Journal of General Microbiology (1991), **137**, 1375–1385. *Printed in Great Britain*

Bioconversion of 2-hydroxy-6-oxo-6-(4′-chlorophenyl)hexa-2,4-dienoic acid, the *meta*-cleavage product of 4-chlorobiphenyl

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(*Received 26 March 1990; revised 18 July 1990; accepted 28 August 1990*)

Bacterial conversion of 4-chlorobiphenyl (4-CB) usually proceeds through a pathway involving an initial oxidation of the unsubstituted ring in the 2,3 position followed by a 1,2 *meta*-cleavage. The *meta*-cleavage product (MCP) is converted through a single hydrolysis step into chlorobenzoic acid. However, several other acidic metabolites that were not expected as part of this pathway have already been described. In this paper, we used strains of *Pseudomonas putida* carrying cloned genes from *Pseudomonas testosteroni* B-356 that are involved in polychlorinated biphenyl (PCB) degradation to demonstrate that several acidic metabolites found in the culture media of various bacteria grown in the presence of 4-CB result from alternative novel bioconversion pathways of MCP. The degradation products of MCP through these pathways were identified as analogues with saturated or shorter side chains or as 4′-chlorophenyl-2-picolinic acid; pathways leading to their formation are proposed.

Introduction

Polychlorinated biphenyls (PCBs) are very resistant to biodegradation. However, many bacterial strains able to degrade lower chlorinated PCBs have now been described (Furukawa & Matsumura, 1976; Bedard *et al.*, 1986; Furukawa & Miyazaki, 1986; Khan *et al.*, 1988; Parsons *et al.*, 1988; Sylvestre & Fauteux, 1982; Massé *et al.*, 1984; Ahmad *et al.*, 1990). The postulated steps for the conversion of such PCBs into the corresponding chlorobenzoic acids are illustrated in Fig. 1. Analysis of metabolites generated in the growth medium of bacteria grown in the presence of biphenyl or PCBs and cloning of the genes involved in this pathway have provided evidence that the degradation is initiated by the hydroxylation of the unsubstituted (or less substituted) ring in the 2,3 position followed by a *'meta'* 1,2 fission of the ring to generate the acidic *meta*-cleavage product (MCP).

However, a number of questions still remain to be answered since there are many other metabolites in the culture medium of bacteria grown in the presence of biphenyl, chlorobiphenyls or hydroxybiphenyls (Massé *et al.*, 1984; Sylvestre *et al.*, 1982; Yagi & Sudo, 1980; Bedard *et al.*, 1986; Takase *et al.*, 1986; Sondossi *et al.*, 1991) that one would not expect to find if the proposed pathway was the only one operating. These unexpected metabolites were described either as saturated analogues of MCP or benzoyl compounds substituted with an aliphatic side chain of five, four or three carbon atoms instead of six as in the MCP. The presence of some acidic metabolites with shorter side chains has been used as evidence for 3,4-dihydroxylation of the biphenyl ring (Yagi & Sudo, 1980; Massé *et al.*, 1984; Bedard *et al.*, 1986). Although 3,4-dihydro-3,4-dihydroxychlorobiphenyl has indeed been detected in some bacterial cultures grown in the presence of chlorobiphenyls (Massé *et al.*, 1989; Nadim *et al.*, 1987), the direct formation of these acidic compounds from 3,4-dihydroxybiphenyls has never been demonstrated. It is, therefore, possible that at least some of these metabolites may have been dead-end products generated from the degradation of unstable intermediates of the pathway or that they may have been derived chemically during the procedure followed to extract the metabolites from the growth medium.

Although it is generally believed that the *bphD* gene controls the single final hydrolysis step between the MCP of 4-chlorobiphenyl (4-CB), i.e. 2-hydroxy-6-oxo-6-(chlorophenyl)hexa-2,4-dienoic acid, and 4-chlorobenz-

Abbreviations: BP, biphenyl; 4-CB, 4-chlorobiphenyl; MCP, *meta*-cleavage product [of 4-CB, viz. 2-hydroxy-6-oxo-6-(4′-chlorophenyl)-hexa-2,4-dienoic acid]; PCB, polychlorinated biphenyl; 2,3-OH-4′-CBP, 2,3-dihydroxy-4′-chlorobiphenyl; BSTFA, *N,O*-bis(trimethylsilyl)trifluroacetamide; TIC, total ion chromatogram; TMS, trimethylsilyl. Other abbreviations are listed in Table 2.

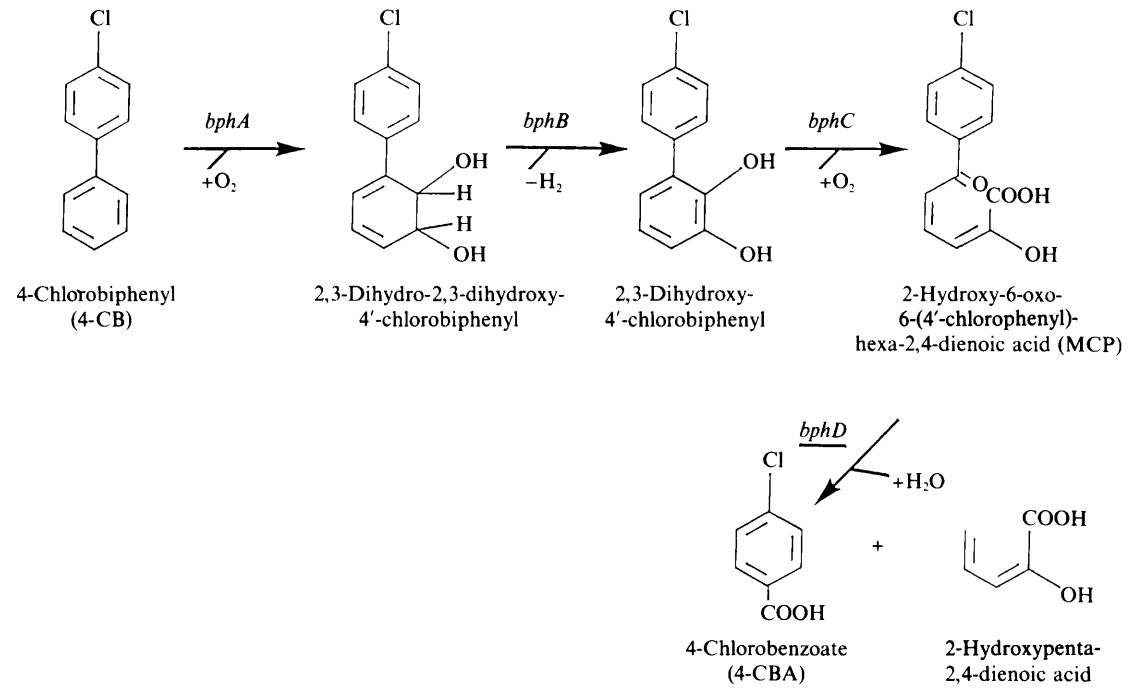

Fig. 1. Main pathway for the degradation of 4-chlorobiphenyl.

oate (4-CBA), alternative pathways involving stepwise oxidative decarboxylation of the aliphatic side chain of the MCP have also been proposed to explain the formation of some of the benzoyl compounds with short aliphatic side chains (Massé *et al.*, 1984, 1989). Since the *bphD* gene product(s) has not yet been studied in detail, this last question still remains unanswered.

We have recently cloned, in *Pseudomonas putida* KT2440, the whole series of genes involved in the biphenyl/chlorobiphenyl (BP/PCB) catabolic pathway from *Pseudomonas testosteroni* strain B-356. Two clones were isolated, *P. putida* DA1, which is able to transform 4-CB directly into 4-CBA with almost no accumulation of any other metabolite, and *P. putida* DA2, which accumulates many acidic metabolites including several that are not expected through the pathway shown in Fig. 1 (Ahmad *et al.*, 1990). All the enzymes of this pathway showed a relaxed specificity toward substrate so that the pathway is also able to degrade hydroxybiphenyls and hydroxychlorobiphenyls. The degradation of these substrates by *P. putida* DA2 gave similar results as with 4-CB, whereby saturated and unsaturated *meta*-cleavage compounds were detected as well (Sondossi *et al.*, 1991). Sangodkar *et al.* (1988) have pointed out the importance of mutational studies in the determination of the catabolic pathways rather than simple analysis of compounds accumulating in the growth medium. In the work described in this paper, we used strains DA1 and DA2, along with a few of the subclones derived from them which carried only part of the pathway, to study the origin of some of these unexpected acidic metabolites, and the pathways for their formation are proposed. To achieve this goal, 4-CB was used as a model compound since bacterial metabolites produced during its degradation have been studied and characterized extensively in our laboratory (Massé *et al.*, 1984, 1989), thus enabling us to identify all the metabolites produced.

Methods

Bacterial strains, culture media and chemicals. The bacterial strains used in this study and their phenotypic characteristics are listed in Table 1. The strains were kept lyophilized in bovine serum or frozen in 1% (w/v) dimethyl sulphoxide and they were transferred into minimal medium no. 30 (MM 30) (Sylvestre, 1980) or LB broth (Maniatis *et al.*, 1982) containing the appropriate selective agents for propagation. The 4-CB used in this study was from Aldrich; 4-CB metabolites used as substrates were produced as described below; all other chemicals and solvents were of the highest purity commercially available.

Production of 2,3-dihydroxy-4'-chlorobiphenyl (2,3-OH-4'-CBP). This compound was produced using *P. putida* DA261, which accumulates this intermediate in the growth medium (unpublished result). Cells from cultures grown overnight in 1 litre of MM 30 plus 1% (w/v) glucose and 150 μg streptomycin (Sm) ml^{-1} were centrifuged, washed in MM 30 and transferred into the same volume of MM 30 plus 1% glucose, 150 μg Sm ml^{-1} and 0·1% (w/v) 4-CB. The cultures were incubated with shaking at 29 °C for 12–18 h, the cells were spun down and the supernatant was extracted with ethyl acetate. The extract was

Table 1. *Bacterial strains and plasmids*

Strain or plasmid	Genotype or phenotype*	Source or reference
Bacterial strains		
P. testosteroni		
B-356	BP$^+$ PCB$^+$	Ahmad *et al.* (1990)
P. putida		
KT2440	BP$^-$	Bagdasarian *et al.* (1981)
DA1	KT2440(pDA1) Ap^R Sm^R Su^R Mob$^+$ *bpbhA,B,C,D*	Ahmad *et al.* (1990)
DA2	KT2440(pDA2) Ap^R Sm^R Su^R Mob$^+$ *bphA,B,C*	Ahmad et al. (1990)
DA261	KT2440(pDA261) Ap^R Sm^R Sp^R *bphA,B*	This laboratory
E. coli		
DA251	JM105(pDA251) Ap^R *bphC*	This laboratory
JM105	*supE endA sbcB15 hsdR4 rpsL thi* Δ(*lac-proAB*)	Yanisch-Perron *et al.* (1985)
Plasmids		
pDA1	Ap^R Sm^R Su^R Mob$^+$; pPSA842 with 21 kb *P. testosteroni* B-356 DNA insert; *bphA*$^+$, *B*$^+$, *C*$^+$, *D*$^+$	Ahmad *et al.* (1990)
pDA2	Ap^R Sm^R Su^R Mob$^+$; pPSA842 with 25 kb *P. testosteroni* B-356 DNA insert; *bphA*$^+$, *B*$^+$, *C*$^+$	Ahmad *et al.* (1990)
pDA261	Ap^R Sm^R Sp^R; pMMB66 with 8·2 kb *P. testosteroni* B-356 DNA insert; *bphA*$^+$, *B*$^+$	This laboratory
pDA251	Ap^R; pUC18 with 3·7 kb *P. testosteroni* B-356 DNA insert; *bph*C$^+$	This laboratory

* BP$^+$, degrades biphenyl; PCB$^+$, degrades polychlorinated biphenyls; BP$^-$, does not carry BP/PCB-degrading genes; Ap^R, ampicillin resistance; Sm^R, streptomycin resistance; Su^R, sulphathiazole resistance; Mob$^+$, can be mobilized; Sp^R, spectinomycin resistance; *bphA,B,C* and *D* are genes involved in biphenyl degradation (see Fig. 1).

concentrated by evaporation to yield 50–100 mg product per g substrate. The 2,3-OH-4′-CB was purified by HPLC using a Whatman C-18 semi-preparative column that was eluted with methanol/acetonitrile/water (8:17:75, by vol.) applied as a gradient to reach a final concentration of methanol/acetonitrile (32:64, v/v) in 20 min.

Production of meta-cleavage products derived from 4-CB. The *meta*-cleavage product 2-hydroxy-6-oxo-6-(4′-chlorophenyl)hexa-2,4-dienoic acid (the abbreviation MCP is hereafter used to refer specifically to this compound) was produced from *Escherichia coli* JM105(pDA251), bearing the *bphC* gene. *E. coli* JM105(pDA251) was grown overnight into LB broth containing ampicillin (100 μg ml^{-1}) and IPTG (50 μg ml^{-1}) to induce the enzyme. The cells were harvested, washed in 0·1 M-phosphate buffer pH 7·2, resuspended in the same buffer to an OD_{600} of 1, and then ultrasonified with a Braunsonic sonifier. The cell extracts were centrifuged at 20000 *g* for 20 min, and adjusted to a protein concentration of approximately 2 mg ml^{-1} using the Lowry protein assay method (Stauffer, 1975). A 25 ml volume of this preparation was used directly as a reaction mixture to which 0·5 μmol substrate (2,3-OH-4′-CB) was added to initiate the reaction. The substrate was almost completely converted to MCP when the reaction mixture was incubated for 1–2 h at 37 °C, producing 15–20 nmol MCP ml^{-1}. The reaction mixture was extracted twice with ethyl acetate at neutral pH to remove the residual substrate and then three times at pH 4·0. Gas chromatographic (GC) analysis of the acidic ethyl acetate extract showed two major peaks at approximately 34·9 min which were isomers of MCP. No other transformation products from the substrate were detected.

Reduced MCP derivatives with mono- and di-saturated aliphatic side chains, 2-hydroxy-6-oxo-6-(4′-chlorophenyl)hex-4-enoic acid (MS-MCP) and 2-hydroxy-6-oxo-6-(4′-chlorophenyl)hexanoic acid (DS-MCP) respectively, were obtained as the degradation products of 4-CB when *P. putida* strain DA2 was grown in MM 30 in the presence of Sm (150 μg ml^{-1}), 1% glucose and 0·1% 4-CB. Cultures (25 ml in 125 ml Erlenmeyer flasks) were incubated with shaking for 5 d, then filtered through glass wool to remove residual solids of 4-CB and centrifuged. The supernatant was extracted with ethyl acetate first at neutral pH and then at pH 3. The pH 3 extract contained MS-MCP and DS-MCP as major products. Approximately 5–10 μg of combined MCP, MS-MCP and DS-MCP was obtained for each 1 mg of 4-CB added to the medium. Although the yields were low, the amounts produced were sufficient to test for their bioconversion. The pH 3 extract was either used as such or was purified further by HPLC using a Whatman semi-preparative C18 column eluted with methanol/acetonitrile/water as described above. Under these conditions, MS-MCP and DS-MCP were separated as two distinct peaks.

Biotransformation of the metabolites from the 4-CB degradation pathway. The biotransformation of 4-CB metabolites was assayed in growing cultures or resting cell suspensions of *P. testosteroni* B-356 and *P. putida* DA1, DA2 and KT2440. MM 30 containing the substrate was inoculated with a high-density cell suspension to give a final concentration of approximately 1 × 10^9 cells ml^{-1}, and the culture was incubated with shaking at 29 °C for the duration of the experiment. In all cases, appropriate control experiments were run in parallel. At the end of the incubation period, the metabolites were extracted with ethyl acetate first at neutral pH and then at pH 3·0 or 4·0 as described previously (Massé *et al.*, 1984, 1989). The extracts were analysed as trimethylsilyl (TMS) derivatives by GC and gas chromatography/mass spectrometry (GC/MS) using the procedure described by Ahmad *et al.* (1990).

Results

Analysis of acidic metabolites of 4-CB produced by P. putida DA2

P. putida strain DA2, carrying plasmid pDA2, is able to transform 4-CB as well as other chlorinated (Ahmad *et al.*, 1990) or hydroxylated biphenyl (Sondossi *et al.*, 1991) analogues into the corresponding acidic derivatives. pDA2 was obtained by cloning, in the vector plasmid pPSA842, a 25 kbp DNA fragment of *P. testosteroni* strain B-356 (Ahmad *et al.*, 1990). pDA2 does not carry the *bphD* gene which is required to carry out the last step

Table 2. *Metabolites produced from 4-CB by P. putida DA2*

No.*	Name and structure†	Abbreviation	M^+ TMS‡	M–15 TMS‡
1	4-Chlorobenzoate R-COOH	4-CBA	228	213
2	4-Chlorocinnamic acid R-CH=CH-COOH	CCA	254	239
3	4-Chlorobenzoylpropionic acid R-C(=O)-CH_2-CH_2-COOH	CBP	284	269
4	4-Chlorobenzoylbutanoic acid R-C(=O)-CH_2-CH_2-CH_2-COOH	CBB	298	283
5	2-Hydroxy-5-oxo-5-(4′-chlorophenyl)pentanoic acid R-C(=O)-CH_2-CH_2-CH(OH)-COOH	OHCBB	(–)	371
7	2-Hydroxy-6-oxo-6-(4′-chlorophenyl)hexanoic acid R-C(=O)-CH_2-CH_2-CH_2-CH(OH)-COOH	DS-MCP	400	385
8	2-Hydroxy-6-oxo-6-(4′-chlorophenyl)hex-4-enoic acid R-C(=O)-CH=CH-CH_2-CH(OH)-COOH	MS-MCP	398	383
9	2-Hydroxy-6-oxo-6-(4′-chlorophenyl)hexa-2,4-dienoic acid R-C(=O)-CH=CH-CH(OH)=C-COOH	MCP	396	381

* Metabolite numbers as they appear in Figs 2 and 3 (metabolite 6 is discussed later).
† R- stands for *p*-chlorophenyl ring.
‡ Metabolites were identified as TMS derivatives.

of the pathway, i.e. transformation of MCP into the corresponding benzoic acid (Ahmad *et al.*, 1990). The acidic metabolites listed in Table 2 were produced when 4-CB was added to resting cell suspensions of strain DA2; they were identified using GC/MS analysis, by comparison with published data from our laboratory and mass spectral characteristics of authentic reference compounds (Massé *et al.*, 1989, 1984). Except for metabolites 1 (4-CBA) and 9 (MCP), all other metabolites were not expected as being part of the BP/PCB degradation pathway shown in Fig. 1. These metabolites were not exclusively produced by strain DA2 since they were also detected in cultures of the parental strain B-356 grown on 4-CB. Moreover, these metabolites (CCA, CBP, CBB, OHCBB, MS-MCP and DS-MCP) have also been detected in the growth medium of other bacterial strains, including strain B-206 (Massé *et al.*, 1989), *Achromobacter* sp. strain B-218 and *Bacillus brevis* strain B-257 (Massé *et al.*, 1984). Thus many PCB-degrading bacteria produce these unexpected metabolites. However, it is not clear whether these metabolites are part of other existing unknown pathways for PCB degradation, are dead-end metabolites, or are metabolites generated through spontaneous reactions from intermediates of the pathway during their extraction from the medium.

We felt that strain DA2, and other clones from our collection, that carry selected genes for the BP/PCB degradation pathway, could help understand the origin of these acidic metabolites. The total ion chromatogram (TIC) profile in Fig. 2 gives an idea of the relative proportion of the acidic metabolites produced from 4-CB by resting cell suspensions of strain DA2. The major metabolites were MS-MCP (metabolite 7) and DS-MCP (metabolite 8). Younger cultures (1–6 h old) contained more MS-MCP while older cultures (18–72 h old) contained almost exclusively DS-MCP. The other benzyl or benzoyl metabolites with a shorter acidic aliphatic side chain, viz. CCA, CBP, CBB and OHCBB, were detected in lesser amounts. Metabolite 9 (MCP) was always found in trace amounts. Therefore, it appeared that MCP was either rapidly converted by the cells or transformed during extraction.

Strain DA2 was unable to grow on 4-CB. However, interestingly, when this strain was grown on MM 30 in

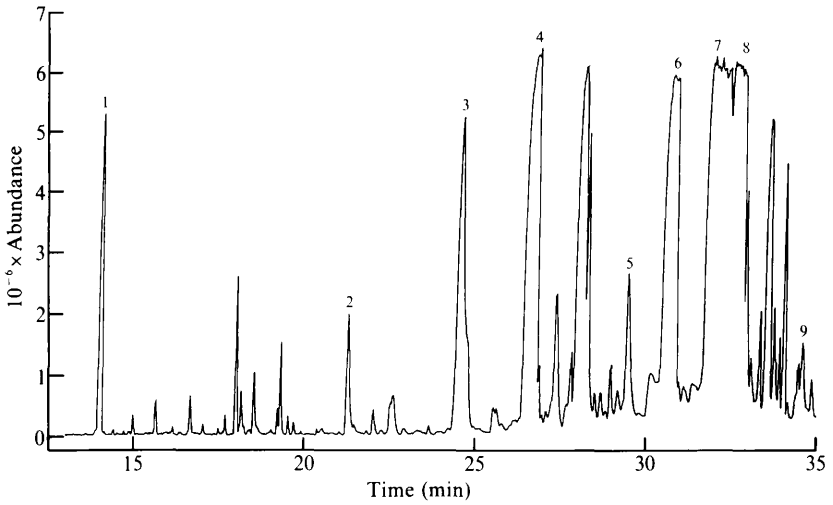

Fig. 2. TIC of acidic ethyl acetate extracts of a *P. putida* DA2 resting cell suspension incubated in the presence of 4-CB. Cells from an overnight culture in LB broth plus 150 μg Sm ml^{-1} were washed and resuspended (10^9 cells ml^{-1}) in MM 30 plus 0·1% 4-CB. The culture was incubated for 18 h with shaking. The cells were spun down; the medium was first extracted at neutral pH with 3 vols ethyl acetate, then acidified (pH 3) and extracted with a further 3 vols ethyl acetate. The ethyl acetate extracts were evaporated and the residues reacted with BSTFA for GC/MS analysis. The identity of each numbered metabolite is given in Table 2.

the presence of 4-CB plus glucose, the production of other acidic metabolites seemed to be almost totally inhibited, and only MS-MCP and DS-MCP plus traces of MCP were produced. This is an important observation, since it allowed us to produce the two saturated forms of MCP for further assays required to study their biotransformation pathways.

Production of MCP from 2,3-dihydroxy-4′-chlorobiphenyl

When 2,3-OH-4′-CBP was fed to resting cell suspensions of *P. putida* DA1 or DA2, or *P. testosteroni* B-356, essentially the same results were obtained as with 4-CB. In all these cultures, the medium immediately turned yellow after addition of 2,3-OH-4′-CBP, indicating the production of MCP. While cultures of strain DA2 remained yellow, with no change in A_{434} for several hours, the colour rapidly disappeared and large amounts of 4-CBA were produced in cultures of strains B-356 and DA1, which carry the whole series of genes required for the transformation of 4-CB into 4-CBA (Ahmad *et al.*, 1990). However, in suspensions of strain DA2, only trace amounts of MCP were detected by GC/MS, and MS-MCP and DS-MCP were the major metabolites.

Because of the low copy number and the large size of pDA2, the gene *bphC* was not very well expressed in *E. coli* DHI(pDA2). However, *E. coli* DA251 carrying a 3·7 kbp *Sma*I fragment of pDA2 corresponding to the *bphC* gene subcloned in pUC18, was able to rapidly convert 2,3-OH-4′-CBP to MCP. Cell-free extracts of *E. coli* DA251 were able to transform 20 nmol ml^{-1} of the substrate into almost stoichiometric amounts of MCP during 1 h of incubation at 37 °C. Although we noticed, as will be discussed later, that MCP was not stable for long periods in aqueous solutions, it became evident that MCP was not degraded or transformed into its saturated analogues during extraction with ethyl acetate since the expected amounts of MCP could be extracted from the medium in the above-mentioned experiments.

Further transformation of MCP

The TICs in Fig. 3(*a*, *b*) show that *P. putida* DA1 rapidly transformed MCP into 4-CBA, while *P. putida* DA2, which does not bear the *bphD* gene, transformed MCP into MS-MCP and DS-MCP. In these experiments, the conversion was not stoichiometric. In the case of DA1, when 0·5 μmol MCP was added to 10 ml medium, only 150–200 nmol 4-CBA was produced within 30 min, while no substrate could be re-extracted from the medium. For strain DA2, only 40% of the added substrate was recovered as DS-MCP plus small amounts of other acidic metabolites. These data are not surprising, considering, as we will discuss later, that MCP is also chemically converted into molecular species (probably polymers) that are not detected in GC/MS analysis. However, the important point about this experiment is that the host strain *P. putida* KT2440 also converted MCP into MS-MCP and DS-MCP (Fig. 3*c*), which provided evidence that the genes controlling further reduction of MCP are not carried on plasmid pDA2 and that they are not part of the BP/PCB degradation pathway. In fact, a 2-hydroxy-6-oxo-6-phenylhex-2,4-dienoic acid-reducing enzyme has recently been purified from a BP degrading *Pseudomonas cruciviae* (Omori *et al.*, 1986*a*). However, this enzyme did not appear to play any specific role in BP degradation.

The data presented in Fig. 3 were obtained from cultures grown in broth medium containing glucose in

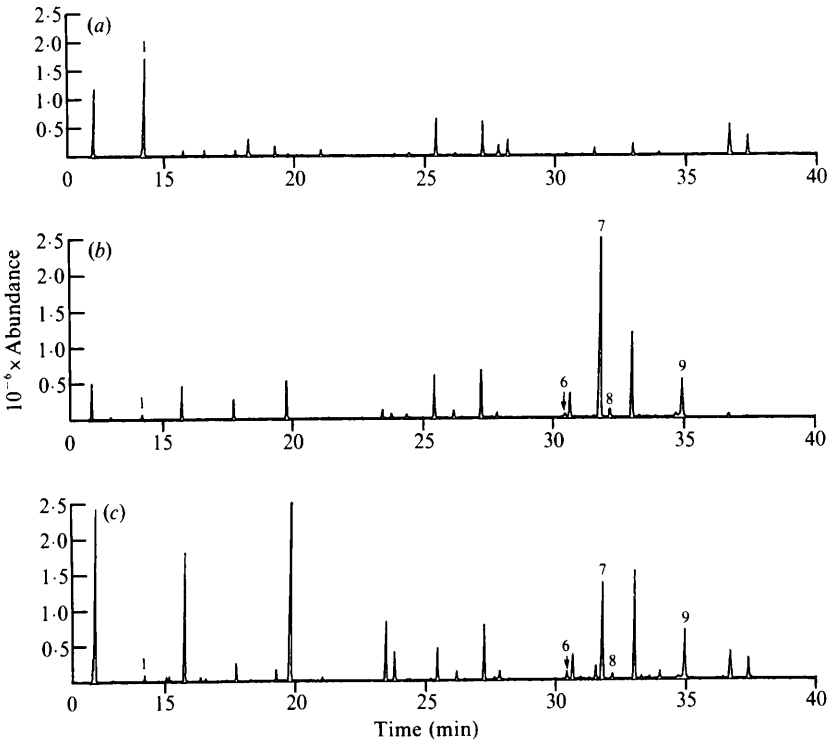

Fig. 3. TICs of acidic ethyl acetate extracts of cells of *P. putida* strain DA1 (*a*), DA2 (*b*) or KT2440 (*c*) grown for 30 min in the presence of MCP. Cells grown overnight on MM 30 plus glucose (1%, w/v) (and 150 μg Sm ml^{-1} for DA1 and DA2) were washed and resuspended in MM 30 (3×10^9 cells ml^{-1}) containing 0·1% glucose and 0·5 μmol MCP in 10 ml medium. The cell suspensions were incubated for 30 min at 29 °C then acidified (pH 3) and extracted with 3 vols of ethyl acetate. Metabolites 1, 7, 8 and 9 are described in Table 1. The structure of metabolite 6 is presented in Fig. 4.

addition to MCP. When the experiment was carried out in the absence of glucose, besides MS-MCP and DS-MCP, the metabolites CCA, CBP, CBB and OHCBB were also produced from MCP by both *P. putida* KT2440 and DA2 and the proportions of metabolites were also about the same in both strains. Therefore, the acidic compounds with a shorter aliphatic side chain that were produced when strain DA2 was grown in the presence of 4-CB (Fig. 2) appeared to be generated from MCP or its saturated analogues through biotransformation reactions involving enzymes that are not related to the main BP/PCB pathway. These enzymes appear to be under some glucose-mediated control in *P. putida*.

Conversion of MCP into metabolite 6

The case of metabolite 6 is special since it is a nitrogen-containing derivative. This metabolite was detected in cultures of strains DA2 (Fig. 2) and B-356 (not shown) grown in the presence of 4-CB. Metabolites having a structure similar to metabolite 6 were also detected when other chlorobiphenyls were used as substrates. Metabolite 6 was produced from MCP since it was also detected in resting cell suspensions of *P. putida* DA2 or KT2440 and of *E. coli* DA251 incubated with 0·5 μmol MCP per 10 ml.

From the mass-spectral characteristics of metabolite 6-TMS (Fig. 4), it was tentatively identified as the 2-picolinic acid derivative of MCP. The TMS derivative exhibits a molecular ion at *m/z* 305 and fragment ions at *m/z* 290 ($M-CH_3$), 277 (M–CO), 188 (M–CO-TMS), 153 (M–CO-TMS-Cl), 127 (M–CO-TMS-Cl-NC), which are in accordance with the proposed structure.

Further evidence for the structure of metabolite 6 was provided from the observation that in the presence of 6 M-NH_4OH, MCP was converted into a derivative with retention time and mass spectral characteristics identical to metabolite 6 detected in the culture broth of cells grown with 4-CB. Such a conversion into a 2-picolinic acid derivative has also been observed in the case of the *meta*-cleavage product generated from 3,4-dihydroxy-9,10-secoandrosta-1,3,5,(10),triene-9,17-dione (Gibson *et al.*, 1966) and in the case of 2-hydroxy-6-carboxy-muconate semi-aldehyde, which is the *meta*-cleavage product of protocatechuate (Rossi & Shing, 1948), when these compounds were incubated in 6 M-ammoniacal solution. Since in these two examples, the cleavage occurs in a manner analogous to 2,3-OH-4'-CBP, resulting in the formation of an acidic metabolite possessing a 2-hydroxy-6-oxo-hexa-2,4-dienoic moiety, the mechanism of formation of metabolite 6 when MCP is incubated in ammoniacal solution is probably similar.

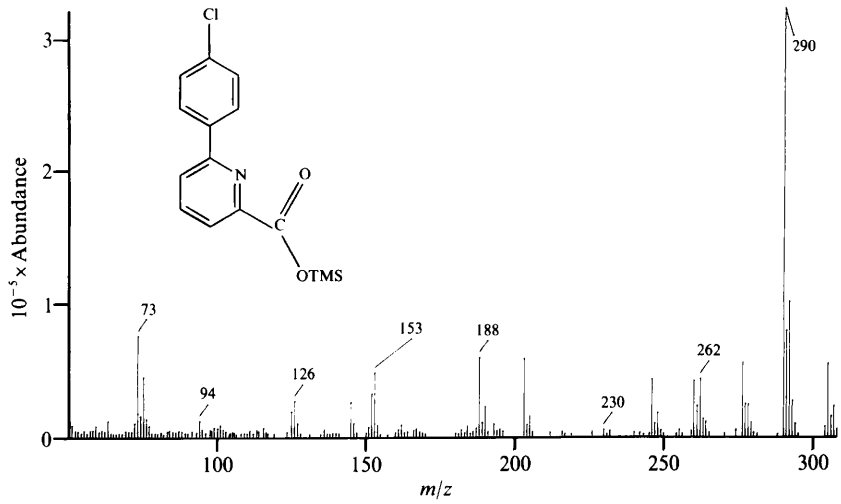

Fig. 4. Mass spectrum of metabolite 6-TMS.

Transformation of DS-MCP to MS-MCP

In order to further understand the interrelationship between the various acidic derivatives of 4-CB, HPLC-purified DS-MCP was fed to cell suspensions of strains B-356, DA1, DA2 and KT2440. These suspensions were incubated for either 18 or 48 h. Part of the results are given in Table 3. *P. testosteroni* strain B-356 rapidly converted DS-MCP – 70% of the substrate disappeared within 18 h of incubation; metabolites 6 and 8 were the major products of this conversion. In 48 h old cultures, metabolites 7 and 8 had completely disappeared and metabolites 1 to 6 were all detected.

Table 3. *Production of metabolites from purified DS-MCP*

Cells were grown for 18 or 48 h in the presence of purified DS-MCP (20 nmol per 25 ml). The cultures were extracted at pH 3 with 3 vols ethyl acetate. The extracts were evaporated; the residue was solubilized in 1 ml BSTFA/acetonitrile (1 : 1, v/v) and analysed by GC/MS.

Strain	Time (h)	Recovery* of metabolite no.: 1	2	3	4	5	6	7	8
Control†	0	0	0	0	0	0	0	49	0
	48	0	0	0	0	0	0	47	0
B-356	18	0	0·2	0	1·7	0	7	4	31
	48	5	4	3·5	16	5	10	0	0
DA-1	18	0·3	1	0	0·7	0	0	43	0
	48	2	4	0·1	6	0·1	3	40	0·2
DA-2	18	0·1	2	0	0·2	0	1	44	0
	48	2	4	0·3	3	0	5	41	0
KT2440	18	0·1	2	0	0	0	2	40	0
	48	2	5	0	2	0·5	8	36	0

* Metabolite recovery was evaluated as the area (in arbitrary units) under the peak representing each metabolite. Metabolite 9 was not detected in any preparation.

† Culture medium plus DS-MCP incubated without bacterial cells.

Table 4. *Production of metabolites from non-purified DS-MCP*

P. putida DA-2 was incubated for 48 h in MM 30 plus 0·1% 4-CB. The culture was extracted at neutral pH with ethyl acetate, acidified at pH 3 then extracted again with ethyl acetate. The acid extract contained large amounts of DS-MCP with small amounts of metabolites 1, 3 and 4. Resting cell suspensions of strains KT2440 and B-356 were prepared from exponential-phase cultures, and were adjusted to an OD_{600} of 1. Suspensions (25 ml) were fed 60 μg of the acid extract, containing 210 μmol DS-MCP, then incubated for 18 h at 29 °C followed by extraction at pH 3 with ethyl acetate.

Strain	Recovery* of metabolite no.: 1	2	3	4	5	6	7	8
Control†	5	0	15	35	0	0	360	0
KT2440	14	23	19	56	0	186	14	240
B-356	42	64	42	83	0	160	73	248

* Metabolite recovery was evaluated as the area (in arbitrary units) under the peak representing each metabolite. Metabolite 9 was not detected in any preparation.

† Culture medium plus acid extract incubated without bacterial cells.

All three *P. putida* strains tested were also able to carry out this transformation, but the rate of transformation was slower than in the case of *P. testosteroni* strain B-356 (Table 3). All three strains produced approximately equal amounts of metabolites 2, 3, 4, 5 and 6, which slowly increased with time, suggesting that these reactions are controlled by genes carried by the host strain *P. putida* KT2440.

One interesting observation is that a trace amount of MS-MCP (metabolite 8) was detected in cultures of DA1 grown with DS-MCP (Table 3). In further experiments using as substrate an acidic extract of DA2 cultures grown with 4-CB, DS-MCP, which was the major component of the extract, was rapidly converted into MS-MCP and metabolite 6 by both *P. testosteroni* B-356

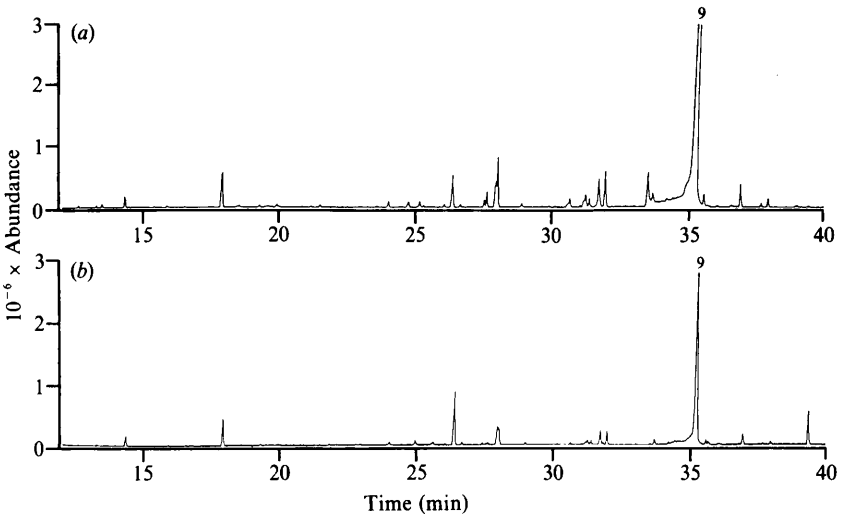

Fig. 5. Stability of MCP in distilled water at 29 °C. Purified MCP (0·5 μmol) was dissolved in 10 ml distilled water and immediately re-extracted with ethyl acetate or incubated for various periods of time before re-extraction. (*a*) TIC of extracts obtained at time 0; (*b*) TIC of extracts obtained after 7 h incubation.

and *P. putida* KT2440 (Table 4). It is noteworthy that unpurified DS-MCP was transformed into MS-MCP as fast in strain KT2440 as in strain B-356. Some undetected regulatory factor present in culture extracts of strain DA2 could possibly be controlling this transformation.

The important point about these experiments is that they clearly show that DS-MCP is transformed into MS-MCP and metabolite 6. The fact that metabolite 6 is derived from MCP shows that DS-MCP can be converted back into MS-MCP and MCP. Since hydrolysis or oxidative decarboxylation of aliphatic carbon chains cannot occur directly on a reduced chain, it is not surprising that transformation of DS-MCP into metabolites 2, 3, 4 and 5 included an oxidative step converting DS-MCP back into MS-MCP and/or MCP. Moreover, analysis of controls of DS-MCP, MS-MCP and MCP without cells clearly shows that the production of these acids occurs through a biological process. Hence, MS-MCP and DS-MCP seem to be very stable and under neutral conditions are not spontaneously cleaved to compounds with a shorter acidic side chain. When 0·5 μmol purified MCP was dissolved in distilled water, the A_{434} remained stable for at least 7 h. However, although 100% of MCP was re-extracted immediately after solubilization of the compound in water, when analysed by GC/MS, the amount of MCP recovered decreased with the time of incubation. After 2 h, 75% of the added MCP was re-extracted, and after 7 h, only 35% was recovered. However, as shown in Fig. 5, metabolites 2, 3, 4 and 5 were not detected in these preparations. MCP was also solubilized in MM 30 and in 0·1 M-phosphate buffer pH 7·0, and similar results were observed. Therefore, when MCP was incubated in aqueous solution under neutral conditions, it was converted to unrecoverable coloured derivatives, possibly polymers as suggested by Catelani *et al.* (1973), that were neither detected by GC/MS analysis nor were cleaved spontaneously to benzoyl compounds with a shorter acidic side chain.

Discussion

The pathway shown in Fig. 1 summarizes the various enzymic steps presumed to be involved in the catabolism of biphenyls and chlorobiphenyls. This pathway was deduced from analysis of metabolites produced from the biphenyl substrates (Catelani *et al.*, 1973; Ahmed & Focht, 1973; Furukawa & Matsumura, 1976; Omori *et al.*, 1986*b*; Ishigooka *et al.*, 1986) and it was further supported by recent cloning of the genes controlling these enzymes (Mondello, 1989; Khan & Walia, 1989; Hayase *et al.*, 1990; Ahmad *et al.*, 1990). Several reports have suggested the existence of other biodegradation routes involved in the biodegradation of biphenyls or chlorinated biphenyls (Yagi & Sudo, 1980; Sylvestre *et al.*, 1982; Massé *et al.*, 1984; Bedard *et al.*, 1986; Barton & Crawford, 1988; Omori *et al.*, 1986*b*). However, very little information on the step-by-step reactions occurring in these pathways, or on their relative importance in the metabolism of biphenyls, is available. For example, it was postulated that the initiation of oxidation of the biphenyl molecule through a 3,4-dihydroxylation step is a prerequisite for the degradation of some PCB congeners (Bedard *et al.*, 1987). However, although 3,4-dihydro-3,4-dihydroxychlorobiphenyls and possible metabolites deriving from it have been detected in some bacterial cultures (Massé *et al*, 1989; Nadim *et al.*, 1987), details of the transformation steps are still unknown.

In the present work, using bacterial clones carrying only part of the PCB degradation pathway, we have determined the origin of formation of some of the acidic

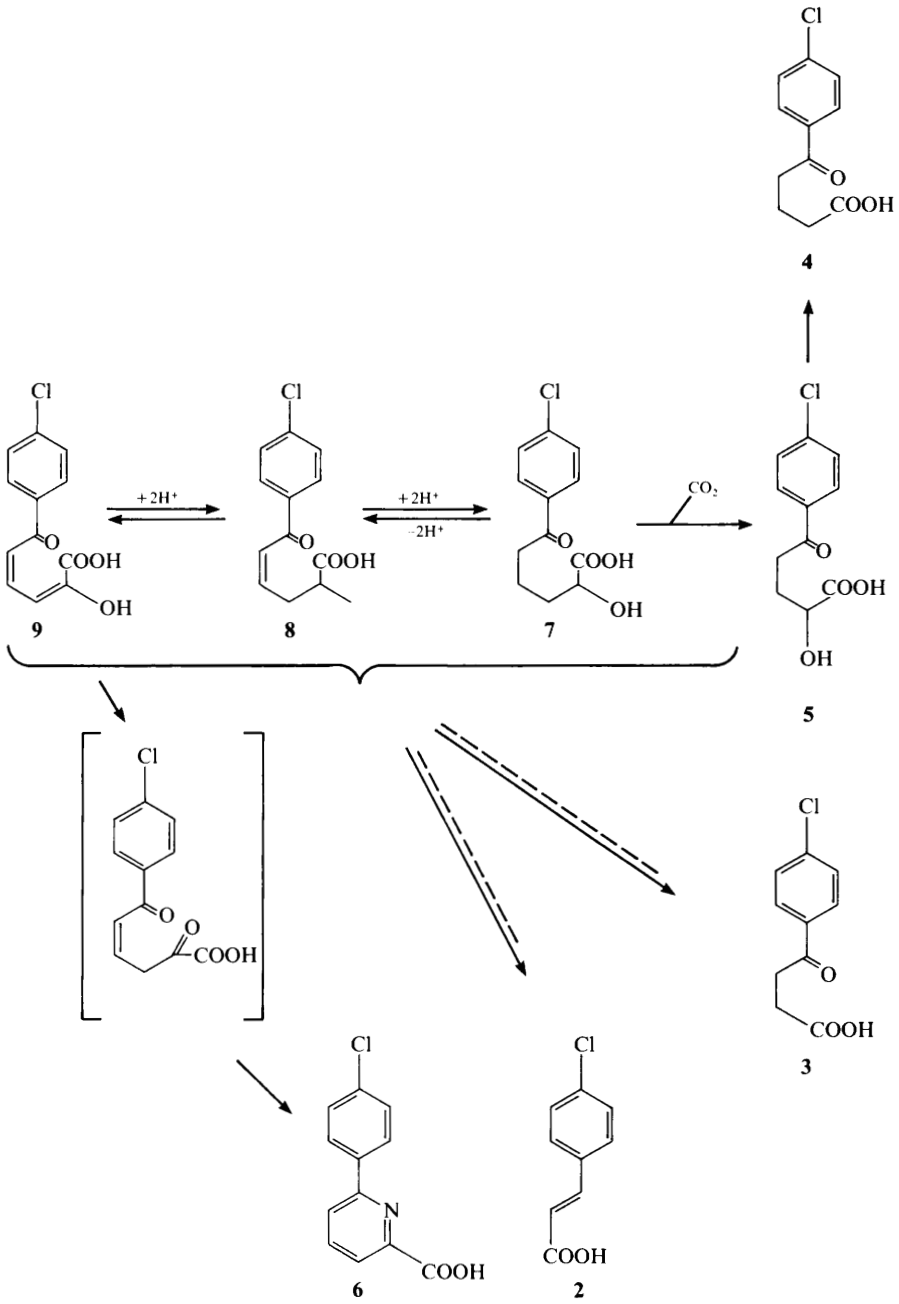

Fig. 6. Proposed pathways for the formation of the bacterial acidic compounds produced from the MCP of 4-CB. Dotted lines indicate uncertainty about the conversion being a single or multi-step.

metabolites that have been encountered in many bacterial cultures grown in the presence of chlorobiphenyls (Massé *et al.*, 1984, 1989). Using 4-CB as a model, we have shown that MCP deriving from it is rapidly converted into derivatives with mono- and di-saturated side chains. The genes responsible for this conversion are not part of the *bph* operon, and are found in the host strain *P. putida* KT2440 which is unable to degrade biphenyl. DS-MCP can be re-oxidized and then cleaved to generate acid metabolites with a shorter aliphatic side chain. It is interesting that these biotransformation reactions are very efficient in strain B-356, where all saturated MCP added was degraded into other acidic derivatives within 48 h (Table 3). It has previously been shown that 4-CBA affects the regulation of the BP/PCB degradation pathway, where accumulation of 4-CBA favours the production of acidic metabolites that are unexpected as intermediates of this pathway (Sylvestre

et al., 1985). Although we do not know whether the enzymic system controlling the transformation of MCP into other acidic metabolites is the same in all strains, our results clearly show that the same metabolites are produced when MS-MCP is fed to *P. putida* strain KT2440, which does not bear the *bph* genes. The difference between *P. testosteroni* strain B-356 and *P. putida* strain KT2440 when purified substrate was used, was in the rate of transformation of DS-MCP and MS-MCP. Strain B-356 degraded all the added substrate within 48 h, while under the same conditions, strain KT2440 only transformed a fraction of the added substrate. This observation could possibly reflect the fact that in strain B-356 these biotransformation reactions have evolved to drain the MCP that is produced under conditions favouring 4-CBA accumulation. But more importantly, it provides strong evidence that the acidic metabolites 2, 3, 4, 5, 7 and 8 are produced through a series of enzymic steps that are genetically controlled. These reactions are summarized in Fig. 6. Further support for the biological nature of these chlorobiphenyl derivatives is provided by the fact that neither MCP, MS-MCP nor DS-MCP was transformed into the shorter side chain metabolites when incubated in water or buffer. In fact, an enzymic system able to reduce the MCP derived from biphenyl has been purified from *Pseudomonas cruciviae* (Omori *et al.*, 1986*a*), showing that biological conversion of MCP into DS-MCP can occur. Finally, our results show that in *P. putida*, a glucose effect seems to prevent the conversion of MCP, MS-MCP or DS-MCP into the benzoyl derivatives with a shorter side chain.

Metabolite 6 reported in this work has never been reported before as a product of chlorobiphenyl metabolism. In our laboratory, this metabolite has been detected in many bacterial cultures grown in the presence of biphenyl analogues. However, until recently, when MCP could be produced in large amounts, we did not have enough of this metabolite to show a direct conversion to metabolite 6. The observed transformation of MCP into a compound with identical mass spectral characteristics to metabolite 6 in ammoniacal solution strongly suggests that this 4-CB derivative is the 2-picolinic acid derived from MCP (Fig. 4). Since this derivation usually occurs under strongly basic conditions (Gibson *et al.*, 1966) it is not clear whether under neutral pH conditions it is produced spontaneously or as a result of some biologically catalysed reaction. However, the fact that it is produced under neutral conditions in all growth media raises the question of toxicity of these derivatives when PCBs of low chlorine content are degraded. Further work is in progress to answer these questions.

Since 3,4-dihydro-3,4-dihydroxychlorobiphenyl has been detected in several instances as a bacterial metabolite of chlorobiphenyl (Yagi & Sudo, 1980; Massé *et al.*, 1986; Nadim *et al.*, 1987), some of the acidic chlorobenzoyl derivatives of chlorobiphenyl such as chloroacetophenone and CBP or CBB have been suggested to be generated through a different initial mechanism of oxidation and cleavage of the aromatic ring (Bedard *et al.*, 1987; Massé *et al.*, 1984). However, the results presented in this paper strongly suggest that several of the acidic metabolites of chlorobiphenyl that are not expected as part of the BP/PCB degradation pathway (Fig. 1) are rather produced from transformation reactions (biocatalytically or not) involving the *meta*-cleavage product resulting from ring opening of 2,3-OH-CBP. None of the strains we used in this study produced chloroacetophenone from 4-CB. However, they all produced chlorocinnamic acid (metabolite 2), which was recently shown to be transformed into chloroacetophenone through an alternative route branching from the chlorocinnamic degradation pathway (Hilton & Cain, 1990). Therefore, caution should be exercised when new PCB metabolites are detected before attributing their presence to a new mechanism of initial oxidation of the biphenyl molecule.

This paper was supported by grant nos. G1966 and 0GP003579 from the Natural Sciences and Engineering Research Council of Canada. We thank Carole Corriveau for typing this manuscript.

References

AHMAD, D., MASSÉ, R. & SYLVESTRE, M. (1990). Cloning and expression of genes involved in 4-chlorobiphenyl transformation by *Pseudomonas testosteroni*: homology to polychlorobiphenyl-degrading genes in other bacteria. *Gene* **86**, 53–61.

AHMED, M. & FOCHT, D. D. (1973). Degradation of polychlorinated biphenyls by two species of *Achromobacter*. *Canadian Journal Microbiology* **19**, 47–52.

BAGDASARIAN, M., LURZ, R., RIICKERT, B., FRANKLIN, F. C. H., BAGDASARIAN, M. M. & TIMMIS, K. N. (1981). Specific-purpose plasmid cloning vectors, and a host vector system for gene cloning in *Pseudomonas*. *Gene* **16**, 237–247.

BARTON, M. & CRAWFORD, D. L. (1988). Novel biotransformations of 4-chlorobiphenyl by a *Pseudomonas* sp. *Applied and Environmental Microbiology* **54**, 594–595.

BEDARD, D. L., UNTERMAN, R., BOPP, L. H., BRENNAN, M. J., HABERL, M. L. & JOHNSON, C. (1986). Rapid assay for screening and characterising microorganisms for the ability to degrade polychlorinated biphenyls. *Applied and Environmental Microbiology* **51**, 761–768.

BEDARD, D. L., HABERL, M. L., MAY, R. J. & BRENNAN, M. J. (1987). Evidence for novel mechanism of polychlorinated biphenyl metabolism in *Alcaligenes eutrophus* H850. *Applied and Environmental Microbiology* **53**, 1103–1112.

CATELANI, D., COLOMBI, A., SORLINI, C. & TRECCANI, V. (1973). Metabolism of biphenyl, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate: the *meta*-cleavage product from 2,3-dihydroxybiphenyl by *Pseudomonas putida*. *Biochemical Journal* **134**, 1063–1066.

FURUKAWA, K. & MATSUMURA, F. (1976). Microbial metabolism of polychlorinated biphenyls. Studies on the relative degradation of polychlorinated biphenyl components by *Alcaligenes* sp. *Journal of Agricultural and Food Chemistry* **42**, 543–548.

FURUKAWA, K. & MIYAZAKI, T. (1986). Cloning of gene cluster encoding biphenyl and chlorobiphenyl degradation in *Pseudomonas pseudoalcaligenes*. *Journal of Bacteriology* **166**, 392–398.

GIBSON, D. T., WANG, K. C., SIH, C. J. & WHITOCK, H. (1966). Mechanism of steroid oxidation by microorganisms. IX. Mechanism of ring cleavage in the degradation of 9,10-seco steroids. *Journal of Biological Chemistry* **241**, 591–559.

HAYASE, N., TAIRA, K. & FURUKAWA, K. (1990). *Pseudomonas putida* KF715 *bph*ABCD operon encoding biphenyl and polychlorinated biphenyl degradation: cloning, analysis, and expression in soil bacteria. *Journal of Bacteriology* **172**, 1160–1164.

HILTON, D. M. & CAIN W. J. (1990). Bioconversion of cinnamic acid to acetophenone by a pseudomonad: microbial production of a natural flavor compound. *Applied and Environmental Microbiology* **56**, 623–627.

ISHIGOOKA, H., YSHIDA, Y., OMORI, T. & MINODA, Y. (1986). Enzymatic dioxygenation of biphenyl-2,3-diol and 3-isopropylcatechol. *Agricultural and Biological Chemistry* **90**, 1045–1046.

KHAN, A. & WALIA, S. (1989). Cloning of bacterial genes specifying degradation of 4-chlorobiphenyl from *Pseudomonas putida* 0083. *Applied and Environmental Microbiology* **55**, 798–805.

KHAN, A., TEWARI, R. & WALIA, S. (1988). Molecular cloning of 3-phenylcatechol dioxygenase involved in the catabolic pathway of chlorinated biphenyl from *Pseudomonas putida* and its expression in *Escherichia coli*. *Applied and Environmental Microbiology* **54**, 2664–2671.

MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

MASSÉ, R., MESSIER, F., PÉLOQUIN, L., AYOTTE, C. & SYLVESTRE, M. (1984). Microbial biodegradation of 4-chlorobiphenyl, a model compound of chlorinated biphenyls. *Applied Environmental Microbiology* **47**, 947–951.

MASSÉ, R., MESSIER, F., AYOTTE, C., LÉVESQUE, M. F. & SYLVESTRE, M. (1989). A comprehensive gas chromatographic/mass spectrometric analysis of 4-chlorobiphenyl bacterial degradation products. *Biomedical and Environmental Mass Spectrometry* **18**, 27–47.

MONDELLO, F. J. (1989). Cloning and expression in *Escherichia coli* of *Pseudomonas putida* LB400 genes encoding polychlorinated biphenyl degradation. *Journal of Bacteriology* **171**, 1725–1732.

NADIM, L., SCHOCKEN, M. J., HIGSON, F. J., GIBSON, D. T., BEDARD, D. L., BOPP, L. H. & MONDELLO, F. J. (1987). Bacterial oxidation of polychlorinated biphenyls. In *Proceedings of the 13th Annual Research Symposium on Land Disposal, Remedial Action, Incineration, and Treatment of Hazardous Wastes*, pp. 395–402. Cincinnatti, Ohio: US Environmental Protection Agency. EPA/600/9–87/015.

OMORI, T., ISHIGOOKA, H. & MINODA, Y. (1986*a*). Purification and some properties of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA) reducing enzyme from *Pseudomonas cruciviae* 593B1 involved in the degradation of biphenyl. *Agricultural and Biological Chemistry* **50**, 1513–1518.

OMORI, T., SUGIMURA, K., ISHIGOOKA, H. & MINODA, Y. (1986*b*). Purification and some properties of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid hydrolyzing enzyme from *Pseudomonas cruciviae* 593B1 involved in the degradation of biphenyl. *Agricultural and Biological Chemistry* **50**, 931–937.

PARSONS, J. R., SIJM, D. T. H. M., VAN LOOR, A. & HUTZINGER, O. (1988). Biodegradation of chlorinated biphenyls and benzoic acids by a *Pseudomonas* strain. *Applied Microbiology and Biotechnology* **29**, 81–84.

ROSSI, A. & SHING (1948). 75. Alcuni a-cheti-y-lattoni con sastituenti alchilici in posizione y. *Helvetia Chimica Acta* **31**, 473–492.

SANGODKAR, U. M. X., CHAPMAN, P. J. & CHAKRABARTY, A. M. (1988). Cloning, physical mapping and expression of chromosomal genes specifying degradation of the herbicide 2,4,5-T by *Pseudomonas cepacia* AC1100. *Gene* **71**, 267–277.

SONDOSSI, M., SYLVESTRE, M., AHMAD, D. & HASSI, R. (1991). Metabolism of hydroxybiphenyl and chloro-hydroxybiphenyl by biphenyl/chlorobiphenyl degrading *Pseudomonas testosteroni* strain B-356. *Journal of Industrial Microbiology* (in the Press).

STAUFFER, C. E. (1975). A linear standard curve for the Folin–Lowry determination of proteins. *Analytical Biochemistry* **69**, 646–648.

SYLVESTRE, M. (1980). Isolation methods for bacterial isolates capable of growth on *p*-chlorobiphenyl. *Applied and Environmental Microbiology* **39**, 1223–1224.

SYLVESTRE, M. & FAUTEUX, J. (1982). A new facultative anaerobe capable of growth on chlorobiphenyls. *Journal of General and Applied Microbiology* **28**, 61–72.

SYLVESTRE, M., MASSÉ, R., MESSIER, F., FAUTEUX, J., BISAILLON, J.-G. & BEAUDET, R. (1982). Bacterial nitration of 4-chlorobiphenyl. *Applied and Environmental Microbiology* **44**, 871–877.

SYLVESTRE, M., MASSÉ, R., AYOTTE, C., MESSIER, F. & FAUTEUX, J. (1985). Total degradation of 4-chlorobiphenyl by a bacterial mixed culture. *Journal of Applied Microbiology and Biotechnology*, **21**, 193–197.

TAKASE, I., OMORI, T. & MINODA, Y. (1986). Microbial degradation products from biphenyl-related compounds. *Agricultural and Biological Chemistry* **50**, 681–686.

YAGI, O. & SUDO, R. (1980). Degradation of polychlorinated biphenyls by microorganisms. *Water Pollution Control Federation* **52**, 1035–1043.

YANISCH-PERRON, C., VIEIRA, J. & MESSING, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences and pUC19 vectors. *Gene* **33**, 103–119.