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Ring Hydroxylation of *N*-Methylcarbamate Insecticides by *Rhodococcus* TE1[†]

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Rhodococcus TE1, a soil bacterial isolate shown previously to metabolize several thiocarbamate herbicides, organophosphorus insecticides, and *s*-triazine herbicides, was found to degrade carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl *N*-methylcarbamate), propoxur (2-isopropoxyphenyl methylcarbamate), and carbaryl (1-naphthyl methylcarbamate) in a defined growth medium. The insecticides were cometabolized, serving neither as a carbon nor as a nitrogen source for growth. Oxygen was required for metabolism of the insecticides. Carbofuran, carbaryl, and propoxur were each converted to hydrophilic end-products 16 mass units larger in size than the respective parent compounds. On the basis of NMR and GC–MS analysis, the end-product of carbofuran metabolism was identified as 5-hydroxycarbofuran. Hydroxylation of the aryl *N*-methylcarbamate insecticides was associated with a 77 kb plasmid whose loss resulted in the inability to metabolize these compounds. Given the ubiquity of the rhodococci in soils, and the variety of pesticides degraded by strain TE1, it is likely that these Gram-positive bacteria contribute significantly to the dissipation of pesticides in the environment.

INTRODUCTION

Carbofuran is widely used as a broad-spectrum insecticide in agriculture. The fate of the insecticide in soil has been studied intensively since it is one of a number of pesticides subject to enhanced or accelerated degradation following repeated applications to soil (Felsot et al., 1981; Rajagopal et al., 1984; Harris et al., 1984; Camper et al., 1987; Felsot, 1989; Turco and Konopka, 1990). A number of microorganisms able to metabolize carbofuran have been described including several *Pseudomonas* and *Flavobacterium* strains (Chaudhry and Ali, 1988), an *Achromobacter* sp. (Karns et al., 1986), an *Arthrobacter* sp. (Ramanand et al., 1991), and an unidentified Gram-negative methylotroph (Topp et al., 1993). Carbofuran metabolism is most frequently initiated by hydrolysis of the labile carbamate linkage (Chaudhry and Ali, 1988; Karns et al., 1986; Topp et al., 1993), producing carbon dioxide, methylamine, and 7-phenol carbofuran. Growth on carbofuran is usually at the expense of the methylamine hydrolysis product, although in some cases the 7-phenol carbofuran is metabolized as well (Chapalamadugu and Chaudhry, 1992). The carbofuran hydrolase enzyme from *Achromobacter* WM111 has been purified and characterized (Karns and Tomasek, 1991), and the plasmid-borne methylcarbamate degradation (*mcd*) gene encoding it has been cloned (Tomasek and Karns, 1989). Although the carbofuran hydrolase carrying plasmid of WM111 is found in another carbofuran-degrading bacterium, strain ER2 (Topp et al., 1993), the *mcd* gene is not universally conserved among carbofuran-hydrolyzing bacteria since a fragment cloned from it did not hybridize to DNA isolated from various carbofuran-degrading isolates (Chapalamadugu and Chaudhry, 1992).

A number of hydroxylated carbofuran metabolites produced in soils and in bacterial cultures have been isolated and identified, indicating that hydrolysis is not the only mechanism of degradation. These include 3-hydroxycarbofuran, 3-hydroxycarbofuran 7-phenol, 3-ketocarbofuran, and 3-ketocarbofuran 7-phenol (Camper et al., 1987; Chaudhry and Ali, 1988).

Rhodococcus strains are ubiquitous in the environment and have diverse biodegradative capabilities, breaking down a variety of alkanes, halogenated aliphatics and aromatics, and other xenobiotic pollutants (Cain, 1981; Peczynska-Czoch and Mordarski, 1983; Finnerty, 1992). We previously reported the isolation of *Rhodococcus* TE1 from a soil subject to enhanced degradation of the herbicide EPTC (Tam et al., 1987). This organism degrades a variety of thiocarbamate herbicides, organophosphorus insecticides, and *s*-triazine herbicides including atrazine (Tam et al., 1988; Behki, 1991; Behki and Khan, 1990, 1991; Behki et al., 1993). The degradation of the herbicides EPTC and atrazine was found to be associated with a 77 kb plasmid (Tam et al., 1987; Behki et al., 1993). The purpose of the study reported here was to determine if the organism could also degrade the *N*-methylcarbamate insecticides.

MATERIALS AND METHODS

Growth and Maintenance of Bacteria. The *Rhodococcus* strains used in this study are listed in Table 1. Bacteria were routinely grown at 30 °C with shaking in basal salts medium (BMN; Behki and Khan, 1986) adjusted to pH 6.8 and supplemented with 1 mg/mL glucose or glycerol (BMNG) as previously described (Tam et al., 1987). Growth was measured by the increase in optical density at 600 nm.

Metabolic Studies. The ability of the bacterium to degrade the *N*-methylcarbamate insecticides was tested in batch cultures consisting of 20 mL of BMNG or BMNGY (BMNG supplemented with 1 mg/mL yeast extract) incubated in 100-mL flasks with agitation at 30 °C. Cell-free controls to account for abiological transformations were included with all incubations. Samples (1 mL) were periodically taken from the batch culture and the bacteria sedimented by centrifugation in a microfuge (12000*g*, 10 min). Cell-free supernatants were analyzed for residual parent compound and metabolites by HPLC as described below. Possible anaerobic metabolism of carbofuran was tested under an atmosphere of helium as previously described (Behki et al., 1993). Possible induction of carbofuran-degrading activity was tested with resting cell suspensions prepared from cultures of *Rhodococcus* TE1 cells grown in BMNG in the presence or

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Table 1. ***Rhodococcus* TE1 and Related Strains**

strain	plasmids (kb sizes)	reference or origin
TE1	100, 91, 77, 3.8	Tam et al., 1987
TE3	100, 91, 3.8	Tam et al., 1987; cured derivative of TE1
TE7	100, 3.8	Tam et al., 1987; cured derivative of TE1
TE10	100, 91, 77, 3.8	Tam et al., 1987; transconjugant TE1 × TE3
TE12	100, 77, 3.8	Tam et al., 1987; transconjugant TE1 × TE7
TE13–TE18	100, 91, 3.8	Behki et al., 1993; spontaneous cured derivatives of TE1

absence of 50 mg/L carbofuran. Cells were harvested from the mid-logarithmic phase of growth, washed, and resuspended in fresh BMNG medium containing 50 mg/L carbofuran in the presence or absence of 50 mg/L chloramphenicol to block protein synthesis (Behki et al., 1993). The degradation of EPTC was determined in BMNG medium containing 60 mg/L EPTC as previously described (Tam et al., 1987).

Chemicals and Analytical Methods. Pesticides and metabolites were purchased from Chem Service (Westchester, PA). Uniformly aromatic ^{14}C-ring-labeled carbofuran (16.7 mCi/mmol, >98% pure) was purchased from Sigma Chemical Co. (St. Louis, MO). Pesticide stock solutions were prepared in acetone and dispensed into containers, and the solvent was evaporated under a stream of sterile air before growth media or cell suspensions were added.

Residual pesticides and metabolites were identified and quantified in cell-free aqueous supernatants by isocratic reversed-phase HPLC analysis as previously described (Topp and Akhtar, 1990). Ethyl ether extracts of spent culture fluid were prepared and analyzed by gas chromatography–mass spectrometry (GC–MS) according to the method of Topp and Akhtar (1990). Metabolites were purified by thin-layer chromatography (TLC) according to the method of Topp et al. (1993) for nuclear magnetic resonance (NMR) analysis. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AM 500-MHz NMR spectrometer at 500.13 and 125.78 MHz, respectively. Chemical shifts are referenced with respect to deuteriomethanol (CD_3OD) at 3.33 (^{1}H) and 49.0 ppm (^{13}C) and are reported relative to tetramethylsilane (TMS). Chemical shift assignments were made via homonuclear decoupling, DEPT, and $^{1}H/^{13}C$ correlation experiments and by consideration of substituent effects. Ethyl ether extracts prepared from cultures incubated with radiolabeled carbofuran were analyzed by HPLC coupled to a radioactivity monitor (Model LB504; Berthold Instruments, Pittsburgh, PA).

RESULTS AND DISCUSSION

Degradation of Carbofuran. *Rhodococcus* TE1 degraded carbofuran when incubated in BMNG or BMNGY medium supplemented with 50 mg/L of the insecticide (Figure 1). Carbofuran was degraded by *R.*TE1 in all phases of cell growth, and the degradation was largely dependent upon cell density in the incubation medium. However, up to 150 mg/L carbofuran as the sole source of carbon in BMN medium did not support the growth of the organism (data not shown). Likewise, a basal medium consisting of BMNG modified by omitting the inorganic nitrogen source and supplemented with 150 mg/L carbofuran as the sole nitrogen source did not support the growth of strain TE1. There was no carbofuran degradation when *Rhodococcus* TE1 was incubated under an atmosphere of helium, indicating that the transformation reaction required oxygen.

Identification of Carbofuran Metabolite. HPLC analysis of supernatants from cultures of *Rhodococcus* TE1 grown in BMNG medium supplemented with 50 mg/L carbofuran revealed that the disappearance of carbofuran (retention time, 4.0 min) was accompanied by the accumulation of a more hydrophilic metabolite with a

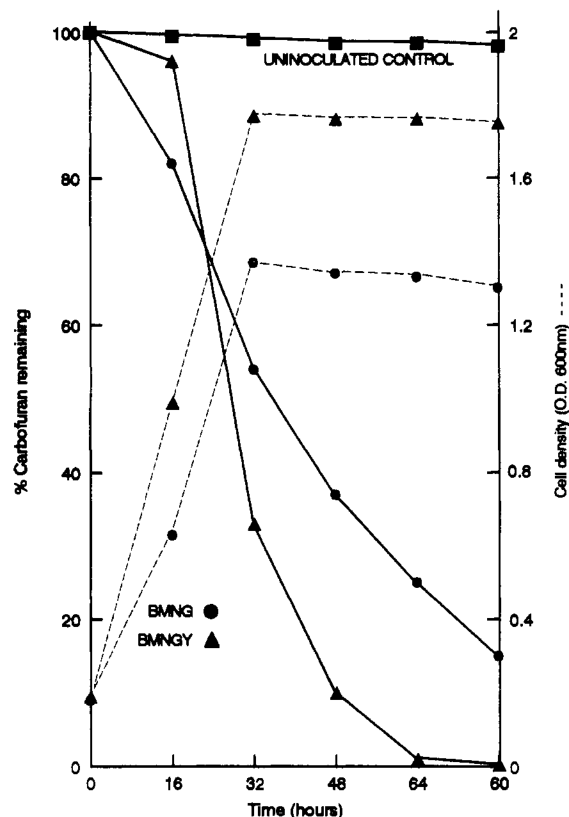


Figure 1. Degradation of carbofuran (50 μg/mL) with time and cell growth by *Rhodococcus* TE1 in BMNG and BMNGY media.

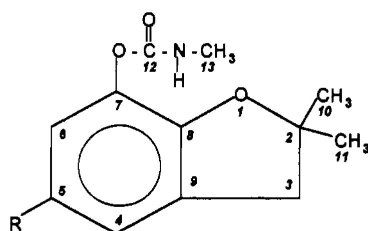
retention time of 3.3 min. No other metabolites were observed. Ring-labeled carbofuran was not mineralized nor was carbofuran–carbon assimilated into sedimented cells. At the end of a 96-h incubation, 93% of the radioactivity in culture supernantants was extractable into ethyl ether. Of this, 89% comigrated with the hydrophilic metabolite when analyzed by HPLC radioactivity detector. GC–MS analysis of ethyl ether extracts prepared from spent culture fluid following carbofuran degradation revealed a compound (retention time, 9.2 min) with a molecular weight of 237 (M^+), 16 mass units greater than that of carbofuran (Table 2). The fragmentation pattern had all major peaks 16 mass units larger than the corresponding fragments in the mass spectrum of carbofuran. The fragmentation pattern of the metabolite was also quite different from that of a 3-hydroxycarbofuran standard which had peaks at m/z 237 (M^+), 180, 162, 147, and 137 (base peak). A comparison of the two spectral data strongly suggested that hydroxylation by *R.*TE1 occurred at the aromatic ring rather than at the furyl ring. This was supported by ^{1}H and ^{13}C NMR analysis as described below.

The aryl *N*-methylcarbamate insecticides carbaryl and propoxur were also transformed by *Rhodococcus* TE1. GC–MS analysis of the metabolites indicated that, as with carbofuran, in both cases the benzene ring was substituted with a single hydroxyl group (Table 2). However, the metabolites were not subjected to NMR analysis, and the location of the substituent was not determined.

NMR Analysis of Carbofuran Metabolite. Comparison of the ^{1}H NMR spectrum of the metabolite with that of carbofuran showed that the three adjacent aromatic protons at 6.98, 6.82, and 6.75 ppm were replaced by two aromatic protons at 6.32 and 6.49 ppm whose resonances showed coupling typical of meta protons (2.4 Hz). This indicates that the hydroxyl substitution occurred at position C-5 of the benzene ring. This conclusion was

Table 2. HPLC Retention Times, GC Retention Times, and Mass Spectral Data of *N*-Methylcarbamate Insecticides Transformed by *Rhodococcus* TE1

	parent compound			end-product		
substrate	HPLC rt	GC rt	mass spectral data (rel intensity)	HPLC rt	GC rt	mass spectral data (rel intensity)
carbofuran	4.0	6.3	221 (M^+, 3), 164 (100), 149 (65), 131 (20), 122 (50), 103 (10), 91 (10), 77 (9)	3.3	9.2	237 (M^+,15), 180 (100), 165 (50), 147 (8), 138 (24)
carbaryl	4.2	5.3	201 (M^+, 3), 144 (100), 127 (8), 115 (70), 89 (10)	3.9	7.4	217 (M^+, 16), 160 (100), 143 (4), 131 (59), 115 (8)
propoxur	4.0	3.3	209 (M^+, 7), 168 (3), 153 (38), 152 (88), 137 (18), 111 (100), 109 (60), 91 (8), 81 (87), 65 (40), 63 (53)	3.6	5.5	225 (M^+, 49), 168 (100), 149 (21), 127 (91), 125 (81), 97 (53), 80 (23), 58 (34)



Compound	R =
1	H
2	OH

Figure 2. Structure of carbofuran (compound 1) and the hydroxy metabolite (compound 2).

Table 3. ^{1}H and ^{13}C NMR Chemical Shift Assignments (Parts per Million from TMS)

position	compd 1 (Figure 2)		compd 2 (Figure 2)	
no	^{1}H	^{13}C	^{1}H	^{13}C
2		89.2		88.6
3	3.04 (2H, s)	43.9	2.95 (2H, s)	43.8
4	6.82 (1H, br d, $J_{4,5}$ = 8.0 Hz)	122.7	6.32 (1H, d, $J_{4,6}$ = 2.4 Hz)	109.5
5	6.75 (1H, dd, $J_{4,5}$ = 8.0 Hz, $J_{5,6}$ = 7.4 Hz)	121.2		152.0
6	6.98 (1H, dd, $J_{6,5}$ = 7.4 Hz, $J_{6,4}$ = 1.2 Hz)	123.3	6.49 (1H, d, $J_{4,6}$ = 2.4 Hz)	110.5
7		136.2		135.6
8		151.8		144.9
9		131.0		131.1
10, 11	1.44 (6H, s)	28.2	1.40 (6H, s)	28.2
12		157.3		157.2
13	2.77 (3H, s)	27.7	2.76 (3H, s)	27.7

confirmed by the ^{13}C NMR spectrum of the metabolite. The ^{13}C resonances associated with these two aromatic protons were shifted upfield from 122.7 and 123.3 ppm in carbofuran to 109.5 and 110.5 ppm in the metabolite, values typical of the ortho carbons in a phenol. Furthermore, the resonance due to C-5 of carbofuran (121.2 ppm) was shifted downfield to 152.0 ppm, typical of direct OH substitution, and that of C-8 was slightly shifted due to the para effect of the OH. All other resonances in the metabolite were similar to those in carbofuran, indicating that the heterocyclic ring was unmodified. These data are consistent with the assigned structure in Figure 2. The ^{1}H and ^{13}C chemical shift assignments are summarized in Table 3.

Effect of Carbon Sources on Carbofuran Biotransformation. Various carbon sources were tested for their effects on the kinetics of carbofuran degradation by *Rhodococcus* TE1. Carbon sources that supported growth, such as glucose and glycerol, accelerated carbofuran degradation, at least in part because they supported the growth of more biomass. Growth on organic acids such as succinate and acetate was accompanied by alkalinization of the media, and consequently chemical hydrolysis of carbofuran to 7-phenol carbofuran occurred. Mannitol completely repressed carbofuran hydroxylation, presumably by catabolite repression.

Expression of carbofuran degradation activity by *Rhodococcus* TE1 did not require the insecticide as an inducer. Cells harvested from cultures grown overnight in BMNG degraded carbofuran in the presence of 50 mg/L chloramphenicol at the same rate as cells grown under identical conditions but in BMNG supplemented with 50 mg/L carbofuran. *Rhodococcus* TE1 grown overnight in BMNG supplemented with 50 μg/mL carbofuran transformed carbofuran at the same rate as the bacteria grown only in BMNG, indicating that the hydroxylation process is constitutive. The oxidation process, therefore, is not inducible, unlike the results reported with *Pseudomonas* 50432 (Mateen et al., 1993).

Involvement of a Plasmid in Carbofuran Hydroxylation. The 77 kb plasmid previously observed to be required for EPTC and atrazine degradation (Tam et al., 1987; Behki et al., 1993) is also involved in carbofuran hydroxylation. Strains that had been cured of the plasmid (TE3, TE7, and TE13–18) (Behki et al., 1993) were unable to transform carbofuran. Cured derivatives that had reacquired the plasmid by conjugation with TE1, strains TE10 and TE12 (Table 1) (Behki et al., 1993), also reacquired the ability to hydroxylate carbofuran.

In summary, under aerobic conditions *Rhodococcus* TE1 degraded the aryl *N*-methylcarbamate insecticides carbofuran, carbaryl, and propoxur. The end-product of metabolism of these compounds was a ring-hydroxylated derivative, in the case of carbofuran metabolism, 5-hydroxycarbofuran. This is the first report of hydroxylation of carbofuran at C-5 by a bacterial species. Carbofuran was not utilized as either a carbon or nitrogen source. Alternate carbon sources that supported the growth of strain TE1 accelerated degradation, with the exception of mannitol, which repressed the hydroxylation activity. Finally, a 77 kb plasmid was required for methylcarbamate transformation. Given the ubiquity of rhodococci in soils, it is probable that they contribute to the degradation of carbofuran *in situ*.

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