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Formation of Chlorocatechol *meta* Cleavage Products by a Pseudomonad during Metabolism of Monochlorobiphenyls

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***Pseudomonas cepacia* P166 was able to metabolize all monochlorobiphenyls to the respective chlorobenzoates. Although they transiently accumulated, the chlorobenzoate degradation intermediates were further metabolized to chlorocatechols, which in turn were *meta* cleaved. 2- and 3-Chlorobiphenyl both produced 3-chlorocatechol, which was transformed to an acyl halide upon *meta* cleavage. 3-Chlorocatechol metabolism was toxic to the cells and impeded monochlorobiphenyl metabolism. In the case of 2-chlorobiphenyl, toxicity was manifested as a diminished growth rate, which nevertheless effected rapid substrate utilization. In the case of 3-chlorobiphenyl, which generates 3-chlorocatechol more rapidly than does 2-chlorobiphenyl, toxicity was manifested as a decrease in viable cells during substrate utilization. 4-Chlorobenzoate was transformed to 4-chlorocatechol, which was metabolized by a *meta* cleavage pathway leading to dehalogenation. Chloride release from 4-chlorocatechol metabolism, however, was slow and did not coincide with rapid 4-chlorocatechol turnover. Growth experiments with strain P166 on monochlorobiphenyls illustrated the difficulties of working with hydrophobic substrates that generate toxic intermediates. Turbidity could not be used to measure the growth of bacteria utilizing monochlorobiphenyls because high turbidities were routinely measured from cultures with very low viable-cell counts.**

Polychlorinated biphenyls (PCBs) constitute a class of 209 structurally related congeners comprising a biphenyl nucleus substituted with 1 to 10 chlorine atoms. Although PCBs as a group are generally regarded as recalcitrant to biodegradation, bacterial isolates have been described which can metabolize specific congeners. A commonality among PCB-transforming strains is that they are all biphenyl utilizers which metabolize PCBs with the same suite of enzymes employed in biphenyl catabolism (3, 12). Oxidative catabolism of biphenyl proceeds in four steps through a *meta* cleavage pathway (11, 13, 15, 24) producing benzoate and a five-carbon fragment (2-oxopent-4-enoate). The fate of these products has not been well studied. PCB congeners containing two or more chlorines are aerobically transformed only by cometabolism (7, 14), which requires biphenyl as a carbon source and inducer of the requisite enzymes. Isolates have been reported which can utilize monochlorobiphenyls (MCBPs) as growth substrates. Isolates which can utilize 4-chlorobiphenyl (4-CBP) are common (2, 3, 5, 12, 13, 18, 22, 23, 27). Reports of isolates which utilize 2- and 3-CBP are less common (7, 16, 25).

The transformation of PCBs results in the accumulation of chlorinated dead-end metabolites, usually chlorobenzoates (14). Moreover, chlorobenzoates have generally been regarded as dead-end products in the utilization of MCBPs. However, further transformation of these intermediates has occasionally been reported (6, 21, 25, 28). 4-CBP-utilizing *Alcaligenes* and *Acinetobacter* isolates transformed 4-chlorobenzoate (4-CBa) to 4-hydroxybenzoate (21, 27). *Pseudomonas* isolates have been reported to transform chlorobenzoates to several products, including chlorocatechols and their cleavage products (25, 28). In one case, these products were demonstrated to inhibit enzymes of the biphenyl pathway (28). The toxicity of 3-chlorocatechol (3-CC) has been demonstrated in a toluene-utilizing pseudomonad (4). *meta* cleavage transformed 3-CC into a highly reactive acyl halide (5-chloroformyl-2-hydroxypenta-2,4-dienoic acid) which quickly bound irreversibly to and inactivated macromolecules.

We have investigated the transformations of chlorobenzoate intermediates produced in the biodegradation of MCBPs by *Pseudomonas cepacia* P166. We present evidence that although chlorobenzoates transiently accumulated, they were further oxidized to chlorocatechols which were *meta* cleaved. Both 2- and 3-CBP were transformed to the 3-CC *meta* cleavage product, which was toxic and restricted growth. 4-CBP, which generated the 4-CC *meta* cleavage product, supported unrestricted growth.

MATERIALS AND METHODS

Isolation and culture conditions. *P. cepacia* P166 was isolated by enrichment on biphenyl from an industrial sewage effluent in Panama City, Republic of Panama. The isolate was a motile gram-negative rod with one polar flagellum, oxidase positive, catalase positive, lysine decarboxylase positive, arginine dihydrolase negative, nitrate reductase positive, and gelatin hydrolase negative. It metabolized carbohydrates oxidatively only, did not denitrify, did not fluoresce on King's Medium B, and did not require growth factors. In accordance with standard taxonomic criteria (8), the isolate was classified as *Pseudomonas cepacia* and designated strain P166. Strain P166 was grown in a mineral salts medium (1) supplemented with biphenyl (2 to 3 mM) or cultured on inverted mineral salts medium agar plates with biphenyl crystals added to the bottom lid.

Chemicals. Biphenyl, benzoate, 2-CBa, 3-CBa, 4-CBa, and catechol were purchased from Aldrich Chemical Co., Inc. (Milwaukee, Wis.). 2-CBP and 3-CBP were purchased from Lancaster Synthesis Inc. (Windham, N.H.). 4-CBP was purchased from Pfaltz & Bauer, Inc. (Waterbury, Conn.). 3- and 4-CC were obtained from Helix Biotech Corporation (Rich-

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mond, British Columbia, Canada). 2,3-Dihydroxybiphenyl was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Growing cell incubations. Growth studies were performed as previously described (10) for each of the MCBPs (1 mM) except that 10 g of glass beads was added to each flask. For viable cell counts, 50-μl aliquots were removed, serially diluted, and plated in triplicate on King's Medium B plates (19). At the end of each growth experiment, small aliquots were acidified 1:50 with 10 N H_2SO_4, centrifuged to remove cells, analyzed by high-performance liquid chromatography (HPLC), and quantified for chloride. After addition of Triton X-100 (0.7%), sodium sulfate, and a second MCBP (internal standard) to the remaining medium, residual MCBPs were extracted into an equal volume of hexane and analyzed by gas chromatography (GC).

Resting cell incubations. Resting cells were prepared as previously described (17) to a final density of 70 mg (wet wt) of cells ml^{-1}. Incubations were carried out in 1-ml aliquots in scintillation vials which were shaken (225 rpm) at 28°C. Incubations were started with the addition of a substrate(s) from 10-mg/ml methanol stock solutions. At each time point, duplicate incubations were terminated by the addition of 20 μl of 10 N H_2SO_4. One replicate was centrifuged to remove cells, analyzed for polar metabolites by HPLC, and quantified for chloride. For quantification of residual MCBPs, the second replicate was extracted with four volumes of hexane as described above and analyzed by GC.

Enzyme assays. *meta*-Pyrocatechase (catechol 2,3-dioxygenase; EC 1.13.11.2) and *ortho*-pyrocatechase (catechol 1,2-dioxygenase; EC 1.13.11.1) activities in cell extracts were determined as previously described (1); the cell extracts were also prepared as previously described (1). The protein content of cell extracts was determined by the Bradford method (9) using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Richmond, Calif.).

Analytical methods. (i) Oxygen uptake analysis. Oxygen consumption by resting cells was measured with an oxygen electrode as previously described (17). The protein content of cell suspensions was determined by the biuret method (20).

(ii) Chloride determination. Chloride was quantified with a model 94-17B chloride electrode (Orion Research, Inc., Boston, Mass.) by reference to a calibration curve constructed with NaCl standards. Aqueous samples (0.5 ml) were first diluted with an equal volume of an ionic strength adjusting solution (0.25 M HNO_3, 1.75 M KNO_3).

(iii) GC. Hexane extracts of MCBPs were quantified on an HP 5890A gas chromatograph (Hewlett-Packard Co., Palo Alto, Calif.) fitted with a DB-5 capillary column (length, 30 m; inner diameter, 0.24 mm; film thickness, 0.25 μm) (J&W Scientific, Folsom, Calif.). Manual injections were made in the split mode (split ratio, 10) with an injector temperature of 250°C. Helium was employed as the carrier gas (flow rate, 0.75 $ml \cdot min^{-1}$). An electron capture detector (320°C) was employed with nitrogen as the make-up gas (40 $ml \cdot min^{-1}$). The temperature program ran from 180°C to 200°C (2°C · min^{-1}) with an initial holding time of 2 min. For biphenyl quantification, a flame ionization detector (300°C) was employed with nitrogen as the makeup gas (45 $ml \cdot min^{-1}$).

(iv) GC-mass spectroscopy (MS). A Hewlett-Packard 5989A gas chromatograph-mass spectrometer in the 70-eV electron impact mode was used. The injector, ion source, and detector temperatures were 250, 200, and 280°C, respectively. Metabolites were separated on a DB-5 capillary column as described above, with a temperature program of 100°C (1-min initial wait) to 220°C (10°C · min^{-1}).

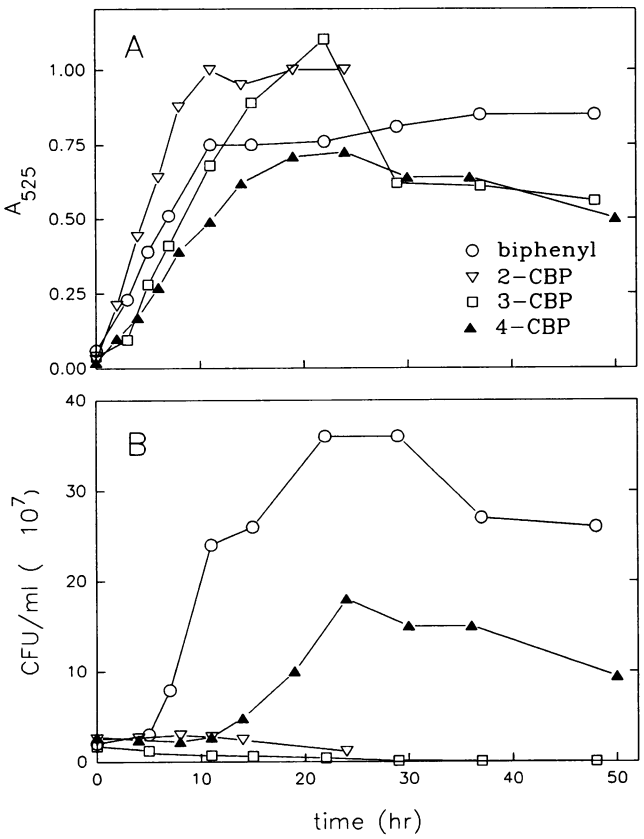

FIG. 1. Comparative growth of strain P166 on biphenyl (1 mM) and on MCBPs (1 mM) as measured by A_{525} (A) and viable cell count (CFU) per milliliter (B).

(v) HPLC. Polar metabolites were analyzed on a System Gold model 126 pump module connected to a model 166 UV detector module (Beckman Instruments, Inc., Fullerton, Calif.). Metabolites were detected at 254 nm and were separated on a C_{18} reverse-phase column (length, 250 mm; inside diameter, 4.5 mm; 5-μm [particle size] Spherisorb ODS-2 packing) (Alltech Associates Inc., Deerfield, Ill.). A mobile phase of 45% acetonitrile and 55% acetic acid (40 mM) was used with a flow rate of 1 $ml \cdot min^{-1}$. Residual MCBPs were purged from the column by ramping the solvent system to 90% acetonitrile after 5.5 min and holding it there for 15 min.

RESULTS

Growth studies. *P. cepacia* P166 utilized biphenyl, benzoate, and all three MCBPs but not monochlorobenzoates. The optical density increased rapidly during incubation with all MCBPs (Fig. 1A), but in the case of 2- and 3-CBP, a white precipitate was formed. As measured by viable cell count, biphenyl supported the highest growth rate (doubling rate [t_d] = 2.1 h) and final cell density (Fig. 1B). During growth on 4-CBP, the t_d was 5.24 h, and a cell density was achieved which was approximately half of that achieved on biphenyl. After incubation, the medium contained 0.53 mM 4-CBa and 0.34 mM chloride. No residual 4-CBP was detected.

Incubation with 2-CBP produced a negligible increase in viable cells. Nevertheless, over 90% of the 2-CBP was transformed (0.08 mM remaining). Several metabolites accumulated in the medium, most notably 0.55 mM 2-CBa. Incubation

TABLE 1. Rates of oxygen uptake by whole resting cells of strain P166 previously grown on biphenyl or 4-CBP

Test substrate	Oxygen uptake rate[a] (nmol of O_2 min^{-1} mg of $protein^{-1}$) from whole cells grown on:	
	Biphenyl	4-CBP
Biphenyl	466 (100)	36 (100)
2-CBP	248 (53)	18 (50)
3-CBP	518 (111)	23 (64)
4-CBP	423 (91)	24 (67)
Benzoate	306 (100)	151 (100)
2-CBa	12 (4)	0 (0)
3-CBa	54 (18)	19 (13)
4-CBa	15 (5)	5 (3)
Catechol	794 (100)	187 (100)
3-CC	77 (10)[b]	4 (2)
4-CC	111 (14)[b]	55 (29)[b]

[a] Values in parentheses are rates expressed as a percentage of that measured on biphenyl, benzoate, or catechol.

[b] Represents the initial rate only as rates on chlorocatechols decreased with time. In the case of 3-CC, the rate dropped to zero in less than 3 min.

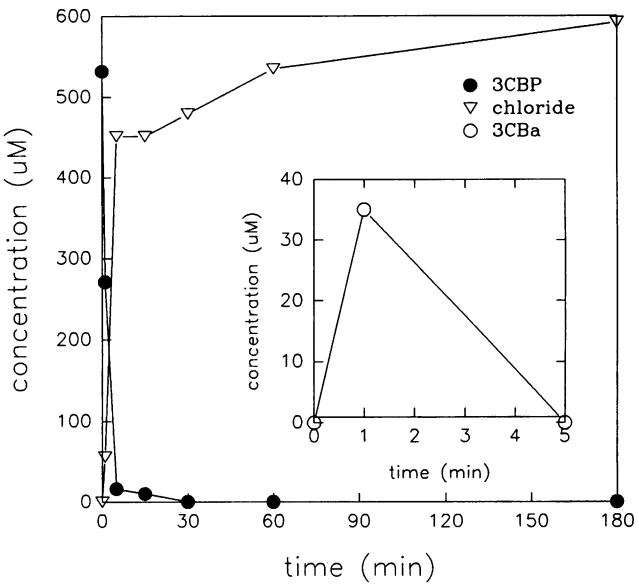

FIG. 2. Disappearance of 3-CBP (540 μM) upon incubation with resting cells of strain P166. The inset shows transient accumulation of 3-CBa.

with 3-CBP effected a slow decline in viable cells. Several metabolites were detected in the medium, including a trace of 3-CBa, but untransformed 3-CBP remained (0.71 mM).

Oxygen uptake studies. For resting cells grown on biphenyl, high rates of metabolism were observed for biphenyl and all of the MCBPs (Table 1). The three monochlorobenzoates were all metabolized, but at rates an order of magnitude lower than those for MCBPs. Catechol and monochlorocatechols were metabolized at higher rates than those for chlorobenzoates. Similar relative rates for biphenyl-, 4-CBP-, and 2-CBP-grown resting cells (data not shown) indicated that the same enzymes were working in both biphenyl- and MCBP-grown cells.

Determination of metabolic pathways. Because oxygen uptake data provided no evidence for the induction of a different set of enzymes between the biphenyl- and MCBP-grown cells, biphenyl-grown resting cells were used to study the metabolism of MCBPs.

3-CBP was quickly transformed by resting cells, with a corresponding stoichiometric release of chloride (Fig. 2). 3-CBa was a transient intermediate detected at a low level (Fig. 2 [inset]). The transformation of 3-CBa was oxidative, as evidenced by the buildup of 3-CBa in nonaerated resting cell incubations (data not shown). The most likely oxidative metabolites of 3-CBa are chlorocatechols, namely 3- or 4-CC. In order to identify metabolites, acidified resting cell incubations were clarified by centrifugation and extracted three times with an equal volume of ethyl acetate. The organic phases were pooled, dried over sodium sulfate, concentrated to dryness by rotary evaporation, resuspended in a small volume of methanol, and analyzed by GC-MS. A metabolite was detected that had a mass spectrum consistent with the structure of 3-CC. The molecular ion (M) at *m/z* 144 showed the characteristic 3:1 M- to M+2 isotope ratio of a single Cl atom. Major fragment ions were *m/z* 108 (M-Cl) and 80 (M-Cl-CO). The mass spectrum was consistent with that of an authentic standard but differed from that of a 4-CC standard. When incubated directly with resting cells, 3-CC was rapidly metabolized, with the coincident accumulation of chloride (Fig. 3). No products were detected by HPLC analysis. *ortho*-Pyrocatechase had no activity against 3-CC, as evidenced by direct assay of enzyme activity. *meta*-Pyrocatechase activity on 3-CC cannot be assayed directly because the product irreversibly binds to the enzyme and cannot be detected. However, when 3-CC was added to cell extracts, *meta* cleavage activity toward other catecholic substrates was destroyed, as determined by the absence of typical yellow color formation. *meta* cleavage activity toward 2,3-dihydroxybiphenyl, a catecholic substrate of the upper biphenyl pathway, was also destroyed, indicating that 2,3-dihydroxybiphenyl dioxygenase is inactivated by 3-CC. The effect of 3-CC on the transformation of biphenyl was studied by coincubating

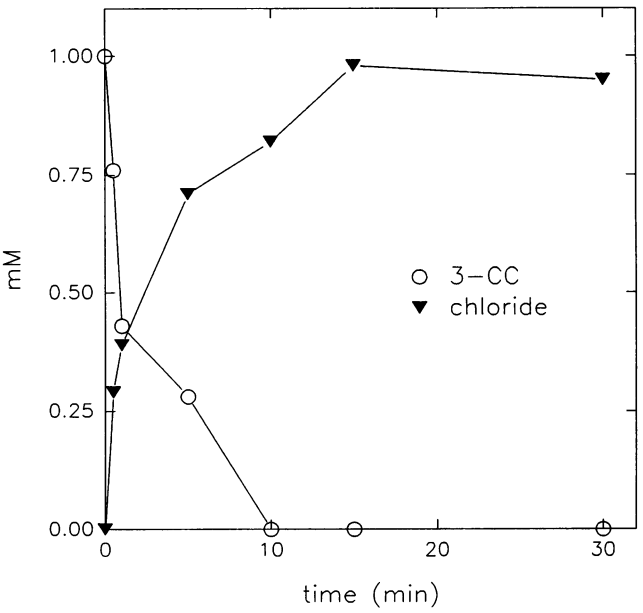

FIG. 3. Incubation of 3-CC (1 mM) with resting cells of strain P166. Chloride release was stoichiometric and coincident with 3-CC depletion.

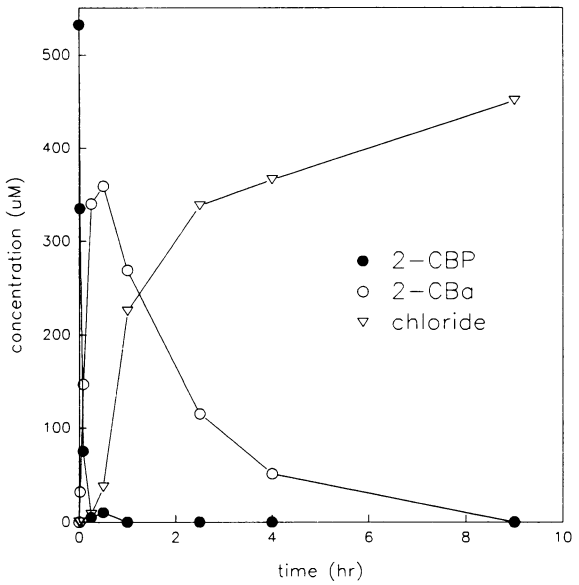

FIG. 4. Resting cells of strain P166 incubated with 2-CBP (540 μM).

resting cells with biphenyl (3 mM) and 3-CC (1 mM) (see Fig. 7). In the presence of 3-CC, biphenyl metabolism was obstructed and complete transformation was not achieved.

2-CBP was also metabolized quickly by resting cells (Fig. 4). 2-CBa accumulated to high levels but was slowly metabolized, with a corresponding stoichiometric release of chloride. GC-MS analyses of culture extracts identified 2-CBa, but no other intermediates were detected.

Resting cells of strain P166 also rapidly metabolized 4-CBP (Fig. 5). 4-CBa accumulated transiently at low levels (Fig. 5 [inset]). When resting cells were incubated with 4-CBP under

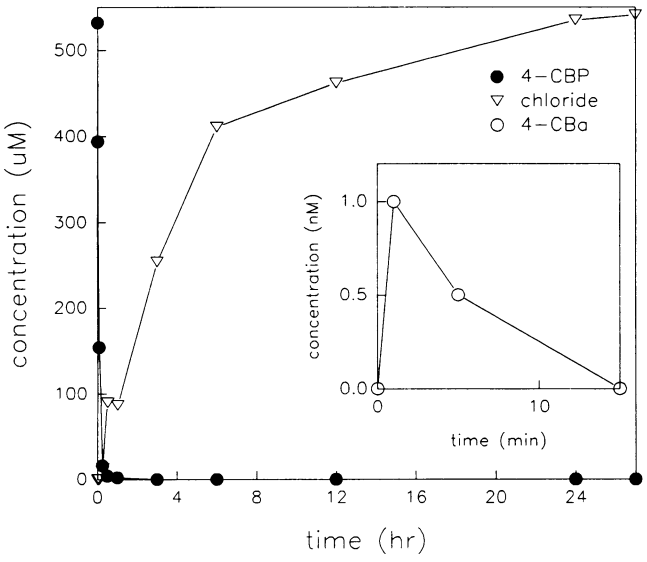

FIG. 5. Disappearance of 4-CBP (540 μM) upon incubation with resting cells of strain P166. The inset shows transient accumulation of 4-CBa.

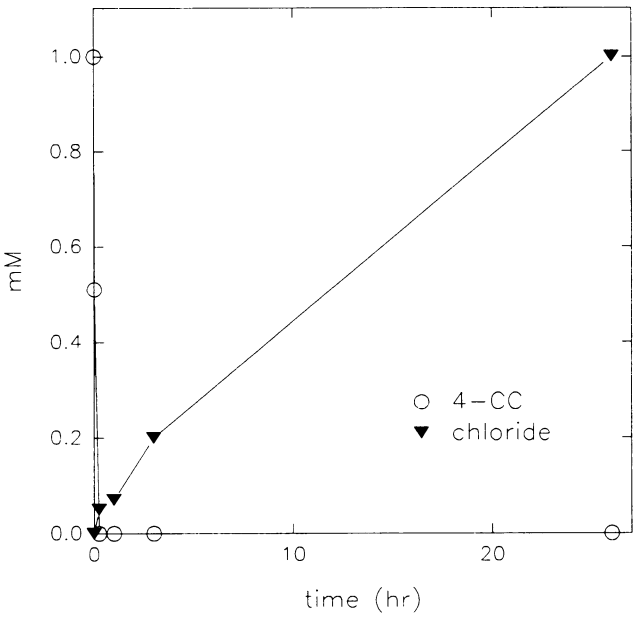

FIG. 6. Incubation of 4-CC (1 mM) with resting cells of strain P166. Chloride release was stoichiometric but not coincident with 4-CC depletion.

conditions of lower oxygen tension (no shaking and minimum head space), 4-CBa accumulated to higher levels and for a longer period (data not shown). Chloride release from resting cells incubated with 4-CBP was stoichiometric but did not coincide with 4-CBP depletion. Rather, chloride accumulated slowly over 24 h. HPLC analyses revealed the transient accumulation of several polar metabolites. When 4-CBa was added to stationary-phase cultures grown on biphenyl, the media turned yellow, indicating *meta* cleavage of a catecholic product, namely 4-CC. When 4-CC was added directly to resting cells, a yellow product was again immediately formed and the rapid transformation of 4-CC was followed by the slow release of chloride (Fig. 6). For extracts of biphenyl-grown cells, *meta* cleavage activity toward 4-CC was determined to be 250 nmol min^{-1} mg of $protein^{-1}$. However, *ortho* cleavage activity toward 4-CC was also present (100 nmol min^{-1} mg of $protein^{-1}$). When resting cells were coincubated with biphenyl and 4-CC (Fig. 7), the presence of 4-CC slowed the metabolism rate but did not prevent the complete transformation of biphenyl.

DISCUSSION

On the basis of the evidence presented, we propose the pathways illustrated in Fig. 8 for the metabolism of MCBPs by strain P166. Most reports characterizing growth of bacteria on MCBPs have described chlorobenzoates as dead-end products. Although chlorobenzoates did accumulate in the medium of strain P166 when the strain was incubated with each of the three MCBPs, these intermediates were further oxidized to chlorocatechols, which were quickly transformed by *meta* cleavage. In the case of 3-CBP, 3-CC was identified as a product. Strain P166 oxidized 3-CC by *meta* cleavage, the product of which has been reported to produce a reactive acyl halide (5-chloroformyl-2-hydroxypenta-2,4-dienoic acid) (4, 26), which can quickly condense to macromolecules. This

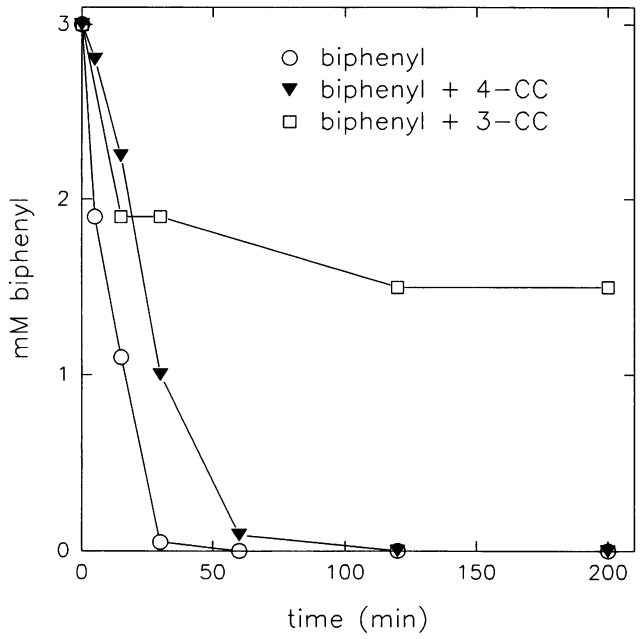

FIG. 7. Effect of CCs (1 mM) on the ability of strain P166 resting cells to transform biphenyl (3 mM). Catechol (as a control) slightly delayed, but did not otherwise interfere with, the transformation of biphenyl (data not shown).

condensation inactivates proteins, releases chloride, and eliminates free metabolites. Resting cell metabolism of both 3-CBP and 3-CC released chloride stoichiometrically, and no products were detected. A rapid decrease in the rate of oxygen uptake on 3-CC suggests just such an inactivation of *meta*-pyrocatechase. 3-CC interfered with the utilization of biphenyl or MCBP as a carbon source, as evidenced by its arrest of biphenyl transformation (Fig. 7). A likely mechanism for this phenomenon is the inactivation by 3-CC of 2,3-dihydroxybiphenyl dioxygenase (1), another *meta*-pyrocatechase and a key enzyme in the biphenyl pathway. This explains why 3-CBP does not support an increase in viable cell counts even though there is an efficient attack of substrate to an extent that should have effected an increase in cell mass. When strain P166 was incubated with 3-CBP, chloride was never definitively detected, but the amount released by low-density populations would be below detection limits. Stoichiometric chloride release could be monitored, however, with high-density resting cells.

In the case of 2-CBP, growing cells of strain P166 utilized almost all of the starting material and accumulated much 2-CBa but experienced only a negligible increase in viable cells. Further transformation of 2-CBa to 3-CC (29) with subsequent dehalogenation is consistent with our observations. 3-CC was never detected from cells incubated with 2-CBP, but chloride release coincided with 2-CBa disappearance, and no products were observed. Since the oxidation of 3-CC is much faster than the oxidation of 2-CBa (Table 1), the accumulation of 3-CC is not predicted. Chloride release from 2-CBa might also result from direct dioxygenation at the 1,2 position, with liberation of chloride to form catechol (30). The latter transformation, however, would introduce no toxic metabolites, and the cell yield would be expected to approach that achieved by growth on biphenyl. The poor growth of strain P166 during incubation with 2-CBP is explained by formation of 3-CC. The slow transformation of 2-CBa (Table 1, Fig. 4) prevents 3-CC production from proceeding as rapidly during metabolism of 2-CBP as during metabolism of 3-CBP. Thus, a small net increase in viable cells is observed during incubation with 2-CBP but not with 3-CBP.

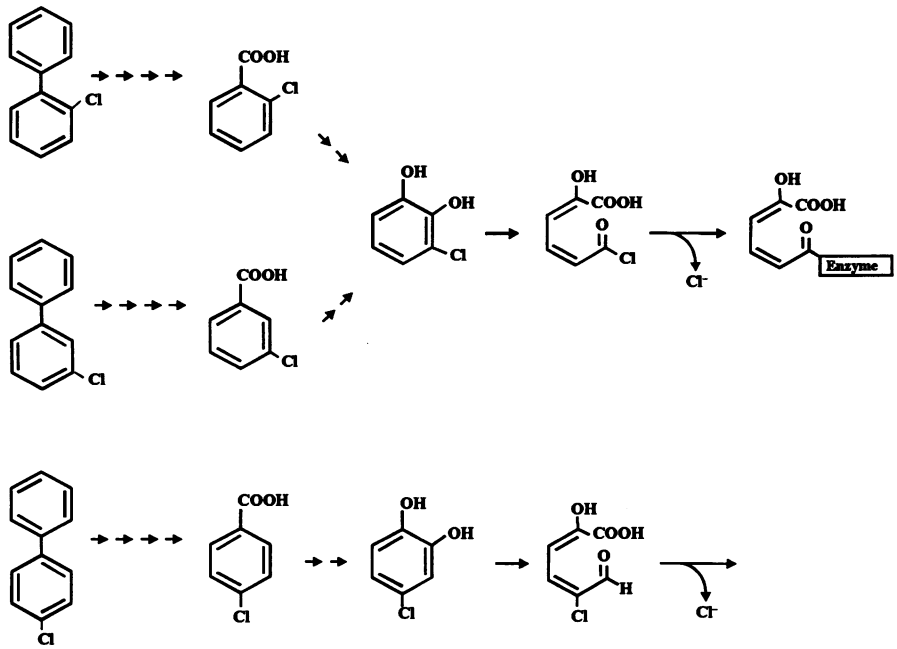

FIG. 8. Proposed pathways for the metabolism of 2-, 3-, and 4-CBP by strain P166. 2- and 3-CBP are transformed to 3-CC, which is *meta* cleaved to form a reactive acyl halide, which condenses to cellular macromolecules. 4-CBP is transformed to 4-CC, which is metabolized via a *meta* cleavage pathway, with the eventual release of chloride.

Although 4-CC was never detected as a product of 4-CBP, yellow color formation upon oxidation of 4-CBa strongly suggests the *meta* cleavage of 4-CC. In addition, 4-CC transformation by resting cells effected the same characteristic lag in chloride release as that observed on 4-CBP. Figure 8 depicts proximal *meta* cleavage, but distal cleavage cannot be excluded. The *meta* cleavage product, which is not an acyl halide, does not block biphenyl metabolism. The slow release of chloride from 4-CC indicates that one (or more) of the enzymes in the pathway has low affinity for chlorinated substrates.

The formation of chlorobenzoates from MCBPs is assumed to be coincident with the formation of 2-oxopent-4-dienoate, a five-carbon fragment derived from the nonchlorinated ring. This five-carbon fragment is a common product of all three MCBPs and is a labile carbon source. It is of interest that the scientific literature contains many reports of isolates which do not grow on 2- and 3-CBP but which produce chlorobenzoates from these same substrates. The present work offers an explanation for this dilemma. It is possible that the transformation of 3-CBa to 3-CC is common among biphenyl degraders but has gone unnoticed because any accumulation of 3-CBa was taken to be the end of metabolism. Reports of growth on 4-CBP, which does not generate a reactive acyl halide intermediate, are much more common.

The construction of recombinant strains which can grow on and mineralize more-chlorinated PCB congeners has met with only limited success. The strategy typically involves pooling the biphenyl pathway of PCB cometabolizers with the chlorobenzoate pathway of chlorobenzoate degraders (1). The latter pathway contains the modified *ortho* cleavage enzymes for transformation of chlorocatechols. The competing conversion of chlorocatechols to toxic metabolites by *meta* cleavage precludes the use of strain P166 in the construction of PCB degraders unless the responsible *meta*-pyrocatechase can be inactivated (26).

ACKNOWLEDGMENTS

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