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# Isolation and Characterization of an *N*-Methylcarbamate Insecticide-Degrading Methylotrophic Bacterium†

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**A gram-negative bacterium which hydrolyzed aryl *N*-methylcarbamate insecticides was isolated from an agricultural soil which quickly degraded these pesticides. This organism, designated strain ER2, grew on carbofuran as a sole source of carbon and nitrogen with a doubling time of 3 h in a mineral salts medium. The aromatic nucleus of the molecule was not metabolized, and carbofuran 7-phenol accumulated as the end product of metabolism. The insecticides carbaryl, bendiocarb, and propoxur were similarly hydrolyzed, with each yielding the corresponding phenol. Strain ER2 contained two plasmids (120 and 130 kb). A probe cloned from the pDL11 plasmid of *Achromobacter* sp. strain WM111, which encodes the carbofuran hydrolase (*mcd*) gene (P. H. Tomasek and J. S. Karns, J. Bacteriol. 171:4038–4044, 1989), hybridized to the 120-kb plasmid. Restriction fragment profiles of pDL11 and strain ER2 plasmid DNAs suggested that the 120-kb plasmid of strain ER2 is very similar to pDL11. On the basis of the results of biochemical tests, 16S rRNA sequence analysis, and membrane lipid analyses, strain ER2 was found to be a phylogenetically unique type II methylotroph. The constitutive carbofuran hydrolase activity in glucose-grown cells increased sevenfold when strain ER2 was grown in the presence of 100 mg of carbofuran per liter as the sole source of carbon and nitrogen or as the sole nitrogen source in the presence of glucose. Growth on carbofuran resulted in the induction of enzymes required for methylamine-dependent respiration and the serine pathway of formaldehyde assimilation. These results indicate that the carbofuran hydrolase *mcd* gene is conserved on a plasmid found in organisms from different geographic areas and that the specific activity of carbofuran degradation may increase in response to carbofuran treatment.**

Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranoyl *N*-methylcarbamate) is an insecticide used to control a variety of insect pests that infest a number of crops, including canola, corn, alfalfa, potatoes, and strawberries (34). In North America several million kilograms of this pesticide per year are used in agriculture (50). Carbofuran is in the *N*-methylcarbamate class of insecticides, which also includes carbaryl (1-naphthyl methylcarbamate), propoxur (2-isopropoxyphenyl methylcarbamate), bendiocarb (2,2-dimethyl-1,3-benzodioxol-4-yl methylcarbamate), and methomyl [S-methyl *N*-(methylcarbamoyloxy)thioacetimidate] (Fig. 1). Carbofuran is one of several pesticides which in some soils are subject to accelerated or "enhanced" degradation following repeated application. This phenomenon reduces pesticide efficacy and causes economic loss (8). Enhanced degradation of pesticides is presumably the result of an increase in the numbers or specific activities of soil pesticide-degrading bacteria (1, 14, 22, 42), but the ecological, physiological, or genetic basis for the adaptation of the microflora is not known. Although in some circumstances carbofuran is subject to accelerated degradation, it is sufficiently mobile in some soils to be considered a potential groundwater and surface water pollutant (32, 50, 60).

The enhanced degradation problem and the potential environmental impact of *N*-methylcarbamate pollution have prompted studies on the biodegradation of carbofuran. A number of carbofuran-degrading organisms have been described, including several *Pseudomonas* and *Flavobacterium* strains (9), an *Achromobacter* sp. (23), and an *Arthrobacter* sp. (36). Such bacteria may be useful for decontaminating pesticide waste and spills (3, 24). Although a number of carbofuran-degrading pathways have been observed, carbofuran metabolism is most often initiated by hydrolysis of the labile methylcarbamate linkage, yielding carbofuran 7-phenol (2,3-dihydro-2,2-dimethyl-7-benzofuranol) and methylamine. Growth generally occurs at the expense of the methylamine, although in some cases the aromatic moiety is metabolized. An enzyme that catalyzes carbofuran hydrolysis has been isolated from the *Achromobacter* sp. (25), and the plasmid-borne gene encoding carbofuran hydrolase in this organism has been cloned (46).

In this study, we isolated and characterized a bacterium, designated strain ER2, from an agricultural soil that exhibited enhanced degradation of carbofuran. This organism was very similar to the *Achromobacter* sp. described by Karns and Tomasek (25, 46) in its biochemical properties and some genetic properties related to carbofuran degradation. However, DNA probing showed that the two organisms were different, and, on the basis of the results of 16S rRNA sequencing and membrane lipid analyses, strain ER2 was shown to be a phylogenetically unique methylotroph. Enzymatic activities required for growth on carbofuran were identified, and the regulation of these activities in response to nutritional conditions was studied.

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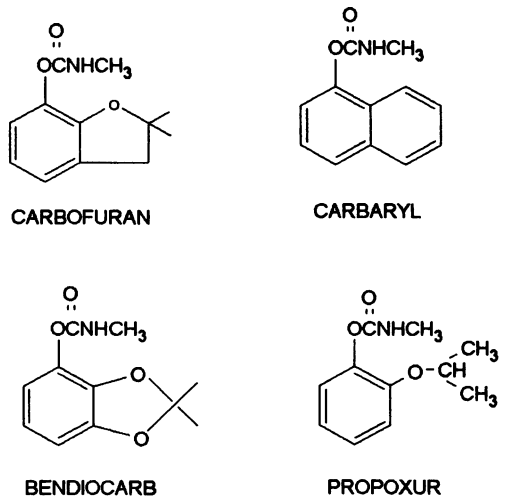


FIG. 1. Structures of aryl *N*-methylcarbamate insecticides used in this study.

## MATERIALS AND METHODS

**Chemicals and analytical methods.** Analytical standards for carbofuran and its hydrolysis product, carbofuran 7-phenol, were gifts from FMC Corp., Middleport, N.Y. Other pesticides were purchased from Chromatographic Specialties, Brockville, Ontario, Canada (Riedal de Haen Pestanal Standards) and were more than 99% pure according to the manufacturer. Naphthol and 2-isopropoxyphenol were purchased from Aldrich, Milwaukee, Wis. Pesticides and metabolites were dispensed into containers from stock solutions prepared in acetone, the solvent was evaporated with a gentle stream of filter-sterilized air, and cell suspensions, buffers, or extracts were then added.

The concentrations of pesticides and metabolites in aqueous samples were determined by high-pressure liquid chromatography (HPLC) as described by Topp and Akhtar (48). The samples subjected to thin-layer chromatography (TLC) analysis were applied to 60A LK60DF silica gel plates (20 by 5 cm) containing a fluorescent indicator (Whatman, Maidstone, England). The plates were prerun and developed in ethyl ether-hexane (75:25), and dark spots were visualized by illumination at 254 nm. Gas chromatography-mass spectrometry (GC-MS) was performed as previously described, as was sample preparation for the HPLC, GC-MS, and TLC analyses (47).

The protein contents of cell suspensions and cell extracts were determined by the Bradford assay (5). Protein standards were prepared with bovine serum albumin.

**Enrichment, isolation, and maintenance of strain ER2.** The bacterium described in this paper was isolated from a composite Prince Edward Island soil sample provided by D. C. Read. The soil was observed to catalyze the rapid degradation of a number of pesticides, including carbofuran, methomyl, and aldicarb (37). The soil samples were stored at 4°C for about 2 years prior to this study. The soil was enriched for carbofuran-degrading microorganisms by perfusing it continuously for 4 months at room temperature with distilled water containing 100 mg of carbofuran per liter. The carbofuran concentration in the perfusate was not monitored, but the perfusate was fortified at 2-week intervals by the addition of 100 mg of carbofuran per liter.

Enrichment preparations consisting of a mineral salts (MS) medium (45) containing 25 mg of carbofuran per liter as the sole carbon source were inoculated with a small amount of enriched soil and perfusion fluid. Before the isolation of strain ER2, an enrichment culture was serially transfered 19 times at about weekly intervals when it became slightly turbid and the carbofuran was removed, as determined by HPLC. The isolation medium was MS medium containing 50 mg of carbofuran per liter and spent enrichment culture supernatant as a source of potential growth factors. MS medium containing 25 g of agar per liter was autoclaved, cooled, and then mixed at a 1:1 ratio with filter-sterilized (0.2-μm-pore-size cellulose nitrate membrane; type S filter unit; Nalge Co., Rochester, N.Y.) spent enrichment culture medium fortified with 100 mg of carbofuran per liter. Samples from the enrichment culture were streaked onto the agar medium, and colonies developed after 1 week of incubation at 30°C. These colonies were purified by repeated restreaking on the isolation medium and were tested for the ability to degrade 25 mg of carbofuran per liter when it was provided as the sole carbon source in MS medium. This procedure yielded the isolate designated strain ER2 described in this paper.

Strain ER2 was routinely maintained on nutrient agar or on nitrate-free mineral salts (NFMS) medium supplemented with 2 g of methylamine hydrochloride per liter as the sole source of carbon and nitrogen and solidified with 12 g of agar per liter. NFMS medium consisted of MS medium (45) modified by replacing the $NaNO_3$ with 0.34 g of NaCl per liter and adjusting the pH to 6.2. At this pH chemical hydrolysis of carbofuran was negligible, whereas at pH 7.0 8.3% of a 25-mg/liter solution was hydrolyzed after 72 h of incubation at 30°C (data not shown).

**Characterization of strain ER2.** Substrate utilization and biochemical reactions were tested by using API 20E, Rapid CH, and Rapid NFT strips as recommended by the manufacturer (API Laboratory Products, Ltd., St. Laurent, Quebec, Canada). Transmission electron microscopy was performed by using cells negatively stained with 1% phosphotungstate or by using thin sections prepared after staining with osmium tetroxide (17). All other tests and procedures were performed as described in the *Manual of Methods for General Bacteriology* (17).

The ability of strain ER2 to degrade various *N*-methylcarbamate insecticides was tested with cells grown in NFMS medium containing 2 g of methylamine per liter. Cells were harvested by centrifugation at 12,000 × *g* for 10 min, washed, and resuspended in NFMS medium. Aliquots (3 ml) of the cell suspension were supplemented with each insecticide tested at a concentration of 10 mg/liter, and the preparations were incubated in screw-cap tubes under three different nutritional conditions: with the test insecticide as the sole source of carbon and nitrogen; in the presence of 10 g of glucose per liter; and in the presence of 25 mg of carbofuran per liter as a potential inducer of *N*-methylcarbamate-degrading enzymes. The cultures were incubated with agitation at 30°C for 48 to 72 h. The cultures were then visually examined for turbidity, and samples were removed for an HPLC analysis of the residual pesticide and accumulated metabolites. Cell-free controls were included in all incubations to account for chemical hydrolysis.

Growth of biomass was measured by monitoring $A_{600}$ or by determining the viable counts for samples serially diluted in MS medium and plated onto nutrient agar.

**Regulation of carbofuran metabolism.** The effects of nutritional conditions on carbofuran metabolism were determined by measuring enzyme activities in cells grown in NFMS

medium supplemented with various carbon and nitrogen sources, including 2 g of $CH_3NH_2$ HCl per liter, 5 g of glucose per liter plus 0.2 g of $CH_3NH_2$ HCl per liter, 0.1 g of carbofuran per liter, 5 g of glucose per liter plus 0.1 g of carbofuran per liter, or 5 g of glucose per liter plus 0.1 g of $NH_4Cl$ per liter. Cells were grown in 2-liter Erlenmeyer flasks containing 0.5 liter of medium incubated at 30°C with agitation (200 rpm). The media were inoculated with cells harvested by centrifugation at 12,000 × *g* for 12 min from 50-ml starter cultures grown in the same medium.

For some experiments involving measurement of enzyme activities involved in the fixation of formaldehyde, methylotrophs known to possess the serine pathway (*Methylobacterium organophilum* XX) or the ribulose monophosphate (RuMP) pathway (*Methylomonas methylovora*) (2, 28, 33, 44, 58) were included as controls. *Methylobacterium organophilum* XX and *Methylomonas methylovora* were grown in 2-liter Erlenmeyer flasks containing 0.75 liter of MacLennan's mineral salts medium supplemented with 2 ml of methanol per liter incubated at 30°C with agitation (200 rpm) (28).

Cells were harvested from batch cultures in the midlogarithmic phase of growth, washed once, and resuspended in 10 ml of 50 mM phosphate buffer (pH 7.0). A portion of each cell suspension was reserved for respiration measurements, and the remainder was used for preparing cell extracts by passing the cell suspension twice through a chilled (4°C) French pressure cell operated at 10,000 lb/in$^2$. Undisrupted cells were removed by centrifugation at 12,000 × *g* for 15 min. Cells and cell extracts were kept on ice and used within 2 h of preparation.

Carbofuran hydrolase activity was determined in cell extracts. The reaction was initiated by mixing equal volumes of prewarmed (30°C) cell extract and 50 mM phosphate buffer (pH 7.0) containing 20 mg of carbofuran per liter and then incubating the preparation with agitation (200 rpm) in a water bath shaker. Samples (150 μl) were removed at the start of the reaction and at 15-min intervals thereafter. Autoclaved (5 min, 121°C) cell extract similarly prepared and incubated was included in all experiments to account for possible adsorption and chemical transformation of carbofuran, phenomena which actually proved to be insignificant. Protein-free supernatants from reaction mixtures were prepared for HPLC analysis by adding samples directly to 1 N HCl (1:1) and then centrifuging the preparations at 14,000 × *g* for 4 min. Samples were subjected to HPLC analysis within 30 min.

The rate of methylamine metabolism was estimated by measuring methylamine-dependent respiration by whole cells of strain ER2 with a Clark type oxygen electrode (model 53 biological oxygen monitor; Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio). Cells suspended in 50 mM phosphate buffer (pH 7.0) were incubated with agitation in a water-jacketed reaction vessel maintained at 30°C. The rates of oxygen consumption by cell suspensions following the addition of 10 mM methylamine hydrochloride were determined and corrected for endogenous respiration. The oxygen electrode was calibrated by setting full-scale deflection with air-saturated phosphate buffer (the dissolved oxygen concentration was assumed to be 237 μM) and zero response with buffer made anaerobic by the addition of sodium dithionite (12).

The presence of the two C-1 assimilation pathways in strain ER2 was determined by measuring the activities of the following key enzymes: for the serine pathway, hydroxypyruvate reductase (HPR) and serine glyoxylate aminotransferase (SGAT); and for the RuMP pathway, hexulose phosphate synthase (HPS). HPR activity was determined by measuring hydroxypyruvate-dependent NADH oxidation as described by Blackmore and Quayle (4), except that the buffer used was 50 mM phosphate (pH 7.0) and the incubation temperature was 30°C. SGAT activity was determined by measuring the glyoxylate-dependent formation of hydroxypyruvate from serine as described by Blackmore and Quayle (4). HPS activity was determined by measuring the rate of ribulose 5-phosphate-dependent consumption of HCHO as described by Ferenci et al. (15). Formaldehyde concentrations were determined by the colorimetric method of Chrastil and Wilson (10), as modified by Topp and Knowles (49).

The data reported below are the means of two replicate measurements obtained from each of two batch cultures.

**Nucleic acid methods.** Total DNA was isolated from 5-ml cultures, restricted with endonucleases, and prepared for probing in Southern blots as previously described (54). Supercoiled plasmids were visualized in 0.8% agarose gels by the method of Wheatcroft and Williams (56), modified by extraction of the alkaline lysate with phenol-chloroform-isoamyl alcohol (25:24:1) prior to electrophoresis. Fractions enriched for DNAs of smaller plasmids for restriction analysis were isolated by fractionation of alkaline sodium dodecyl sulfate cell lysates on a sucrose density gradient (56), followed by purification on a cesium chloride-ethidium bromide density gradient and ethanol precipitation (40). DNA restriction, ligation, and transformation of *Escherichia coli* were performed as described elsewhere (40). DNA probes were labelled with [$^{32}$P]dCTP (Dupont Canada) by nick translation (38) and were used to probe Southern blots (43). Autoradiograms were prepared with Kodak XAR-2 film.

pJH5 was used to probe for the methylcarbamate hydrolase (*mcd*) gene. This plasmid contains a 3.1-kb *Cla*I fragment of the *mcd* gene cloned from *Achromobacter* sp. strain WM111 in pBluescript-SKII (46). No homology to the vector was detected in the DNA of strain WM111 or strain ER2. A mixture of genomic fragments cloned from WM111 was used to test for homologous sequences in the genome of ER2. Total cellular DNA of WM111 was digested with *Bgl*II, and fragments ranging in size from 1.6 to 2.0 kb were collected on NA45 paper (Schleicher & Schuell) during agarose gel electrophoresis. The fragments were eluted as previously described (55) and ligated with *Bam*HI-cut pUC19. The mixture was used to transform *E. coli* DH5αMCR (Bethesda Research Laboratories) and plasmid DNAs from six randomly chosen clones isolated as described previously (53). The mixture of cloned inserts used as a multiple probe was excised by double digestion with *Eco*RI and *Sal*I and was purified from the vector by using NA45 paper during gel electrophoresis.

An analysis of 16S rRNAs was performed to examine the phylogeny of strain ER2 with respect to other methylotrophs (6, 16, 21, 51). Isolation of total RNA, radiolabelling of signature probes, and hybridization of membrane-bound RNA on slot blots with $^{32}$P-labelled signature probes were performed precisely as described by Tsien et al. (51). Isolation of RNA for sequencing of 16S rRNAs was performed as described by Tsuji et al. (52). The primers used for sequencing have been described by Tsuji et al. (52). The primer sequences were elongated by using reverse transcriptase (27), and the resulting DNA sequences were determined by the dideoxynucleotide sequencing method of Sanger et al. (41). Homology values based on 16S rRNA sequences were determined by using programs of DeSoete (13) as described

by Tsuji et al. (52). The sequences of the 16S rRNAs of previously described methylotrophs and the phylogenetic relationships of these bacteria to organisms other than strain ER2 have been published previously (52).

**Membrane lipid analyses.** Approximately 20 to 25 mg (dry weight) of bacterial cells was extracted with a chloroform-methanol, single-phase solvent system modified to include phosphate buffer (57); care was taken to prevent artifacts and contamination as recommended by Guckert et al. (19). The total lipid extract was fractionated on silicic acid columns into neutral lipids, glycolipids, and phospholipids (35). The polar lipid fraction recovered in methanol was transesterified by mild alkaline methanolysis (57), and the methyl esters were separated, quantified, and tentatively identified after injection by capillary gas chromatography (GC) onto a 50-m HP-1 (nonpolar methyl silicone) column, using the conditions described previously (39). The phospholipid ester-linked fatty acid (PLFA) structures were verified by using GC-MS with the same GC column, and a Hewlett-Packard model 5996A GC/MS as described previously (39).

Mono- and dienoic PLFA double-bond positions and conformations were determined by performing a GC-MS analysis of the dimethyl disulfide adducts (30). Cyclopropyl PLFA ring positions were determined by GC-MS after hydrogenation (19). Equivalent chain lengths were calculated as described previously (11).

The lipopolysaccharide (LPS) hydroxy fatty acids from lipid A were recovered from the lipid-extracted residue (31). This material was hydrolyzed in 1 N HCl for 2 to 4 h at 100°C. The residue was then reextracted with 2 volumes of methanol and 3 volumes of chloroform so that the final preparation contained chloroform, methanol, and 1 M HCl at a ratio of 5:2:3, (vol/vol/vol). After 24 h of extraction and centrifugation the chloroform phase was recovered, evaporated to dryness, and methylated with "magic" methanol (methanol-chloroform-concentrated HCl, 10:1:1) (29) at 100°C for 1 h. The methylated hydroxy fatty acids (OHFA) were recovered in hexane-chloroform (4:1, vol/vol); the volatile solvent was then removed with a stream of nitrogen, and the OHFA were purified on precleaned (hexane-ethyl ether, 1:1) thin-layer plates. The plates were developed in the same solvent, and the OHFA band was recovered after location with a standard run simultaneously. Before GC-MS analysis the hydroxyl groups were derivatized with *N,O*-bis(trimethylsilyl)trifluoroacetamide (Pierce, Rockford, Ill.).

Fatty acids were designated by the total number of carbon atoms, the number of double bonds, and the position of the double bond closest to the methyl end (w) of the molecule. The configuration of the double bonds was indicated by c (*cis*) or t (*trans*). For example, 16:1w7c is a PLFA with 16 carbons atoms and one double bond 7 carbon atoms from the methyl end in the *cis* configuration. Branched fatty acids were designated by i (iso) or a (anteiso) if the methyl branch was one or two carbon atoms from the methyl end (i15:0), or the position with respect to the methyl end of the molecule was indicated (10Me16:0). Cyclopropyl (cy) fatty acids were designated by the total number of carbon atoms (cy17:0), and the position of the hydroxyl group with respect to the carboxyl end of the fatty acid was indicated by the position, with OH as a prefix (3OH16:0).

## RESULTS

**Metabolism of *N*-methylcarbamate insecticides by strain ER2.** The degradation of carbofuran in enrichment cultures or cell suspensions of isolate ER2 was accompanied by the accumulation of a metabolite which comigrated with a carbofuran 7-phenol standard when the preparations were analyzed by TLC or HPLC (Table 1). A GC-MS analysis of enrichment culture or pure culture extracts revealed a compound with a retention time and mass spectrum which corresponded to those of the carbofuran 7-phenol standard. The carbofuran 7-phenol metabolite accumulated in stoichiometric amounts and was not degraded when it was added to either the enrichment culture or pure cultures of strain ER2 (data not shown).

Strain ER2 grew in NFMS medium containing 100 mg of carbofuran per liter as the sole source of carbon and nitrogen with a doubling time of 3 h, as determined by viable counts (data not shown). No extracellular carbofuran hydrolase activity was detected. Carbofuran was not transformed in culture supernatants prepared by centrifugation of log-phase batch cultures.

Strain ER2 was able to utilize carbaryl, propoxur, or bendiocarb as a sole nitrogen source when glucose was provided as the carbon source. Methomyl, however, did not support growth. Bendiocarb, carbaryl, and propoxur, but not methomyl, were metabolized, and each yielded an end product corresponding in its properties to the anticipated phenolic hydrolysis product (Table 1). A comparison of the mass spectra and chromatographic properties of the accumulated metabolites with those of standards indicated that 1-naphthol and 2-isopropoxyphenol were the end products of carbaryl and propoxur metabolism, respectively. No standard for the anticipated metabolite of bendiocarb hydrolysis was available. There was no obvious difference in HPLC chromatograms between cell-free controls and cell suspensions incubated with this compound. However, a hydrophilic end product with an $R_f$ of 0.95, a GC retention time of 4.15 min, and a mass spectrum consistent with the anticipated hydrolysis product was found. There was no transformation of methomyl, as revealed by TLC or GC-MS. The results of the TLC analysis of ethyl ether extracts of cell extracts incubated with the various pesticides were in agreement with the whole-cell results; namely, the aryl substrates were hydrolyzed, and methomyl was not metabolized (data not shown).

**Morphological and biochemical properties of strain ER2.** Colonies of strain ER2 growing on nutrient agar were circular, 3 mm in diameter, raised with entire margins and smooth surfaces, opaque, dull, butyrous, and buff. Colonies growing on methylamine-containing NFMS agar were similar except that they were whiter. Strain ER2 was a gram-negative, oxidase-positive, catalase-positive, nonmotile, rod-shaped organism. Cells were 1 by 2.5 μm, and no flagella were visible by electron microscopy. Thin sections prepared from cells grown on methylamine plates did not contain any visible intracytoplasmic membranes (Fig. 2). Strain ER2 was an obligate aerobe and did not grow under denitrifying conditions. The following tests or reactions were positive: growth on methylamine, D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-D-glucoseamine, maltose, and L-malate; urease activity; and esculin hydrolysis. The following tests or reactions were negative: growth on methanol, D-gluconate, caprate, adipate, citrate, and phenyl acetate; nitrate reduction; glucose fermentation; arginine dihydrolase activity; gelatin hydrolysis; and β-galactosidase activity.

**rRNA phylogeny.** The signature probe 9-alpha hybridized to filter-bound RNA isolated from strain ER2 (data not shown). This probe does not hybridize to RNAs purified from RuMP pathway methylotrophs. Signature probe 10-gamma, which has been shown to hybridize only to RNAs

TABLE 1. TLC relative mobilities, HPLC retention times, GC retention times, and mass spectral characteristics of insecticide substrates and end products of insecticide metabolism by strain ER2[a]

| Substrate | Metabolism | Characteristics of substrate | | | | Characteristics of product | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | $R_f$ | HPLC retention time (min) | GC retention time (min) | m/z (% intensity) | $R_f$ | HPLC retention time (min) | GC retention time (min) | m/z (% intensity) |
| Carbofuran | + | 0.78 | 4.0 | 6.3 | 221 ($M^+$, 3), 164 (100), 149 (65), 131 (20), 123 (18), 103 (10), 91 (10), 77 (9) | 0.97 | 4.4 | 2.3 | 164 ($M^+$, 100), 149 (86), 131 (45), 123 (38), 103 (30), 91 (20), 77 (23) |
| Carbaryl | + | 0.71 | 4.2 | 9.3 | 201 ($M^+$, 12), 144 (100), 127 (8), 115 (70), 89 (10) | 0.92 | 4.2 | 6.2 | 144 ($M^+$, 100), 115 (70), 89 (12) |
| Propoxur | + | 0.80 | 4.0 | 4.6 | 209 ($M^+$, 7), 168 (3), 153 (38), 152 (32), 137 (18), 110 (100), 91 (8), 81 (87), 65 (40), 63 (53) | 0.99 | 4.4 | 1.3 | 152 ($M^+$, 60), 110 (100), 92 (18), 81 (40), 65 (24), 63 (38) |
| Bendiocarb | + | 0.78 | 5.0 | 7.4 | 223 ($M^+$, 47), 166 (75), 151 (100), 126 (85), 123 (25), 108 (18), 79 (22) | 0.95 | 5.0 | 4.2 | 166 ($M^+$, 86), 157 (100), 126 (85), 123 (45), 108 (65), 79 (35) |
| Methomyl | − | 0.24 | ND[b] | ND | ND | NA[c] | NA | NA | NA |

[a] The procedures and conditions used for TLC, HPLC, GC, and GC-MS analyses are described in Materials and Methods.
[b] ND, not done.
[c] NA, not applicable.

from type I methylotrophs (51), did not hybridize with RNA purified from strain ER2 or other type II methylotrophs (data not shown).

The sequence of the 16S rRNA from strain ER2 was determined and compared with the sequences of 16S rRNAs from several previously described methylotrophs (Table 2). All serine pathway methylotrophs whose 16S rRNAs have been sequenced by us except strain ER2 exhibit homologies at positions 139 to 150 on the *E. coli* 16S rRNA map. Strain ER2 16S rRNA has three mismatches within the consensus sequence (Table 2). Because the mismatches are located at the end of the signature sequence and there are 15 homologous positions within the consensus sequence, probe 9-alpha hybridized to filter-bound RNA from strain ER2 under the conditions used. It is also obvious from the data in Table 2 that the sequence of the 16S rRNA from strain ER2 in the region containing the consensus sequence for RuMP pathway methylotrophs more closely resembles the sequence of the serine pathway methylotrophs than the sequence of the RuMP methylotrophs. Several nucleotides are deleted in the 16S rRNA molecules of serine pathway methylotrophs within this region. The phylogenetic relationship between strain ER2 and other methylotrophs is shown in Fig. 3.

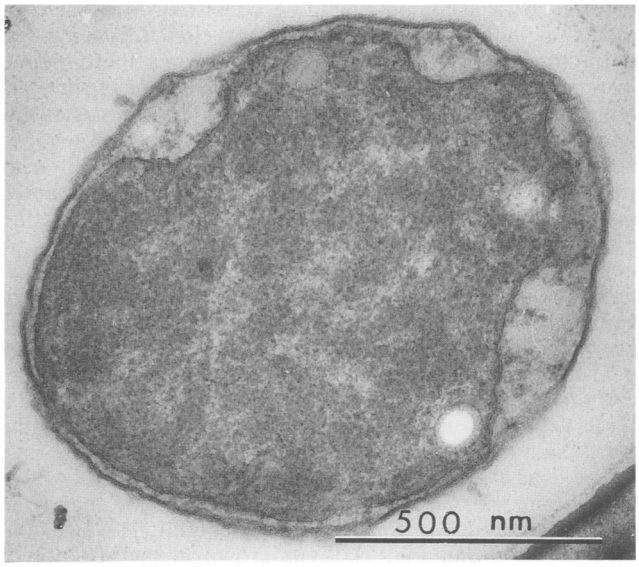


FIG. 2. Thin section of a methylamine-grown strain ER2 cell. Magnification, ×50,000.

**Lipid analyses.** The levels of individual PLFAs detected in extracts prepared from strain ER2 are shown in Table 3. The major PLFAs detected exhibit the w7 monounsaturation characteristic of synthesis by the anaerobic desaturation pathway, the pathway utilized by most methylotrophs. The most prominent PLFA was the monoenoic acid 18:1w7c (43.5%). Also, an unusual PLFA, br19:1w6, was detected. The derivitization of this PLFA was not complete, suggesting that another acid (possibly a cyclopropyl acid) coeluted with this branched monoenoic acid. The other interesting fatty acid was cy19:0 (4.99%), which was most likely formed from the monoenoic acid 18:1w7c by addition of a methyl group. This phenomenon occurs in cultures as they pass from the log phase to the stationary phase of growth.

Table 3 also shows the LPS OHFAs detected. The OHFA profile is dominated by 3OH18:0, an observation common in other methylotrophs. The level of 3OH19:0 (5.22%) was quite high.

Figure 4 shows the relationship of strain ER2 to other methylotrophs as determined by cluster analysis of the total PLFA profiles. Strain ER2 is most similar to group 1 methylotrophs (type II, pink-pigmented, facultative methylotrophs), which are characterized by high levels of 18:1w7c, but is distinct enough to be classified in a separate group (similarity index, less than 0.75). This distinction is most certainly related to the presence of br19:1w6, which was not found in any other isolate (20).

**Plasmid content, location of carbofuran hydrolase gene, and similarity of strain ER2 to *Achromobacter* sp. strain WM111**

TABLE 2. Comparison of nucleotides at positions 131 to 161 (*E. coli* numbering) in 16S rRNAs from methylotrophic bacteria[a]

| Organism or sequence | Nucleotide sequence |
|---|---|
| Serine pathway methanotrophs | |
| Target sequence 9-alpha | GGUUCGG AAUAACUCAGG |
| *Methylosinus* sp. strain B | UCGGUUCGG AAUAACUCAGG GAAA |
| *Methylosinus trichosporium* OB3b | UCGGUUCGG AAUAACUCAGG GAAA |
| *Methylosinus methanica* 81Z | UCGGUUCGG AAUAACUCAGG GAAA |
| *Methylosinus sporium* | UCGGUUCGG AAUAACUCAGG GAAA |
| *Methylocystis parvis* | UCGGUUCGG AAUAACUCAGG GAAA |
| *Methylocystis pyriformis* | UCGGUUCGG AAUAACUCAGG GAAA |
| *Methylosinus echinoides* | UCGGUUCGG AAUAACUCAGG GAAA |
| *Methylosinus minimus* | UCGGUUCGG AAUAACUCAGG GAAA |
| Strain B-3060 | UCGGUUCGG AAUAACUCAGG GAAA |
| Serine pathway methylotrophs that do not use methane | |
| *Methylobacterium* sp. strain PR-6 | UCGGUUCGG AAUAACUCAGG GAAA |
| *Methylobacterium* sp. strain PK-1 | UCGGUUCGG AAUAACUCAGG GAAA |
| *Methylobacterium* sp. strain M27 | UCGGUUCGG AAUAACUCAGG GAAA |
| *Methylobacterium* sp. strain DM4 | UCGGUUCGG AAUAACUCAGG GAAA |
| *Methylobacterium organophilum* XX | UCGGUUCGG AAUAACUCAGG GAAA |
| *Methylobacterium extorquens* | UCGGUUCGG AAUAACUCAGG GAAA |
| *Methylobacterium extorquens* AM1 | UCGGUUCGG AAUAACUCAGG GAAA |
| Strain ER2 | GCUCUACGG AAUAACUCAGG GAAA |
| RuMP pathway methylotrophs | |
| *Methylococcus capsulatus* Bath | UUCUGGGGG AAUAACUCGGG GAAA |
| *Methylococcus flagellatum* | UAAUGGGGG AAUAACUAGU GAAA |
| *Methylococcus methylophilus* AS1 | UCGUGGGGG A-CAACUAGUC GAAA |
| *Methylococcus methanolica* | UAAUGGGGG A-NAACUAGUC GAAA |
| *Methylomonas methylovora* | UAAUGGGGG AN-AACUAGUC GAAA |
| *Methylomonas glycogenes* | UAAUGGGGG AN-AACUAGUC GAAA |
| *Methylomonas rubra* | UAGUGGGGG AU-AACGUGGG GAAA |
| *Methylomonas* sp. strain A4 | UAGUGGGGG ACGAACUUGGG GAAA |
| *Methylomonas albus* BG8 | UAGUGGGGG NC-AACUUGGG GAAA |
| *Methylomonas methanica* | UGGUGGGGG AU-AACUUGGG GAAA |
| *Methylomonas luteus* | UAGUGGGGG AC-AACUUGGG GAAA |
| *Methylobacter vinelandii* | UAGUGGGgg uC-AACUUGGG GAAA |
| *Methylobacter bovis* | UAGUGGGgg aC-AACUUGGG GAAA |
| Lake Mendota clone | UAGUGGGGG AU-AACCCGGG GAAA |

[a] Most of the bacteria and their sources are described in references 6, 21, 51, and 52. The exceptions are *Methylobacter bovis*, *Methylobacter vinelandii*, *Methylosinus echinoides*, and *Methylosinus minimus*, which were obtained from the culture collection of V. F. Galchenko and are described in reference 16.

**based on genetic background.** Two plasmids, pER2a and pER2b, were detected in strain ER2 (Fig. 5A, lane 2); these plasmids were estimated to be 120 and 130 kb, respectively, from their electrophoretic mobilities in agarose gels compared with the mobilities of standard-size plasmids. Thus, pER2a was comparable in size to pDL11 of *Achromobacter* sp. strain WM111 (46). We also detected a faint band, suggesting that there is a 160-kb plasmid in WM111 which has not been described previously (Fig. 5A, lane 1). Probe pJH5 hybridized to pER2a but not to pER2b in blots of the gels, indicating that pER2a contained sequences homologous to the *mcd* gene of *Achromobacter* sp. strain WM111 (Fig. 5A, lane 4). Sucrose gradient fractions enriched for pER2a and pDL11 were obtained and, after digestion with *Eco*RI or *Cla*I, gave strikingly similar restriction fragment profiles in ethidium bromide-stained gels (Fig. 5B, lanes 1 through 4). The distributions of restriction fragments hybridizing to pJH5 in Southern blots of these gels were identical (Fig. 5B, lanes 5 through 8). Total cellular and plasmid-enriched fractions were digested with *Bgl*II or *Hin*dIII, and Southern blots of the gels were probed with pJH5 (Fig. 6A). The total cellular and plasmid-enriched preparations yielded the same restriction fragment profiles, indicating that there were no chromosomal copies of the *mcd* gene. The similarity of these plasmids was confirmed and at the same time contrasted with the dissimilarity in other regions of the strain ER2 and *Achromobacter* sp. strain WM111 genomes by probing blots of restricted plasmid and total cellular DNAs digested with a compound probe consisting of *Bgl*II fragments (including one plasmid fragment) cloned from a digest of the total genome of WM111 (Fig. 6B). From these hybridization results we concluded that strain ER2 and *Arthrobacter* sp. strain WM111 are genotypically distinct organisms which contain similar plasmids containing the *mcd* gene.

**Carbofuran hydrolysis, methylamine oxidation, and serine pathway activity in strain ER2 grown under various nutritional conditions.** Hybridization with 16S rRNA signature probes indicated that strain ER2 was a type II methylotroph which used the serine pathway of formaldehyde assimilation. This result was confirmed by enzyme assays. Methylamine-grown strain ER2 clearly expressed HPR and SGAT, but not HPS, indicating that it possessed the serine pathway of C-1 carbon assimilation but not the RuMP pathway of C-1 carbon assimilation (Table 4). On the basis of these results, HPR activity was assayed in experiments in which the expression of the serine pathway was examined.

The expression of enzymes required for hydrolysis of carbofuran and metabolism of the methylamine moiety in cells grown under various nutritional conditions was examined (Table 5 and Fig. 7). Cells grown in the absence of carbofuran exhibited significant constitutive carbofuran hydrolase activity (about 225 to 292 ng of carbofuran hydro-

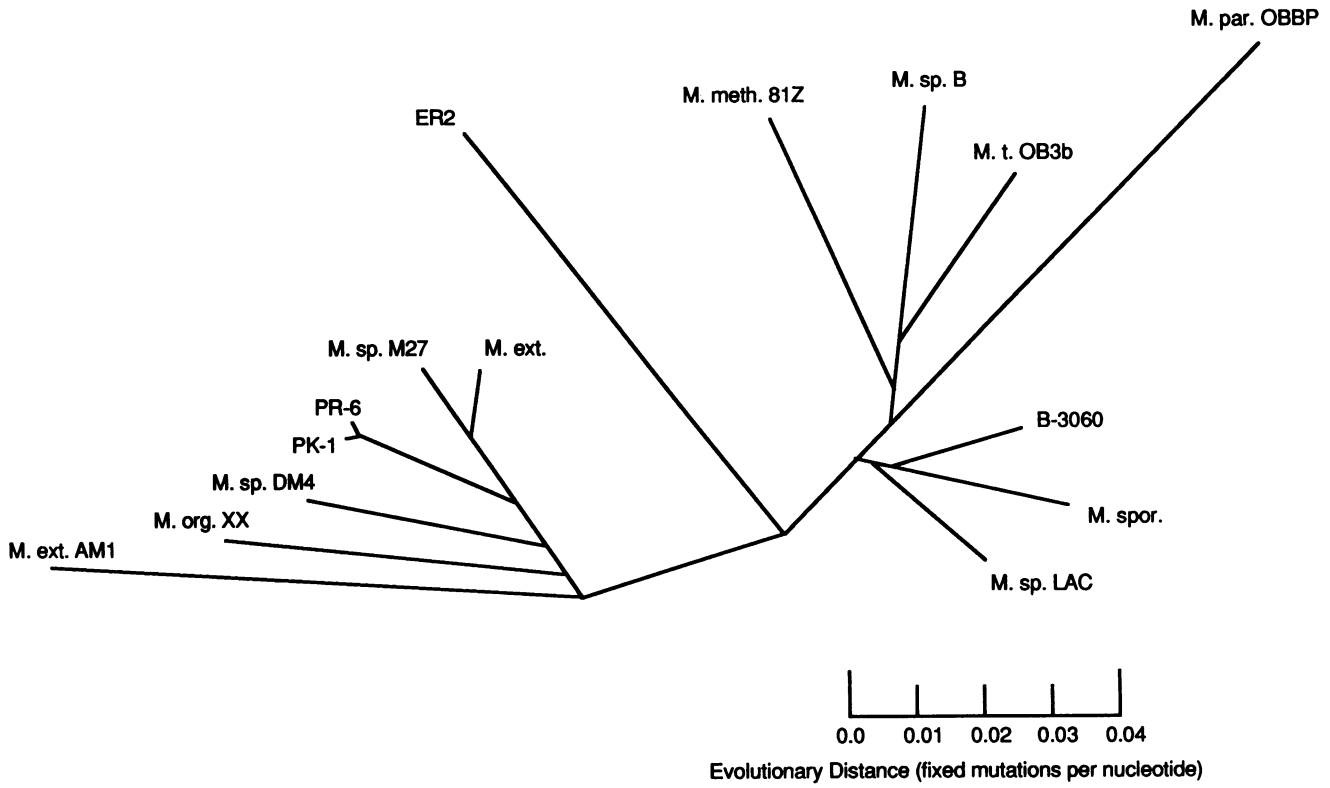


FIG. 3. Phylogenetic tree based on a comparison of 16S rRNA sequences, indicating relationships among methylotrophic bacteria that utilize the serine pathway for formaldehyde assimilation. Abbreviations: M. ext. AM1, *Methylobacterium extorquens* AM1 (GenBank accession no. M29027); M. org. XX, *Methylobacterium organophilum* XX (GenBank accession no. M29028); M. sp. DM4, *Methylobacterium* sp. strain DM4 (GenBank accession no. M29029); PK-1 and PR-6, *Methylobacterium* sp. strains PK-1 (GenBank accession no. M95654) and PR-6 (GenBank accession no. M95655), respectively; M. sp. M27, *Methylobacterium* sp. strain M27 (GenBank accession no. M95653); M. ext., *Methylobacterium extorquens* (GenBank accession no. M95656); ER2, strain ER2 (GenBank accession no. L20802); M. meth. 81Z, *Methylosinus methanica* 81Z (GenBank accession no. M29025); M. sp. B, *Methylosinus* sp. strain B (GenBank accession no. M95662); M. t. OB3b, *Methylosinus trichosporium* OB3b (GenBank accession no. M29024); M. par. OBBP, *Methylocystis parvis* OBBP (GenBank accession no. M29024); B-3060, strain B-3060 (GenBank accession no. L20845); M. spor., *Methylosinus sporium* (GenBank accession no. M95665); M. sp. LAC, *Methylosinus* sp. strain LAC (GenBank accession no. M95664). The strains used and their sources (except strain ER2 and its source) are described in references 6 and 21. Approximately 1,400 nucleotides in each 16S rRNA from positions 1 to 1,410 were determined. The nucleotide sequences have been deposited in the GenBank data base.

lyzed per min per mg of protein). This activity increased to about 1,800 ng of carbofuran hydrolyzed per min per mg of protein when carbofuran was provided either as the sole source of carbon and nitrogen or as the sole source of nitrogen in the presence of glucose.

Methylamine-dependent respiration rates were very low in cells grown on glucose and $NH_4Cl$. These rates increased 15- to 20-fold in the presence of carbofuran and 25-fold in the presence of methylamine. The presence of glucose in the growth medium had little effect on methylamine-dependent respiration regardless of whether the nitrogen source was methylamine or carbofuran.

HPR activity was barely detectable in cells grown on glucose and $NH_4Cl$. The highest levels of activity were observed with cells grown on methylamine as the sole source of carbon and nitrogen; these levels were sevenfold higher than the levels in cells grown on glucose plus methylamine. Cells grown in the presence of carbofuran exhibited levels of activity which were intermediate between the levels in methylamine-grown cells and the levels in glucose-grown cells.

## DISCUSSION

Strain ER2 hydrolyzed aryl *N*-methylcarbamate insecticides and grew at the expense of the methylamine produced. In this respect this organism is similar to other carbofuran-degrading bacteria, including *Achromobacter* sp. strain WM111 (23) and a number of *Pseudomonas* and *Flavobacterium* isolates (9).

Strain ER2 is remarkably similar to *Achromobacter* sp. strain WM111 with respect to its appearance, biochemical properties, and DNA homology characteristics. However, these organisms are not identical since *Achromobacter* sp. strain WM111 is motile, whereas strain ER2 is not. Since *Achromobacter* strains are motile by means of peritrichous flagella (59), strain ER2 does not belong in this genus. Furthermore, pER2a and pDL11 are similar in size and contain sequences which hybridize to the *mcd* gene on restriction fragments of the same size. Although it has been pointed out by Chapalamadugu and Chaudhry (7) that a number of carbofuran-degrading isolates do not hybridize to the *mcd* probe, our results indicate that the carbofuran

TABLE 3. Levels of PLFAs and LPS OHFAs in strain ER2

| Fatty acid | pmol/mg (dry wt) | mol% |
|---|---|---|
| PLFAs | | |
| 16:1w7c | 347 | 0.36 |
| 16:0 | 6,037 | 6.26 |
| i17:0 | 890 | 0.92 |
| cy17:0 | 244 | 0.25 |
| 18:1w7c | 41,964 | 43.50 |
| 18:0 | 6,476 | 6.71 |
| br19:1w6 | 4,629 | 4.80 |
| cy19:0 | 4,812 | 4.99 |
| Total | 96,475 | 100.00 |
| Residue OHFAs | | |
| 3OH12:O | 405 | 27.76 |
| 3OH18:O | 814 | 55.79 |
| 3OH19:O | 76 | 5.22 |
| 3OH20:O | 164 | 11.23 |
| Total | 1,459 | 100.00 |

hydrolase gene and the plasmid on which it is borne are not unique to *Achromobacter* sp. strain WM111.

Strain ER2 could not be identified on the basis of the results of biochemical tests since its properties do not match those of previously described genera of gram-negative bacteria (26). Strain ER2 is an unusual methylotroph in that it can utilize methylamine but not methanol or methane. A 16S rRNA sequence analysis indicated that strain ER2 is phylogenetically related to serine pathway methylotrophs. Figure 3 shows the relationships between strain ER2 and other methylotrophic bacteria that employ the serine pathway of formaldehyde assimilation. The bacteria to the left of strain ER2 in Fig. 3 are pink-pigmented facultative methylotrophs (18) that are not able to use methane as a carbon and energy source, whereas the bacteria to the right of strain ER2 are all obligate methanotrophs. All of the pink-pigmented facultative methylotrophs are capable of growth on methanol or methylamine. *Methylobacterium* sp. strain DM4 can also use dichloromethane as a carbon and energy source. It is clear that strain ER2 is not closely related to either subgroup of methylotrophs found in the alpha subdivision of the *Proteobacteria* (Fig. 3). The target sequence identified as 9-alpha (Table 2) is found in all sequenced 16S rRNAs from methylotrophic bacteria that use the serine pathway except the 16S rRNA from strain ER2. Probe 9-alpha did not hybridize to filter-bound RNA from strain ER2 under high-stringency conditions (wash temperature of 53°C) but did hybridize under lower-stringency conditions (wash temperature of 28°C). Probe 9-alpha does not hybridize to RNAs from the RuMP pathway organisms even under low-stringency conditions. It is possible that a probe complementary to the sequence for strain ER2 RNA shown in Table 2 will specifically hybridize to RNAs extracted from strain ER2 and related methylamine utilizers. Lipid analyses also indicate that strain ER2 is distinct from, but related to, the group 1, type II, pink-pigmented, facultative methylotrophs. It may be that methylotrophs limited to the methylamines for C-1 substrates are phylogenetically related.

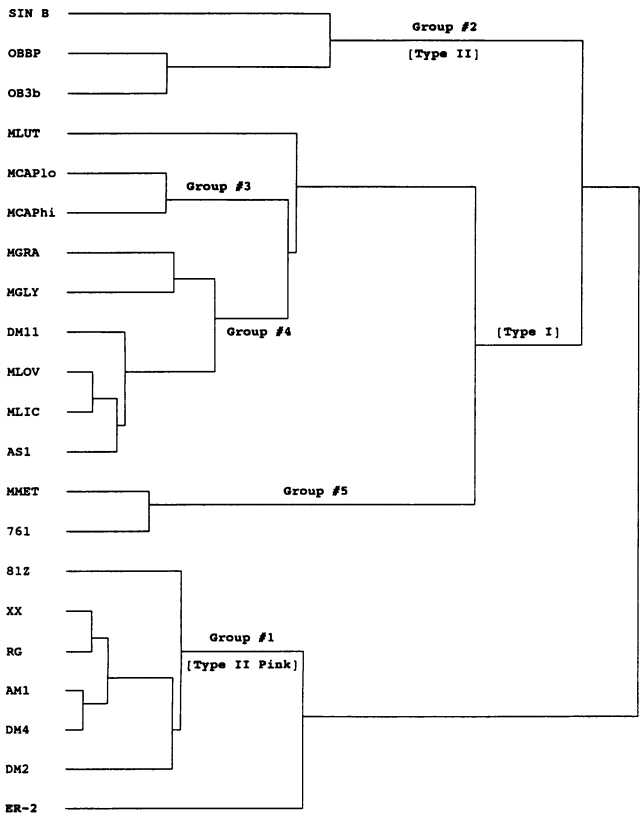


FIG. 4. Phylogenetic relationship among methylotrophs based on a comparison of membrane lipids, as determined by a hierarchical cluster analysis (complete linkage method). The strains used and their sources are described in reference 20.

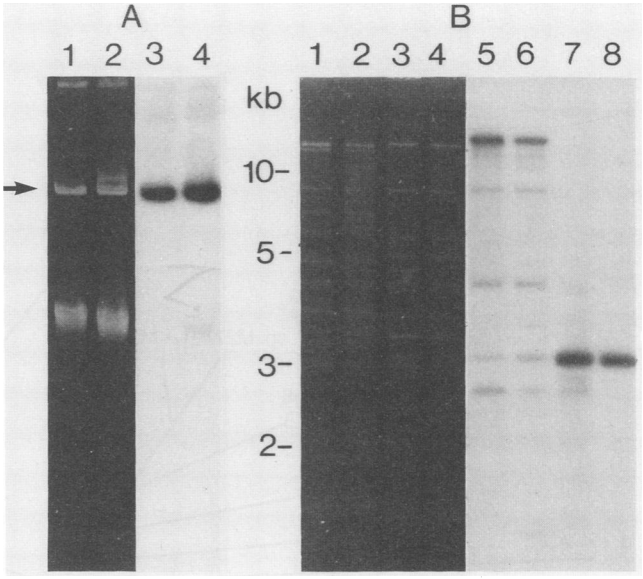


FIG. 5. Comparison of plasmids in *Achromobacter* sp. strain WM111 and strain ER2. (A) Supercoiled plasmids. Lanes 1 and 3 contained WM111, and lanes 2 and 4 contained ER2. Lanes 1 and 2, agarose gel after staining with ethidium bromide, containing two plasmid bands; lanes 3 and 4, autoradiogram of a Southern blot of the same gel probed with *mcd* gene probe pJH5. The arrow indicates the position of pDL11 and pER2a. (B) Restricted plasmid DNA from sucrose gradient fractions. Lanes 1, 3, 5, and 7 contained *Achromobacter* sp. strain WM111(pDL11), and lanes 2, 4, 6, and 8 contained strain ER2(pER2a). Lanes 1 to 4, agarose gel after staining with ethidium bromide; lanes 5 to 8, autoradiogram of a Southern blot of the same gel probed with pJH5. Lanes 1, 2, 5, and 6 contained *Eco*RI digests, and lanes 3, 4, 7, and 8 contained *Cla*I digests.

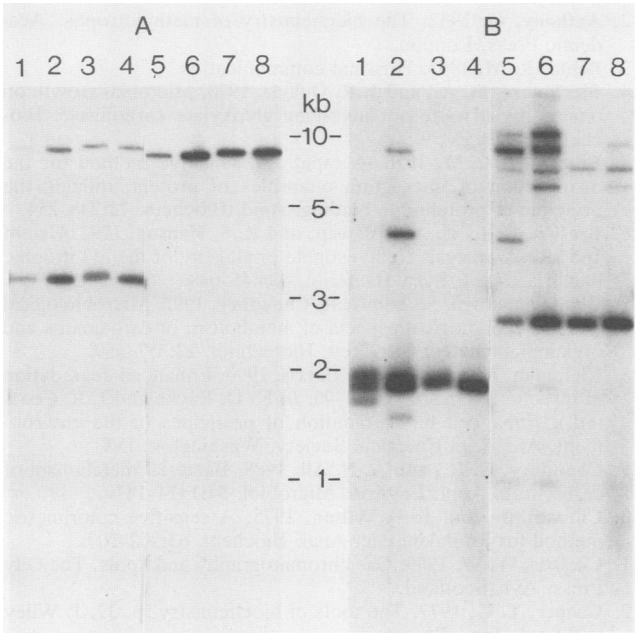


FIG. 6. Hybridization of restricted DNAs from total cell extracts and sucrose gradient fractions enriched for plasmids. (A) *mcd* gene probe pJH5. (B) Compound probe consisting of *Bgl*II fragments (1.6 to 2.0 kb), including one plasmid fragment, cloned from a digest of the total genome of *Achromobacter* sp. strain WM111. Lanes 1, 2, 5, and 6 contained total cellular extracts, and lanes 3, 4, 7, and 8 contained plasmid-enriched fractions. Lanes 1, 3, 5, and 7, *Achromobacter* sp. strain WM111; lanes 2, 4, 6, and 8, strain ER2. Lanes 1 to 4 contained *Bgl*II digests, and lanes 5 to 8 contained *Hin*dIII digests.

The similarity of pER2a and pDL11 found in bacteria isolated from geographic areas separated by great distances suggests that these plasmids may have widespread significance in *N*-methylcarbamate metabolism in the environment. The nearly identical restriction patterns of these plasmids suggest that genes other than carbofuran hydrolase have been conserved. The plasmid and the carbofuran-degrading phenotype were stably maintained in the absence of selective pressure in strain ER2 serially transferred several dozen times on nutrient agar. Attempts to cure the plasmid by treatment with mitomycin, acridine orange, and an elevated temperature failed. Thus, the plasmid may be required by the bacterium under the growth conditions used.

TABLE 4. Specific activities of HPR, SGAT, and HPS in methylamine-grown strain ER2 and methanol-grown *Methylobacterium organophilum* XX and *Methylomonas methylovora*

| Organism | Sp act | | |
|---|---|---|---|
| | HPR[a] | SGAT[a] | HPS[b] |
| Strain ER2 | 0.71 | 0.33 | BDL[c] |
| *Methylobacterium organophilum* XX | 0.40 | 0.22 | BDL |
| *Methylomonas methylovora* | BDL | BDL | 16.3 |

[a] Specific activities are expressed in micromoles of NADH oxidized per minute per milligram of protein.
[b] Specific activities are expressed in micromoles of HCHO consumed per minute per milligram of protein.
[c] BDL, below detection limit.

TABLE 5. Carbofuran hydrolase, methylamine-dependent respiration, and HPR activities in strain ER2 cells grown on different substrates

| Substrate(s) | Carbofuran hydrolase activity[a] | Methylamine-dependent respiration activity[b] | HPR activity[c] |
|---|---|---|---|
| $CH_3NH_2$ | 288 | 320 | 0.71 |
| Glucose + $CH_3NH_2$ | 225 | 324 | 0.11 |
| Carbofuran | 1,728 | 246 | 0.07 |
| Glucose + carbofuran | 1,868 | 194 | 0.04 |
| Glucose + $NH_4Cl$ | 292 | 12 | 0.02 |

[a] Expressed in nanograms of carbofuran hydrolyzed per milligram of protein per minute.
[b] Expressed in nanomoles of oxygen per milligram of protein per minute.
[c] Expressed in micromoles of NADH oxidized per milligram of protein per minute.

rRNA signature probes and enzyme measurements showed that strain ER2 is a type II methylotroph that fixes HCHO via the serine pathway. The specific activities of HPR and SGAT in cell extracts prepared from cells of strain ER2 grown in the presence of methylamine were somewhat higher than the specific activities in extracts prepared from methanol-grown cells of *Methylobacterium organophilum* XX. The HPR and SGAT specific activities in *Methylobacterium organophilum* XX were somewhat lower than, but comparable to, the specific activity of 0.58 μmol/min/mg of protein previously reported for this organism grown under similar conditions (33). There was no detectable HPS activity in either strain ER2 or *Methylobacterium organophilum* XX, whereas *Methylomonas methylovora* had an HPS specific activity of 16.3 μmol of HCHO consumed per min per mg of protein, indicating that strain ER2 does not possess the RuMP pathway.

Strain ER2 had significant constitutive carbofuran hydrolase activity. This activity was induced sevenfold in the presence of carbofuran. The relationship between carbofuran concentration and the degree of induction was not examined. Carbofuran hydrolase was not repressed by glucose and was not induced by methylamine.

Methylamine-dependent respiration was induced in the presence of methylamine or carbofuran. The induction of methylamine metabolism in the presence of carbofuran strongly suggests that methylamine is the intermediate through which carbofuran carbon enters metabolism. Methylamine metabolism was not subject to repression by glucose. We have not examined which of the three mechanisms of methylamine oxidation (via methylamine dehydrogenase,

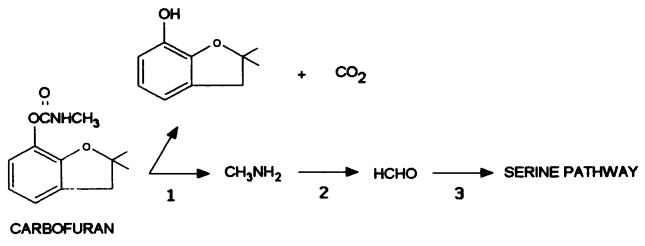


FIG. 7. Pathway of carbofuran metabolism proposed for strain ER2. The specific activities of carbofuran hydrolysis (1), methylamine oxidation (2), and HPR (3), an enzyme of the serine pathway of formaldehyde assimilation, were measured in cells grown under various nutritional conditions.

via methylamine oxidase, and via methylated amino acids) is used by strain ER2.

HPR activity was induced in the presence of methylamine or carbofuran. Glucose significantly repressed methylamine-induced HPR activity. Glucose partially repressed carbofuran-induced activity. In the presence of glucose, methylamine is required only as a nitrogen source, and therefore the rate of methylamine utilization would be expected to be lower. Calculations showed that the HPR specific activity was more than sufficient to handle the production of methylamine from carbofuran given that the maximum rate of carbofuran hydrolysis was roughly 2 μg of carbofuran hydrolyzed per min per mg of protein. This rate corresponds to 9 nmol of carbofuran hydrolyzed per min per mg of protein. Assuming stoichiometric production of methylamine and complete oxidation of methylamine to HCHO, 9 nmol of HCHO per min per mg of protein would be produced. If all of the HCHO was to be assimilated through the serine pathway, the HPR specific activity would have to be at least 9 nmol of NADH oxidized per min per mg of protein. The lowest specific activity observed in cells grown in the presence of carbofuran was 40 nmol of NADH oxidized per min per mg of protein, at least four times greater than the minimum required rate.

Glucose repressed HPR activity but not methylamine-dependent respiration or carbofuran hydrolysis. The data suggest that readily metabolizable carbon in the environment would not influence the synthesis of enzymes required for carbofuran hydrolysis and the oxidation of the resulting methylamine to carbon dioxide. However, since C-1 assimilation for biosynthesis is not required in the presence of an additional carbon source, serine pathway activity could be reduced.

In summary, strain ER2 is very similar in its properties to *Achromobacter* sp. strain WM111 but is a phylogenetically distinct methylotroph. The *mcd* gene and the plasmid on which it resides are highly conserved in these two bacteria isolated from widely separated areas. Strain ER2 expresses constitutive carbofuran hydrolase activity, but the full complement of enzymes required to hydrolyze carbofuran, oxidize methylamine, and assimilate formaldehyde are expressed at higher levels in the presence of the insecticide. Carbofuran-degrading bacteria with elevated specific activities of catabolic enzymes induced after exposure to the insecticide may therefore potentially contribute to enhanced degradation in soil. We are currently examining other carbofuran-degrading bacteria to assess the distribution of the *mcd* gene and to determine whether this gene would be a useful probe for studying enhanced degradation of carbofuran in soils.

## ACKNOWLEDGMENTS

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