Biodegradation of 2,4-Dichlorophenol through a Distal meta-Fission Pathway

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Alcaligenes eutrophus JMP222, a derivative of A. eutrophus JMP134 which has lost plasmid pJP4 (encoding the tfd genes for the ortho fission pathway), was induced for the meta fission pathway when grown on o-cresol. Resting cell suspensions, grown on o-cresol, oxidized 2,4-dichlorophenol (2,4-DCP), a degradation product of 2,4-dichlorophenoxyacetic acid, to 3,5-dichlorocatechol. Further degradation of 3,5-dichlorocatechol was observed by the production of a yellow ring fission product with liberation of chloride. Oxidation of 2,4-DCP (305 μM) in 47 hs resulted in 69% dehalogenation through this pathway. The ring fission product was characterized as 2-hydroxy-3,5-dichloro-6-oxo-hexa-2,4-dienoic acid by gas chromatography-mass spectrometry and gas chromatography-Fourier transform infrared spectroscopy. These data indicate that 2,4-DCP is degraded through a distal meta ring fission pathway, in contrast to either a suicidal proximal fission or the standard ortho fission pathway.

2,4-Dichlorophenoxyacetic acid (2,4-D) was the first xenobiotic compound demonstrated to be biodegradable to innocuous products of the tricarboxylic acid cycle (5) and one of the first to provide the molecular basis for the organization of catabolic genes (15). The tfdAB genes encode the respective cleavage of the ether linkage to 2,4-dichlorophenol (2,4-DCP) and the subsequent monoxygenation to 3,5-dichlorocatechol. Plasmid pJP4 in Alcaligenes eutrophus JMP134 contains the tfdCDEF genes, which encode the complete chlorocatechol pathway downstream from the central metabolite, 3,5-dichlorocatechol (15).

Although the tfdA and tfdB genes, which encode the respective cleavage of the ether bond and the oxidation of the ring, are commonly found in soil, the tfdCDEF genes encoding the chlorocatechol pathway are not (1, 11). Fulthorpe et al. (7) recently observed that 2,4-D-utilizers were not as readily isolated from soils, throughout the world, as 3-chlorobenzoate degraders. The possibility that the central metabolite, 3,5-dichlorocatechol, is not metabolized in soil through the ortho fission pathway merits consideration because o-cresol and other phenols induce meta fission (catechol 2,3-dioxygenase) in JMP134 (4). Thus, the proposal that an alternative pathway may exist was considered in the present study by examining how A. eutrophus JMP222, lacking the clc genes, metabolizes 2,4-DCP.

A. eutrophus JMP222 was a kind gift from P. Fortnagel (University of Hamburg, Hamburg, Germany). It was cured of the 2,4-D-degradative plasmid pJP4 (4). This strain does not grow on 2,4-D but readily grows on phenol and 2-methylphenol (i.e., o-cresol), which both induce the meta-cleavage pathway. Plate or liquid cultures were routinely incubated at 27°C in an incubator or on a rotary shaker (120 rpm). Stock cultures were maintained on mineral salts agar with phenol (250 ppm) as the growth substrate and stored at 4°C or kept in phenol mineral salts medium containing 20% glycerol in a deep freezer (−80°C) for permanent preservation. The isolate was grown in a defined mineral salts medium (6) containing o-cresol (2.3 mM).

Resting-cell suspensions were prepared from cells harvested at midexponential growth, washed twice with 50 mM phosphate buffer (pH 7.5), and resuspended to a final optical density at 525 nm of 10. The cells were incubated at initial concentrations of 305 and 610 μM 2,4-DCP, which were measured by high-pressure liquid chromatography (2). Formation of the ring fission product was measured spectrophotometrically at 375 nm, and chloride production was detected by a specific ion electrode (2). Another flask, incubated under the same condi-

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tions, was used to extract the reaction mixture at the time of peak production of the ring fission product (Fig. 1). The product was methylated with diazomethane and analyzed separately by combined gas chromatography-mass spectrometry and gas chromatography-Fourier transform infrared spectroscopy (14).

_A. eutrophus_ JMP222 readily grew on phenol or o-cresol as the sole carbon source but not on 2,4-DCP or chloroacetate. Resting cells grown on o-cresol released 69 and 42% of the total chloride contained in 305 and 610 μM 2,4-DCP after 47 h (Table 1). The disappearance of 2,4-DCP was concomitant with the production of a yellow ring fission product and the liberation of chloride (Fig. 1). Analysis of cell extracts from resting cells showed no activity for catechol 1,2-dioxygenase, as measured by monitoring the absorbance at 260 nm, in contrast to the rapid formation of a yellow ring fission product at 375 nm, which was indicative of catechol 2,3-dioxygenase.

The methylated derivative of the ring fission product was analyzed by gas chromatography-mass spectrometry and gas chromatography-Fourier transform infrared spectroscopy. The molecular ion was not noticeable in the main scan (Fig. 2). Hence, selective ion monitoring at a sensitivity 500-fold higher than that shown in Fig. 2 was performed (Fig. 3). The 3:2:0.3 ^35^Cl/^37^Cl isotope ratio is consistent for a compound having two chlorine atoms, and the masses of the M (238), M+2 (240), and M+4 (242) ions are consistent with the molecular ion postulated in Fig. 2. Although dimethylated derivatives of the ortho fission and meta fission products have identical molecular masses, the fragment ion (m/z = 59) shown in Fig. 2 is characteristic of dimethyl muconic acids, which give a −OCH₃ (m/z = 31) fragment from rupture of the ester bond. In contrast, the electron-withdrawing oxygen atom on each vicinal carbon of the semialdehyde product (Fig. 2) causes a positive repulsion between them and facilitates COOCH₃ cleavage. Characteristically weak molecular ions with a major fragmentation of the COOCH₃ moiety have been observed for other meta fission products (2, 10). The Fourier transform infrared spectrum of the methylated product (Fig. 4) is similar to that observed from meta fission of 4-methylcatechol fission (2). Two C—O stretching bands, characteristic of an unsaturated aliphatic ester, are observed at 1,184 and 1,319 cm⁻¹, and the higher than usual frequency (1,764 cm⁻¹) for an aldehyde C—O stretch is characteristic of a vicinal chlorine (20).

Reineke and Knackmuss (17, 18) noted that all bacteria which utilize chlorinated aromatic acids and phenols split the catechol intermediate between the two hydroxyl groups (ortho-pyrocatechase; EC 1.13.11.1), in contrast to aromatic hydrocarbon utilizers, which split catechols adjacent to one of the hydroxyl groups (meta-pyrocatechase; EC 1.13.11.2). 3-Chlorocatechol is consequently a suicidal substrate for bacteria which use the meta fission pathway, because it generates an acyl halide that reacts with the enzyme (3, 9, 19). However, suicidal inactivation is irrelevant for distal meta-pyrocatechase attack, as reported many years ago by Horvath (12). Productive meta fission pathways (oxidation and dehalogenation) have been reported for 3-methyl-4-chlorocatechol in _Pseudomonas cepacia_ MB2 (10) and for 4-chlorocatechol in _P. cepacia_ P166 (2). It should be noted that fission of 4-chlorocatechol, in contrast to 3-chlorocatechol, does not generate an acyl halide. Likewise, distal cleavage of 3,5-dichlorocatechol, in contrast to

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**TABLE 1. Oxidation of 2,4-DCP by resting cells of _A. eutrophus_ JMP222 grown on o-cresol**

<table>
<thead>
<tr>
<th>2,4-DCP concn (μM)</th>
<th>Time (h)</th>
<th>Chloride concn (μM)</th>
<th>% Stoichiometry</th>
</tr>
</thead>
<tbody>
<tr>
<td>305</td>
<td>24</td>
<td>257 ± 8</td>
<td>42.2</td>
</tr>
<tr>
<td></td>
<td>47</td>
<td>422 ± 35</td>
<td>69.2</td>
</tr>
<tr>
<td>610</td>
<td>24</td>
<td>118 ± 1</td>
<td>9.7</td>
</tr>
<tr>
<td></td>
<td>47</td>
<td>509 ± 68</td>
<td>41.7</td>
</tr>
</tbody>
</table>

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**FIG. 2.** Mass spectrum of the methylated ring fission product (observed from Fig. 1) of gas chromatographic separation (retention time, 30.8 min).
proximal cleavage, would not produce an acyl halide. Further metabolism of the 2,4-DCP ring fission product would logically proceed through formate, chloropyruvate, and chloroacetate to be consistent with the results of recent studies on meta fission pathways of chlorocatechols (2, 10).

The inability of JMP222 to utilize 2,4-DCP as the sole carbon source, although it is able to dehalogenate it, is not surprising inasmuch as chlorophenols are classic cytochrome uncouplers and are not always used as sole carbon sources because of toxicity at low (100 ppm) substrate concentrations (16, 21–23). Likewise, chloroacetate is not used as the sole carbon source by cells able to dehalogenate it, because of substrate toxicity (8) or failure to induce its own dehalogenation (2).

We have shown that a derivative of the archetype 2,4-D degrader JMP134 can metabolize 2,4-DCP through the meta fission pathway by liberating chloride. Strain JMP134 also contains genes for both meta and ortho fission pathways (4), which raises the possibility that either pathway could be used. Likewise, Pseudomonas acidovorans M3GY oxidizes either ring of the growth substrate 3,4′-dichlorobiphenyl to yield 3- and 4-chlorobenzoate, which are metabolized, respectively, through the ortho and meta fission pathways (13, 14). The results of these studies and ours have interesting implications concerning the recent discovery by Fulthorpe et al. (7). Although the two gene cassettes encoding 2,4-D (fdd) and 3-chlorobenzoate (ccl) are essentially identical, the authors concluded that different microorganisms were involved in the metabolism of the two substrates. Thus, the concept that 2,4-D is metabolized exclusively through the ortho fission pathway needs to be reassessed.

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REFERENCES