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Microbial Biodegradation of 4-Chlorobiphenyl, a Model Compound of Chlorinated Biphenyls

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The biodegradation products of 4-chlorobiphenyl were analyzed in an *Achromobacter* sp. strain and a *Bacillus brevis* strain. Both strains generated the same metabolites, with 4-chlorobenzoic acid as the major metabolic product. Our results corroborate previous observations whereby most bacterial strains degrade the chlorobiphenyls via a major pathway which proceeds by an hydroxylation in position 2,3 and a *meta*-1,2 fission. However, we also detected several metabolites whose structure suggests the existence of other routes for the degradation of chlorinated biphenyls.

Many bacterial strains able to degrade low-chlorine-content polychlorinated biphenyls (PCBs) have already been described in the literature (1, 7, 18–21). However, extensive studies of the metabolism of PCBs has been reported only in some of these strains. Ahmed and Focht (1) postulated that the degradation of low-chlorine-content PCBs in two species of *Achromobacter* proceeds via the formation of a 2,3-dihydroxy derivative which is open in a *meta*-1,2 position to ultimately generate the corresponding chlorobenzoate (Fig. 1). Furukawa et al. (6) postulated the same pathway for the degradation of PCBs in an *Acinetobacter* sp. strain and an *Alcaligenes* sp. strain. The steps between the 6-oxo-6-(4'-chlorophenyl)-2-hydroxy-2,3-hexadienoic acid and the 4-chlorobenzoate are still not known. Although this pathway is the major route for the degradation of PCBs by a strain of *Alcaligenes*, Yagi and Sudo (21) identified several acidic chlorophenyl derivatives of PCBs with aliphatic side chains containing two, three, or four carbons which were not expected as intermediates of the metabolic pathway shown in Fig. 1. They suggest that some of these metabolites were generated through a *meta* or *ortho* fission of the 3,4-dihydroxylated derivatives.

Strain B-206 also generated other unexpected metabolites of 4-chlorobiphenyl (4CB), such as the hydroxynitrochlorobiphenyls and the monohydroxynitrochlorobiphenyls (19) (which are probably the product of side reactions involving intermediates of the 4CB biodegradation pathway).

To extend our knowledge of the bacterial metabolism of 4CB, we further studied and compared the metabolites of 4CB generated by two other bacterial strains belonging to different genera which are able to grow on this substrate. The precise question asked was whether the degradation of 4CB could also involve, as in the examples cited above, metabolites other than the one expected as intermediates of the pathway shown in Fig. 1.

MATERIALS AND METHODS

Bacterial strains. Strain B-218 was isolated in our laboratory from an activated sludge sample by the method of Sylvestre (17). Strain B-257 was isolated by the same method from a soil sample. The strains were identified by the Laboratoire de Santé Publique du Québec (Ste. Anne de Bellevue, Quebec) as being an *Achromobacter* sp. strain (B-218) and a *Bacillus brevis* strain (B-257). The strains were kept lyophilized in beef serum or frozen at −80°C in dimethyl sulfoxide. The culture media used in this study were nutrient broth (Difco Laboratories, Detroit, Mich.) and medium number 30 (MM 30) in which sodium nitrate or ammonium sulfate was used as the nitrogen source (17).

Growth of strains B-218 and B-257 on 4CB. Each strain was grown on 4CB as the sole growth substrate in MM 30 to evaluate its ability to oxidize low-chlorine-content chlorobiphenyls. The incubation was done in 125-ml Erlenmeyer flasks containing 30 ml of broth, and the cultures were incubated at 29°C with agitation (250 rpm). The concentration of the growth substrate added to the medium was 0.25% (wt/vol) (13.54 mM). The inocula were prepared from log-phase cultures of the strains in MM 30. Growth was measured by the increase in viable counts, and the disappearance of the substrate was monitored by gas chromatographic analysis. The conditions for the gas chromatographic analysis of 4CB and 4-chlorobenzoate (4CBA) have been previously described (18).

Analysis of 4CB metabolites. The growth conditions for the analysis of the metabolites were the same as those described above. After various incubation periods, the content of a whole culture was filtered through a Whatman no. 40 paper and centrifuged at 8,000 × *g* for 10 min. The supernatant was first extracted three times at neutral pH with ethyl acetate and extracted three more times with the same solvent at pH 3.0. The extracts were analysed by gas chromatography-mass spectrometry, using conditions already described (19). The chloride released in the growth medium was followed by using a chloridometer (Buchler Instrument Inc., Fort Lee, N.J.), with which the chloride ion concentration is measured by a pair of silver electrodes immersed in an acid solution.

Plasmid DNA detection. The plasmid detection methods of Eckhardt (4), Casse et al. (3), and Wheatcroft and Williams (20) combined with agarose gel electrophoresis (12) were used for the detection of plasmid DNA. The technique of Hansen and Olsen (10) was used for the isolation of plasmid DNA in strain B-218. Extraction by the technique of Hansen and Olsen was followed by a cesium chloride-ethidium bromide density gradient ultracentrifugation. Agarose gel electrophoresis was performed in a horizontal 0.7% agarose

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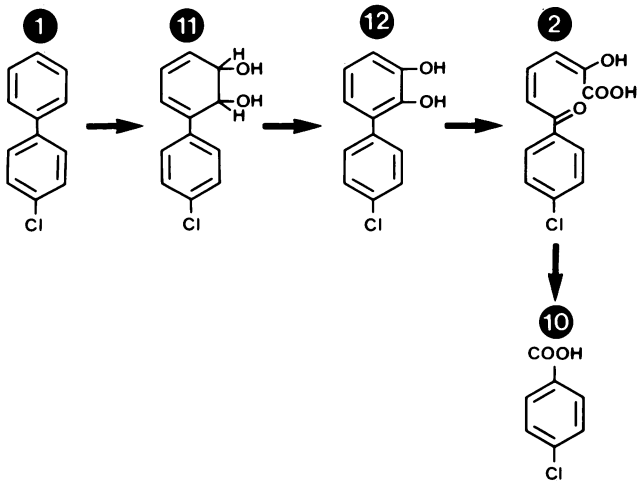

FIG. 1. Major pathway for the bacterial degradation of 4CB. 1, 4CB; 10, 4CBA; 2, 2-hydroxy-6-oxo-6-(4'-chlorophenyl)hexa-2,4-dienoic acid; 11, 2,3-dihydro-2,3-dihydroxy-4'-chlorobiphenyl; 12, 2,3-dihydroxy-4'-chlorobiphenyl.

gel using a Bio-Rad subcell unit, with tris-acetate (40 mM)–EDTA (21 mM) (pH 7.8) used as the buffer system. The restriction endonucleases used in this study were purchased from BRL (Gaithersburg, Md.), and the digestions were carried out following the recommendation of the supplier. For the determination of the molecular weight of strain B-218 plasmid DNA, DNA was digested with *Hin*dIII, *Eco*RI, *Sal*I, and *Bam*HI, and fragments of bacteriophage lambda DNA were used as molecular weight markers.

RESULTS

Growth on 4CB. 4CB added to the growth media was slowly metabolized during the first 48 h of incubation. However, it was much more rapidly and extensively degraded between 48 and 144 h of incubation. Figure 2 shows the disappearance of 4CB and the appearance of 4CBA, the ultimate biodegradation product of 4CB by both bacterial strains. After 144 h of incubation, nearly 50% of the initial amount of 4CB had been transformed into 4CBA and several other minor metabolites.

Analysis of the metabolites. To identify the metabolites of 4CB generated by strains B-218 and B-257, separate experiments were conducted in which bacteria were incubated for 24, 48, 72, 96, 144, and 168 h in the presence of the chlorinated substrate. Gas chromatographic-mass spectrometric analysis of the individual bacterial extracts demonstrated that the metabolic profiles of 4CB in both microbial organisms are identical throughout the biodegradation period (48 to 168 h).

The proposed structures of the minor metabolites are shown in Fig. 3. The individual components were characterized by the mass spectral features of their corresponding trimethylsilyl (TMS) ether and ester derivatives (Table 1) and by comparison with the mass spectra of analogous compounds (6, 8, 21).

Acidic metabolites 2, 3, and 4 showed molecular ions at *m/z* 396, 398, and 400, respectively, and diagnostically important ions at *m/z* 279, 281, and 283 (M-COOTMS). These ions are characteristic of 2-hydroxy 2,4-hexen (8, 21), 4-hexen and hexanoic acid 2, 3, and 4 TMS derivatives. The mass spectrum of saturated acid 4 TMS derivative is dominated by an intense ion at *m/z* 129 (CH_2=CH—CH=$^+$OTMS) resulting from a McLafferty rearrangement of the intermediate M-COOTMS ion at *m/z* 283. Compounds 3 and 4 or any other analogous monounsaturated and saturated acids have never been reported previously in the microbial biodegradation of PCBs.

The mass spectrum of metabolite 5 TMS derivative is shown in Fig. 4. Two structurally informative ions at *m/z* 173 and 213 clearly establish that this compound bears a *p*-chlorine benzylic hydroxyl group and a C_5 saturated carboxylic acid moiety. Although no molecular ion was observed at *m/z* 386, it was indicated by the M-15 ion at *m/z* 371 which was shifted up to *m/z* 386 in the mass spectrum of its d_9-TMS derivative.

Metabolite 6 was identified as 2-hydroxy-5-oxo-5-(4'-chlorophenyl) pentanoic acid. The mass spectrum of its TMS derivative (Table 1) was similar to that of compound 4, with characteristic ions at *m/z* 371 (M-15) and 269 (M-COOTMS), indicating the presence of a terminal α-hydroxycarboxylic acid moiety (8, 21). The ions at *m/z* 111 and 139 are characteristic of the 4-chlorobenzoyl group.

When trimethylsilylated, diketonic metabolite 7 was transformed into its corresponding 2,5-diTMS enol derivative, the mass spectrum exhibited ions at *m/z* 456 (M^+), 441 (M-15), 147, and 73. The structure of this minor metabolite was ascertained by the formation of the corresponding 2,5-

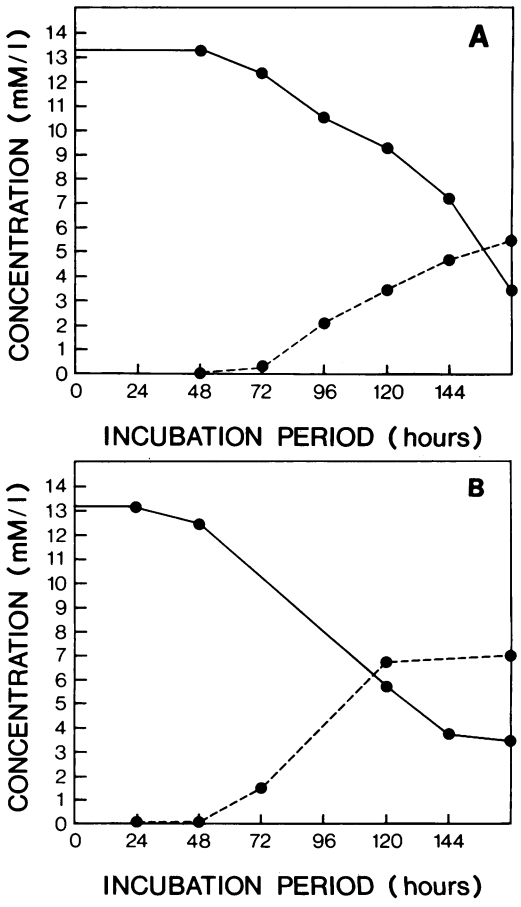

FIG. 2. Degradation of 4CB and production of 4CBA by (A) *Achromobacter* sp. strain B-218 and (B) *B. brevis* B-257. ●——●, 4CB; ●-----●, 4CBA.

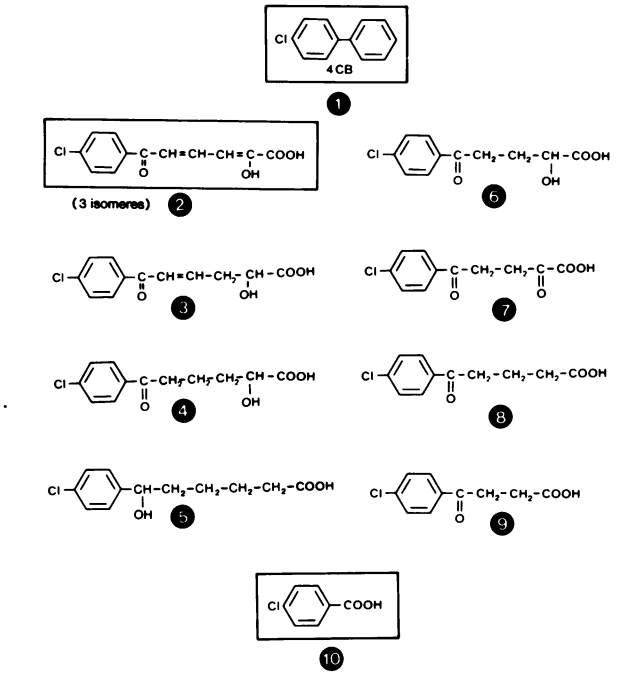

FIG. 3. Metabolites generated in the growth medium of strain B-218 and strain B-257 grown on 4CB in MM 30. For 1, 2, and 10, see the legend to Fig. 1. 3, 2-hydroxy-6-oxo-6-(4'-chlorophenyl)-4-hexenoic acid; 4, 6-oxo-6-(4'-chlorophenyl)-2-hydroxy-hexanoic acid; 5, 6-hydroxy-6-(4'-chlorophenyl)-hexanoic acid; 6, 2-hydroxy-5-oxo-5-(4'-chlorophenyl)-pentanoic acid; 7, 2,5-oxo-5-(4'-chlorophenyl)-pentanoic acid; 8, 5-oxo-5-(4'-chloro-phenyl)-pentanoic acid; 9, 4-oxo-4-(4'-chlorophenyl)-butanoic acid.

dimethyloxime-TMS derivative (M^+; m/z 370) and by d_9-trimethylsilylation (M^+; m/z 474).

The mass spectrum of metabolite 8 TMS derivative is shown in Fig. 4. From the series of ions at m/z 298 (M^+), 283 $(M-15)^+$, 241 (M-59), 208 (M-$TMSOH^+$), and 139 (4-chlorobenzoyl moiety) and by comparison with the mass spectrum of an authentic 5-oxo-5-phenyl pentanoic acid TMS derivative, compound 8 was identified as 5-oxo-5-(4'-chlorophenyl) pentanoic acid. Its structure was further ascertained by the mass spectra of the corresponding 5-methyloxyme-TMS (M^+; m/z 327) and d_9-TMS derivatives (M^+; m/z 307).

Finally, the mass spectrum of metabolite 9 was shown to be identical to that of authentic 4-oxo-4 (4'-chlorophenyl)butanoic acid (Table 1). A similar acidic compound has already been reported as a metabolite of 4CB in *Alcaligenes* sp. strain BM-2 (21).

Plasmid detection. Strain B-218 was shown to carry two plasmids which were detected as separate bands in the electrophoretic gel. However, by using the plasmid detection methods mentioned above (3, 4, 10, 19), we were unable to detect any plasmid in strain B-257. The molecular weight of strain B-218 plasmid DNA was calculated to be 16 and 72 kilobases, respectively, from the sum of the endonuclease digestion fragments. We were able to relate each of the plasmid DNA fragments to one of the two plasmid bands because of the relative abundance of each plasmid. Unfortunately, the frequency of $4CB^-$ mutant formation was very low, and all the $4CB^-$ mutants we isolated after plasmid-curing treatments (using either mitomycin or ethydium bromide) were able to revert to the wild-type phenotype. Thus, we cannot easily relate any of these plasmids to the 4CB catabolic pathway in strain B-218. We also compared the *Eco*RI and *Bam*HI fragmentation pattern of these plasmids with the fragments of pKF1 which carry a PCB biodegradation pathway (5). The results indicate that strain B-218 plasmids did not generate any fragments that comigrate with fragments of pKF1.

DISCUSSION

The results reported here indicate that *Achromobacter* sp. strain B-218 and *B. brevis* B-257 have the ability to grow on 4CB and degrade this model substrate into 4CBA. The latter is not further metabolized by the bacteria and accumulates in the growth media as the major and ultimate biodegradation product of 4CB. Similar observations have been reported for other bacterial strains grown in pure culture in the presence of various PCB isomers (6, 18, 21). 4CBA also appears to be the major metabolite in the case of the environmental biodegradation of PCBs (2, 15). The identification of 2-hydroxy-6-oxo-6-(4'-chlorophenyl)hexa-2-4-dienoic acid (compound 2) as one of the biodegradation products of 4CB by both bacterial strains suggests that the conversion of 4CB to 4CBA can be rationalized according to the pathway shown in Fig. 1. However, besides compound 2 and 4CBA, several minor degradation products were detected in the growth media of both bacterial strains grown on 4CB (Fig. 3). It is apparent from these data that enzymatic reactions other than those shown in Fig. 1 are likely to occur in the bacterial strains.

First, we characterized a series of three acidic metabolites (compounds 3, 4, and 5) which are likely to derived from the

TABLE 1. Mass spectrometric data of the TMS derivatives of bacterial metabolites of 4CB[a]

Metabolite	m/z[b]										
	M^+	M-15	Other ions								
2	396 (5)	381 (8)	279[c] (100)	263[d] (3)	189 (11)	147 (21)	75 (16)	73 (53)			
3	398 (20)	383 (15)	281[c] (17)	280 (26)	169 (10)	147 (80)	139 (44)	75 (64)	73 (100)		
4	400 (1)	385 (3)	283[c] (36)	267[d] (3)	203 (13)	151 (11)	147 (27)	139 (17)	129 (100)	75 (19)	73 (69)
5	386 (−)	371 (4)	253[d] (3)	213 (100)	173 (28)	147 (6)	75 (25)	73 (74)			
6	386 (−)	371 (8)	269[c] (100)	253[d] (10)	151 (18)	147 (42)	139 (31)	111 (17)	75 (41)	73 (92)	
7	456 (5)	441 (4)	147 (23)	75 (8)	73 (100)						
8	298 (8)	283 (14)	241 (12)	208 (11)	180 (13)	139 (100)	111 (29)	75 (79)	73 (48)		
9	284 (5)	269 (43)	241 (5)	213 (12)	195 (6)	169 (4)	139 (100)	111 (18)	75 (85)	73 (30)	

[a] Only ions containing ^{35}Cl are presented.
[b] Numbers in parentheses are relative abundance in terms of percentage of base peak.
[c] $(M\text{-}COOTMS)^+$.
[d] $(M\text{-}HCO_2\ TMS\text{-}CH_3)^+$.

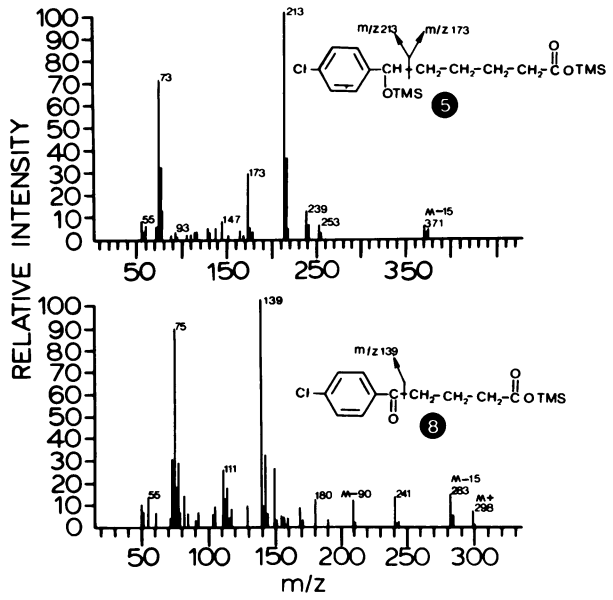

FIG. 4. Mass spectra of compound 5 [6-hydroxy-6-(4'-chlorophenyl)-hexanoic acid] (above) and compound 8 [5-oxo-5-(4'-chlorophenyl)pentanoic acid] (bottom).

enzymatic reduction of compound 2 double bonds. It is probable that compound 5 also originates from compound 2, although its precise route of formation is not known. The major structural feature which distinguished compound 5 from its structurally related analogs 2, 3, and 4 is the absence of an hydroxy group at C-2. Enzymatic or nonenzymatic dehydration of compounds 3 and/or 4, followed by the hydrogenation of the resulting double bonds at C-2 and C-4 and reduction of the 6-keto function into the 6-hydroxy group, may be a route for the formation of this metabolite. One must emphasize that metabolites 3, 4, and 5 have never been reported as biodegradation products of chlorinated biphenyls in bacteria. Although these compounds disappeared in an older culture, whether they are intermediate metabolites in the conversion of 4CB to 4CBA is still a matter of speculation and remains to be demonstrated.

Another significant point about the bacterial degradation of 4CB is the detection of the substituted pentanoic acids 6, 7, and 8 and that of 4-keto-4-(4'-chlorophenyl) butanoic acid (Fig. 3). Although 3,4-dihydroxy-4'-chlorobiphenyl was not detected in the bacterial broths, the presence of the above-mentioned acids in both bacterial extracts gives strong support to 3,4-hydroxylation of 4CB by *Achromobacter* sp. strain B-218 and *B. brevis* B-257. The detection of 3,4-hydroxy-4'-chlorobiphenyl and of 4-chlorocinnamic acid in strain B-206 (M. Sylvestre, R. Massé, F. Messier, and M. Levesque, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, Q143, p. 284; R. Massé, F. Messier, and M. Sylvestre, Abstr. Annu. Meet. Am. Soc. Mass Spect. 1982, RPA-13) is also a strong indication that ring fission mechanisms other than the *meta*-1,2 cleavage of 2,3-dihydroxy chlorobiphenyl can also occur in bacteria. Thus, it is likely that compounds 6 and 9 could have been generated through *meta* and *ortho* fission of 3,4-dihydroxy-4'-chlorobiphenyl, as shown in Fig. 5. For example, its 3,4-*ortho* cleavage could yield 3-(4-chlorophenyl)muconic acid, which would be subsequently oxydized to 4-keto-4-(4'-chlorophenyl)-2-butenoic acid. The latter would be enzymatically hydrogenated to 4-keto-4-(4'-chlorophenyl)butanoic acid (compound 9; Fig. 5). Alternatively, a 3,4-*meta* cleavage at the 2,3 position could yield 2-hydroxy-5-(4'-chlorophenyl) muconic acid, which would be further converted to 2-hydroxy-4-keto-4-(4'-chlorophenyl) pentanoic acid (compound 6).

Similar pathways have been proposed by Yagi and Sudo (21) to account for the formation of chlorinated cinnamic, phenylacetic, and benzoyl propionic acids in *Alcaligenes* sp. strain BM-2.

Whether there is a sequential oxidation of the ring fission product is still a matter of speculation. Such sequential oxidation has already been postulated for the degradation of biphenyl by nocardiae (14) and could also contribute to the formation of compounds 6 and 9.

The release in the environment of intermediate metabolites and/or side metabolites of xenobiotics, whose toxicity is still unknown, should be of much concern for environmentalists, even if xenobiotics are produced in very low

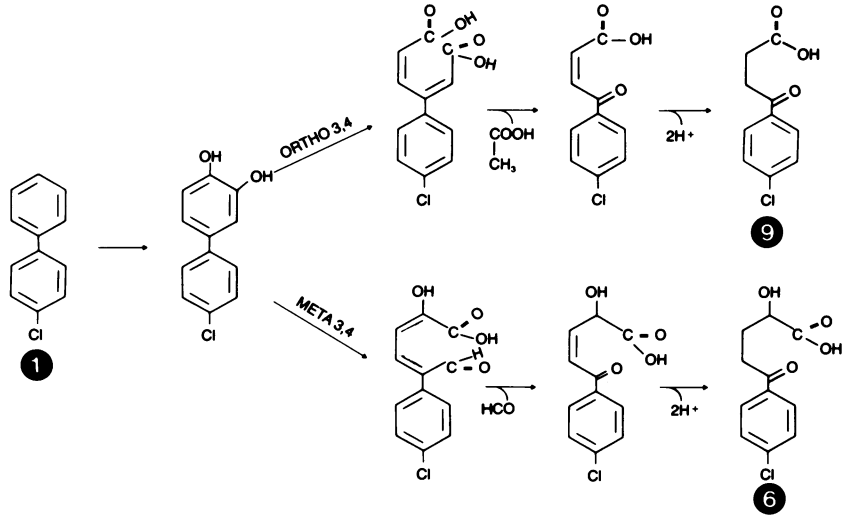

FIG. 5. Postulated routes for the formation of compounds 6 and 9.

amounts. This is even more true for side metabolites like the nitroderivatives, which are produced from 4CB (19) and other xenobiotics (9, 11, 13).

Contrary to results for strain B-206 (19), *Achromobacter* sp. and *B. brevis* B-257 were unable to generate detectable amounts of hydroxynitro derivatives of 4CB. Since the production of these derivatives is believed to be related to the presence of a monooxygenase in strain B-206 (19), it suggests that both strains possess only dioxygenases. This is in agreement with the traditional belief that the oxidation of aromatic rings in bacteria mostly involves dioxygenases (16).

The PCB degradation pathways in *Acinetobacter* sp. strain P6 and in *Arthrobacter* sp. strain M5 have been shown to be coded by plasmid genes (5). Strain B-218 was shown to carry a large and a smaller plasmid, but we were unable to relate these plasmids to 4CB biodegradation.

ACKNOWLEDGMENTS

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