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Degradation of 2-Methylbenzoic Acid by *Pseudomonas cepacia* MB2

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We report the isolation of *Pseudomonas cepacia* MB2, believed to be the first microorganism to utilize 2-methylbenzoic acid as the sole carbon source. Its growth range included all mono- and dimethylbenzoates (with the exception of 2,5- and 2,6-dimethylbenzoates) and 3-chloro-2-methylbenzoate (but not 4- or 5-chloro-2-methylbenzoate) but not chlorobenzoates lacking a methyl group. 2-Chlorobenzoate, 3-chlorobenzoate, and 2,3-, 2,4-, and 3,4-dichlorobenzoates inhibited growth of MB2 on 2-methylbenzoate as a result of cometabolism to the corresponding chlorinated catechols which blocked the key enzyme catechol 2,3-dioxygenase. A metapyrocatechase-negative mutant, MB2-G5, showed accumulation of dimethylcatechols from 2,3- and 3,4-dimethylbenzoates, and phenols were detected in resting-cell transformation extracts bearing the same substitution pattern as the original substrate, presumably following thermal degradation of the intermediate dihydrodiol. 2-Methylphenol was also found in extracts of the mutant cells with 2-methylbenzoate. These observations suggested a major route of methylbenzoate metabolism to be dioxygenation to a carboxy-hydrodiol which then forms a catechol derivative. In addition, the methyl group of 2-methylbenzoate was oxidized to isobenzofuranone (by cells of MB2-G5) and to phthalate (by cells of a separate mutant that could not utilize phthalate, MB2-D2). This pathway also generated a chlorinated isobenzofuranone from 3-chloro-2-methylbenzoate.

The aerobic biodegradation of aromatic hydrocarbons involves the enzymatic incorporation of both atoms of molecular oxygen into the aromatic nucleus. The toluene dioxygenase of *Pseudomonas putida* F1, for example, oxidizes toluene to a *cis*-dihydrodiol which then undergoes NAD-dependent oxidation to form 3-methylcatechol (8). The toluene dioxygenase and *cis*-toluene dihydrodiol dehydrogenase both have a relaxed specificity that generally allows bacteria to convert a wide range of aromatic compounds, including *m*- and *p*-xylenes, to catechols (7, 9). In contrast, the methyl group of toluene can be attacked by *P. putida* mt-2 to produce sequentially benzyl alcohol, benzaldehyde, and benzoate (30); benzoate is oxidized to catechol via the carboxyhydrodiol (5, 23, 31). The ability to degrade toluene is coded by a transmissible plasmid designated TOL in strain mt-2 (29), whereas it is chromosomally encoded in strain F1 (28). Strain mt-2 oxidized *m*- and *p*-xylenes by way of the methylbenzoates (MBs) and methylcatechols (30). Certain disubstituted benzoates, including 3,4-dimethylbenzoate (3,4-diMB), which is an intermediate in the degradation of pseudocumene, were also oxidized by this organism (17). The pseudomonad (strain Pxy) isolated by Davey and Gibson (3) also utilized *p*-xylene by way of 4-MB, which was accumulated by mutant Pxy-40.

Perhaps for steric reasons, 2-MB and *ortho*-xylene are not growth substrates for either strain F1 or mt-2. Indeed, while *m*- and *p*-xylene-degrading bacteria are readily obtained by enrichment, Omori et al. (22) were unable to isolate a single *o*-xylene utilizer from 364 different soil samples. However, microbial degradation of *o*-xylene is facilitated when an additional carbon source is available (13, 14, 19). Gibson and Subramanian (10) reported a *Nocardia* sp. which utilized *o*-xylene by an initial ring dioxygenation that produced 3,4-dimethylcatechol. The strain failed to grow on *m*- or *p*-xylene or on any of the MBs. A similar phenotype was found for the strain of *Pseudomonas stutzeri* isolated by Baggi et al. (1) and *Corynebacterium* sp. strain C125 of Schraa et al. (24); the dimethylcatechol was assumed to undergo *meta* fission (20) to 2-hydroxy-5-methyl-6-oxo-2,4-heptadienoate. Oxygen uptake measurements for *o*-xylene-grown cells of strain C125 indicated much higher specificity for *ortho*-xylene than for *meta*- or *para*-xylenes and no activity with these substrates in acetate-grown cells.

We report here the isolation of a pseudomonad that utilizes 2-MB and suggest routes by which this and other methylated benzoates are degraded.

MATERIALS AND METHODS

Isolation and growth of strains used. Strain MB2 was isolated from activated sewage sludge by enrichment and serial transfer with 2-MB (0.05% [wt/vol]) as the growth substrate. (The benzoic acid derivatives occur very largely as potassium salts under the approximately neutral conditions of cell growth and substrate transformation. The concentration of substrate [say, 500-ppm 2-MB], however, refers to the acid form obtained as a commercial stock.) A minimal salts medium of pH 7.25 containing 50 mM KH_2PO_4, 3 mM NaH_2PO_4, 10 mM $(NH_4)_2SO_4$, 1 mM $MgSO_4$, and 10 ml of a trace element stock solution per liter was used. The latter contained (per liter) 0.2 g of $CaSO_4$, 0.2 g of $FeSO_4 \cdot 7H_2O$, 20 mg of $MnSO_4 \cdot 7H_2O$, 20 mg of $CuSO_4$, 20 mg of $ZnSO_4 \cdot 7H_2$), 10 mg of $CoSO_4 \cdot 7H_2$), 10 mg of $NaMoO_4 \cdot 2H_2O$, and 5 mg of H_3BO_3. The cells were grown in 2.8-liter Fernbach flasks agitated at 120 rpm on a gyratory shaker to provide suitable aeration. The strain was characterized by the procedures described by Starr et al. (26) and Gerhardt et al. (6) and by the Micro-station system of Biolog Inc. (Hayward, Calif.). Growth determinations were

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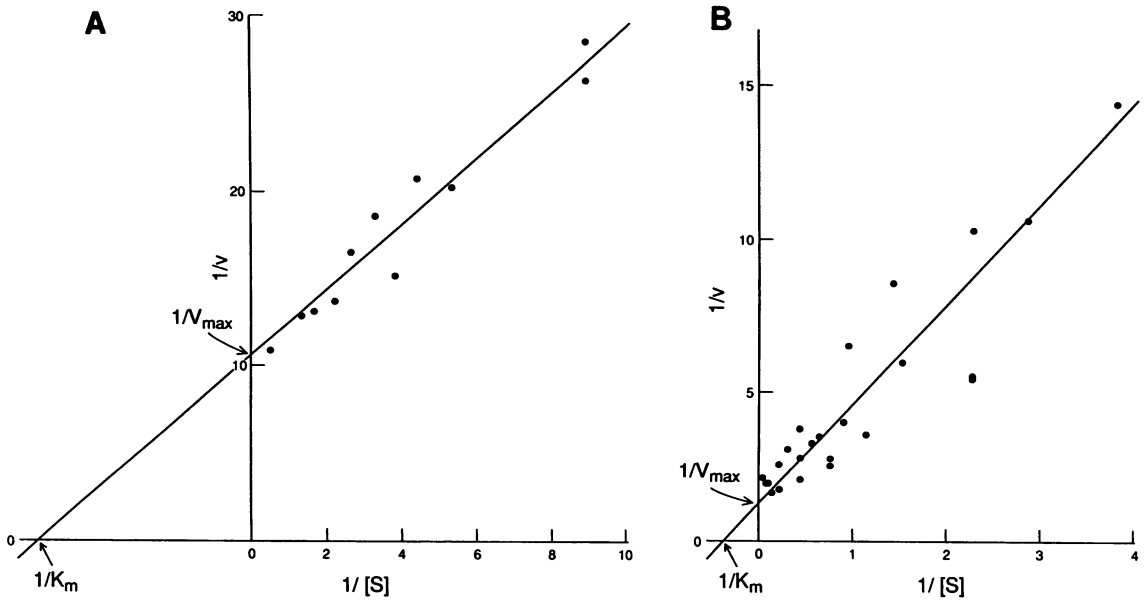

FIG. 1. Lineweaver-Burk plots for strain MB2 extract 4-chlorocatechol 2,3-dioxygenase activity (A) and catechol 2,3-dioxygenase activity (B). v, reaction rate in micromoles of substrate per milligram of protein per minute; [S], micromolar substrate concentration.

made after 7 days with a substrate concentration of 500 mg/liter. The strain was sensitive to kanamycin (50 mg/liter).

Isolation of mutants. Cells of strain MB2 grown on Luria agar and cells of *Escherichia coli* C600 pGS9::Tn*5* grown on Luria agar containing kanamycin (50 mg/liter) were combined on a Luria agar plate and incubated at 30°C for 20 h. A sample of the mated cells was then suspended in water and applied to plates of minimal medium containing agar (15 g/liter), sodium acetate (0.1% [wt/vol]), and kanamycin (50 mg/liter). Colonies (1,542) were removed with the aid of sterile toothpicks and replica plated from a grid arrangement on acetate-kanamycin medium onto 2-MB–kanamycin and benzoate-kanamycin media. Several colonies, such as MB2-A8 and MB2-G5, that grew on acetate and benzoate but showed very weak growth on 2MB were identified; these were routinely grown on benzoate-kanamycin medium. In a second selection system, 1,000 Tn*5*-mutant colonies were screened for the inability to grow on phthalate; one negative mutant (MB2-D2) was selected for further study. MB2-D2 grew well on benzoate and 2-MB.

Chemicals. 2-MB, 3-chloro-2-MB, 4-chloro-2-MB, and 5-chloro-2-MB were purchased from Pfaltz and Bauer (Stamford, Conn.), and chlorinated catechols were purchased from Helix (Vancouver, British Columbia, Canada), with the exception of 3-methyl-5-chlorocatechol, which was a kind gift from J. Knuutinen, Department of Chemistry, University of Jyväskylä, Jyväskylä, Finland. All other chemicals were obtained from Aldrich (Milwaukee, Wis.).

Preparation of cell suspensions and extracts. Cells were harvested at the end of the logarithmic growth phase by centrifugation (15 min at 12,000 × *g*), washed with potassium phosphate buffer (50 mM, pH 7.5), and resuspended in the same buffer. Cell extracts were prepared from suspensions by passage through a French press (20,000 lb/in^2, i.e., 1,400 kg/cm^2) and centrifugation at 40,000 × *g* for 40 min at 4°C.

Analytical methods. Chloride in culture supernatants was measured turbidimetrically (4) from the increase in A_{525} in a Uvikon 860 spectrophotometer (Kontron, Everett, Mass.) after addition of 0.2 ml of 0.1 M silver nitrate solution in 5 M phosphoric acid to 1 ml of sample to which 0.2 ml of 5 M phosphoric acid had already been added. Optical densities of cell suspensions were measured at 600 nm.

Protein was assayed by the biuret method (11), and catechol 1,2- and 2,3-dioxygenase activities were determined spectrophotometrically by the increase in A_{260} and A_{375}, respectively, as described by Spain and Nishino (25). The extinction coefficients of the *meta*-cleavage product of catechol and 3- and 4-methylcatechols at a common 375 nm were determined to be 48.4, 17.0, and 30.6 mM^{-1} cm^{-1}, from the values stated for other wavelengths by McClure and Venables (18). The extinction coefficient of 4-chlorocatechol's *meta*-cleavage product was assumed to be similar to that of catechol. The 2,3-dioxygenase was blocked without reducing the 1,2-dioxygenase activity by the incubation of extract with 7.35 μM hydrogen peroxide for 2 min at 30°C before monitoring the change in absorbance. Autoxidation of catechol substrates was not significant over the assay period.

Transformations of substrates were carried out at 28°C in

TABLE 1. Rates of catechol 1,2- and 2,3-dioxygenase activity in kanamycin-resistant mutants of strain MB2[a]

Mutant colony	Activity (nmol/mg of protein/min)	
	1,2-Dioxygenase	2,3-Dioxygenase
None (2MB-positive control)	1,100	137
MB2-A8	37	213
MB2-B2	35	172
MB2-B8	229	132
MB2-G5	0	190
MB2-G8	63	158
MB2-G9	204	117
MB2-G12	52	183

[a] Extracts were prepared from cells grown on benzoate in the presence of 50 mg of kanamycin per liter.

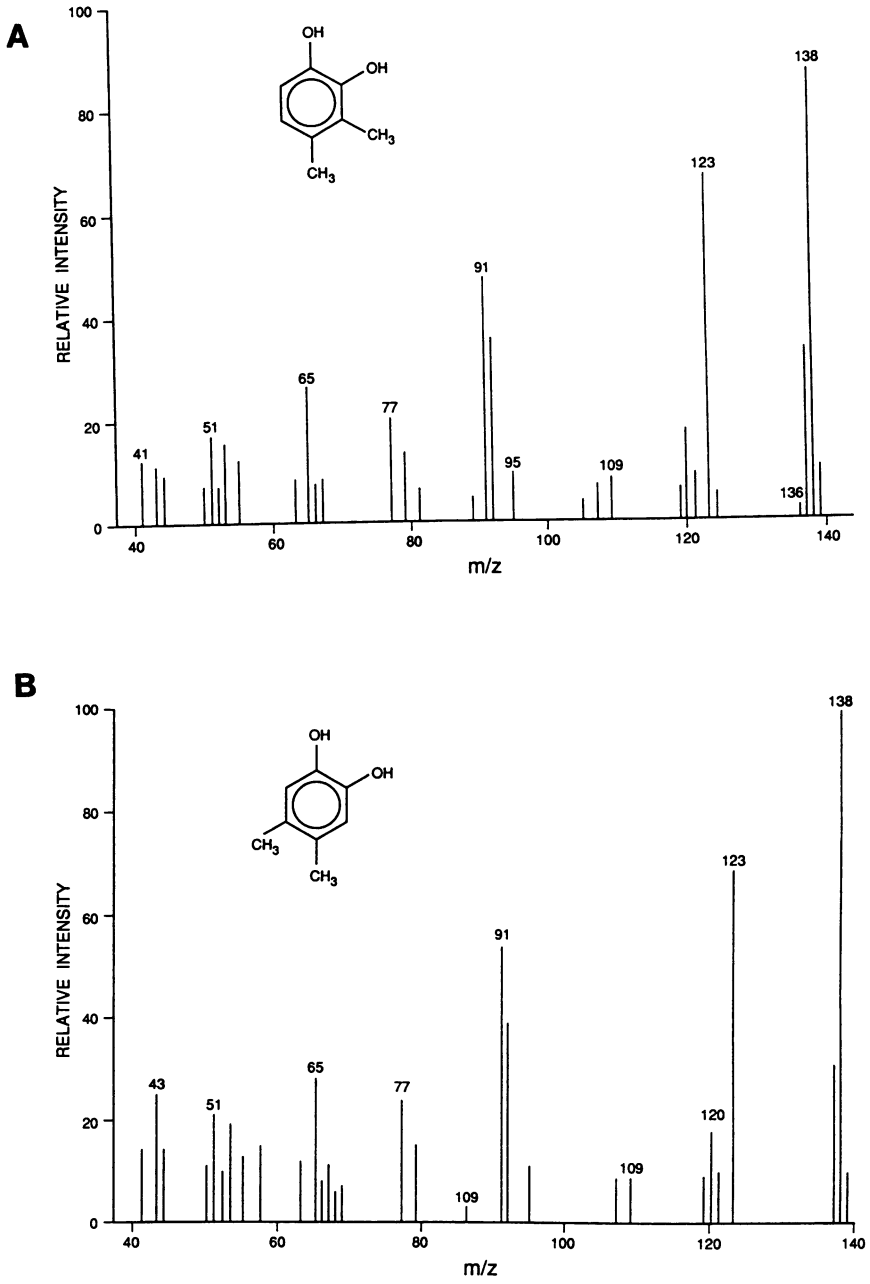

FIG. 2. Mass spectra of dimethylcatechols generated from 2,3-diMB (A) and 3,4-diMB (B) by resting cells of mutant MB2-G5.

glass vials crimp-sealed with a Teflon-lined rubber septum. Suspensions of resting cells in 5 ml of phosphate buffer were aerated on a platform shaker. Substrate was added from an acetonitrile stock (100 mg/ml), and the reaction was terminated with 0.5 ml of 10 N sulfuric acid. The suspensions were clarified by centrifugation in the Eppendorf Microfuge and analyzed by high-performance liquid chromatography. A Spherisorb ODS2 5-μm-diameter column was used, with an acetonitrile-water–0.04 M acetic acid mobile phase, typically run at a 40% isocratic condition. When extracts were prepared, ethyl ether was used, and the organic phase was dried with sodium sulfate and concentrated by rotary evaporation. The residue was redissolved in methanol and concentrated by passage of nitrogen for injection into a Hewlett-Packard 5989 gas chromatograph-mass spectrometer, operated normally in the electron impact mode with 70 eV of ionizing energy. The injector, ion source, and detector temperatures were 230, 200, and 280°C, respectively, and separation was achieved on a 15-m 0.25-mm-diameter HP1 capillary column (Hewlett-Packard), with a temperature program of 70°C (2-min initial wait) and then 8°C/min through 240°C (1-min stand). The quadrupole mass filter was scanned over the range of *m/z* 40 to 450 each second.

Methylation was carried out on extract residues by using ethereal diazomethane (produced by the base-catalyzed degradation of Diazald) at 25°C for 1 h.

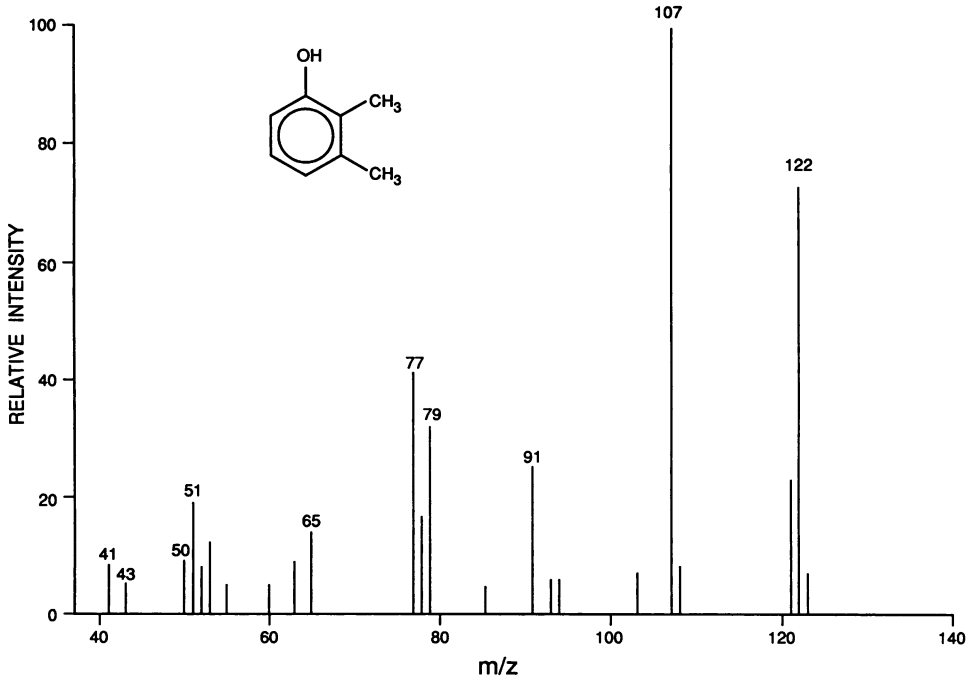

FIG. 3. Mass spectrum of 2,3-dimethylphenol produced from 2,3-diMB by resting cells of mutant MB2-G5.

RESULTS

Characterization of bacterial strain MB2. The enrichment procedure generated a pure culture designated MB2 that was able to grow on 2-MB as the sole carbon source. It was a rounded rod, motile by polar flagella, that formed small, circular, colorless, convex, smooth, opaque, butyrous colonies on 2-MB agar. The organism did not form fluorescent pigments on King media; was oxidase and catalase positive and arginine dihydrolase, gelatinase, and denitrification negative; and did not produce levan from sucrose. It did not hydrolyze starch and used glucose oxidatively and not fermentatively. These observations, together with the application of the Biolog identification system, indicated that the organism belongs to the species *Pseudomonas cepacia*, and it was designated strain MB2.

Strain MB2 grew on benzoate and all MBs and diMBs, with the exception of 2,5- and 2,6-diMBs. It grew on 3-chloro-2-MB and chloroacetate but not on 4- or 5-chloro-2-MB or 6-chloro-3-MB or on chlorobenzoates (CBs) without a methyl group. The growth rates (μ, h^{-1}) measured at 28°C and 500-ppm substrate were found to be as follows: benzoate, 0.39; 2-MB, 0.15; 3-MB, 0.21; 4-MB, 0.145; 2,3-diMB, 0.21; 2,4-diMB, 0.026; 3,4-diMB, 0.18; and 3,5-diMB, 0.037. The growth rate on 300-ppm 3-chloro-2-MB was 0.016 h^{-1}. The slow growth of strain MB2 on 2,4- and 3,5-diMBs produced a deep brown color not seen with other substrates. Strain MB2 grew on chloroacetate, phthalate, and phthalic anhydride, but there was no growth on hydrocarbons such as toluene or the xylenes.

Influence of CBs on growth of MB2. Certain chlorinated benzoates affected the growth of strain MB2 on 500-ppm 2-MB. At a 300-ppm concentration of 2- or 3-CB or of 2,3-dichlorobenzoate (2,3-diCB), 2,4-diCB, or 3,4-diCB, there was no growth at all. The addition of 300-ppm 3,5-diCB slightly reduced the growth rate (μ, 0.11); 4-CB, 2,6-diCB, or 6-chloro-3-MB had no effect (μ, 0.155); and 2,5-diCB slightly accelerated growth (μ, 0.18). When the concentration of 2-CB added was varied, inhibition of growth was found to begin at about 50 ppm; there was essentially no growth with 100 ppm.

The inclusion of 4-CB in the medium gave rise to a bright yellow color (λ_{max}, 380 nm), which was lost on acidification (HCl). The inclusion of 2,5- or 3,5-diCB or 6-chloro-3-MB in the medium gave rise to a brown color; addition of 50-ppm 2-CB gave a particularly pronounced brown coloration. The other cultures remained white.

Growth on 2-MB was unaffected by the inclusion of 300 ppm of 2,4-, 2,5-, or 2,6-diMB in the medium, but 3,5-diMB reduced the growth rate somewhat (μ, 0.14). There was a higher final yield of cells when 2,4- or 3,5-diMB was added. A brown color (with a broad absorbance shoulder at about 380 nm) was produced when 3,5-diMB (and, to a lesser extent, 2,4-diMB) was present in the growth culture. The other two diMBs had no effect on culture coloration.

Catechol dioxygenase data. Strain MB2 displayed both 1,2- and 2,3-dioxygenase activities with catechol, 3- and 4-methylcatechols, and 4-chlorocatechol, but neither was observed with 3-chlorocatechol or dichlorocatechols or with 5-chloro-3-methylcatechol. The yellow *meta*-fission product from 4-chlorocatechol was stable for several hours and showed absorption maxima in neutral solution of 283 and 379 nm. *meta* cleavage of 4-chlorocatechol was inhibited in a concentration-dependent fashion by 3-chlorocatechol and 3,4-, 3,5-, 3,6-, and 4,5-dichlorocatechols. The 4-chlorocatechol 2,3-dioxygenase activity showed a K_m of 0.175 μM and a V_{max} of 89 nmol/min/mg of protein; catechol 2,3-dioxygenase activity, by comparison, had a K_m of 2.0 μM and a V_{max} of 682 nmol/min/mg of protein. The double-reciprocal plots are presented in Fig. 1.

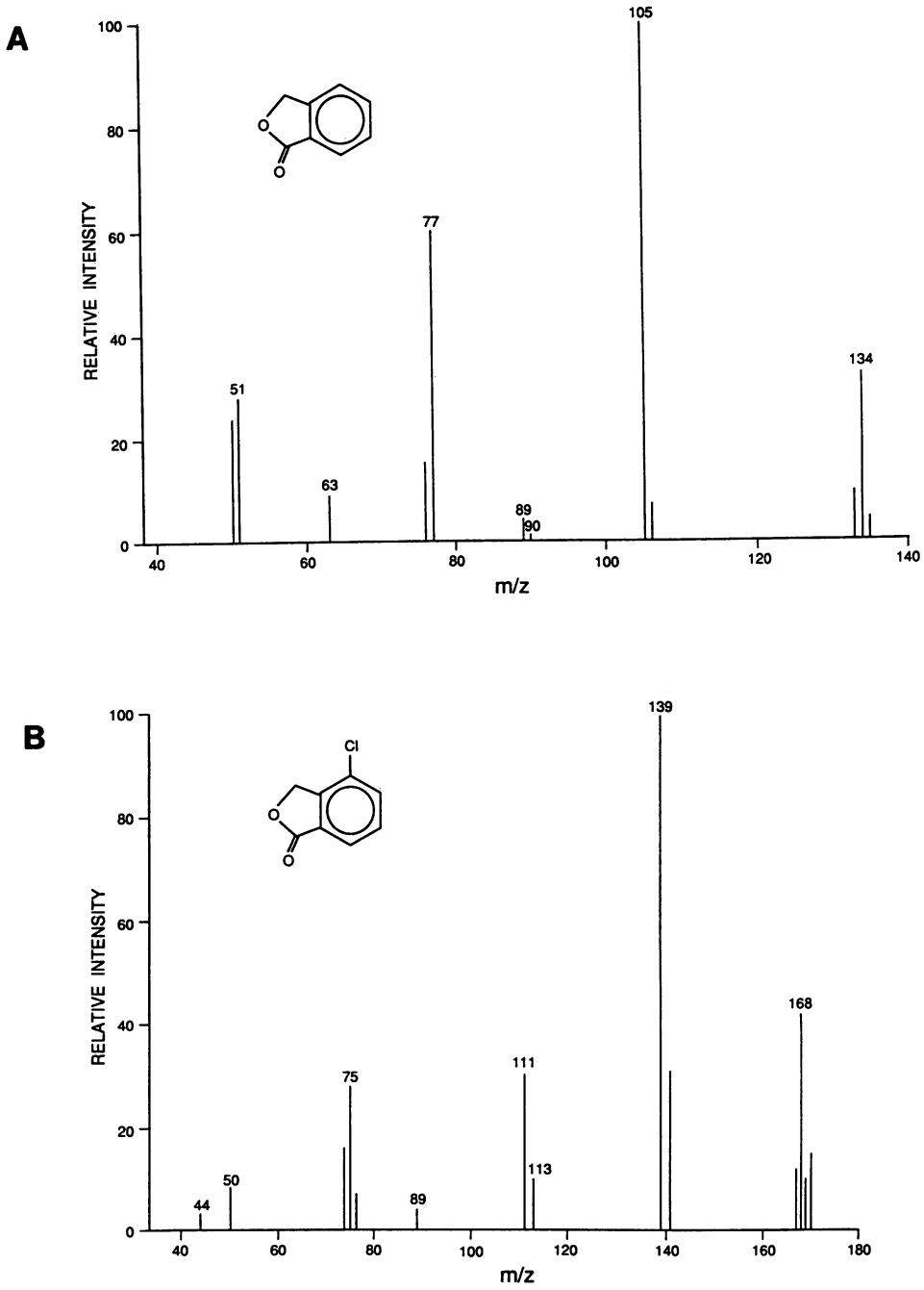

FIG. 4. Mass spectra of isobenzofuranone (A) and 3-chloro-isobenzofuranone (B) produced from 2-MB and 3-chloro-2-MB, respectively, by resting cells of mutant MB2-G5.

After growth of cells on benzoate-kanamycin medium, catechol 2,3-dioxygenase was greatly reduced in certain mutant colonies, compared with a kanamycin-resistant colony that showed normal growth on 2-MB (the 2-MB-positive control mutant), while *ortho* activity was approximately normal in each (Table 1). In one mutant, MB2-G5, no metapyrocatechase was detected, but the *ortho* activity was unimpaired, showing a preference for catechol, 4-methylcatechol, and 4-chlorocatechol over 3-methylcatechol (rates were 173, 55, 12, and 6 nmol/mg/min, respectively) and virtually no response to 3-chlorocatechol, 3,5-dichlorocatechol, 3-fluorocatechol, or 5-chloro-3-methylcatechol. Indeed, 3-chlorocatechol and 3,4-dichlorocatechol blocked the *ortho* fission of catechol or 4-chlorocatechol, although 3,5- and 3,6-dichlorocatechol were not inhibitory.

When the mutant MB2-G5 was shaken in 2-MB medium for an extended period, growth and metapyrocatechase activity were eventually produced. The transposon was presumed to relocate under the selection pressure of a substrate that demands *meta* enzyme expression.

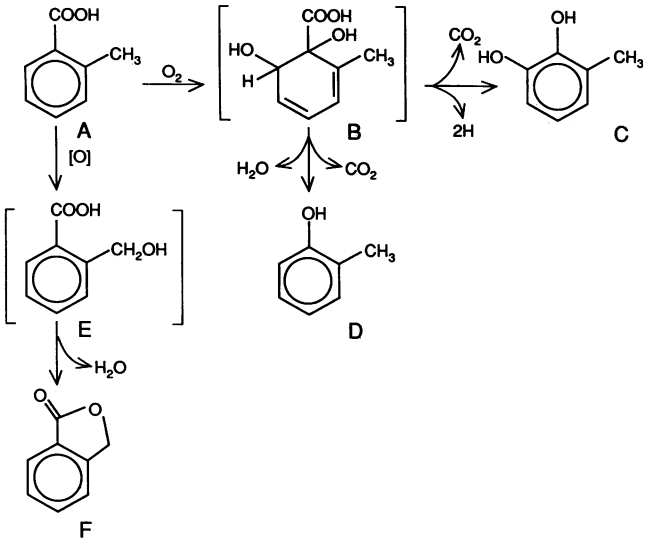

FIG. 5. Pathway of 2-MB metabolism by *P. cepacia* MB2, based on identification of products. Hypothetical intermediates in brackets would spontaneously degrade. Addition of oxygen to 2-MB (A) gives rise to a carboxyhydrodiol (B), which normally generates 3-methylcatechol (C) by decarboxylation and dehydrogenation but can thermally decompose to 2-methylphenol (D). Another proposed branch from compound A is to form 2-hydroxymethylbenzoate (E), which would spontaneously produce isobenzofuranone (F).

Transformation of benzoate derivatives. Resting cells of MB2 grown on 2-MB rapidly metabolized each of the mono- and diMBs; significant metabolites could be seen by high-performance liquid chromatography analysis of acidified supernatants only for transformations of 2,5-, 2,6-, and 3,5-diMBs.

In contrast, benzoate-kanamycin-grown cells of mutants MB2-A8 and MB2-G5 showed very little transformation of 2-, 2,4-, and 3,5-diMBs and greatly reduced attack on 2,3-, 2,5-, and 2,6-diMBs; they metabolized benzoate, 3- and 4-MBs, and 3,4-diMBs at a rate similar to that of cells of the 2MB-positive control mutant, also grown on benzoate-kanamycin.

Gas chromatography-mass spectrometry analysis of neutral ether extracts of overnight transformations by wild-type MB2 cells indicated large accumulations of 3-chlorocatechol from 2-CB. Resting cells of mutant MB2-G5 produced a dimethylcatechol from 2,3- and 3,4-diMBs (Fig. 2). The extracts from the MB2-G5 transformations also contained significant amounts of phenols: 2-MB produced 2-methylphenol, and 2,3-diMB produced 2,3-dimethylphenol (Fig. 3). Isobenzofuranone was identified in the extracts of MB2-G5 transformations of 2-MB, and the corresponding chlorinated derivative was obtained from 3- and 4-chloro-2-MBs. The mass spectra of these products are presented in Fig. 4. Neither catechol nor isobenzofuranone metabolites were evident in extracts of wild-type cells incubated with 2-MB or 3-chloro-2-MB.

When benzoate-kanamycin-grown cells of the phthalate-negative mutant MB2-D2 were incubated with 2-MB and ether extracts at 0 h, 15 min, and 24 h were reacted with ethereal diazomethane, gas chromatography-mass spectrometry analysis identified the dimethyl ester of phthalic acid at 15 min and 24 h (with similar yields), but none was found to be present at zero time.

DISCUSSION

This is the first report of a microorganism that utilizes 2-MB as the sole carbon source. A scheme for the degradation of this compound (Fig. 5) whereby either dioxygenation produces a carboxyhydrodiol that is then converted to 3-methylcatechol or monooxygenation of the methyl group occurs to form an intermediate that rapidly lactonizes to isobenzofuranone is proposed. The latter compound is assumed to give rise to the readily metabolized phthalic acid in the wild-type strain. Strain MB2 has a wide growth range, even including a benzoate derivative bearing both methyl and chlorine substituents. CBs lacking a methyl substituent, in contrast, fail to support growth. The rate of cell increase on 2,4- and 3,5-diMBs and 3-chloro-2-MB is an order of magnitude less than on the other methylated benzoates, and the three substrates give rise to a brown, flocculant culture which may reflect the accumulation of catecholic polymers. A likely common intermediate of the degradation of both 2,4- and 3,5-diMBs would be 3,5-dimethylcatechol, and this may be more difficult for the organism to oxidize. There have been few reports on the utilization of diMBs, although Zeyer et al. (32) found that *P. putida* mt-2 toluate oxidase could also act on 2,3-diMB.

As a general rule, organisms utilizing a CB oxidize the chlorocatechol intermediate by *ortho* fission and those degrading MBs employ *meta* cleavage for the methylcatechol intermediate. Cells of strain MB2 grown on 2-MB show both activities. Rather surprisingly, 4-chlorocatechol is *meta* cleaved by extracts of such cells, and so the cometabolism of 4-CB by resting cells produces a bright yellow color.

4-Chlorocatechol is not inhibitory to growth on 2-MB; thus, the presence of 4-CB in the medium has no effect. In contrast, catechols bearing a chlorine substituent adjacent to a hydroxyl are potent inhibitors of the 2,3-dioxygenase involved in methylcatechol oxidation and so of growth on 2-MB. Chlorocatechol formation presumably accounts for the inhibition of growth on 2-MB by certain chlorinated benzoates. The lack of *meta* fission of 4,5-dichlorocatechol was rather surprising, since this compound could be viewed as having two chlorines *para* to the two hydroxyls and might have been expected to be cleaved in a way similar to that of 4-chlorocatechol. Cometabolism of 4-chlorocatechol to a hydroxymuconic semialdehyde was reported for benzoate-grown cells of an *Achromobacter* sp. (12); the 1,6-cleavage described by Horvath also applied to 3,5-dichlorocatechol, which is not a substrate for the MB2 metapyrocatechase.

The inactivation of catechol 2,3-dioxygenase by 3-halocatechols has been previously described. Klecka and Gibson (15) found that catechol, 3- and 4-methylcatechols, and 4-fluorocatechol were all substrates for the partially purified enzyme from toluene-grown preparations of *P. putida* (with varying values of K_m and V_{max}), while 3- and 4-chlorocatechols were inhibitory (K_i, 0.14 and 50 μM, respectively). Bartels et al. (2) reported 3-fluorocatechol to be even more inhibitory to another catechol 2,3-dioxygenase, from *P. putida* mt-2, than 3-chlorocatechol (K_i, 17 and 23 μM, respectively). The fluorinated analog also showed a higher rate of inactivation. The crystallized mt-2 enzyme was found to oxidize 4-chlorocatechol at 51% of the catechol rate by Nozaki and coworkers (21), who observed negligible activity with 3,5-dichlorocatechol. The metapyrocatechase of toluene degrader *P. putida* R5-3 oxidized 4-chlorocatechol, catechol, and 3- and 4-methylcatechols at essentially equal rates (16). The catechol 2,3-dioxygenase of aniline-grown *Alcaligenes faecalis* showed 17% activity on 4-chlorocate-

chol relative to catechol (27), although the chlorinated analog became inhibitory at concentrations exceeding 0.4 mM. The organism MB2 thus resembles strain mt-2 or R5-3 more than the *P. putida* described by Klecka and Gibson (15).

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