

1. **Chatterjee, D. K., S. T. Kellogg, S. Hamada, and A. M. Chakrabarty.** 1981. Plasmid specifying total degradation of 3-chlorobenzoate by a modified ortho pathway. J. Bacteriol. **146**:639–646.
2. **Chaudhry, G. R., A. N. Ali, and W. B. Wheeler.** 1988. Isolation of a methyl parathion-degrading *Pseudomonas* sp. that possesses DNA homologous to the *opd* gene from a *Flavobacterium* sp. Appl. Environ. Microbiol. **54**:288–293.
3. **Don, R. H., and J. M. Pemberton.** 1985. Genetic and physical map of the 2,4-dichlorophenoxy acetic acid-degradative plasmid pJP4. J. Bacteriol. **161**:466–468.
4. **Dorough, H. W.** 1968. Metabolism of Furadan (NIA-10241) in rats and house flies. J. Agric. Food Chem. **16**:319–325.
5. **Dunn, N. W., and I. C. Gunsalus.** 1973. Transmissible plasmid coding early enzymes of naphthalene oxidation in *Pseudomonas putida*. J. Bacteriol. **114**:974–979.
6. **Fahmy, M. A., T. R. Fukuto, R. O. Myers, and R. B. March.** 1970. The selective toxicity of N-phosphorothioylcarbamate esters. J. Agric. Food Chem. **18**:793–796.
7. **Felsot, A., J. V. Maddox, and W. Bruce.** 1981. Enhanced microbial degradation of RA 565-A1BB carbofuran in soils with histories of carbofuran use. Bull. Environ. Contam. Toxicol. **26**:781–788.
8. **Inouye, S., A. Nakazawa, and T. Nakazawa.** 1986. Nucleotide sequence of the regulatory gene *xylS* on the *Pseudomonas putida* TOL plasmid and the identification of the protein product. Gene **44**:235–242.
9. **Inouye, S., A. Nakazawa, and T. Nakazawa.** 1987. Overproduction of the *xylS* gene product and activation on the *xylDLEGF* operon on the TOL plasmid. J. Bacteriol. **169**:3587–3592.
10. **Kandasaym, D., K. Chendrayan, K. Rajukkannu, and M. Balasubramanian.** 1977. On the variations in the degradation of carbofuran by three soil fungi. Curr. Sci. **46**:280–281.
11. **Karns, J. S., W. W. Mulbry, J. O. Nelson, and P. C. Kearney.** 1986. Metabolism of carbofuran by a pure bacterial culture. Pest. Biochem. Physiol. **25**:211–217.
12. **Krieg, N. R., and J. G. Holt (ed.).** 1984. Bergey's manual of systematic bacteriology. The Williams & Wilkins Co., Baltimore.
13. **Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurements with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
14. **Pemberton, J. M., and P. R. Fisher.** 1977. 2,4-D plasmids and persistence. Nature (London) **268**:732–733.
15. **Searle, C. E. (ed.).** 1984. Chemical carcinogens. American Chemical Society, Washington, D.C.
16. **Spooner, R. A., K. Lindsay, and F. C. H. Franklin.** 1986. Genetic functional and sequence analysis of the *xylR* and *xylS* genes of the TOL plasmid pWWO. J. Gen. Microbiol. **132**:1347–1358.
17. **Stanier, R. Y., N. J. Palleroni, and M. Doudoroff.** 1966. The aerobic *Pseudomonas*: a taxonomic study. J. Gen. Microbiol. **43**:159–271.
18. **Streber, W. R., K. N. Timmis, and M. H. Zenk.** 1987. Analysis, cloning, and high-level expression of 2,4-dichlorophenoxyacetate monooxygenase gene *tfdA* of *Alcaligenes eutrophus* JMP134. J. Bacteriol. **169**:2950–2955.
19. **Venkateswarlu, K., T. K. Siddarame Gowda, and N. Sethunathan.** 1977. Persistence and biodegradation of carbofuran in flooded soils. J. Agric. Food Chem. **25**:533–536.
20. **Williams, I. H., H. S. Pepin, and M. J. Brown.** 1976. Degradation of carbofuran by soil microorganisms. Bull. Environ. Contam. Toxicol. **15**:244–248.
21. **Williams, P. A., and K. Murray.** 1974. Metabolism of benzoate and the methylbenzoate by *Pseudomonas putida* (*arvilla*) mt-2: evidence for the existence of a TOL plasmid. J. Bacteriol. **120**:416–423.
22. **Wong, C. L., and N. W. Dunn.** 1974. Transmissible plasmid coding for the degradation of benzoate and m-toluate in *Pseudomonas putida*. Genet. Res. **23**:227–232.
23. **Worsey, M. J., F. C. H. Franklin, and P. A. Williams.** 1978. Regulation of the degradative pathway enzymes coded for by the TOL plasmid (pWWO) from *Pseudomonas putida* mt-2. J. Bacteriol. **134**:757–764.
24. **Worsey, M. J., and P. A. Williams.** 1975. Metabolism of toluene and xylene by *Pseudomonas putida* (arvilla) mt-2: evidence for a new function of the TOL plasmid. J. Bacteriol. **124**:7–13.