APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1994, p. 691–696
0099-2240/94/$04.00+0

Vol. 60, No. 2

Recombination of the *bph* (Biphenyl) Catabolic Genes from Plasmid pWW100 and Their Deletion during Growth on Benzoate

GARETH LLOYD-JONES,[1] CAROLINE DE JONG,[2] RICHARD C. OGDEN,[1]
WOUTER A. DUETZ,[2] AND PETER A. WILLIAMS[1]*

School of Biological Sciences, University of Wales, Bangor, Gwynedd LL57 2UW, United Kingdom,[1] and Laboratory for Waste Materials and Emissions (LAE), National Institute of Public Health and Environmental Protection (RIVM), Bilthoven, The Netherlands[2]

Received 16 August 1993/Accepted 24 November 1993

***Pseudomonas* sp. strain CB406 was isolated from polychlorinated biphenyl-contaminated soil and harbors a nontransmissible plasmid, pWW100, of approximately 200 kb which carries the genes required for biphenyl and 4-chlorobiphenyl catabolism. The catabolic phenotype was mobilized following the construction in vivo of a cointegrate plasmid containing functional upper and lower biphenyl operons inserted into the broad-host-range R plasmid RP4. The Bph$^+$ phenotype carried by pWW100 was stable in nonselective media but was unstable during growth on benzoate, where the sequential selection of two species of *bph* deletion derivatives occurs at high frequency. This mirrors observations made with TOL plasmids (encoding toluene and xylene catabolism) grown under similar conditions. Subcloning of dioxygenase genes involved in biphenyl catabolism confirmed the localization of the *bph* genes on the wild-type plasmid and the RP4 cointegrate plasmid.**

Genes encoding the aerobic microbial catabolism of biphenyl and polychlorinated biphenyls (PCBs) are widespread on bacterial chromosomes (12, 13, 23), plasmids (11, 14), and transposons (30). The obvious similarities between many of the *bph* genes studied by DNA hybridization (1, 12, 27, 40) and nucleotide sequencing (8, 10, 18, 33) suggest a common ancestry and the possibility that recombination or transposition between strains may be significant in the evolution and spread of biphenyl catabolic operons. The study of specific genetic events, such as in vivo recombination and plasmid segregational instability, which occur within biphenyl-degrading strains should help cast light upon how such catabolic operons behave in the environment and hence how they evolve.

The loss of plasmid-borne catabolic phenotypes probably occurs at a low but detectable frequency and may often be manifested as the spontaneous loss of catabolic abilities observed during laboratory culture (2). Deletion of catabolic plasmid DNA from biphenyl plasmids, which results in the curing of the Bph$^+$ phenotype during growth on nutrient-rich media, has been observed (11). Many TOL plasmids, for example, pWW0 from *Pseudomonas putida* mt-2, undergo spontaneous and irreversible changes during growth on benzoate but not on other carbon sources. These consist of deletions in the wild-type TOL plasmids (termed "benzoate curing") (2) and cause total or partial loss of the plasmid-encoded phenotype (3, 17, 31, 38).

In this paper, we demonstrate that *Pseudomonas* strain CB406 carries a plasmid, pWW100, which is responsible for its ability to utilize biphenyl and which undergoes deletion of its *bph* genes during growth on benzoate but is otherwise stable when grown on either succinate or nutrient-rich growth media. We have adopted the same nomenclature for the later genes of biphenyl catabolism as previously proposed by Carrington et al. (4), that is, those which encode the further catabolism of benzoate have the same letter as the isofunctional *xyl* genes from TOL plasmids (2) but with a *bph* prefix (i.e., *bphXYZ LTEGFJQKIH*). Thus, we have called the catechol 2,3-dioxygenase protein BphE.

* Corresponding author. Mailing address: School of Biological Sciences, University of Wales, Bangor, Gwynedd, LL57 2UW, United Kingdom. Phone: (44) 248 351151. Fax: (44) 248 370731. Electronic mail address: BSS003@BANGOR.AC.UK.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Pseudomonas* strain CB406 was isolated from industrial soil heavily contaminated with PCBs by selective enrichment in batch culture with 4-chlorobiphenyl as the sole carbon source. It can grow on biphenyl, 4-chlorobiphenyl, 2-, 3-, and 4-methylbiphenyls, and benzoate but not on *m*- or *p*-toluates (methylbenzoates). It produces a yellow-green pigment on King's medium B (19) typical of fluorescent *Pseudomonas* species *P. putida* or *P. fluorescens*. Other strains and plasmids used during the course of this work are listed in Table 1.

Conjugation. Attempts to transfer the Bph$^+$ phenotype by conjugation into PaW340 were carried out by standard procedures: (i) incubating donor and recipient cultures together in stationary liquid media, (ii) growing mixed donor and recipient cultures on membranes resting on a nutrient agar surface, and (iii) by cross-streaking donor and recipient cultures directly on agar plates. Incubation temperatures of 30, 20, and 15°C and incubation times from 2 to 24 h were used. After conjugation, the mixtures of donor and recipient were plated on to biphenyl minimal plates containing 200 μg of streptomycin per ml to select for transconjugants and against donor and recipient.

Sequential batch growth on benzoate. Batch growth on benzoate-minimal medium was carried out at 30°C with 50 ml of medium in 250-ml Erlenmeyer flasks shaken at 200 rpm. *Pseudomonas* strain CB406 was grown overnight on biphenyl, and 50 μl of the culture was inoculated into fresh biphenyl medium. At the end of the exponential phase (8 to 12 h), 10 μl of this cell culture was inoculated into a batch culture containing 5 mM benzoate. After the culture reached turbidity (about 10^8 cells per ml), fresh 5 mM benzoate batch cultures were

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics[a]	Source or reference
Bacterial strains		
Pseudomonas sp. CB406	Bph^+ 4Cbph$^+$ Ben^+ $Mtol^-$	This study
P. putida AC34(RP4)	Tc^r Ap^r Km^r	9
P. putida PaW340	Sm^r Trp^- $Mtol^-$	15
P. putida PaW95	Ben^-	16
E. coli XL1-Blue	Tc^r	Stratagene Ltd.
Plasmids		
pWW100	Wild type; Bph^+ Ben^+	This study
pWW100-1	RP4::pWW100; Bph^+ Ben^+ Tc^s Km^r	This study
pWW100-10	Deleted derivative of pWW100; Bph^+ Ben^+ BphC$^+$ BphE$^-$	This study
pWW100-20	Deleted derivative of pWW100; Bph^- Ben^+ BphC$^-$ BphE$^-$	This study
RP4	Tc^r Ap^r Km^r	
pUC18	Ap^r	39
pG6	5.5-kb *Eco*RI fragment of pWW100 in pUC18; Ap^r BphC$^+$	This study
pG7	2-kb *Eco*RI-*Pst*I fragment of pWW100; Ap^r BphE$^+$	This study

[a] Phenotype designations: Bph, biphenyl; 4Cbph, 4-chlorobiphenyl; Ben, benzoate; Mtol, *m*-toluate (3-methylbenzoate).

inoculated with 10 μl from the previous culture before stationary phase was reached.

Growth of *Escherichia coli* containing recombinant plasmids and preparations of cell extracts. *E. coli* XL1-Blue carrying the cloned dioxygenase genes was grown at 37°C in 100 ml of Luria-Bertani medium in 250-ml Erlenmeyer flasks shaken at 200 rpm. Cells were harvested in late logarithmic phase by centrifugation and washed in 50 mM phosphate buffer (pH 7.5), and the cell pellet was resuspended in 50 mM phosphate buffer (pH 7.5) containing 10% acetone (vol/vol) at 0.5 g/ml (wet weight). The cells were disrupted by sonication (three bursts of 30 s separated by 1 min of cooling on ice), and the cell debris were removed by spinning at 50,000 rpm in a TY65 Beckman rotor.

Continuous culture. Chemostat fermenters (100 ml), stirred magnetically at 550 rpm, were fed with basal medium containing the appropriate carbon source and aerated at 100 ml/min. They were supplied through a common inlet; the temperature was maintained at 28 ± 1°C (6, 7). Persistence experiments were carried out under conditions of carbon limitation, with 5 mM benzoate supplied at a dilution rate of $D = 0.05\ h^{-1}$. The inoculum for chemostat experiments was always from batch cultures of biphenyl-grown cells.

Detection of loss of Bph^+. Samples were withdrawn from batch cultures, diluted, and plated onto peptone agar plates (8 g of Bacto Peptone per liter, 4 g of NaCl per liter, 1.5% agar). After 1 to 2 days of incubation at 30°C, colonies were screened for the presence of either or both of the plasmid-encoded catechol 2,3-dioxygenase (BphE) and 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC) activities by spraying the agar plates with sterile solutions of 100 mM catechol. Positive colonies became yellow within 1 min as a result of the formation of the ring-fission product 2-hydroxymuconic semialdehyde, whereas negative colonies remained white. The observation that BphC showed no activity against 4-methylcatechol (see Table 2) was used to determine whether only the BphE activity was lost during growth on benzoate. Colonies which turned yellow after spraying with catechol but which failed to go yellow on spraying with 4-methylcatechol had no BphE (indicative of the lower pathway) but may have retained BphC (indicative of the upper pathway). Representative colonies of each type were screened by replica plating onto biphenyl and succinate minimal agar plates to correlate the yellow and white colonies with the Bph^+ and/or Bph^- phenotypes.

Formation of cointegrate plasmids. The RP4 cointegrates were constructed by transferring RP4 from *P. putida* AC34(RP4) into *Pseudomonas* strain CB406 by conjugation on filters. A Cb^r Tc^r Km^r Bph^+ transconjugant, containing both RP4 and pWW100, was used as a donor in a mating on filters with *P. putida* PaW340 as the recipient; selection was made for Bph^+ Str^r Trp^- transconjugants on minimal plates containing streptomycin at 200 μg/ml and tryptophan, with solid biphenyl in the inverted lid. All transconjugants screened contained single plasmids.

Assays of extradiol dioxygenases. Catechol 2,3-dioxygenase was assayed in 50 mM phosphate buffer (pH 7.5) containing 10% acetone (vol/vol) and 10 mM catechol (25). The A_{375} increase was measured, and reaction rates were calculated by using a molar extinction coefficient of 36,000 $M^{-1}\ cm^{-1}$. For 3-methylcatechol and 4-methylcatechol, the corresponding values used were A_{388} with 15,000 $M^{-1}\ cm^{-1}$ and A_{382} with 31,500 $M^{-1}\ cm^{-1}$, respectively. 2,3-dihydroxybiphenyl 1,2-dioxygenase was assayed by the method of Taira et al. (32) in 50 mM phosphate buffer (pH 7.5) containing 10% acetone (vol/vol) and 100 μM 2,3-dihydroxybiphenyl. The A_{434} increase was measured, and a molar extinction coefficient of 22,000 $M^{-1}\ cm^{-1}$ was used to calculate initial rates. 1,2-Dihydroxynaphthalene dioxygenase was assayed as detailed by Kuhm et al. (20). Protein was determined as described by Lowry et al. (21).

Assay of β-ketoadipate enzymes. Cell extracts were prepared as described above but in 33 mM Tris-HCl, pH 8.0. Catechol 1,2-dioxygenase was assayed by measuring the A_{260} increase in 100 mM Tris-HCl (pH 7.5) containing 20 μM catechol. *cis,cis*-Muconate lactonizing enzyme was determined by measuring the A_{260} decrease in 33 mM Tris-HCl (pH 8.0) containing 100 μM *cis,cis*-muconate and 1 mM $MnCl_2$.

Test for formation of β-ketoadipate. A modification of the method of Rothera (28) was used. Cells were grown overnight in benzoate-minimal medium. The culture was harvested by centrifugation, and the cell pellet was washed and resuspended in 3 ml of 33 mM Tris-HCl buffer (pH 8.0). Subsequently, 2 drops of toluene followed by 0.1 ml of 0.2 M catechol were added. The mixture was incubated with shaking for 1 h at 30°C. The solution was saturated with excess solid $(NH_4)_2SO_4$, and a few drops of concentrated aqueous NH_3 followed by 5 drops of freshly prepared 5% sodium nitroprusside were added. The

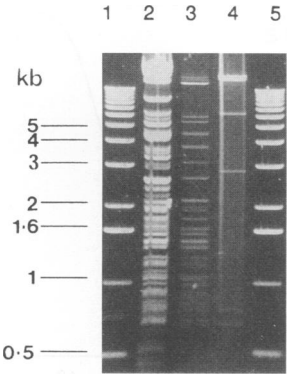

FIG. 1. Agarose electrophoresis of *Pst*I restriction digests of pWW100, pWW100-1, and RP4. Lanes: 1, 1-kb ladder (Gibco-BRL); 2, pWW100; 3, pWW100-1; 4, RP4; 5, 1-kb ladder (Gibco-BRL).

development of a purple color within 5 min was indicative of the presence of β-ketoadipate.

Sources of substrates. 2,3-Dihydroxybiphenyl was obtained from Wako Chemicals (W-4040; Neuss, Germany), 3,4-dihydroxybiphenyl was from Promochem Ltd. (Welwyn Garden City, England), and catechol, 3-methylcatechol, and 4-methylcatechol were from BDH Ltd. (Poole, Dorset, England). Any catechol substrates which were discolored, indicating a degree of oxidation, were purified by sublimation in vacuo at 60 to 70°C to yield pure white crystals. *cis,cis*-Muconate was a gift from L. Nicholas Ornston.

Plasmid and DNA manipulations. Catabolic plasmids were extracted by alkaline lysis-sucrose gradient purification as described by Wheatcroft and Williams (37), and recombinant DNA techniques were carried out by published protocols (29).

RESULTS

The biphenyl catabolic plasmid pWW100. *Pseudomonas* strain CB406 was found to carry a large plasmid of approximately 200 kb, designated pWW100 (Fig. 1). Attempts to transfer pWW100 by direct conjugation into *P. putida* PaW340 selecting for Bph^+ transconjugants by a number of different procedures were unsuccessful. This suggested that the plasmid did not conjugate into that particular recipient at a detectable frequency ($<10^{-8}$) or that it did not encode the Bph^+ phenotype.

Subcloning and analysis of dioxygenase genes. Two separate recombinant plasmids carrying cloned dioxygenase genes, pG6 and pG7, were obtained from digests of pWW100 ligated into pUC18 (Table 1). Southern blotting with these recombinant plasmids as probes revealed that they were present on the wild-type plasmid pWW100 (Fig. 2, lanes 1 and 3).

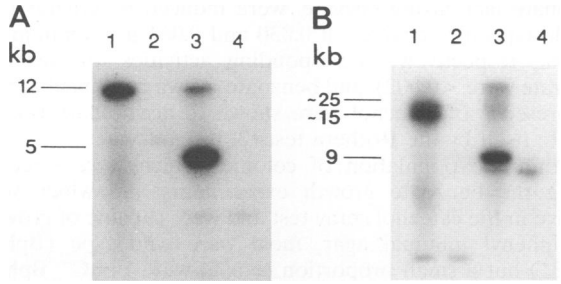

FIG. 2. Southern blot hybridizations of plasmid DNA of pWW100 restricted with *Bam*HI (lanes 1) and *Pst*I (lanes 3); and pWW100-20 restricted with *Bam*HI (lanes 2) and *Pst*I (lanes 4). The ^{32}P-labeled probes for the two blots were the *Eco*RI insert of pG6 (BphC) (A) and the *Eco*RI-*Pst*I insert of pG7 (BphE) (B).

Analysis of the substrate specificities of the two dioxygenases expressed from the plasmids showed that pG6 encoded an enzyme with the substrate specificity characteristic of a 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC), while that encoded by pG7 had a substrate specificity characteristic of a catechol 2,3-dioxygenase (BphE) (Table 2). The BphC expressed by plasmid pG6 showed no activity towards 4-methylcatechol, while the BphE encoded by pG7 was active against this substrate.

Growth of CB406 on biphenyls and benzoate. After growth on biphenyl, benzoate, and acetate, cells of strain CB406 were assayed for *meta* cleavage activity with both catechol and 2,3-dihydroxybiphenyl as substrates (Table 3). The ratio of activities against the two substrates can be taken as a measure of the relative degree of expression of the two dioxygenase activities. During growth on biphenyl, both BphC and BphE are expressed, resulting in a specificity intermediate between those of the two cloned enzymes. In acetate-grown (uninduced) cells, there is a high level of activity but the specificity is much closer to that of BphE, indicating a high constitutive level of this enzyme possibly with a low basal level of the BphC. In benzoate-grown cells there is a specific activity similar to that in uninduced cells but with a higher relative activity against 2,3-dihydroxybiphenyl. This could indicate a low level of induction of BphC activity during growth on benzoate (Table 3).

These results indicate that biphenyl is being metabolized by the pathway originally postulated by Catelani et al. (5), where it is converted to benzoate which is then further metabolized by the *meta* pathway with catechol 2,3-dioxygenase for ring cleavage. This is analogous to the TOL plasmid pathway for toluene catabolism through benzoate and identical to the pathway we have recently described for biphenyl catabolism in a different strain, *Pseudomonas* strain IC, which also contains a large plasmid (pWW110) implicated in biphenyl catabolism (4). The constitutive expression of the BphE activity (and possible also the other *meta* pathway enzymes) in CB406 distinguishes it from the inducible *meta* pathway activities in strain IC.

Strain CB406 grows on 4-chlorobiphenyl, but we have shown

TABLE 2. Substrate specificities of the dioxygenase clones

Substrate	Relative % of sp act[a] in *E. coli* extracts containing:	
	pG6 (BphC)	pG7 (BphE)
Catechol	28 (± 2.4)	100
3-Methylcatechol	57	42 (± 2.1)
4-Methylcatechol	0	90 (± 4.2)
2,3-Dihydroxybiphenyl	100	6.6 (± 0.6)
3,4-Dihydroxybiphenyl	0	0
2,3-Dihydroxynaphthalene	0	0

[a] Substrate specificities of the extradiol dioxygenases encoded by the subcloned dioxygenase gene derived from pWW100. Values are expressed as a relative percentage of specific activities for each extradiol dioxygenase normalized to the substrate showing the highest value (i.e., its natural substrate). Values are the means of four replicates with standard deviations (in parentheses). The actual specific activities were as follows: pG6, 230 ± 5 mmol/min/mg of protein (on 2,3-dihydroxybiphenyl); pG7, 18.0 ± 0.5 mmol/min/mg of protein (on catechol).

TABLE 3. Extradiol dioxygenase activities present in *Pseudomonas* strains CB406 and PaW340(pWW100-1)

Strain	Growth substrate	Sp act (μmol/min/mg of protein) in cell extracts[a]	
		Catechol	2,3-Dihydroxybiphenyl
CB406	Biphenyl	221 ± 27	360 ± 25
	Benzoate[b]	104 ± 49	98 ± 7
	Acetate	188 ± 14	24 ± 13
PaW340(pWW100-1)	Biphenyl	192 ± 14	290 ± 14
	Benzoate[b]	162	86 ± 8
	Acetate	156 ± 2	40 ± 12

[a] Extradiol dioxygenase activities ± standard deviations present in *Pseudomonas* sp. strains CB406 and PaW340(pWW100-1) grown at the expense of different carbon sources supplied at 10 mM.

[b] The absence of any significant level of plasmid deletion during growth on benzoate was avoided by using a substantial inoculum of cells for the batch culture and was confirmed after growth by screening the cells by plating dilutions of the cultures out onto peptone plates and spraying them with catechol to confirm the presence of BphC and BphE.

that it accumulates 4-chlorobenzoate stoichiometrically in the culture medium and therefore has no pathway for mineralization of chloro-substituted aromatics and merely utilizes the products from the fission of the unsubstituted first ring of the chlorobiphenyl as the carbon and energy source. We were unable to detect the release of any chloride ions during growth on 4-chlorobiphenyl.

Formation of RP4 cointegrate plasmids. The *Inc*P1 plasmid RP4 (Cb^r Tc^r Km^r) was introduced into *Pseudomonas* strain CB406 to mobilize pWW100 into a recipient strain, PaW340. All Bph^+ transconjugants obtained from CB406(pWW100, RP4) had the phenotype Km^r Tc^s Bph^+ and contained a conjugative plasmid, designated pWW100-1, in which a 50- to 60-kb fragment from pWW100 had been inserted into RP4 (Fig. 1). The insertional inactivation of the tetracycline resistance determinant suggests that insertion of the *bph* genes is within this coding region of the RP4 plasmid. The catabolic transposon Tn*4371* which encodes only *bphABCD* in *Alcaligenes eutrophus* A5 appears to be inserted into either the tetracycline or kanamycin resistance genes of RP4 (30).

Conjugation of pWW100-1 into *P. putida* PaW95, a mutant strain unable to grow on benzoate, restored this ability, thus confirming that a gene (or genes) required for benzoate utilization were also present on the cointegrate.

Extradiol dioxygenase activities in cell extracts from PaW340(pWW100-1) after growth on biphenyl, benzoate, and acetate showed an identical pattern to that found in wild-type CB406, suggesting that the entire structural and regulatory genes for biphenyl catabolism had recombined with RP4 (Table 3).

Loss of *bph* genes during growth on benzoate. When grown at the expense of benzoate, either in batch or chemostat culture, there was a gradual decrease in the fraction of the population capable of growth on biphenyl and expressing genes essential for biphenyl and benzoate catabolism via the *meta*-cleavage pathway (Fig. 3). Analysis of the population showed that the proportion of Bph^- segregants increased only when growing on benzoate. Parallel experiments in the chemostat with succinate as the limiting carbon source over a period of 2 months did not result in any Bph^- segregants.

The culture was screened during growth on benzoate by spreading dilutions on to peptone agar plates. After incubation to allow single colonies to grow, the plates were sprayed with

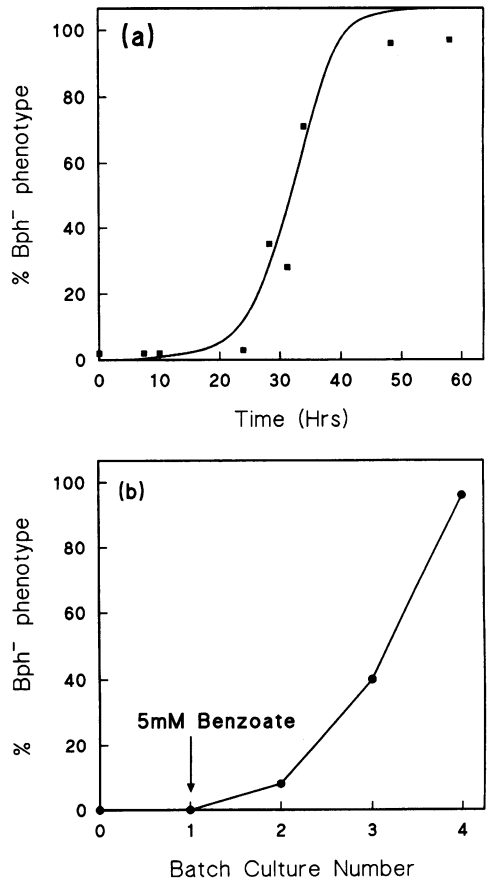

FIG. 3. Persistence of pWW100 in *Pseudomonas* strain CB406 grown in chemostat culture under benzoate-limitation at a dilution rate of 0.05 h^{-1} (a) or grown on 5 mM benzoate in sequential batch culture (b).

catechol to identify clones which had lost both dioxygenase activities BphC and BphE. Screening of the white colonies for the ability to utilize biphenyl revealed a 100% correlation between the loss of the two dioxygenases and the Bph^+ phenotype. A typical plasmid found in such a strain was pWW100-20 (Table 1). CB406(pWW100-20) was grown on benzoate and assayed for catabolic enzymes. In contrast to benzoate-grown wild-type CB406 (Table 3), no *meta*-cleavage pathway enzymes could be detected but activities of two key β-ketoadipate pathway enzymes, catechol 1,2-dioxygenase and muconate lactonizing enzyme, were induced by greater than 15-fold (specific activities of 0.030 and 0.064 μmol/min/mg of protein, respectively; corresponding activities on succinate substrate were <0.002), and benzoate-grown cells incubated in the presence of catechol were shown to accumulate β-ketoadipate itself by the Rothera test (28).

Within the population of colonies which were screened during the benzoate growth experiments and which were positive in the catechol spray test and were capable of growing on biphenyl minimal agar, most were wild type ($BphC^+$ $BphE^+$) but a small proportion (≤6%) were $BphC^+$ $BphE^-$ (typified by plasmid pWW100-10; Table 1). These were identified by spraying replicated colonies with 4-methylcatechol, which BphC does not attack (Table 2), and the colonies therefore remain white. As these strains were still Bph^+, it was assumed that the benzoate formed from the biphenyl was

assimilated by the alternative β-ketoadipate pathway. This was more difficult to prove than for CB406(pWW100-20) (see the results discussed above) because the strain retains an extradiol dioxygenase (BphC) which affects both the assays of catechol 1,2-oxygenase and the Rothera test. However, by treating benzoate-grown cells with H_2O_2 to destroy the BphC activity and then incubating them with catechol we were able to obtain a weak positive Rothera test indicating some formation of β-ketoadipate.

By the end of the batch growth on benzoate, all the colonies were Bph^- and had lost both dioxygenase activities. No segregants which were $BphC^-$ $BphE^+$ were detected at any stage.

Restriction digests and hybridizations of plasmid preparations of both types of segregant showed that the wild-type plasmid pWW100 had undergone deletions of a substantial amount of DNA. All the $BphC^-$ $BphE^-$ strains examined appeared to have plasmids with digests indistinguishable from pWW100-20, suggesting that all have the same or very similar deletions. No plasmid-free segregants with this phenotype were detected in any of our experiments. We confirmed the loss of both *bphC* and *bphE* genes from pWW100-20 by hybridization of Southern blots of digests with the two cloned dioxygenase genes as probes (Fig. 2, lanes 2 and 4).

DISCUSSION

There is strong evidence presented that the *bph* genes of strain CB406 are encoded on the large plasmid pWW100. Although the plasmid itself cannot be transferred by conjugation at a detectable frequency, a cointegrate between pWW100 and RP4 capable of conjugational transfer of the *bph* genes, which are similarly expressed in transconjugants carrying the cointegrate as in the wild-type, can be formed. Additionally, Bph^- segregants can be selected during growth on benzoate and the loss of the growth phenotype correlates with a deletion in the plasmid. Furthermore, two essential catabolic genes have been cloned from pWW100 preparations and their use as hybridization probes on the wild-type plasmid, the RP4 cointegrate, and the deleted derivatives definitively links their presence with the Bph^+ phenotype.

Catabolic transposons have now been demonstrated to be involved in the catabolism of a number of aromatic compounds, including toluene and xylenes (35), naphthalene (34), haloaromatics (24, 36), and PCBs (30). It is possible that a transposable element bearing the genes required for biphenyl and benzoate catabolism is located on pWW100. Recombination of catabolic genes occurred during the formation of the RP4 cointegrate, pWW100-1, in a manner analogous to that observed by Springael et al. (30) with *bph* catabolic transposon Tn*4371*. The possible transposable element from pWW100, however, appears to carry the genes for the further catabolism of benzoate by the *meta*-cleavage pathway in addition to the early genes for catabolism of biphenyl. This is shown by the similarity in the dioxygenase assays of cells carrying the wild-type plasmid and the cointegrate grown under different induction conditions.

The reproducible deletion of the *bph* genes from pWW100 may be related to transposition. The elimination of phenotypes carried on transposons often results from recombination between direct-repeated flanking sequences, with the accompanying loss of any encoded phenotype, as occurs with the chlorobenzoate catabolic transposon Tn*5721* (24). However, the deletion of the *xyl* genes from TOL plasmid pWW0 on a 39-kbp fragment of DNA (22, 38) is due to recombination between two direct 1,275-bp repeats (unpublished data) which are unrelated to and have no functional role in the two larger transposons on the plasmid (35).

Like the TOL plasmids (3, 7, 31, 38), the deletion of the catabolic genes from plasmid pWW100 is selected for during growth on benzoate. The reason appears to be the same. Both toluene and biphenyl have benzoate as a metabolite, and benzoate can be catabolized by way of either the *meta*-cleavage pathway or the alternative β-ketoadipate pathway which appears to be chromosomally encoded in almost all fluorescent *Pseudomonas* strains. In the case of the TOL plasmids, it has been argued that growth by the latter route can only occur when the plasmid-encoded *xyl* genes are no longer functional in the host strain and this loss of function occurs most frequently either by deletion or plasmid loss: spontaneous segregants thus produced can now utilize the β-ketoadipate pathway, outgrow the wild type, and take over the population (38). In the case of biphenyl plasmid pWW100, the situation exactly parallels what happens with TOL plasmids. The Bph^- segregants which take over the benzoate cultures have lost both of the dioxygenases necessary for biphenyl utilization and metabolize the benzoate by the β-ketoadipate pathway. Mathematical analysis of the loss of the Bph^+ phenotype during chemostat growth on benzoate by methods previously used to describe TOL plasmid loss (7) shows that the growth of the Bph^- segregants on benzoate is around 10-fold faster than that of the wild-type.

The transient appearance during growth on benzoate of $BphC^+$ $BphE^-$ segregants shows that at least two different deletions can occur. However, such strains must be outgrown by the $BphC^-$ $BphE^-$ segregants rather than undergo a second sequential deletion, since their phenotype is stably maintained when grown in axenic benzoate culture. A similar transient intermediate phenotype has been reported during benzoate growth of *P. putida* MT53 which carries a TOL plasmid pWW53 (26).

The constitutive expression of BphE and presumably the other *meta* pathway enzymes is unusual and poses an interesting question. CB406 does not grow upon the methylbenzoates, (*m*- or *p*-toluates), as might be expected from the usual tolerance of *meta* pathways for small alkyl substituents on the aromatic ring. The reason for the metabolic block cannot be the inability of the toluates to act as inducers, since the enzymes, as evidenced by BphE, appear to be constitutive. This and other biochemical and structural aspects of the plasmid are being further investigated.

ACKNOWLEDGMENTS

We thank Monsanto plc, Newport, Gwent, Wales, for providing PCB-contaminated soils and analysis of chlorobiphenyl degradation.

This work was supported by the EC STEP program (grant CT90-0102).

REFERENCES

1. **Ahmed, D., R. Masse, and M. Sylvestre.** 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.
2. **Assinder, S. J., and P. A. Williams.** 1990. The TOL plasmids: determinants of the catabolism of toluene and the xylenes. Adv. Microb. Physiol. **31:**1–69.
3. **Bayley, S. A., C. J. Duggleby, M. J. Worsey, P. A. Williams, K. G. Hardy, and P. Broda.** 1977. Two modes of loss of the TOL function from *Pseudomonas putida* mt-2. Mol. Gen. Genet. **154:** 203–204.
4. **Carrington, B., A. Lowe, and P. A. Williams.** The lower pathway operon for benzoate catabolism in biphenyl-utilising *Pseudomonas* sp. strain IC and the nucleotide sequence of the *bphE* gene for

catechol 2,3-dioxygenase. Microbiology, in press.
5. **Catelani, D., C. Sorlini, and V. Treccani.** 1971. The metabolism of biphenyl by *Pseudomonas putida*. Experientia **27:**1173–1174.
6. **Duetz, W. A., and J. G. van Andel.** 1991. Stability of TOL plasmid pWW0 in *Pseudomonas putida* mt-2 under non-selective conditions in continuous culture. J. Gen. Microbiol. **137:**1369–1374.
7. **Duetz, W. A., M. K. Winson, J. G. van Andel, and P. A. Williams.** 1991. Mathematical analysis of catabolic function loss in a population of *Pseudomonas putida* mt-2 during non-limited growth on benzoate. J. Gen. Microbiol. **137:**1363–1368.
8. **Erickson, B. D., and F. J. Mondello.** 1992. Nucleotide sequencing and transcriptional mapping of the genes encoding biphenyl dioxygenase, a multicomponent polychlorinated-biphenyl-degrading enzyme in *Pseudomonas* strain LB400. J. Bacteriol. **174:**2903–2912.
9. **Franklin, F. C. H., and P. A. Williams.** 1980. Construction of a partial diploid for the degradative pathway encoded by the TOL plasmid (pWW0) from *Pseudomonas putida* mt-2: evidence for the positive nature of the regulation by the *xylR* gene. Mol. Gen. Genet. **177:**321–328.
10. **Furukawa, K., N. Arimura, and T. Miyazaki.** 1987. Nucleotide sequence of the 2,3-dihydroxybiphenyl dioxygenase of *Pseudomonas pseudoalcaligenes*. J. Bacteriol. **169:**427–429.
11. **Furukawa, K., and A. M. Chakrabarty.** 1982. Involvement of plasmids in the total degradation of chlorinated biphenyls. Appl. Environ. Microbiol. **44:**619–626.
12. **Furukawa, K., N. Hayase, K. Taira, and N. Tomizuka.** 1989. Molecular relationship of chromosomal genes encoding biphenyl/polychlorinated biphenyl catabolism: some bacteria possess a highly conserved *bph* operon. J. Bacteriol. **171:**5467–5472.
13. **Furukawa, K., and T. Miyazaki.** 1986. Cloning of a gene cluster encoding biphenyl and chlorobiphenyl degradation in *Pseudomonas pseudoalcaligenes*. J. Bacteriol. **166:**392–398.
14. **Hooper, S. W., T. C. Dockendorff, and G. S. Sayler.** 1989. Characteristics and restriction analysis of the 4-chlorobiphenyl catabolic plasmid pSS50. Appl. Environ. Microbiol. **55:**1286–1288.
15. **Jeenes, D. J., and P. A. Williams.** 1982. Excision and integration of degradative pathway genes from TOL plasmid pWW0. J. Bacteriol. **150:**188–194.
16. **Keil, H., S. Keil, R. W. Pickup, and P. A. Williams.** 1985. Evolutionary conservation of genes coding for *meta* pathway enzymes within TOL plasmids pWW0 and pWW53. J. Bacteriol. **164:**887–895.
17. **Keshavarz, T., M. D. Lilly, and P. H. Clarke.** 1985. Stability of a catabolic plasmid in continuous culture. J. Gen. Microbiol. **131:**1193–1203.
18. **Kimbara, K., T. Hashimoto, M. Fukuda, T. Koana, M. Takagi, M. Oishi, and K. Yano.** 1989. Cloning and sequencing of two tandem genes involved in the degradation of 2,3-dihydroxybiphenyl to benzoic acid in the polychlorinated biphenyl-degrading soil bacterium *Pseudomonas* sp. strain KKS102. J. Bacteriol. **171:**2740–2747.
19. **King, E. O., M. K. Ward, and D. E. Raney.** 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. **44:**301–307.
20. **Kuhm, A. E., A. Stolz, K.-L. Ngai, and H.-J. Knackmuss.** 1991. Purification and characterization of a 1,2-dihydroxynaphthalene dioxygenase from a bacterium that degrades naphthalenesulfonic acids. J. Bacteriol. **173:**3795–3802.
21. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
22. **Meulien, P., R. G. Downing, and P. Broda.** 1981. Excision of the 40 kb segment from the TOL plasmid from *Pseudomonas putida* mt-2 involves direct repeats. Mol. Gen. Genet. **184:**97–101.
23. **Mondello, F. J.** 1989. Cloning and expression in *Escherichia coli* of *Pseudomonas* strain LB400 genes encoding polychlorinated biphenyl degradation. J. Bacteriol. **171:**1725–1732.
24. **Nakatsu, C., N. G. James, R. Singh, N. Straus, and C. Wyndham.** 1991. Chlorobenzoate catabolic transposon Tn*5721* is a composite class I element with flanking class II insertion sequences. Proc. Natl. Acad. Sci. USA **88:**8312–8316.
25. **Nozaki, M.** 1970. Metapyrocatechase (*Pseudomonas*). Methods Enzymol. **17A:**522–525.
26. **Osborne, D. J., R. W. Pickup, and P. A. Williams.** 1988. The presence of two complete homologous *meta* pathway operons on TOL plasmid pWW53. J. Gen. Microbiol. **134:**2965–2975.
27. **Pettigrew, C. A., and G. S. Sayler.** 1986. The use of DNA:DNA colony hybridization in the rapid isolation of 4-chlorobiphenyl degradative bacterial phenotypes. J. Microbiol. Methods **5:**205–213.
28. **Rothera, A. C. H.** 1908. Note on the sodium nitroprusside reaction for acetone. J. Physiol. **37:**491–494.
29. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, vol. 1–3. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **Springael, D., S. Kreps, and M. Mergeay.** 1993. Identification of a catabolic transposon, Tn*4371*, carrying biphenyl and 4-chlorobiphenyl degradation genes in *Alcaligenes eutrophus* A5. J. Bacteriol. **175:**1674–1681.
31. **Stephens, G. M., and H. Dalton.** 1987. The effect of lipophilic weak acids on the segregational stability of TOL plasmids in *Pseudomonas putida*. J. Gen. Microbiol. **133:**1891–1899.
32. **Taira, K., N. Hayase, N. Arimura, S. Yamashita, T. Miyazaki, and K. Furukawa.** 1988. Cloning and nucleotide sequence of the 2,3-dihydroxybiphenyl dioxygenase gene from the PCB-degrading strain of *Pseudomonas paucimobilis* Q1. Biochemistry **27:**3990–3996.
33. **Taira, K., J. Hirose, S. Hayashida, and K. Furukawa.** 1992. Analysis of Bph operon from the polychlorinated biphenyl-degrading strain of *Pseudomonas pseudoalcaligenes* KF707. J. Biol. Chem. **267:**4844–4853.
34. **Tsuda, M., and T. Iino.** 1990. Naphthalene degrading genes on plasmid NAH7 are on a defective transposon. Mol. Gen. Genet. **223:**33–39.
35. **Tsuda, M., K. I. Minegishi, and T. Iino.** 1989. Toluene transposons Tn*4651* and Tn*4653* are class II transposons. J. Bacteriol. **171:**1386–1393.
36. **van der Meer, J. R., A. J. B. Zehnder, and W. M. de Vos.** 1991. Identification of a novel composite transposable element, Tn*5280*, carrying chlorobenzene dioxygenase genes of *Pseudomonas* sp. strain P51. J. Bacteriol. **173:**7077–7083.
37. **Wheatcroft, R., and P. A. Williams.** 1981. Rapid methods for the study of both stable and unstable plasmids in *Pseudomonas*. J. Gen. Microbiol. **124:**433–437.
38. **Williams, P. A., S. D. Taylor, and L. E. Gibb.** 1988. Loss of the toluene/xylene catabolic genes of TOL plasmid pWW0 during growth of *Pseudomonas putida* on benzoate is due to a selective growth advantage of "cured" segregants. J. Gen. Microbiol. **134:**2039–2048.
39. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of M13mp18 and pUC19 vectors. Gene **33:**103–119.
40. **Yates, J. R., and F. J. Mondello.** 1989. Sequence similarities in the genes encoding polychlorinated biphenyl degradation by *Pseudomonas* strain LB400 and *Alcaligenes eutrophus* H850. J. Bacteriol. **171:**1733–1735.