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Cloning of a Carbofuran Hydrolase Gene from *Achromobacter* sp. Strain WM111 and Its Expression in Gram-Negative Bacteria

PAUL H. TOMASEK† AND JEFFREY S. KARNS*

Pesticide Degradation Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland 20705

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A 14-kilobase-pair (kbp) *Eco*RI DNA fragment that encodes an enzyme capable of rapid hydrolysis of *N*-methylcarbamate insecticides (carbofuran hydrolase) was cloned from carbofuran-degrading *Achromobacter* sp. strain WM111. When used to probe Southern blots containing plasmid and total DNAs from WM111, this 14-kbp fragment hybridized strongly to a 14-kbp *Eco*RI fragment from the >100-kbp plasmid harbored by this strain but weakly to *Eco*RI-digested total DNA from *Achromobacter* sp. strain WM111, indicating that the gene for *N*-methylcarbamate degradation (*mcd*) is plasmid encoded. Further subcloning localized the *mcd* gene on a 3-kbp *Sca*I-*Cla*I fragment. There was little or no expression of this gene in the alternative gram-negative hosts *Pseudomonas putida*, *Alcaligenes eutrophus*, *Acinetobacter calcoaceticus*, and *Achromobacter pestifer*. Western blotting (immunoblotting) of the protein products produced by low-level expression in *P. putida* confirmed that this 3-kbp fragment encodes the two 70+-kilodalton protein products seen in sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified carbofuran hydrolase.

Microorganisms possess a vast array of enzymatic capabilities that contribute to their major role in biogeochemical cycling of simple and complex chemical compounds. Many organic materials are mineralized by microorganisms, with the resulting products (CO_2, CH_4, NH_4^+, etc.) released for use by other organisms. Man-made organic chemicals, such as the pesticides and plastics developed during the 20th century, have challenged the degradative abilities of microorganisms. There is evidence that continued evolution of microbial genomes has endowed some microorganisms with the ability to degrade various novel or recalcitrant compounds. Hydrocarbons such as naphthalene (14) and toluene (31), as well as man-made halogenated compounds such as chlorophenols (22, 29), chlorophenoxyacetates (12, 25), and haloalkanes (28), are readily degraded by certain strains of bacteria. Although the sources of new genes encoding these unique degradative properties are largely unknown, plasmids are frequently implicated in the dissemination of biodegradation genes (1, 4, 8, 13, 18). Our laboratory is particularly interested in the role plasmids play in the degradation of xenobiotic compounds used as pesticides.

Accelerated microbial degradation of soil-incorporated pesticides in so-called problem or aggressive soils compromises the ability of pesticides to control important agricultural pests (24). Detailed knowledge of the molecular and ecological factors involved in the formation of problem soils is lacking. However, it is likely that bacterial plasmids play a significant role in the evolution and dissemination of certain biodegradation genes within the soil microflora. Thus, it is possible that repeated pesticide application may cause an increase in the number of organisms carrying a particular biodegradation plasmid, resulting in reduced pesticide effectiveness. The soil-incorporated insecticide carbofuran is one compound whose effectiveness has been diminished in several problem-soil areas (2, 15, 19). Carbofuran is used to control various insects, including the corn rootworm (*Diabrotica* sp.), a persistent and destructive pest of corn.

This laboratory previously reported the isolation and characterization of *Achromobacter* sp. strain WM111, which can rapidly hydrolyze the *N*-methylcarbamate linkage of carbofuran while utilizing the compound as a source of nitrogen (23). An enzyme capable of hydrolyzing a wide range of *N*-methylcarbamate insecticides, including carbaryl and aldicarb, was partially purified from this organism (9). Recently, this enzyme was purified to homogeneity (J. S. Karns and P. H. Tomasek, submitted for publication). We now report the molecular cloning of the gene that encodes this *N*-methylcarbamate hydrolase (carbofuran hydrolase [CH]) and the use of the cloned methylcarbamate degradation (*mcd*) gene to confirm its location on the large *Achromobacter* sp. strain WM111 plasmid. We also discuss the expression of the cloned *mcd* gene in various gram-negative bacteria.

MATERIALS AND METHODS

Growth and maintenance of organisms. The strains used are listed in Table 1. Basal salts medium (BSM) was 50 mM potassium phosphate, 15 mM $(NH_4)_2SO_4$, 800 μM $MgSO_4$, 180 μM $CaSO_4$, 8 μM Na_2MoO_4, 5 μM $MnSO_4$, 2 μM $FeSO_4$, and 1 μM each $CoSO_4$, $ZnSO_4$, $Fe_2(SO_4)_3$, $Al(SO_4)_3$, $NiSO_4$, $CuSO_4$, and H_3BO_3. Nitrogen-free BSM (NFB) was BSM without $(NH_4)_2SO_4$. Glucose-supplemented BSM (BSMG) and NFB (NFBG) each contained 0.2% glucose. Solid media were prepared with the addition of 1.5% agar (GIBCO Laboratories, Grand Island, N.Y.) or, in some cases, 1.2% agarose (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). *Achromobacter* sp. strain WM111 was maintained on NFBG agar supplemented with a 200-μg/ml concentration of recrystallized (two times from methanol) carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl-*N*-methylcarbamate; FMC Corp., Princeton, N.J.) at 28°C. For plasmid and CH isolation, the organism was grown in BSMG liquid medium at 28°C. *Escherichia coli* strains were grown on Lennox L broth (LB) (GIBCO) at 37°C. Except where noted, the *Pseudomonas putida* PRS2015R strains were

* Corresponding author.
† Present address: Department of Food Science, Cook College, Rutgers, The State University of New Jersey, New Brunswick, NJ 08903.

TABLE 1. Bacterial strains and plasmids[a]

Strain or plasmid	Characteristics	Reference or source
Bacterial strains		
Achromobacter sp. strain WM111	Carbofuran-degrading soil isolate	23
Achromobacter pestifer		
ATCC 23584	Soil isolate	6
PT297	Rif^r strain of ATCC 23584	This study
Acinetobacter calcoaceticus		
ATCC 33305	Unencapsulated mutant of soil isolate	21
PT298	Rif^r strain of ATCC 33305	This study
Alcaligenes eutrophus		
JMP134	2,4-Dichlorophenoxyacetic acid-degrading strain	12
PT299	Rif^r strain of JMP134	This study
Escherichia coli		
LE392	F$^-$ *hsdR514* (r_k^- m_k^+) *supE44 supF58 lacY1* or Δ(*lacIZY*)*6 galK2 galT22 metB1 trpR55* λ$^-$	30
DH5α	F$^-$ *endA1 hsdR17* (r_k^- m_k^+) *supE44 thi-1 recA1 gyrA96 relA1* φ80 d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 λ$^-$	BRL (*Focus* 8 [#2]:9)
Pseudomonas putida		
PRS2015	*per-1103 catB1123*	30
PRS2105R	Rif^r strain of PRS2015	This study
Plasmids		
pUC18	pMB1 replicon, *lacZ'* Ap^r	32
pUC19	pMB1 replicon, *lacZ'* Ap^r	32
pRK2013	ColE1 replicon, RK2 *tra*$^+$ *mob*$^+$ Km^r	16
pLAFR1	IncP1 (RK2 replicon), *mob*$^+$ *tra* λ *cos*$^+$ Tc^r	17
pVDZ'2	IncP1 (RK2 replicon), *mob*$^+$ *tra lacZ'* Tc^r	10
pPDL11	Plasmid isolated from *Achromobacter* sp. strain WM111; 100 kbp, *mcd*$^+$	This study
pJK6	20.5-kbp partial *Eco*RI-digested insert cloned into the pLAFR1 *Eco*RI site; λ *cos*$^+$ *mcd*$^+$ Tc^r	This study
pJK7	Partial *Eco*RI-digested insert cloned into the pLAFR1 *Eco*RI site; *cos*$^+$ Tc^r	This study
pJK8	Same as pJK7 except *mcd*$^+$	This study
pJK9	Same as pJK8	This study
pJK10	Same as pJK8	This study
pPT300	14-kbp *Eco*RI insert from pJK6 containing the *mcd* gene cloned into the pUC18 *Eco*RI site; Ap^r	This study
pPT301	14-kbp *Eco*RI insert from pPT300 cloned into pVDZ'2; *mcd*$^+$ Tc^r	This study
pPT304	6-kbp *Eco*RI insert from pJK6 cloned into pUC18; Ap^r	This study
pPT305	6-kbp *Eco*RI insert from pPT304 cloned into pVDZ'2; Tc^r	This study
pPT306	0.5-kbp *Eco*RI insert from pJK6 cloned into pUC18; Ap^r	This study
pPT307	0.5-kbp *Eco*RI insert from pPT306 cloned into pVDZ'2; Tc^r	This study
pPT310	7-kbp *Pst*I insert from pPT300 cloned into pUC19; *mcd*$^+$ Ap^r	This study
pPT311	7-kbp *Pst*I insert from pPT310 cloned into pVDZ'2; *mcd*$^+$ Tc^r	This study
pPT320	4.8-kbp *Pst*I-*Sph*I insert from pPT310 cloned into the pUC19 *Pst*I-*Sph*I site; *mcd*$^+$ Ap^r	This study
pPT321	4.8-kbp *Eco*RI-*Hin*dIII insert from pPT320 cloned into the pVDZ'2 *Eco*RI-*Hin*dIII site; *mcd*$^+$ Tc^r	This study
pPT330	4.25-kbp *Sca*I-*Hin*dIII insert from pPT310 cloned into the pUC19 *Sma*I-*Hin*dIII site; *mcd*$^+$ Ap^r	This study
pPT331	4.25-kbp *Eco*RI-*Hin*dIII insert from pPT330 cloned into the pVDZ'2 *Eco*RI-*Hin*dIII site; *mcd*$^+$ Tc^r	This study
pJK340	3.0-kbp *Sca*I-*Cla*I insert from pPT320 cloned into the pUC19 *Sma*I-*Acc*I site; *mcd*$^+$ Ap^r	This study
pJK341	3.0-kbp *Eco*RI-*Hin*dIII insert from pJK340 containing the *Sca*I-*Cla*I fragment cloned into the pVDZ'2 *Eco*RI-*Hin*dIII site; *mcd*$^+$ Tc^r	This study
pJK350	2.0-kbp *Sca*I-*Kpn*I insert from pPT320 cloned into the pUC19 *Sma*I-*Kpn*I site; *mcd* Ap^r	This study
pJK351	2.0-kbp *Eco*RI-*Hin*dIII insert from pJK350 containing the *Sca*I-*Kpn*I fragment cloned into the pVDZ'2 *Eco*RI-*Hin*dIII site; *mcd* Tc^r	This study
pPT360	3.4-kbp *Bgl*II-*Pst*I insert from pPT310 cloned into the pUC19 *Bam*HI-*Pst*I site; *mcd* Ap^r	This study
pPT361	3.4-kbp *Eco*RI-*Hin*dIII insert from pPT360 cloned into the pVDZ'2 *Eco*RI-*Hin*dIII site; *mcd* Tc^r	This study
pPT363	3.5-kbp *Bam*HI insert from pPT310 cloned into the pVDZ'2 *Bam*HI site; *mcd* Tc^r	This study
pPT371	6.5-kbp *Pst*I-*Hin*dIII insert from pPT310 cloned into the pVDZ'2 *Pst*I-*Hin*dIII site; *mcd* Tc^r	This study

[a] Abbreviations: Tc, tetracycline; Ap, ampicillin; Km, kanamycin; Rif, rifampin; BRL, Bethesda Research Laboratories, Inc.

grown on either BSMG or LB at 28°C. *E. coli* and *P. putida* strains containing recombinant plasmids were grown in the presence of ampicillin (100 μg/ml) or tetracycline (30 μg/ml). All liquid cultures were aerated by shaking at 250 rpm.

DNA isolation. WM111 plasmid DNA was isolated by a modified alkaline lysis procedure (3). Cloning vectors and recombinant plasmid DNAs were isolated by the method of Clewell and Helinski (7). Small-scale plasmid preparations

for characterizing recombinant clones were made by either the modified Birnboim-Doly alkaline lysis method (27) or the boiling procedure of Holmes and Quigley (20). Total WM111 DNA was isolated by the method of Deretic et al. (11).

Cosmid cloning. Total DNA or isolated plasmid DNA from WM111 was partially digested at 37°C with *Eco*RI for appropriate lengths of time (established by a trial run and gel electrophoresis) to generate 25- to 35-kilobase-pair (kbp) fragments. The fragments were ligated to an *Eco*RI-digested, alkaline phosphatase-treated pLAFR1 cosmid vector (17), packaged by using a lambda in vitro packaging kit (Packagene; Promega Corp., Madison, Wis.) according to the instructions of the supplier, and transfected into *E. coli* LE392. Cosmid clones from both chromosomal and plasmid WM111 DNA libraries were selected on L-tetracycline agar. Random clones from both libraries were analyzed by *Eco*RI digestion of plasmid minipreparations. All cosmid clones tested contained large DNA inserts. The cosmid libraries were then transferred to *P. putida* PRS2015R by triparental conjugation (see below).

DNA subcloning. DNA fragments derived from the cosmid libraries were separated by agarose gel electrophoresis and isolated by electroelution (27) from the gel matrix. In general, restriction fragments were cloned into pUC18 or -19 and then recloned into pVDZ'2, a pRK290-based broad-host-range plasmid containing the pUC12 *lacZ'* multiple-cloning site (10). Recombinant plasmids were introduced into *E. coli* DH5α competent cells (Bethesda Research Laboratories). Recombinant pLAFR1 or pVDZ'2 carried in *E. coli* LE392 or DH5α donor cells was transferred to rifampin-resistant (Rif^r) *P. putida* PRS2015R recipients by triparental mating (16). *E. coli* HB101 carrying pRK2013 (16) provided the transfer functions. Transconjugants were selected on either L agar or *Pseudomonas* isolation agar (Difco Laboratories, Detroit, Mich.) supplemented with rifampin (200 μg/ml) and tetracycline (30 μg/ml). Donor, helper, or recipient cells individually did not grow on the selective media.

CH HPLC assay in resting cells. Individual *P. putida* colonies containing recombinant constructs were transferred to tetracycline-supplemented LB, and the cultures were allowed to grow for 6 h at 28°C. Samples (10 μl) of these cultures were added to 100 ml of BSMG liquid medium. The cultures were incubated at 28°C for 16 h. A 10-ml amount of each culture was harvested by centrifugation, and the cell pellets were individually suspended in 1 ml of NFB buffer containing 100 μg of carbofuran per ml. The cell suspensions were continuously mixed by inversion at 28°C for 9 h. The cells were then removed by centrifugation, and the supernatant solutions were acidified to pH 2 by addition of dilute sulfuric acid. The acid treatment reduced nonenzymatic carbofuran hydrolysis before high-performance liquid chromatography (HPLC) analysis.

Each supernatant solution was then tested by an HPLC method designed to separate carbofuran from its hydrolysis product 2,3,-dihydro-2,2-dimethyl-7-benzofuranol (7-phenol). The HPLC column was a Resolve C_8 Radial Pak (8 mm by 10 cm; 10-μm particle size [non-end capped]) in an RCM-100 radial compression module (Waters Associates, Inc., Milford, Mass.) preceded by an ODS-GU guard column (4.6 mm by 3 cm) and holder (Brownlee). The isocratic mobile phase was 75% HPLC-grade water acidified to pH 2 with HPLC-grade phosphoric acid (Fisher Scientific Co., Pittsburgh, Pa.)–25% acetonitrile delivered at 2.5 ml/min. Retention times were 6.3 min for 7-phenol and 7.4 min for carbofuran.

Spectrophotometric CH assays of cell extracts. Cell extracts of *Achromobacter* sp. strain WM111 and recombinant strains of *P. putida*, *Alcaligenes eutrophus*, *Acinetobacter calcoaceticus*, and *Achromobacter pestifer* were prepared by French pressure cell (SLM Aminco, Urbana, Ill.) disruption, followed by low-speed centrifugation (10,000 × *g*) and then ultracentrifugation (105,000 × *g*) as described elsewhere (Karns and Tomasek, submitted). CH activity was assayed at 413 nm, using 1 mg of the colorimetric substrate *o*-nitrophenyl dimethylcarbamate (Sigma Chemical Co., St. Louis, Mo.) per ml (9).

Detection of CH in *P. putida* subclones by Western blot (immunoblot). Native CH was purified from strain WM111 by the HPLC method of Karns and Tomasek (submitted). Four-month-old New Zealand White rabbits were immunized intramuscularly (0.5 ml) and subcutaneously (five 0.1-ml injections) with a total of 1 ml of native CH (232 μg of protein) in Freund complete adjuvant. The rabbits were reimmunized subcutaneously 14 days later with 500 μl of native CH (116 μg) in Freund incomplete adjuvant and again 19 days later with 1.5 ml of native CH (357 μg) in Freund incomplete adjuvant (intramuscularly and subcutaneously). After 34 days, both rabbits were reimmunized subcutaneously with 1 ml of 0.8% sodium dodecyl sulfate (SDS)-denatured CH (191 μg) in Freund complete adjuvant and again 10 days later with the same SDS-treated protein in Freund incomplete adjuvant (subcutaneously). Serum was collected 10 days later. One antiserum (CH7) detected low concentrations of both SDS-denatured and native CH and was used for further studies.

A 1.5-ml sample of late-log-phase, BSMG-grown PRS2015R containing fragments subcloned in pVDZ'2 was centrifuged, and the cell pellet was suspended in 500 μl of 10 mM Tris hydrochloride (pH 7.0). The cells were lysed by addition of 500 μl of 2× sample buffer (20% glycerol, 10% 2-mercaptoethanol, 6% SDS, 0.02% bromophenol blue, 125 mM Tris hydrochloride [pH 6.8]), rapidly mixed, and placed in a boiling water bath for 3 min. They were then quickly frozen on dry ice and thawed just before electrophoresis.

Polyacrylamide gel electrophoresis was performed by the method of Laemmli (26). The cell lysates were diluted 1:10 in 1× sample buffer and immediately loaded on 8% gels. The samples were electrophoresed at 30 mA per gel in a Hoefer SE 600 vertical slab gel device. Upon completion of electrophoresis, the gels were immediately equilibrated for 30 min in transfer buffer (192 mM glycine, 25 mM Tris [pH 8.3], 20% [vol/vol] methanol) and then electroblotted onto 0.45-μm-pore-size nitrocellulose sheets (Micron Separations, Inc.) by using a Hoefer TE 52 Transphor electroblotting apparatus.

The nitrocellulose sheets were agitated for 5 to 12 h in a blocking solution of 5% skim milk powder in TBST (10 mM Tris hydrochloride [pH 8.0], 150 mM NaCl, 0.05% Tween 20). The blocked nitrocellulose sheets were agitated in 50 ml of diluted CH7 rabbit antibody (1:600) in TBST for 1 h. After three 10-min washes in TBST, the primary antibodies were reacted with 30 ml of goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (Sigma) diluted 1:2,500 in TBST. The blots were again treated with three 10-min washes. The Western blots were submerged in 20 ml of alkaline phosphatase buffer (100 mM Tris hydrochloride [pH 9.5], 100 mM NaCl, 5 mM $MgCl_2$) containing 5-bromo-4-chloro-3-indolyl phosphate (165 μg/ml; Sigma) and Nitro Blue Tetrazolium (330 μg/ml; Sigma) as colorimetric substrates. Color development was stopped by rinsing the nitrocellulose sheets in distilled water. Normal color development was achieved in 15 to 20 min.

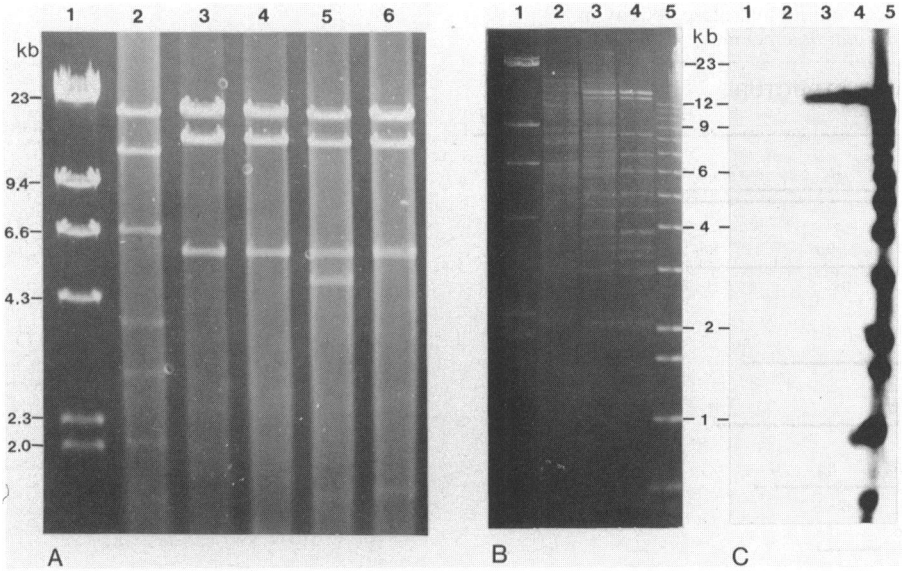

FIG. 1. Cosmid clones of the *mcd* gene from *Achromobacter* sp. strain WM111 and hybridization of an *mcd*-containing fragment to the WM111 plasmid. (A) *Eco*RI digests of putative *mcd*$^+$ cosmid clones in *P. putida* PRS2015R. Lanes: 1, *Hin*dIII-digested lambda DNA standard; 2, clone 26 (pJK7) (an *mcd*-lacking cosmid clone); 3, clone 27 (pJK6); 4, clone 40 (pJK8); 5, clone 46 (pJK9); 6, clone 59 (pJK10). (B) WM111 chromosomal and plasmid DNA digests separated by agarose gel electrophoresis. Lanes: 1, *Hin*dIII-digested lambda DNA standard; 2, WM111 chromosomal DNA digested with *Eco*RI; 3, WM111 chromosomal DNA enriched with WM111 plasmid DNA (top band of CsCl-ethidium bromide density gradient); 4, WM111 plasmid (pPDL11) digested with *Eco*RI; 5, 1-kbp ladder (Bethesda Research Laboratories) standard. (C) Southern blot autoradiogram of the gel shown in panel B probed with the *mcd*$^+$ 14-kbp *Eco*RI fragment of pPT301. Lanes are as in panel B. The probe also contained a radiolabeled 1-kbp ladder DNA for hybridization to the corresponding standard in lane 5.

RESULTS

Cosmid cloning of the methylcarbamate hydrolase gene. WM111 chromosomal and plasmid fragments cloned in the pLAFR1 cosmid vector were mobilized into *P. putida* PRS2015R through triparental matings as described above. Transconjugants were selected by growth on *Pseudomonas* isolation agar containing tetracycline and rifampin. Since *P. putida* can utilize methylamine as a nitrogen source, we attempted to select clones that would generate methylamine from *N*-methylcarbamate pesticides by plating the same mating mixtures on tetracycline- and rifampin-supplemented NFBG agarose plates containing 200 μg of carbaryl (1-naphthyl-*N*-methylcarbamate; Sigma) per ml as a nitrogen source. After 11 days, 62 colonies (approximately 2 mm in size) on the antibiotic- and carbaryl-amended plates were transferred to *Pseudomonas* isolation agar-tetracycline-rifampin plates and to a 96-well plate containing BSMG liquid medium supplemented with carbaryl. After 18 days, four of the wells were light gray in color, indicating the accumulation of 1-naphthol due to hydrolysis of carbaryl. The supernatant solutions from these four wells and six colorless wells (including two control wells that had been inoculated with cosmid-free PRS2015R) were tested by the HPLC methylcarbamate separation method described above. Supernatants from light-gray wells contained approximately 3.5 to 5.5 times more 1-naphthol than the average level found in non-gray wells.

These four clones (derived from the cosmid library of WM111 plasmid DNA) were scored as positive for the methylcarbamate degradation (*mcd*) gene. *Eco*RI restriction digests of these cosmids indicated that they contained 14- and 6-kbp *Eco*RI fragments in addition to the pLAFR1 vector (Fig. 1A). The smallest of the recombinant cosmids, designated pJK6, contained an additional 0.5-kbp *Eco*RI fragment (not visible on Fig. 1A) and was selected for further subcloning studies.

Subcloning of the CH gene. The 14-, 6-, and 0.5-kbp *Eco*RI fragments from pJK6 were each cloned first into the *Eco*RI site of pUC18 and then into the broad-host-range plasmid pVDZ'2. Recombinant *E. coli* DH5α strains containing the subcloned pJK6 14-, 6-, or 0.5-kbp *Eco*RI fragment in either orientation with respect to the *lac* promoter of pUC18 or pVDZ'2 did not hydrolyze carbofuran in resting-cell assays. When the pVDZ'2 subclones were transferred to *P. putida* PRS2015R and resting-cell CH assays were performed, the *P. putida* recombinant strains containing the pJK6 14-kbp *Eco*RI fragment in either orientation with respect to the *lac* promoter of pVDZ'2 hydrolyzed carbofuran, whereas the 6- and 0.5-kbp *Eco*RI subclones were inactive.

Hybridization of the 14-kbp *Eco*RI fragment encoding CH activity to the WM111 plasmid pPDL11. Total and plasmid DNAs isolated from *Achromobacter* sp. strain WM111 were digested with *Eco*RI and separated by agarose gel electrophoresis. The DNA fragments were transferred to nitrocellulose by Southern blotting and then hybridized to a radiolabeled DNA probe prepared by nick translation of the cloned 14-kbp *Eco*RI fragment containing *mcd*. The probe strongly hybridized to a single 14-kbp *Eco*RI band in the plasmid lanes (Fig. 1C), but only a weak signal, visible after prolonged exposure, was observed in the lane containing total WM111 DNA. This result suggested that the *mcd* gene carried on the 14-kbp fragment was found on the WM111 plasmid, pPDL11.

Restriction mapping of the 14-kbp *Eco*RI fragment and further subcloning of the *mcd* gene. A physical map of the 14-kbp fragment showing the *Hin*dIII, *Pst*I, *Bgl*II, *Bam*HI,

TABLE 2. Carbofuran hydrolase activity in *P. putida* subclones[a]

PLASMID	RELEVANT STRUCTURE	CH ACTIVITY[b]	72-kDa PROTEIN[c]
pJK6	E 6kbp E 7 kbp 7 kbp E; P P	+	ND
pPT311	Bg C Bg C Bm; P Sc Bm K Sp H P	+	+
pPT321	P Sp	+	+
pPT331	Sc H	+	+
pJK341	Sc Bg C; Bm K	+	+
pJK351	Sc K	—	—
pPT361	Bg P	—	—
pPT363	Bm Bm	—	—

1 kbp

[a] Abbreviations: ND, not determined; Bg, *Bgl*II; Bm, *Bam*HI; C, *Cla*I; E, *Eco*RI; Sc, *Sca*I; K, *Kpn*I; Sp, *Sph*I; H, *Hin*dIII; P, *Pst*I. Arrowheads denote the 3.0-kbp *Sca*I-*Cla*I structure that is the smallest subclone carrying *mcd* intact.

[b] As detected by the resting-cell–HPLC assay described in Materials and Methods.

[c] The presence of the 72-kDa protein in SDS-polyacrylamide gels was detected by Western blot immunoassay (see Fig. 2).

*Cla*I, *Sca*I, *Sph*I, and *Kpn*I sites was generated (Table 2) and used to develop *mcd* subcloning strategies. Various fragments were subcloned in pUC19 and then pVDZ'2. The pVDZ'2 clones developed in *E. coli* DH5α were transferred to *P. putida* PRS2015R, and the transconjugants were tested by the resting-cell assay for the ability to hydrolyze carbofuran (Table 2). The *mcd* gene was found to be located within a 3-kbp *Sca*I-*Cla*I segment. Clones with segments deleted at the internal *Kpn*I, *Bgl*II, and *Bam*HI sites of this 3-kbp fragment were inactive.

Western blot analysis of recombinant strains. The results of Western blot immunoassays of several *P. putida* subclones containing complete or partial sequences of the putative *mcd* gene are shown in Fig. 2. The rabbit polyclonal antiserum reacted with several proteins present in PRS2015R cells regardless of whether the cells contained the cloned *mcd* gene. The preimmune rabbit serum did not cross-react with these proteins (data not shown). In lanes from recombinant PRS2015R strains that hydrolyzed carbofuran in the resting-cell–HPLC assay, two additional protein bands were observed. These bands corresponded in size (72 and 77 kilodaltons [kDa]) to those found for HPLC-purified WM111 CH. These bands were absent in lanes from recombinant strains that did not hydrolyze carbofuran. They were also absent in control lanes from PRS2015R cells containing only the nonrecombinant pVDZ'2 vector or from PRS2015R cells alone. These results confirmed that the 3-kbp *Sca*I-*Cla*I DNA fragment cloned from the carbofuran-degrading *Achromobacter* sp. strain WM111 plasmid encodes CH.

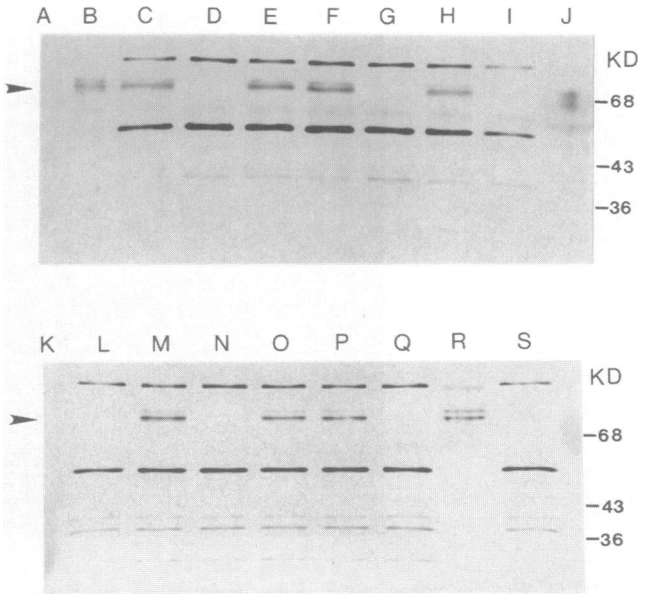

FIG. 2. Western blot immunoassay detection of CH in *P. putida* containing subclones of the *Achromobacter* sp. strain WM111 plasmid. Protein bands that reacted with rabbit antiserum prepared against purified *Achromobacter* sp. strain WM111 CH were visualized as described in Materials and Methods. Lanes: A, prestained molecular size standard; B, purified CH (positive control); C, pPT321 (4.8-kbp *Pst*I-*Sph*I fragment); D, pPT361 (3.4-kbp *Bgl*II-*Pst*I fragment); E, pPT321 (4.8-kbp *Pst*I-*Sph*I fragment); F, pPT371 (6.5-kbp *Pst*I-*Hin*dIII fragment); G, pVDZ'2 vector only (negative control); H, pPT331 (4.25-kbp *Sca*I-*Hin*dIII fragment); I, *P. putida* alone, no plasmid (negative control); J, prestained bovine serum albumin standard; K, prestained molecular size standard; L, pVDZ'2 vector only (negative control); M, pPT321 (4.8-kbp *Pst*I-*Sph*I fragment); N, pJK351 (2.0-kbp *Sca*I-*Kpn*I fragment); O, pJK341 (3.0-kbp *Sca*I-*Cla*I fragment); P, pJK341 (3.0-kbp *Sca*I-*Cla*I fragment); Q, pPT361 (3.4-kbp *Bgl*II-*Pst*I fragment); R, purified CH (positive control); S, pJK351 (2.0-kbp *Sca*I-*Kpn*I fragment). In some cases, independent transconjugants were assayed (lanes C, E, and M, D and Q, O and P, and N and S). Arrowheads indicate the 72-kDa protein band.

Expression of *mcd* clones in *P. putida* and *E. coli*. The active *mcd P. putida* recombinant strains produced significantly lower levels of the enzyme than did wild-type WM111 grown under similar conditions. No CH activity was observed in either strain when a rich medium such as LB was used for growth. Introduction of the same recombinant plasmids into *E. coli* resulted in no detectable CH activity when the cells were grown in LB. This result was observed when the *mcd* gene was placed in both orientations near the strong external *lac* promoter and induced with the *lac* inducer isopropyl-β-D-thiogalactopyranoside (IPTG). To test whether similar or different CH levels would be observed in other gram-negative bacteria, a pVDZ'2 clone of the 14-kbp *Eco*RI fragment containing *mcd*, pPT301, was transferred to Rif^r derivatives of *Alcaligenes eutrophus* JMP134, *Acinetobacter calcoaceticus* ATCC 33305, and *Achromobacter pestifer* ATCC 23584. These exconjugants and the *P. putida* exconjugant were all grown in BSM liquid medium, using 20 mM succinate as a carbon source. Whereas spectrophotometric assays conducted over 4 h failed to detect hydrolysis of the *o*-nitrophenyl dimethylcarbamate substrate, low enzyme activities were observed in cell extracts or intact resting cells from all exconjugants when incubation was extended to 24 to 36 h (slight development of yellow color).

DISCUSSION

The cosmid cloning, subcloning, and Southern hybridization results indicate that the gene encoding CH (*mcd*) lies within a 3-kbp *Sca*I-*Cla*I segment of the 100-kbp *Achromobacter* sp. strain WM111 plasmid pPDL11. The protein bands (72 and 77 kDa) observed in lanes from the active recombinant *P. putida* strains were the same size as and immunologically identical to the bands from HPLC-purified *Achromobacter* sp. strain WM111 CH. These results indicate that the cloned gene does encode the protein(s) shown to be responsible for rapid carbofuran hydrolysis by WM111.

CH isolated and purified from *Achromobacter* sp. strain WM111 yields two unevenly staining protein bands (72 and 77,000 kDa) when run on SDS-polyacrylamide gels (Karns and Tomasek, submitted). This differential staining is also observed in Western blots of CH isolated from either *Achromobacter* sp. strain WM111 or *P. putida* subclones. It is unlikely that both bands would be present in *P. putida* subclones unless they were both encoded on the same DNA segment. However, barring the possibility of overlapping reading frames, the 3-kbp *Sca*I-*Cla*I fragment is not large enough to code for two different proteins. It is more likely that one of the protein bands is derived from the other. This could result from one of the following mechanisms: (i) proteolytic cleavage of the large protein to the smaller; (ii) occasional transcription from an inefficient upstream promoter, yielding a larger mRNA transcript with an alternative translation start site; (iii) differential translation of the two proteins from the same mRNA because of two distinct translational starts; (iv) posttranslational modification of the smaller protein to a larger one (methylation, phosphorylation, glycosylation, etc.); or (v) differential binding of metal ions, including Mn^{2+}, to the enzyme. Further experimentation will be required to elucidate the mechanism responsible for the two protein bands; however, any posttranslational cleavage or modification mechanism would have to be present in both *Achromobacter* sp. strain WM111 and *P. putida* cells.

Although the CHs produced in WM111 and *P. putida* appear to be identical, the specific activity is significantly lower in *P. putida* and other gram-negative bacteria than in WM111 and is undetectable in *E. coli*. This fact probably cannot be attributed to the lack of a carbofuran transport system in the non-WM111 strains, since the activity is also very low or nonexistent in cell extracts prepared from these strains. The low levels of CH activity in alternative bacterial hosts may result from poor interaction between the *Pseudomonas* or *Escherichia* RNA polymerases and the native *Achromobacter* sp. strain WM111 *mcd* promoter or the inability to utilize WM111 translation signals.

Placement of the *mcd* gene in either orientation with respect to the *lac* promoter of pUC18 or pVDZ'2 had no effect on *mcd* expression in *E. coli* or *P. putida*. This result suggests that there may be strong regulatory sequences (transcriptional or translational terminators or both) upstream of the start of *mcd* that prevent expression from exogenous promoters. The low levels of CH activity in *P. putida* also may be due to the absence of a positively acting regulatory protein encoded by a separate gene located on the *Achromobacter* biodegradative plasmid which is not linked to the segment containing *mcd*.

In any case, this study suggests that maximal expression of CH in *Achromobacter* sp. strain WM111 requires one or several signals that are lacking in other soil microorganisms or enteric bacteria. Experiments designed to increase *mcd* expression in *Pseudomonas* by replacing both the native *mcd* promoter and potential upstream regulatory sequences with an exogenous promoter that would be efficiently recognized by *Pseudomonas* RNA polymerase and sigma factors are under way. We are in the process of sequencing the *mcd* structural gene and promoter structures. These studies may lead to a better understanding of gene regulation in important soil biodegradation bacteria. Use of the *mcd* gene as a probe for similar DNA sequences in problem-soil microflora is also being investigated. This effort may lead to a better understanding of the evolution and environmental dissemination of novel catabolic genes in soil ecosystems. Recently, Chaudhry and Ali (5) isolated several *Pseudomonas* and *Flavobacterium* strains capable of hydrolyzing the *N*-methylcarbamate side chain from carbofuran and utilizing the hydrolysis products as a source of either nitrogen or carbon. It would be interesting to see whether the *mcd* gene isolated from *Achromobacter* sp. strain WM111 is conserved or shows significant structural similarity to the hydrolase genes in these independent isolates.

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