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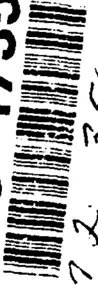
Microorganisms are frequently able to degrade anthropogenic materials using pathways that evolved for the assimilation of related naturally-occurring compounds. Complications can arise, however, during the metabolism of mixtures when incompatible intermediates are formed from different components. The breakdown of chloro- and methyl-aromatics, for example, produces catechols which are oxidized differently: chlorocatechols are normally cleaved by *ortho* fission and methylcatechols by *meta* fission. If both systems act simultaneously, suicide substrates or dead-end metabolites are usually formed. Nevertheless, bacteria differ in their ability to cope with such mixtures. A unique bacterium, *Pseudomonas cepacia* MB2 was isolated by selective enrichment on 2-methylbenzoate, yet was also able to fortuitously utilize 3-chloro-2-methylbenzoate as a sole carbon source. This strain is unique in its ability to utilize an aromatic acid containing both a methyl and chloro substituent via the meta-fission pathway without the production of suicidal products.



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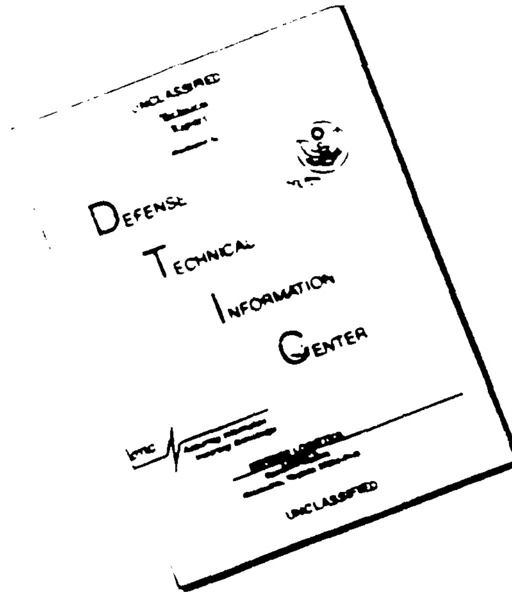

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FINAL REPORT

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Mr. Kozumbo, Program Manager
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Microorganisms are frequently able to degrade anthropogenic materials using pathways that evolved for the assimilation of related naturally-occurring compounds. Complications can arise, however, during the metabolism of mixtures when incompatible intermediates are formed from different components. The breakdown of chloro- and methyl-aromatics, for example, produces catechols which are oxidized differently: chlorocatechols are normally cleaved by *ortho* fission and methylcatechols by *meta* fission. If both systems act simultaneously, suicide substrates or dead-end metabolites are usually formed. Nevertheless, bacteria differ in their ability to cope with such mixtures.

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Utilization of 3-Chloro-2-Methylbenzoic Acid by *Pseudomonas cepacia* MB2 through the *meta* Fission Pathway

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Pseudomonas cepacia **MB2 grew on 3-chloro-2-methylbenzoate as a sole carbon source by metabolism through the *meta* fission pathway with the subsequent liberation of chloride. *meta* pyrocatechase activity in cell extracts was induced strongly by 3-chloro-2-methylbenzoate, but not by nongrowth analogs 4- or 5-chloro-2-methylbenzoate. Although rapid turnover of metabolites precluded direct identification, a mutant strain MB2-G5 lacking *meta* pyrocatechase activity produced 4-chloro-3-methylcatechol when incubated with 3-chloro-2-methylbenzoate. The catecholic product, confirmed by nuclear magnetic resonance and mass spectral analyses, produced a transient *meta* fission product (λ_{max} = 391 nm) from cell extracts of the wild-type MB2 strain. Further confirmation of *meta* pyrocatechase activity was noted by conversion of 4-chlorocatechol to 2-hydroxy-5-chloromuconic semialdehyde, which was not further metabolized. In contrast to 3-chlorocatechol, which was not metabolized and is known to generate suicidal products, 4-chlorocatechols do not generate acyl halides. Thus, further metabolism of the ring fission products is governed in strain MB2 by their suitability as substrates for the hydrolase.**

There have been limited precedents for the degradation of an aromatic ring bearing both methyl and chlorine substituents. Oxidation of 4-chlorotoluene (4-CT) to the *cis*-dihydrodiol and 3-chloro-6-methylcatechol by *Pseudomonas putida* was described by Gibson et al. (5), but the organism could not grow on 4-CT, because the chloromethylcatechol inactivated the strain's catechol 2,3-dioxygenase and accumulated in the medium. Vandenbergh et al. (16) made a reference to a strain purported to use 4-CT, but their growth medium included 500 ppm of yeast extract. A rigorous study of CT utilization was not presented until the work of Haigler and Spain (7) with *Pseudomonas* sp. strain JS6. This culture grew on chlorobenzene, 1,4-dichlorotoluene, or toluene as the sole carbon source (15) and gave rise to a spontaneous mutant, JS21, which additionally used 4-CT. It was proposed that JS21 had an altered regulatory protein such that 4-CT could induce enzymes of its own catabolism. The pathway proceeded by *ortho* ring cleavage of 3-chloro-6-methylcatechol to form 2-chloro-5-methyl-*cis*,*cis*-muconate, which was converted to 2-methyl-4-carboxymethylene-but-2-en-4-olide (2-methyldienelactone) and then hydrolyzed to 2-methylmaleylacetate. The pathway resembled the degradation of 5-chloro-3-methylcatechol described by Gaunt and Evans (4) for 4-chloro-2-methylphenoxyacetate-grown cells of *Pseudomonas* sp. strain NCIB9340 or by Pieper et al. (11) for 4-chloro-2-methylphenoxyacetate-degrading *Alcaligenes eutrophus* JMP134.

Chloro- and methylbenzoates are generally subject to dioxygenation during bacterial growth to a dihydrodiol derivative that gives rise to a chloro- or methylcatechol (8). Catechols are subject to dioxygenolytic cleavage at two sites: at the bond linking hydroxyl-bearing carbons (*ortho*) or to one side of this position (*meta*). Chlorocatechols are metabolized by *ortho* cleavage, whereas methylcatechols generally undergo *meta* fission. Although the *ortho* and *meta* pathways can each act on methyl- and chloroaromatics, dead-end products (methyllactones from *ortho* cleavage of methylcatechols) or reactive intermediates that constitute suicide substrates for the next enzyme in the pathway (acyl chlorides from chlorocatechols that inhibit catechol 2,3-dioxygenase) are generated (1, 10). Such unproductive misrouting is avoided by the expression of either *ortho* or *meta* in response to a specific type of aromatic compound; the addition of mixtures of chloro- and methylaromatics often disrupts bacterial growth (14).

We provide evidence here of conversion of 3-chloro-2-methylbenzoate by *Pseudomonas cepacia* MB2 to a chloromethylcatechol that undergoes *meta* fission instead of the *ortho* cleavage which is normally a requirement in the productive metabolism of chlorinated catechols.

MATERIALS AND METHODS

Isolation, growth of strains, and preparation of cell extracts. Strain MB2 was isolated from activated sewage sludge by enrichment and serial transfer with 2-methylbenzoic acid (0.05%, wt/vol) as the growth substrate and cultured as described previously (9). The isolation of a mutant (MB2-G5) lacking the *meta* pyrocatechase enzyme has been described earlier (9). Cell extracts were prepared by passage through a French press (20,000 lb/in^2) and centrifuged at 40,000 × *g* for 40 min at 4°C (9).

Chemicals. 2-Methyl-, 3-chloro-2-methyl-, 4-chloro-2-methyl-, and 5-chloro-2-methylbenzoic acids were purchased from Pfaltz and Bauer (Stamford, Conn.), and chlorinated catechols were 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 Jyvaskyla, Jyvaskyla 10, Finland.

Analytical methods. Chloride was measured in culture supernatants turbidometrically (3) from the increase in A_{525}

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in a Uvikon 860 spectrophotometer (Kontron, Everett, Mass.) after the 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 (6), 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 (15). Substrate transformations and extractions were done as described previously (9), and analyses were performed on a Hewlett-Packard 5989 gas chromatograph-mass spectrometer, generally in the 70-eV electron-impact (EI) mode. The injector, ion source, and detector temperatures were 230, 200, and 280°C, respectively, and separation was achieved on a [illegible], 0.25-mm-diameter HP1 capillary column (Hewlett-Packard), using a temperature program of 70°C (2-min initial wait), then 8°C/min through 240°C (1-min stand). For investigation of the *meta*-cleavage product of 4-chlorocatechol, the electron energy was reduced to 20 eV for EI, and methane was employed as the reagent gas in positive-polarity chemical ionization (CI), operating at 240 eV. Methylation was done with an ethereal solution of diazomethane (produced by the base-catalyzed degradation of Diazald) for 30 min at 30°C. Proton nuclear magnetic resonance spectra were obtained using a General Electric QE 300-MHz instrument in a deuterochloroform solvent.

RESULTS

[illegible] of strain MB2. The organism grew [illegible] mono- and dimethylbenzoates (500 ppm), with the exception of 2,5- and 2,6-dimethylbenzoate. It grew on 3-chloro-2-methylbenzoate but not on 4- or 5-chloro-2-methylbenzoates or chlorobenzoates lacking a methyl group. Dehalogenation was observed for chloroacetate, which was also utilized for growth, and 3-chloro-2-methylbenzoate but not for other chlorinated benzoates.

Production and metabolism of 4-chloro-3-methylcatechol. Because 4-chloro-3-methylcatechol is not commercially available, it was produced by transformation of 3-chloro-2-methylbenzoate by washed cell suspensions of the mutant MB2-G5, which lacks *meta* pyrocatechase activity (9). After a 24-h incubation on a platform shaker at 28°C, the supernatant was extracted with diethyl ether and fractionated by thin-layer chromatography to obtain a band that became brown on air oxidation, stained purple-brown with Gibb's reagent (2% methanolic solution of 2,6-dichloroquinone-4-chloroimide), and showed UV absorption maxima (in methanol solution) at 224 and 282 nm. Gas chromatographic-mass spectrometric analysis indicated the band to be a chloromethylcatechol. The molecular ion (M) is consistent with the exact molecular mass, and the M:M + 2 ratio of 3:1 is consistent with a single Cl atom. The major fragment at m/z = 123 represents the loss of Cl, and the aromatic nature of the molecule is evident by m/z = 77 (Fig. 1). Nuclear magnetic resonance signals for the compound's methyl protons appeared at δ 2.294 ppm (compare δ 2.223 ppm for 5-methyl-3-chlorocatechol); its aromatic protons showed a doublet of doublets at δ 6.632, 6.660, 6.797, 6.825 ppm, in contrast to a tighter cluster for 5-chloro-3-methylcatechol (δ 6.700, 6.707, 6.727, 6.734 ppm). The hydroxyl proton signals appeared as a broad feature centered at δ 5.4 ppm.

Cell extracts of 2-methylbenzoate-grown wild-type cells of strain MB2 were applied to the chloromethylcatechol prepared from the mutant. There was a transient increase in absorbance centered at 391 nm indicative of *meta* cleavage; this change did not take place with extracts obtained from cells of the mutant MB2-G5. No chlorinated intermediate could be detected by gas chromatography-mass spectrometry [illegible] cells or (cell extracts of wild-type strain MB2, presumably [illegible] due to rapid turnover of the transient *meta*-cleavage product.

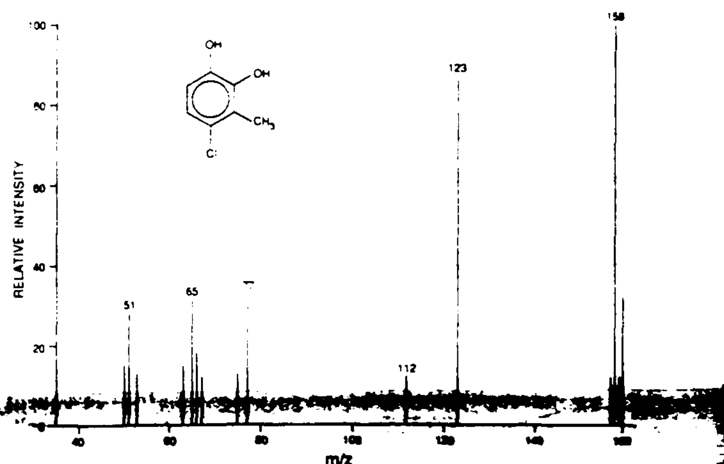

FIG. 1. Mass spectra of transformation product 4-chloro-3-methylcatechol formed from 3-chloro-2-methylbenzoate by resting cells of the mutant *P. cepacia* MB2-G5.

Ring fission of 4-chlorocatechol. To determine the mode of ring fission, cell extracts of wild-type strain MB2 (200 mg of protein in 20 ml of phosphate buffer) were shaken for 10 min at 30°C with 5 mg of 4-chlorocatechol added in 0.2 ml of methanol. The mixture was acidified to pH 2 with HCl, extracted with diethyl ether and, after partial evaporation of solvent, allowed to react with an ethereal solution of about 10 mg of diazomethane for 30 min. The solution was then concentrated by rotary evaporation and analyzed by gas chromatography-mass spectrometry in both EI and CI modes. While a 70-V electron energy setting proved unsuccessful, a 20-eV potential in EI showed peaks with weak molecular ions that lacked the characteristic M + 2 ions from the ^{37}Cl isotope: these ions corresponded to both the dimethylated derivative of 2-hydroxy-5-chloromuconic semialdehyde and the trimethylated derivative of its oxidation product 2-hydroxy-5-chloromuconate (Fig. 2A and B). Loss of a $-COOCH_3$ group was a major fragmentation pattern for both compounds. The CI spectra for these species (Fig. 2C and D) clearly show the 3:1 ratio of the M + 1/M + 3 ions. M + 29/M + 31 ions were also important and represent addition of H and C_2H_5 to the respective molecular ions.

Influence of chloromethylbenzoates on metapyrocatechase activity. Catechol 2,3-dioxygenase showed a significant background activity in extracts of MB2 cells grown for several transfers on sodium acetate (control, Table 1). This base rate was increased approximately fivefold by the presence of 100 ppm of benzoate or 2-methyl- or 3-chloro-2-methylbenzoate in the growth medium. In contrast, 4- and 5-chloro-2-methylbenzoates failed to induce this additional *meta* fission activity. 4-Chlorocatechol 2,3-dioxygenase activity responded similarly, with the same specificity of induction. Whereas 4-chloro-2-methylbenzoate had no sig-

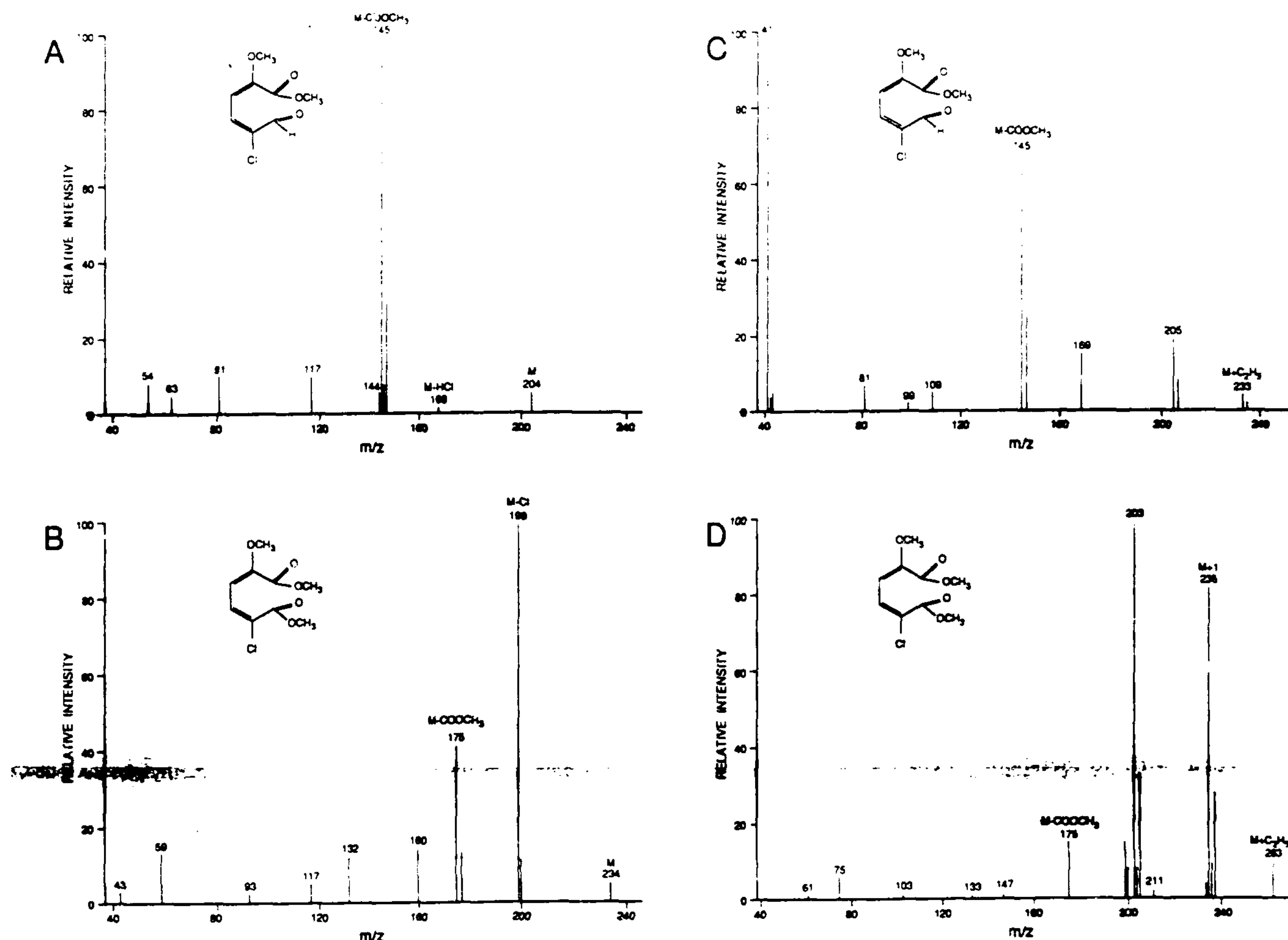

FIG. 2. EI mass spectra of the dimethylated derivative of 2-hydroxy-5-chloromuconic semialdehyde (A) and the trimethylated derivative of its oxidation product, 2-hydroxy-5-chloromuconate (B). Both were obtained from methylated extracts of strain MB2 *meta* pyrocatechase activity on 4-chlorocatechol. The corresponding CI mass spectra are displayed in panels C and D, respectively.

nificant effect on basal (row 4) or induced (rows 6 and 8) *meta* fission activity, the 5-chloroisomer was a strong inhibitor of both basal (row 5) and induced (rows 7 and 9) *meta* fission activity.

TABLE 1. Catechol and 4-chlorocatechol 2,3-dioxygenase data for separate extracts of cells of strain MB2 grown on acetate (1,000 ppm) in the presence of various benzoate derivatives (each 100 ppm)

Added benzoate derivative	Rate of oxidation (nmol of substrate/mg of protein/min)	
	Catechol (2.27 mM)	4-Chlorocatechol (1.72 mM)
Control[a]	198	30
2-MB[b]	1,010	177
3-chloro-2-MB	923	168
4-chloro-2-MB	253	39
5-chloro-2-MB	61	8
2-MB + 4-chloro-2-MB	1,310	216
2-MB + 5-chloro-2-MB	193	20
3-chloro-2-MB + 4-chloro-2-MB	743	120
3-chloro-2-MB + 5-chloro-2-MB	56	9

[a] Cells grown on acetate alone.
[b] 2-Methylbenzoate.

DISCUSSION

Strain MB2 is unique in its ability to grow on and dehalogenate a benzoate derivative bearing both chlorine and methyl substituents through the *meta* fission pathway. Mutant MB2-G5 (*meta* pyrocatechase negative) accumulated 4-chloro-3-methylcatechol despite having a basal level of *ortho* pyrocatechase (9); an *ortho* route for the intermediate chloromethylcatechol is thus not an option for this organism. An initial dioxygenation to produce a carboxydihydrodiol (17) is suggested as the logical intermediate to 4-chloro-3-methylcatechol (Fig. 3). By analogy to the metabolism of the *meta* cleavage product of 4-chlorocatechol (2-hydroxymuconic 4-chloromuconic semialdehyde), we suggest that 4-chloro-3-methylcatechol is cleaved by strain MB2 to 2-hydroxy-5-chloro-6-oxohepta-2,4-dienoic acid, which would in turn be hydrolyzed through the classic *meta* fission pathway (2) to acetate pyruvate and chloroacetaldehyde (Fig. 3). Such intermediates are presumably too transient in this strain to show appreciable concentrations in ether extracts. Moreover, analysis was confounded by the emergence of chloroacetaldehyde with the solvent front.

Utilization of 3-chloro-2-methylbenzoate is highly specific in terms of the arrangement of both methyl and chlorine substituents. Isomers chlorinated at the 4- or 5-position do

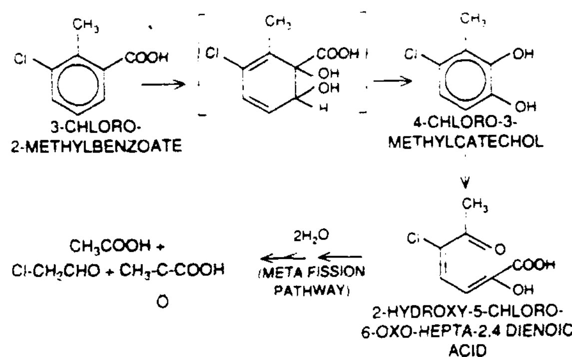

FIG. 3. Proposed scheme for the degradation of 3-chloro-2-methylbenzoate by *P. cepacia* MB2.

not give rise to *meta* fission products, and 4-chlorocatechol undergoes *meta* fission to a yellow product that cannot be further hydrolyzed by strain MB2. It would follow that the initial benzoate dioxygenase and carboxyhydrodiol dehydrogenase enzymes in strain MB2 are of broad specificity, while catechol 2,3-dioxygenase is more restricted, and the hydrolase is the most restrictive enzyme in the pathway.

According to Reineke and Knackmuss (1, 12, 13), the *meta* fission pathway is suicidal because 3-chlorocatechol is converted to an acyl halide that spontaneously reacts with and denatures the catechol 2,3-dioxygenase. The inability of *P. cepacia* MB2 to metabolize 3-chlorocatechol reinforces this point. However, *meta* fission of 4-chloro- and 3-methyl-4-chlorocatechols by strain MB2 does not produce acyl halides, and further metabolism of the ring fission products is apparently dependent on the specificity of the hydrolase.

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