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# Metabolism of hydroxybiphenyl and chloro-hydroxybiphenyl by biphenyl/chlorobiphenyl degrading *Pseudomonas testosteroni*, strain B-356

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## SUMMARY

A biphenyl (BP) and chlorobiphenyl (CBP) metabolizing *Pseudomonas testosteroni*, strain B-356 was also capable of utilizing 2-,3-, and 4-hydroxybiphenyl. Data presented here suggest that utilization of biphenyl and mono-substituted biphenyls involves the enzymes of the same pathway. Chloro-hydroxybiphenyls were also metabolized by strain B-356. The unsubstituted ring is first hydroxylated in position 2 and 3 and then cleaved in a *meta* 1, and 2, position to ultimately generate the benzoic acid derivatives. Since strain B-356 was capable of utilizing benzoic acid and mono-hydroxybenzoic acids, the utilization of biphenyl, 2-,3-, and 4-hydroxybiphenyl is complete at non-toxic concentrations of the substrates. Chlorobenzoic acids and chloro-hydroxybenzoic acids were not metabolized further by this strain. Studies using *Pseudomonas putida*, strain KT2440 carrying cloned BP/CBP genes from strain B-356 provided further evidence for the presence of a common pathway for the metabolism of the above compounds in *P. testosteroni*, strain B-356. Suggestions are made on significance of the broad substrate specificity of the enzymes of biphenyl/chlorobiphenyl pathway in regard to their possible origin and in relation to PCB mixture degradation.

## INTRODUCTION

The involvement of biphenyl (BP) degradation enzymes in chlorinated biphenyl degradation is now well established [6,8]. However, not all BP utilizing organisms are able to degrade PCBs. At best, in most cases, there are strong substrate specificities even among bacterial strains capable of degrading mono-chlorinated biphenyls [30,32]. This is more true when PCBs are considered [10].

Degradation of BP and monochlorinated biphenyls by bacteria generally proceeds by dioxygenation which preferentially occurs on the unsubstituted ring. The dihydroxylated ring is subsequently cleaved at *meta* position. Further degradation by hydrolyzing enzymes produces chlorobenzoic acids.

Hydroxylation of BP and chlorobiphenyls (CBP) generates substituted biphenyls that are more toxic than the parent compounds. Mono-hydroxybiphenyls especially 2-hydroxybiphenyl (2HBP) have widely been used as industrial antimicrobials, agricultural fungicide, and disinfectants for years [23,25,31]. Mono-hydroxybiphenyls could also be produced by yeast, fungi, and mammalian enzyme systems from BP [11,16,22,27]. Catabolism of BP by actinomycetes and accumulation of mono-hydroxybiphenyls has also been reported [25]. Among mono-hydroxybiphenyls, 2HBP is of environmental concern because of its extensive use; moreover, it has been reported to be mutagenic in human cells [29] and as carcinogen in rats [13].

There have been very few reports on bacterial metabolism of hydroxybiphenyls (HBP) [9]. Recently, bacterial growth on some selected HBPs by *Pseudomonas* sp., HBP1 was reported [15]. This organism initiated the metabolism by a NADH-dependent mono-oxygenase that required the presence of a substrate with 2-hydroxyphenyl-R structure. Higson and Focht [12] subsequently reported metabolism of some HBPs by two bacterial strains. *Pseudomonas* sp. strain FH12 mono-oxygenase activity was restricted to 3- and 3,3′-hydroxybiphenyls by introduction of hydroxyl group at *ortho* position. On the other hand, *Pseudomonas* sp. strain FH23 degraded 4-hydroxybiphenyl (4HBP) by using either a mono- or dioxygenase to produce hydroxylated intermediate at 2,3-position. In the above studies neither organism attacked chlorinated hydroxybiphenyls [12].

By measuring oxygen uptake of resting cell suspension,

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Furukawa et al. [9] reported common induction for enzymes of the biphenyl/chlorobiphenyls (BP/CBP) pathway and enzymes involved in the transformation of other BP analogs, including HBPs. These data could suggest that at least the BP/CBP dioxygenase could also transform hydroxylated biphenyls. Recently, some of the enzymes involved in catabolism of BP have been isolated and partially characterized [14,19,20] from soil bacterium *Pseudomonas cruciviae* S93 B1, but activities of these enzymes towards other substituted BP have not been demonstrated.

A BP and CBP metabolizing *Pseudomonas testosteroni*, strain B-356 was isolated earlier from activated sludge. The genes specifying the degradation of BP/4CBP in this strain were cloned into *Pseudomonas putida*, KT2440 [1].

Here we report the degradation of HBP and chlorohydroxybiphenyl by *P. testosteroni*, strain B356, and we provide evidence that enzymes of the BP/CBP pathway are involved in the transformation of these substrates.

## MATERIAL AND METHODS

*Organisms and cultivation conditions.* The CBP degrading *Pseudomonas testosteroni*, strain B-356 was described earlier [1]. *Pseudomonas putida* KT2440 and the two clones deriving from it, *P. putida* DA1 (pDA1) and *P. putida* DA2 (pDA2) carrying cloned BP/CBP degrading genes from strain B-356 were also used.

Lyophilized cultures in bovine serum were kept at − 20 °C and grown in nutrient broth (Difco Laboratories, Detroit, MI) overnight when needed. Minimal medium 30 (MM30) with appropriate carbon source [30] was then inoculated with cells from nutrient broth grown cultures. Concentrations of carbon sources were 0.05–0.1% (w/v) unless indicated otherwise. *P. putida* clones were grown on glucose–MM30 containing streptomycin (150 μg/ml). The incubation was done in Erlenmeyer flasks at 30 °C with rotatory agitation (250 rpm).

*Chemicals.* The chemicals used as substrates were obtained from the following sources: biphenyl (BP), benzoic acid, 4-hydroxy, 2-hydroxybenzoic acids and chlorobenzoic acids from Aldrich Chemical Co. (Milwaukee, WI); 2-hydroxybiphenyl (2HBP), 4-hydroxybiphenyl (4HBP), 2-chloro-4-hydroxybiphenyl (2Cl-4HBP), and 2-chloro-4-hydroxybenzoic acids respectively from ICN Biomedicals Inc. and K & K Laboratories (Cleveland, OH); 3-hydroxybenzoic acid, and chlorobiphenyls (CBP) from Pfaltz & Bauer Inc. (Stanford, CT); 3,4-dihydroxybiphenyl (3,4-DiHBP), 2,5-dihydroxybiphenyl (2,5-DiHBP), 5-chloro-2-hydroxybiphenyl (5Cl-2HBP), 3-chloro-4-hydroxybiphenyl (3Cl-4HBP), 3-hydroxybiphenyl (3HBP) from Ultra Scientific (Hope, RI); organic solvents for extraction from Anachemia (Montréal, Québec); *N,O-bis*-(trimethylsilyl) trifluoracetamide (BSTFA) was obtained from Pierce Chemical Co. (Rockford, IL).

*Toxicity test and MIC determination.* Since the HBPs are toxic, it was necessary to determine the levels at which the experiments could be carried out without greatly affecting the organisms. The minimal inhibitory concentrations of HBPs for each organism were determined by the serial tube dilution method with 2 ml final suspension volume in each tube. Cells grown on BP and/or succinate were challenged with various concentrations of HBPs in MM30 containing BP or succinate as additional carbon source. The final density of challenged cultures were $1–5 \times 10^7$ cfu/ml. The tubes were incubated at 30 °C with shaking for 48–72 h before reading the results. The MIC determinations were done in duplicate and repeated at least twice.

*Preparation of washed and resting bacterial suspensions.* Log phase cultures were filtered through packed glass wool to remove particulates, cell aggregates, and crystals of growth substrate. Cultures were then harvested by centrifugation (12 min at $10000 \times g$), washed with phosphate buffer (30 mM, pH 7.2) and resuspended in MM30 or phosphate buffer.

*Oxygen uptake.* Oxygen uptake rates were measured with oxygen electrodes (YSI model 53 Biological Oxygen Monitor, Yellow Springs Instrument Co., Yellow Springs, OH) at 30 °C. The reaction mixture contained 1.9 ml of phosphate buffer (20 mM, pH 7.1), 1 ml of resting cells suspension ($OD_{600\,nm}$ 2.0), and 0.1 ml substrate dissolved in DMSO to give final concentration of 3 mM in reaction vessel. Further dilution of substrate in DMSO were made when it was needed. The oxygen uptake rate was corrected for endogenous respiration. DMSO did not affect the uptake at concentration used.

*Identification of intermediate metabolites from strain B-356.* Washed cell suspensions ($OD_{600\,nm}$ 2.0) were prepared in MM30 or phosphate buffer (30 mM, pH 7.2) in the presence of sub-toxic levels of the substrates. The final volumes of suspensions were 150 ml and incubated at 30 °C with shaking. After appropriate incubation time (5, 18, 48 h, and one week), the suspensions were extracted at neutral pH and at pH 3 for gas chromatographic/mass spectrometric (CG-MS) analysis. The conditions of extraction process have been previously reported [17,18]. The extracted metabolites were derivatized with BSTFA to generate the TMS derivatives. The GC-MS was performed using a HP5970 mass selective detector. The HP5870 A GC was equipped with an ultra-2 methylsilicone (H.P.), wall-coated fused silicon capillary column (25 m, 0.2 mm i.d.). The conditions and other parameters of analysis have been described earlier [17,18].

*Involvement of BP/CBP degradation pathway genes in hydroxy- and chloro-hydroxybiphenyl metabolism.* Clones DA1 and DA2 were used to confirm the involvement of BP/CBP degradation pathway in metabolism of hydroxy- and chloro-hydroxybiphenyls. Cell suspensions of *P. putida* DA1 that carry all the genes of BP/CBP degradation pathway, *P. putida* DA2 that lacks the gene for hydrolytic conversion of *meta* cleavage compounds to corresponding benzoic acids [1], and *P. putida* KT2440 were incubated in presence of substrates. Subsequently, they were extracted and analyzed for presence of metabolites as described above.

## RESULTS

*Determination of non-toxic levels of substrates*

HBPs are toxic to a variety of organisms and their mode of action, although not clearly defined, are thought to be due to interaction with enzymes vital to organisms [26]. Therefore, it was necessary to determine non-toxic levels of these substrates in order to study their metabolism.

Among HBPs, 2-HBP was most toxic when MICs were determined with a serial tube dilution method. The MIC for 2-HBP in MM30 with BP or succinate as major carbon source was 0.025% (w/v) for strains examined. Consequently, concentrations of 0.015–0.02% were used with resting and washed cell suspensions to study metabolism and intermediate metabolites. Viability of exposed populations to HBPs were also monitored with plate count method to ensure the absence of bactericidal activities at concentrations used. MIC of 2Cl-4HBP for strain B-356 grown on BP was 0.005–0.01% (w/v). Consequently, the concentrations of 0.0025–0.005% were used to study metabolism of this compound.

*Growth substrates and degradation abilities*

The organisms used in this study were tested for their ability to degrade BP, substituted-biphenyls, benzoic acid, and substituted-benzoic acids. The results are shown in Table 1. Strain B-356 is able to grow on and degrade CBPs to corresponding chlorobenzoic acids that were not degraded. On the other hand, growth on BP is extremely efficient and benzoic acid produced is rapidly degraded.

Strain B-356 was also able to degrade HBPs. These monosubstituted BPs, despite of total degradation, were not a good growth substrate due to their toxicity in higher concentrations necessary for adequate growth rate measurements. Moreover, 3,4- and 2,5-DiHBPs were also degraded judged by disappearance from media. Dihydroxybiphenyl metabolites could not be adequately characterized, probably because they were polymerized to tetra-hydroxylated dimers and larger hydroxylated oligomers. Chloro-hydroxybiphenyls were degraded by strain B-356 to produce chloro-hydroxybenzoic acids that were not degraded further. Furthermore, strain B-356 could grow very efficiently on benzoic acid and hydroxybenzoic acids.

*P. putida*, strain KT2440, did not grow on BP and substituted biphenyls. Moreover, this strain did not grow on 2- and 3-hydroxybenzoic acid, but did so on benzoic acid and 4-hydroxybenzoic acid. The clone carrying 4CB degrading genes were able to degrade a variety of substrates as shown in Table 1. These abilities that will be explained further are clearly due to the genes from strain B-356.

*Oxygen uptake*

The rate of oxygen uptake was measured in strain B-356 to obtain relative oxygenase activities that are the initial steps of BP and substituted biphenyls degradation. From the results (Table 2), it is evident that the cells grown on BP are induced for these enzymes when compared to succinate grown cells. This observation suggests that a common set of genes is involved in the oxidation of all the substrates tested. The measured oxygen uptake rates differ depending on the substrates tested and it also suggests a substrate specificity of the oxygenases. Concentrations of 3 mM of 2-HBP, 3-HBP, 2,5-DiHBP, 3,4-DiHBP, 3,4-DiHBP and all chloro-hydroxybiphenyls tested were toxic to the cells since this concentration affected endogenous uptake of oxygen, both in BP and succinate grown cells. However, when concentrations were lowered by further dilutions in DMSO, oxygenase activities were increased over endogenous oxygen uptake in BP grown cells, and this was accompanied by a decrease in toxicity as manifested by the absence of repression of endogenous oxygen uptake in succinate grown cells.

*Identification of intermediate metabolites of HBP in strain B356*

If the BP degrading pathway were used for the degradation of CBP, HBP, and chlorohydroxybiphenyl, we should expect a similar pattern of degradation as shown in Fig. 1.

In order to identify metabolites of HBPs metabolism in strain B-356, extractions were made at various intervals from resting cell suspensions incubated with these substrates. Metabolites were analyzed by GC-MS as TMS derivatives. Relatively short incubation (5–18 h) with HBPs of resting cell suspensions of strain B-356 grown on BP resulted in detection of several intermediate metabolites. Fig. 2A is a total ion-current chromatographic representation of an acidic ethylacetate extract with 2HBP as substrate. Compounds **3a**, **3b**, **4** and **6** are key

TABLE 1

Degradation[a] of related aromatic compounds

| | Bacterial strains | | | |
|---|---|---|---|---|
| | *P. testosteroni* | *P. putida* | | |
| | B-356 | DA1 | DA2 | 2440 |
| Biphenyl | + | + | + | – |
| Hydroxybiphenyl | | | | |
| 2-hydroxy- | + | + | + | – |
| 3-hydroxy- | + | + | + | – |
| 4-hydroxy- | + | + | + | – |
| Chlorobiphenyl | | | | |
| 2-chloro- | + | + | + | – |
| 3-chloro- | + | + | + | – |
| 4-chloro- | + | + | + | – |
| Chlorohydroxybiphenyl | | | | |
| 2-chloro-4-hydroxy- | + | + | + | – |
| 3-chloro-4-hydroxy- | + | + | + | – |
| 5-chloro-4-hydroxy- | + | + | + | – |
| Benzoic acid | + | + | + | + |
| Hydroxybenzoic acid | | | | |
| 2-hydroxy- | + | – | – | – |
| 3-hydroxy- | + | – | – | – |
| 4-hydroxy- | + | + | + | + |
| Chlorobenzoic acids[b] | – | – | – | – |
| Chlorohydroxybenzoic acids[c] | – | – | – | – |

[a] The degradation ability was determined in liquid medium by resting cell suspensions or by growing cultures (where possibe). Degradation measures the disappearance of the substrate and production of metabolites.

[b] Chlorobenzoic acids: 2-, 3- and 4-chlorobenzoic acids.

[c] Chlorohydroxybenzoic acids: 2-, 3-chloro-4-hydroxy- and 4-chloro-2-hydroxybenzoic acids.

metabolites of the 2HBP degradation pathway. Mass spectrum of metabolite **6** from Fig. 2A is identical to authentic 2-hydroxybenzoic acid with same retention time. Metabolites **3a** and **3b** are isomers of the acidic compound generated from the *meta*-cleavage (at 1,2 position). The mass-spectrum of metabolite **3a**, which is identical to metabolite **3b**, is shown in Fig. 3A and it corresponds to the TMS derivative of 2-hydroxy-6-oxo-6-(2′-hydroxyphenyl)-hexa-2,4-dienoic acid. It shows a molecular ion at *m/z* 450 and diagnostically important ion at *m/z* 435 (M-15), *m/z* 333 (M-COOTMS) which is characteristic of the α-hydroxy acids, and *m/z* 193 (hydroxybenzoyl moiety). Similar isomers have also been described as bacterial metabolites of chlorobiphenyl [17,18].

Metabolite **4** is the corresponding mono-unsaturated analog of the acidic open cycle derivative of 2HBP. The TMS derivative of metabolite **4** (Fig. 3B) has a molecular ion at *m/z* 452 and diagnostically important ions at *m/z* 437 (M-15), 335 (M-COOTMS), and 193 (hydroxybenzoyl moiety).

Mono-unsaturated as well as completely saturated analogs of metabolites **3** and **4** have all been detected in bacterial cultures grown in the presence of 4-chlorobiphenyl [17,18]. However, at this time, we do not know whether these *meta*-cleavage analogs are all part of the same pathway, or they are dead end metabolites resulting from non-specific reactions occurring in the cells, and/or if they are produced in the course of the acidic extraction of the metabolites with ethyl acetate. In any event, it should be pointed out that hydrogenase able to reduce the side chain of the biphenyl 1, 2 *meta*-cleavage metabolite have been described in *P. cruciviae* [19].

In resting cell suspension incubated with 3HBP (5–18 h), strain B-356 produced a variety of metabolites (Table 3). Among them was 2,3-3′tri-hydroxybiphenyl with characteristic ions at 418, 403, 330 and 315, respectively. All three types of corresponding *meta*-cleavage intermediates (metabolites **3**, **4**, **5**) were also detected, namely 2-hydroxy-6-oxo-6-(3′-hydroxyphenyl)-hexa-2,4-dienoic acid and the mono-unsaturated and completely

TABLE 2

Oxygen uptake[a] responses of the resting cells of strain B-356 to various substrates

| Substrate | Concentration (mM) | Relative oxygen uptake | |
|---|---|---|---|
| | | BP grown | Succinate grown |
| Biphenyl | 3 | 100 | 4.5 |
| 2CBP | 3 | 44.3 | 6.8 |
| 3CBP | 3 | 35.5 | 4.5 |
| 4CBP | 3 | 47.1 | 9.1 |
| 2HBP | 3 | − 2.3 | − 15.9 |
| | 0.5 | 43.6 | 6.0 |
| | 0.25 | 43.5 | 6.8 |
| 3HBP | 3 | − 11.4 | − 15.9 |
| | 0.5 | 66.5 | 6.3 |
| | 0.25 | 71.7 | 6.8 |
| 4HBP | 3 | 11.8 | 11.3 |
| | 0.5 | 66.9 | 10.5 |
| | 0.25 | 61.5 | 11.1 |
| 2Cl-4HBP | 3 | − 18.2 | − 22.7 |
| | 0.5 | 0 | 4.5 |
| | 0.25 | 13.6 | 4.9 |
| 3Cl-4HBP | 3 | − 21.2 | − 19.1 |
| | 0.5 | 0 | 9.1 |
| | 0.25 | 14.8 | 8.2 |
| 5Cl-2HBP | 3 | − 18.6 | − 8.2 |
| | 0.5 | 27.3 | 0 |
| | 0.25 | 22.7 | 3.5 |

[a] The oxygen uptake was measured as described in MATERIALS AND METHODS. The values represent the means of three separate experiments.

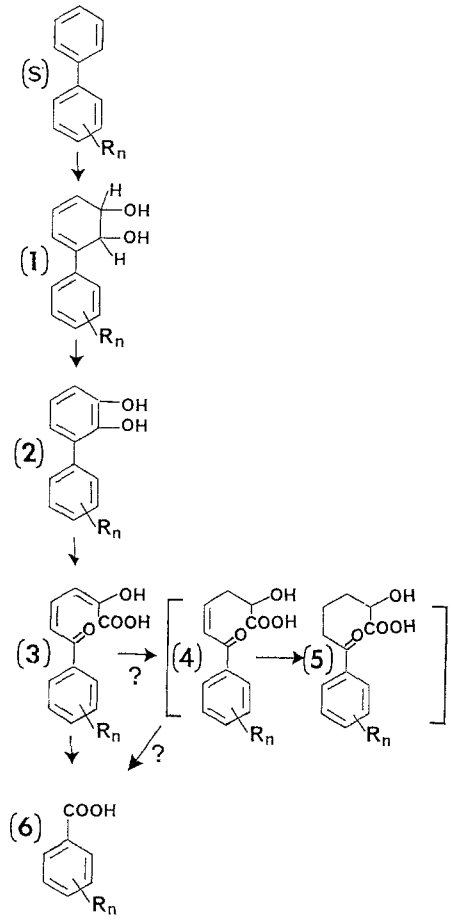


Fig. 1. Major catabolic pathway for bacterial degradation of biphenyl and substituted biphenyls. R = H, Cl, OH.

saturated forms. Again only a small amount of 3-hydroxybenzoic acid was detected in early cultures since strain B-356 is capable of utilizing all hydroxybenzoic acids. Similar results were obtained when 4HBP was fed to resting cell suspensions of strain B-356 (Table 3).

When strain B-356 was incubated with subtoxic levels of 2Cl-4HBP, there was only one major metabolite (Fig. 4A) corresponding to the 2-chloro-4-hydroxybenzoic acid (metabolite **6**) (Fig. 4B) and traces of 2-hydroxy-6-oxo-6-(2′-chloro-4′-hydroxyphenyl)-hex-4-enoic acid. The mass spectral characteristics of latter metabolite is given in Table 4.

From 3Cl-4HBP metabolism by strain B-356, chlorohydroxybenzoic acid was detected as major metabolite. The mass spectrum of TMS derivative of this metabolite is shown in Fig. 4C. The same results were obtained with 5Cl-2HBP, where a large amount of 2,3,2′-trihydroxy-5′-chlorobiphenyl (metabolite **2**) was detected in the early culture extracts at neutral pH (Fig. 5A).

The mass spectrum of TMS derivatized metabolite **2** (in Fig. 5B) has a mass ion at *m/z* 452 and characteristic ions at *m/z* 437 (M-15) and *m/z* 349, which corresponds to the loss of one TMS and subsequent arrangement involving proximal hydroxyls. However, production of 5-chloro-2-hydroxybenzoic acid in late cultures suggests the cleavage and further hydrolysis of this chloro-trihydroxylated biphenyl analog (Fig. 5C,D).

*Involvement of common genes and pathway in degradation*

The conversion of the HBPs and CHBPs into corresponding benzoic acids, suggests the involvement of the enzymes controlling BP/CBP pathway for the degradation of these substrates. To further ascertain this hypothesis, we used cloned strains that carry all or part of the BP/PCB pathway from strain B-356.

*P. putida* KT2440 (pDA1) and (pDA2) are respectively carrying a 21 kb and 24 kb fragment of strain B-356 DNA, each containing a common stretch of about 9.1 kb. Plasmid pDA1 carries all genes for the transformation of BP or CBP into corresponding chlorobenzoic acids while

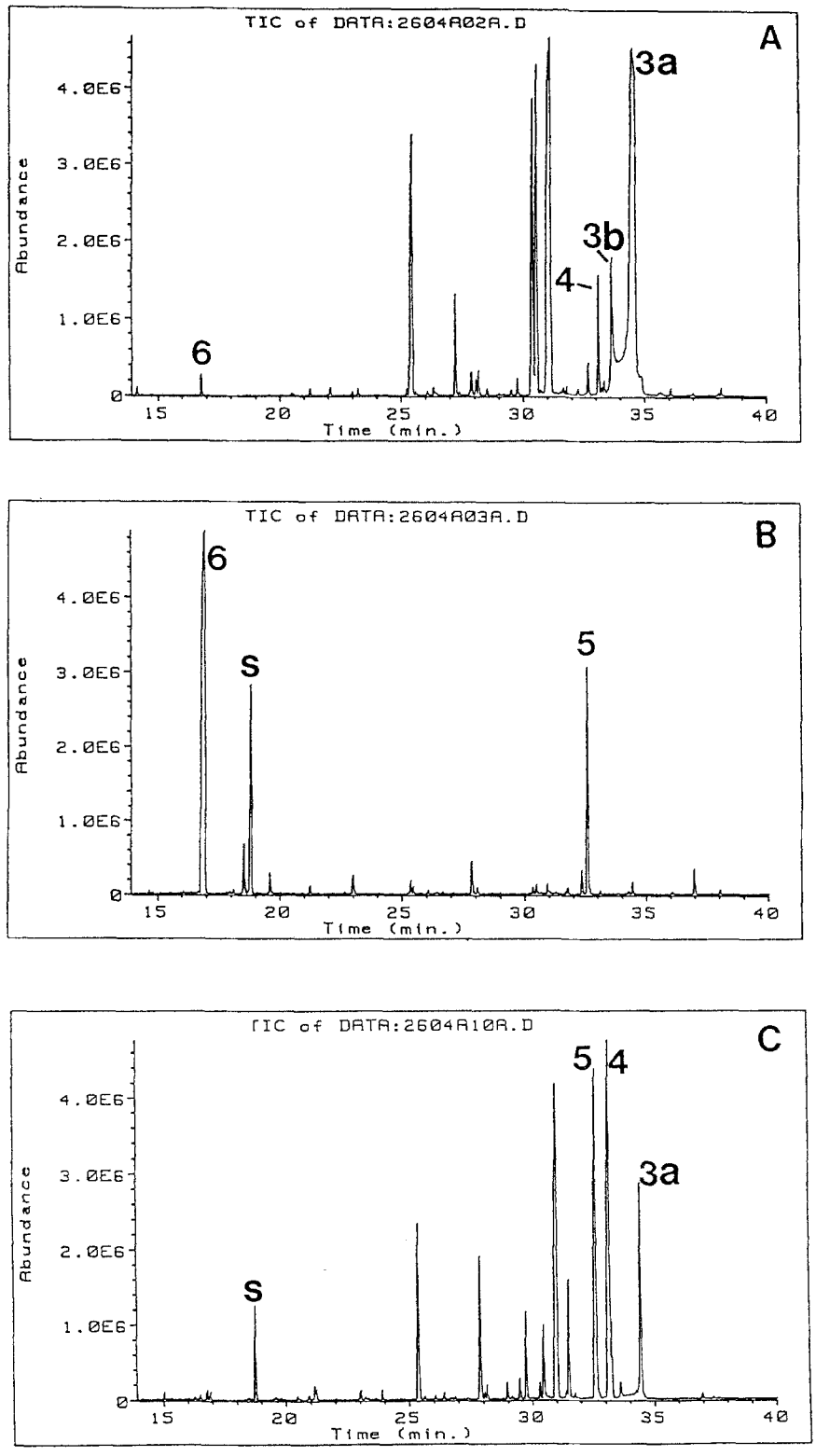


Fig. 2. Total ion-current chromatograms of TMS-derivatized metabolites in acidic ethylacetate extracts produced from degradation of 2-hydroxybiphenyl by: (A) *P. testosteroni* strain B-356; (B) *P. putida* DA1; (C) *P. putida* DA2. Resting cell suspensions were prepared and inoculated as described in MATERIALS AND METHODS. The suspensions were first extracted at neutral pH with ethylacetate and then extracted at pH 3 (acidic extract). The metabolites numbers correspond to the expected metabolites from the pathway shown in Fig. 1 where 2HBP is used as substrate (S). Metabolites **3a** and **3b** are isomers of metabolite **3**.

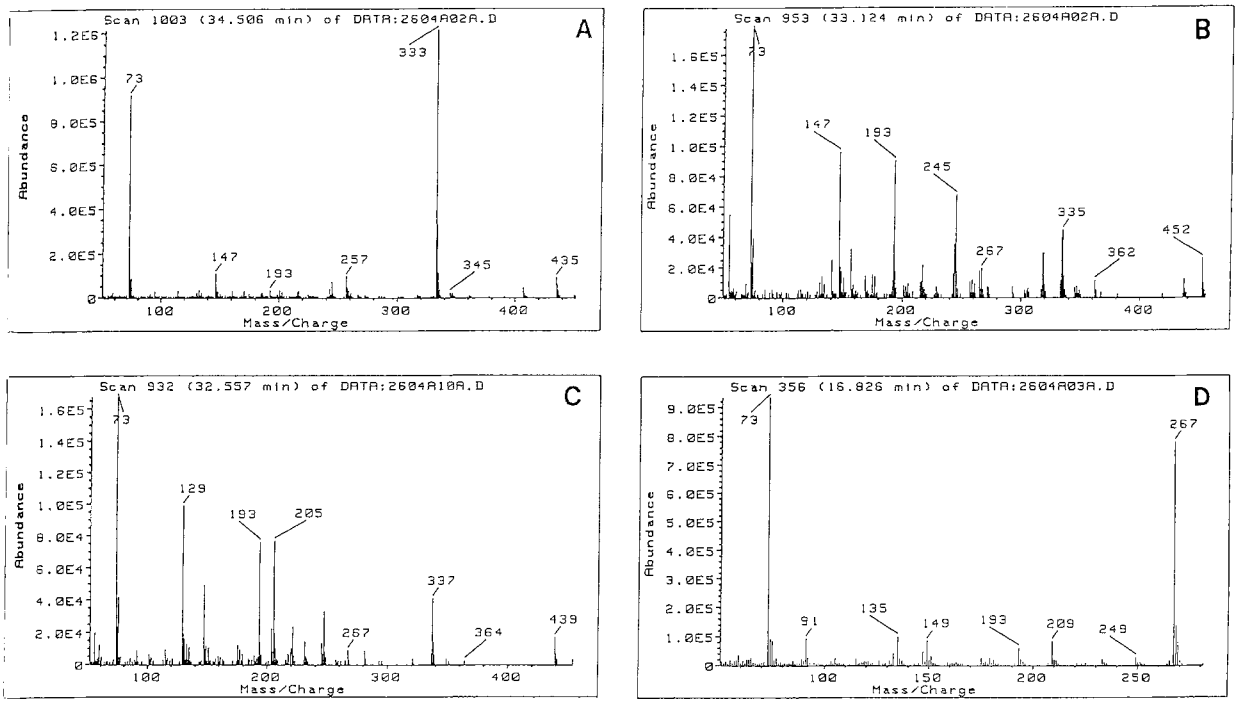


Fig. 3. Mass spectra of major metabolites of 2-hydroxybiphenyl metabolism as TMS derivatives. (A) metabolite **3**: 2-hydroxy-6-oxo-6-(2′-hydroxyphenyl)-hexa-2, 4-dienoic acid. (B) metabolite **4**: 2-hydroxy-6-oxo-6-(2′-hydroxyphenyl)-hex-4-enoic acid. (C) metabolite **5**: 2-hydroxy-6-oxo-6-(2′-hydroxyphenyl)-hexa-noic acid. (D) metabolite **6**: 2-hydroxybenzoic acid. The mass spectra were obtained from the TIC runs presented in Fig. 2.

TABLE 3

Metabolites detected from metabolism of hydroxy- and chloro-hydroxy-biphenyls by strain B-356, clones DA1 and DA2

| Meta-bolite | $M^+$ (TMS) | Organisms | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | B-356 | | | DA1 | | | DA2 | | |
| | | 2HBP | 3HBP | 4HBP | 2HBP | 3HBP | 4HBP | 2HBP | 3HBP | 4HBP |
| 1 | (420)[a] | – | – | – | – | – | – | – | – | – |
| 2 | 418 | + | + | – | – | – | – | – | – | – |
| 3 | 450 | + | + | (+)[b] | – | – | – | + | + | + |
| 4 | 452 | + | + | + | – | – | – | + | + | + |
| 5 | 454 | + | + | – | + | + | + | + | + | + |
| 6 | 282 | (+)[b] | (+)[b] | + | + | + | – | – | (+)[b] | – |
| | | 2Cl-4HBP | 3Cl-4HBP | 5Cl-2HBP | 2Cl-4HBP | | | 2Cl-4HBP | | |
| 1 | (454)[a] | – | – | – | – | | | – | | |
| 2 | 452 | – | – | + | – | | | – | | |
| 3 | (484)[a] | – | – | – | – | | | – | | |
| 4 | 486 | + | – | – | – | | | + | | |
| 5 | (488)[a] | – | – | – | – | | | – | | |
| 6 | 316 | + | + | + | + | | | (+)[b] | | |

[a] Numbers in parentheses are expected molecular ions that were not detected in this study.
[b] A minor metabolite produced in small amount compared to other metabolites.

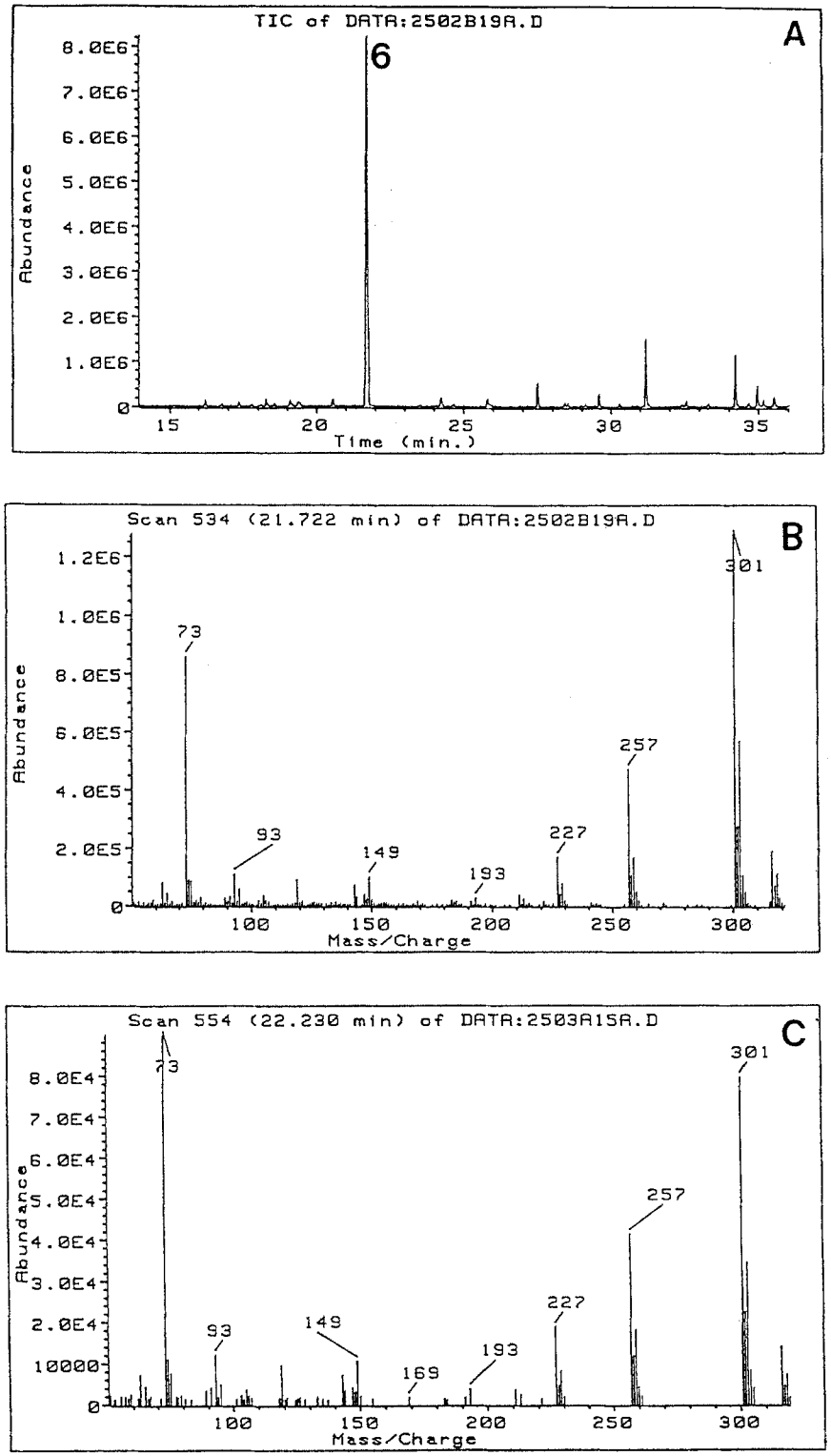


Fig. 4. Total ion-current chromatograms of major metabolites of 2-chloro-4-hydroxybiphenyl metabolism by strain B-356 as TMS-derivatives (A) and corresponding mass spectra of metabolite **6** as TMS derivative, 2-chloro-4-hydroxy-benzoic acid (B). Mass spectra of major metabolite produced from degradation of 3-chloro-4-hydroxybiphenyl by strain B-356 as TMS-derivative, 3-chloro-4-hydroxybenzoic acid (C). The mass spectra of both chloro-hydroxybenzoic acids produced by strain B-356 from corresponding biphenyl analogs were identical to authentic compounds when tested (data not shown). The incubation of the cell suspensions and extractions were made as for 2HBP (Fig. 2).

TABLE 4

Gas chomatographic retention time and partial mass spectral features of hydroxy-biphenyls and chloro-hydroxy-biphenyls bacterial metabolites

| Metabolite | Retention time relative to benzoic acid | | | Spectra characteristics (TMS derivative) | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 2HBP | 3HBP | 4HBP | $M^+$ | $M^{-15}$ | | Other ions | | Benzoyl moiety |
| 1 | – | – | – | (420)[a] | (405) | | | | – |
| 2 | 16.75 | 19.01 | – | 418 | 403 | 330 | 315 | 242 | – |
| 3 | 23.81 | 25.92 | 25.99 | 450 | 435 | 333 | 257 | 245 | 193 |
| 4 | 22.42 | 23.27 | 24.69 | 452 | 437 | 335 | 245 | | 193 |
| 5 | 21.86 | 22.88 | 24.22 | 454 | 439 | 337 | 245 | 205 | 193 |
| 6 | 5.60 | 6.93 | 8.35 | 282 | 267 | | | | 193 |
| | 2Cl-4HBP | 3Cl-4HBP | 5Cl-2HBP | | | | | | |
| 1 | – | – | – | (454) | | | | | – |
| 2 | – | – | 20.03 | 452 | 437 | 379 | 364 | 349 | – |
| 3 | – | – | – | (484) | (469) | – | | – | (227) |
| 4 | 26.97 | – | – | 486 | 471 | 368 | | 279 | 227 |
| 5 | – | – | – | (488) | (473) | – | | – | (227) |
| 6 | 11.25 | 11.17 | 9.13 | 316 | 301 | 257 | | | 227 |

[a] Numbers in parentheses are expected molecular and characteristic ions that were not detected in this study.

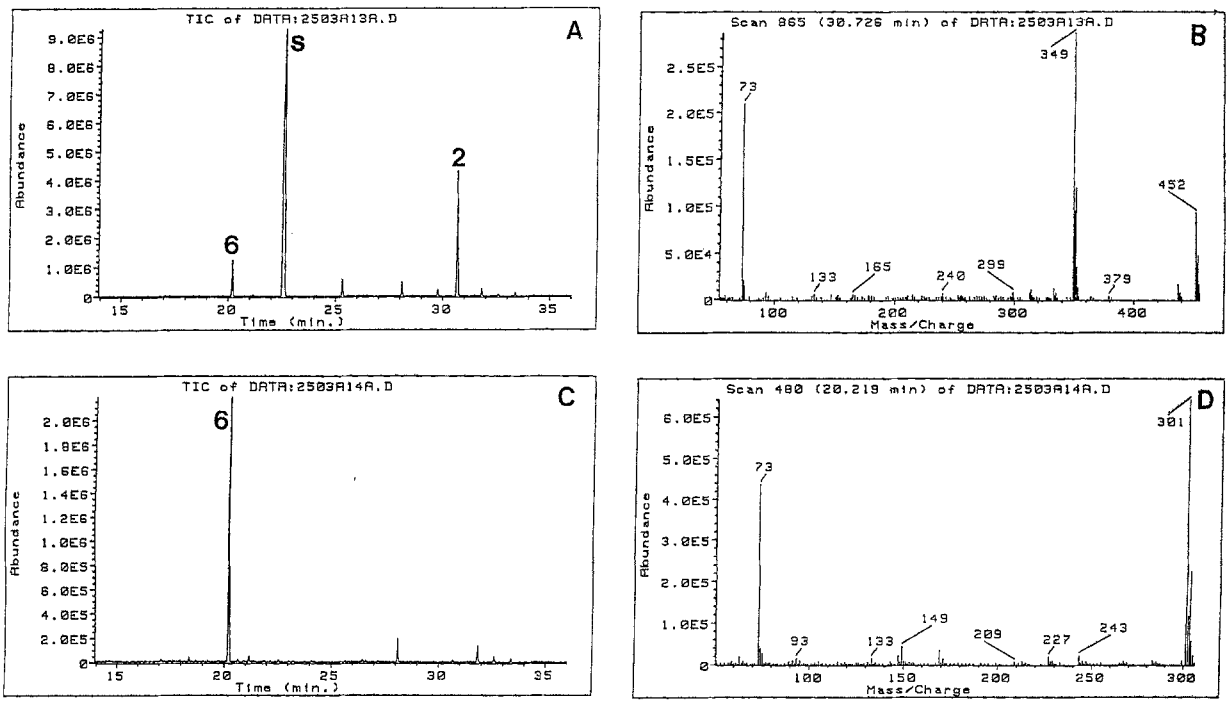


Fig. 5. Total ion-current chromatograms of TMS-derivatized metabolites from degradation of 5-chloro-2-hydroxybiphenyl by strain B-356: (A) TIC of neutral ethyl acetate extract; (B) Mass spectrum of metabolite **2**: 2,3,2′-trihydroxy-5′-chloro-biphenyl; (C) TIC of acidic ethyl acetate extract; (D) mass spectrum of metabolite **6**: 5-chloro-2-hydroxybenzoic acid (M-15 = 301). The incubation of the cell suspensions and extractions were made as for 2HBP (Fig. 2). The metabolites numbers correspond to the expected metabolites from 5-chloro-2-hydroxybiphenyl if the pathway was the same as illustrated in Fig. 1.

pDA2 is lacking the gene which codes for the hydrolysis of the *meta*-cleavage products [1]. Therefore, *P. putida* DA2 accumulates a variety of ring fission products.

Since *P. putida* DA1 is unable to utilize 2-and 3-hydroxybenzoic acids, these products would accumulate if they were generated from HBP metabolism. Therefore, if the cloned BP/CBP degradation pathway was used in strain DA1 and DA2 for the degradation of 2HBP and 3HBP, we should expect accumulation of corresponding hydroxybenzoate in strain DA1 and corresponding *meta* fission products in strain DA2. This is exactly the result we obtained.

When 2HBP was fed to *P. putida* DA1, the major metabolite was the 2-hydroxy-benzoic acid (metabolite **6**, Fig. 2B) with some 2-hydroxy-6-oxo-6-(2′-hydroxyphenyl) hexanoic acid (metabolite **5**, Fig. 2B) whose TMS derivative had the following spectral characteristics (Fig. 3C): The molecular ion was at *m/z* 454 with diagnostically important ions at *m/z* 439 (M-15), at *m/z* 337 (M-COOTMS) and at *m/z* 193 (hydroxybenzoyl moiety). The same type of results were obtained with 3HBP, namely 3-hydroxybenzoic acid was the major metabolite (Fig. 1). Moreover, when 4HBP was used as substrate for *P. putida* DA1, 4-hydroxybenzoic did not accumulate since it was used by *P. putida* KT2440.

Fig. 2C represents a chromatogram of 2HBP degradation metabolite by strain DA2. As expected, 2-hydroxybenzoic acid was not produced by this strain, but metabolites with spectral features corresponding to the unsaturated, mono-unsaturated and saturated derivatives of the *meta*-cleavage metabolites (metabolites **3**, **4** and **5**) were detected in large amounts.

When 3HBP and 4HBP were added to resting cell suspensions of *P. putida* DA2, all three types of *meta*-cleavage intermediates (metabolites **3**, **4** and **5**) accumulated. The same type of results were obtained with the 2Cl-4HBP, where strain DA1 accumulated the corresponding chloro-hydroxybenzoate as only major metabolite and strain DA2, the corresponding mono-unsaturated *meta*-cleavage metabolite (Table 3). Strain DA2 also produced some 2-chloro-4-hydroxybenzoic acid. This was unexpected since *P. putida* KT2440 does not metabolize 2Cl-4HBP. Nevertheless, it is possible that hydrolysis of the ring fission metabolites, produced by strain DA2, could be carried out by non-specific reactions involving enzymes of *P. putida* KT2440 to produce chlorohydroxybenzoic acid.

Therefore, from metabolites produced by strains DA1 and DA2, we have evidence that hydroxybiphenyls and chloro-hydroxybiphenyls are transformed to the corresponding substituted-benzoic acids by the same set of genes that are used for the degradation of BP and CBPs.

## DISCUSSION

In this study, we have shown that bacterial strain B-356 is capable of converting several monohydroxybiphenyls and hydroxychlorobiphenyls into corresponding benzoic acids, through a series of steps that are catalyzed by the same set of enzymes that are used for BP/CBP degradation. This demonstration was made possible by using substrate concentrations at levels that were subinhibitory. Other pathways were described for the degradation of mono-hydroxybiphenyls. In one case, the pathway involves initial hydroxylation of the substituted ring by a mono-oxygenase [15] and this enzymatic step appears to be substrate specific. More recently, Higson and Focht [12] reported the isolation of strain FH23 that is able to convert several hydroxybiphenyl and 4-chlorobiphenyl into metabolite hydroxylated in the unsubstituted ring. They suggested that the same pathway was used for both substrates. However, strain FH23 was unable to degrade chlorohydroxybiphenyls. Using cloned genes of BP/CBP degradation pathway from *P. testosteroni* strain B-356, we substantiated Higson and Focht's suggestion that HBP can be degraded by CBP degradation pathway.

It is not surprising that the 1,2-biphenyl dioxygenase involved in the BP degradation pathway can also oxidize hydroxylated and chlorinated BP analogs. Several examples of broad substrate specificity oxygenases have already been described [24,28]. It is also to be expected that the enzymes involved in BP/CBP degradation pathway of strain B-356 have broad substrate specificity towards chlorinated biphenyl analogs, since this strain can transform several PCB congeners into chlorobenzoic acids. In this respect, Furukawa and Miyazaki [7] have shown that enzymes involved in the BP/CBP pathway can attack many chlorinated biphenyl congeners. However, our data show that besides chlorobiphenyls, hydroxybiphenyl and hydroxychlorobiphenyl analogs can also be completely degraded (to produce corresponding substituted benzoic acids) by this pathway. Some of the end products are metabolized further by different pathways to achieve total degradation.

Only a few other examples have been described of catabolic pathways consisting of a whole set of enzymes with sufficiently broad substrate specificity to allow complete degradation of many analogs carrying substituents of different nature. They are related to the lignin degradation. As a matter of fact, some lignin degraders can also degrade PCBs [5]; aromatic hydrocarbons [3]; and other halogenated aromatic pollutants [4]. However, in these cases, the demonstration has not yet been made that all these substrates are degraded by a single set of enzymes [3]. Based on the fact that BP/CBP genes are conserved

in many stains, Furukawa et al. [7] have suggested that PCB degrading pathway in soil bacteria has evolved for the degradation of aromatic skeleton of plant lignins. A similar conclusion was reached by Ahmad et al. [1]. The observation that hydroxylated biphenyls which are generally very toxic are also degraded by this pathway provided more support to this suggestion. However, although the BP/PCB pathways seem to have a very broad substrate specificity, and that genes of the pathway appear highly conserved with respect to PCB degradation, one important question remains to be answered: "why are there such large differences among PCB degrading strains as to their ability to degrade different PCB congeners?" Since substrate specificity is one of many reasons that prevent degradation of PCB mixtures, development of organisms with broader substrate specificities could be facilitated from the evaluation of changes in the course of adaptation that contributes the most to broaden the substrate specificity of the pathway.

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