RP4::Mu3A-mediated *in vivo* cloning and transfer of a chlorobiphenyl catabolic pathway

Dirk Springael,¹ Jasper van Thor,² Heleen Goorissen,²
Annemie Ryngaert,¹ Raymond De Baere,³ Peter Van Hauwe,³
Laetitia C. M. Commandeur,² John R. Parsons,² Rupert De Wachter³
and Max Mergeay¹

Author for correspondence: Max Mergeay. Tel: +32 14 33 51 06. Fax: +32 14 32 03 72.
e-mail: mergeaym@vito.be

¹ Environmental Technology, Flemish Institute for Technological Research (VITO), Boeretang 200, B-2400 Mol, Belgium

² Department of Environmental and Toxicological Chemistry, University of Amsterdam, Nieuwe Achtergracht 166, N-1018 WV Amsterdam, The Netherlands

³ Department of Biochemistry, University of Antwerp (UIA), Universiteitsplein 1, B-2610 Antwerpen, Belgium

Chromosomal DNA fragments encoding the ability to utilize biphenyl as sole carbon source (*Bph*⁺) were mobilized by means of plasmid RP4::Mu3A from strain JB1 (tentatively identified as *Burkholderia* sp.) to *Alcaligenes eutrophus* CH34 at a frequency of 10⁻³ per transferred plasmid. The mobilized DNA integrated into the recipient chromosome or was recovered as catabolic prime plasmids. Three *Bph*⁺ prime plasmids were transferred from *A. eutrophus* to *Escherichia coli* and back to *A. eutrophus* without modification of the phenotype. The transferred *Bph*⁺ DNA segments allowed metabolism of biphenyl, 2-, 3- and 4-chlorobiphenyl, and diphenylmethane. Genes involved in biphenyl degradation were identified on the prime plasmids by DNA–DNA hybridization and by gene cloning. *Bph*⁺ prime plasmids were transferred to *Burkholderia cepacia*, *Pseudomonas aeruginosa*, *Comamonas testosteroni* and *A. eutrophus* and the catabolic genes were expressed in those hosts. Transfer of the plasmid to the 3-chlorobenzoate-degrading bacterium *Pseudomonas* sp. B13 allowed the recipient to mineralize 3-chlorobiphenyl. Other catabolic prime plasmids were obtained from JB1 by selection on *m*-hydroxybenzoate and tyrosine as carbon sources. 16S rRNA sequence data demonstrated that the *in vivo* transfer of *bph* was achieved between bacteria belonging to two different branches of the β -Proteobacteria.

Keywords: chlorobiphenyl catabolism, β -Proteobacteria, *in vivo* cloning and transfer, RP4::Mu3A

INTRODUCTION

Genetic studies of aerobic PCB degradation by bacteria have focused on the cloning and characterization of the degradative pathway genes in a variety of bacteria (Ahmad *et al.*, 1990; Asturias & Timmis, 1993; Furukawa & Miyazaki, 1986; Khan & Walia, 1989; Kimbara *et al.*, 1989; Mondello, 1989; Peloquin & Greer, 1993; Taira *et al.*, 1992). These studies provide a good basis for an evolutionary comparison between the genes involved in

the individual steps of BP degradation in different PCB degraders and between the various patterns of gene organization or clustering observed. Horizontal gene transfer may be suspected as one of the driving forces in rearranging the genes in the various *bph* clusters and in the subsequent adaptation of a microbial community to the availability of new potential substrates such as PCBs.

In most PCB-degrading bacterial strains the PCB catabolic genes are chromosomally located (Ahmad *et al.*, 1990; Asturias & Timmis, 1993; Furukawa *et al.*, 1989; Hayase *et al.*, 1990; Mondello, 1989). There is some evidence that these genes have been transferred between bacteria. Furukawa *et al.* (1989) demonstrated by DNA–DNA hybridization the occurrence of similar *bphABCD* gene cassettes in PCB-degrading bacteria of different origins. Transfer of PCB catabolic genes has been demonstrated between *Rhodococcus globerulus* P6 and *Pseudomonas* sp. HF1

Abbreviations: PCB, polychlorinated biphenyl; BP, biphenyl; CBP, chlorinated biphenyl; 2CBP, 2-chlorobiphenyl; 3CBP, 3-chlorobiphenyl; 4CBP, 4-chlorobiphenyl; 3CBA, 3-chlorobenzoate; 4CBA, 4-chlorobenzoate; 2CBA, 2-chlorobenzoate.

The GenBank accession number for the sequence of 16S rRNA of strain JB1 is X92188.

Table 1. Bacterial strains and plasmids

Strains	Plasmids and relevant markers*	Relevant chromosomal markers*	Reference or source
<i>Burkholderia</i> sp. JB1	—	Bph ⁺ 2Cbp ⁺ 3Cbp ⁺ 4Cbp ⁺ Mtol ⁺ Ptol ⁺ Nah ⁺ Mhb ⁺ Dpm ⁺	Parsons <i>et al.</i> (1988)
AE1185	RP4::Mu3A (Tc ^r Ap ^r Km ^r)	Bph ⁺ 2Cbp ⁺ 3Cbp ⁺ 4Cbp ⁺ Mtol ⁺ Ptol ⁺ Nah ⁺ Mhb ⁺ Dpm ⁺	This study
AE1236	RP4 (Tc ^r Ap ^r Km ^r)	Bph ⁺ 2Cbp ⁺ 3Cbp ⁺ 4Cbp ⁺ Mtol ⁺ Ptol ⁺ Nah ⁺ Mhb ⁺ Dpm ⁺	This study
<i>A. entrophus</i> CH34	pMOL28 (Nic ⁺), pMOL30 (Zin ⁺)	—	Mergeay <i>et al.</i> (1985)
AE53	pMOL28 (Nic ⁺), pMOL30 (Zin ⁺)	<i>lys-53</i>	Sadouk & Mergeay (1993)
AE7	pMOL28 (Nic ⁺), pMOL30 (Zin ⁺)	<i>tyuB7 ntr-7</i>	Sadouk & Mergeay (1993)
AE992	pMOL28 (Nic ⁺), pMOL30 (Zin ⁺), pSSD51 <i>bpbD1</i>	—	D. Springael
<i>E. coli</i> CM214	RP4::Mu3A (Tc ^r Ap ^r Km ^r)	F ⁻ $\Delta(lac-pro)$ <i>thiA recA galE</i>	Lejeune <i>et al.</i> (1983)
CM330	—	<i>met-803 gal hsdR hsdM⁺ supE</i> (BHB2600)	Springael <i>et al.</i> (1993b)
CM844	RP4::Tn4371 (Tc ^r Ap ^r Km ^r Bph ⁺ 4Cbp ⁺)	<i>met-803 gal hsdR hsdM⁺ supE</i> <i>supF</i> (BHB2600)	Springael <i>et al.</i> (1993b)
CM1073	R- <i>bph1</i> (Tc ^r Ap ^r Km ^r Bph ⁺ 4Cbp ⁺ 3Cbp ⁺ 2Cbp ⁺ Dpm ⁺)	<i>met-803 gal hsdR hsdM⁺ supE</i> <i>supF</i> (BHB2600)	This study
CM1074	R- <i>bph2</i> (Tc ^r Ap ^r Km ^r Bph ⁺ 4Cbp ⁺ 3Cbp ⁺ 2Cbp ⁺ Dpm ⁺)	<i>met-803 gal hsdR hsdM⁺ supE</i> <i>supF rpsL⁺</i> (BHB2600)	This study
CM1168	R- <i>bph3</i> (Tc ^r Ap ^r Km ^r Bph ⁺ 4Cbp ⁺ 3Cbp ⁺ 2Cbp ⁺ Dpm ⁺)	<i>met-803 gal hsdR hsdM⁺ supE</i> <i>supF</i> (BHB2600)	This study
CM887	pRK415 (Tc ^r)	S17/1	Keen <i>et al.</i> (1988)
DH10B	—	F ⁻ <i>mcrA</i> $\Delta(mrr-hsdRMS-mcrBC)$ $\phi 80dlacZ\Delta M15$ $\Delta lacX74$ <i>deoR recA1 endA1 araD139</i> Δara , <i>leu7697</i> , <i>galU</i> <i>galK</i> λ^- <i>rpsL nupG</i>	GIBCO BRL
<i>A. entrophus</i> ATCC 17697	—	—	—
<i>A. entrophus</i> H16	pHG1 (Hox ⁺ Cfx ⁺)	—	Steinbüchel <i>et al.</i> (1983)
<i>A. entrophus</i> JMP222	—	—	Don & Pemberton (1981)
<i>B. cepacia</i> JH230	—	2Cba ⁺ 3Cba ⁺ 4Cba ⁺ 3,4Cba ⁺ 2,5Cba ⁺	Havel & Reineke (1991)
<i>B. cepacia</i> ATCC 17816	—	—	—
<i>A. autotrophicum</i> ATCC 29984	—	—	—
<i>C. testosteroni</i> ATCC 11936	—	—	—
<i>R. meliloti</i> B23	—	—	—
<i>P. aeruginosa</i> JB2	—	2Cba ⁺ 3Cba ⁺ 2,3Cba ⁺ 2,5Cba ⁺	Hickey & Focht (1990)
<i>P. aeruginosa</i> 7NSK2	—	—	Höfte <i>et al.</i> (1990)
<i>P. putida</i> PaW1	pWW0 (Tol ⁺ Xyl ⁺)	—	Assinder & Williams (1990)
<i>P. putida</i> ATCC 12633	—	—	—
<i>P. fluorescens</i> XO150	—	—	—
<i>A. calcoaceticus</i> ATCC 10153	—	—	—
<i>X. maltophilia</i> ATCC 13637	—	—	—
<i>Pseudomonas</i> sp. B13	pB13 (3Cba ⁺)	—	Dorn <i>et al.</i> (1974)

* Phenotypes: Bph⁺, 4Cbp⁺, 2Cbp⁺, 3Cbp⁺, Mtol⁺, Ptol⁺, Nah⁺, Mhb⁺, Dpm⁺, 2Cba⁺, 3Cba⁺, 4Cba⁺, 2,5Cba⁺, 3,4Cba⁺, 2,3Cba⁺, Xyl⁺, Tol⁺, abilities to utilize BP, 4CBP, 2CBP, 3CBP, *m*-toluate, *p*-toluate naphthalene, *m*-hydroxybenzoate; diphenylmethane, 2CBA, 3CBA, 4CBA, 2,5-dichlorobenzoate, 3,4-dichlorobenzoate, 2,3-dichlorobenzoate, xylene and toluene as carbon sources, respectively; Zin⁺, Nic⁺, resistance to zinc and nickel; Tc^r, Km^r, Ap^r, resistance to tetracycline, kanamycin and ampicillin, respectively; Hox⁺, ability to oxidize molecular hydrogen; Cfx⁺, ability to fix carbon dioxide autotrophically.

(Adams *et al.*, 1992), between *Arthrobacter* sp. B1Barc and *Pseudomonas aeruginosa* HF1 (Hickey *et al.*, 1992), between *Pseudomonas putida* JHR and *Burkholderia cepacia* JH230 (Havel & Reineke, 1991), and between *P. putida* BN10 and *Pseudomonas* sp. B13 (Mokross *et al.*, 1990), but the genetic mechanisms involved were not reported. Other reports have described the involvement of plasmids in PCB metabolism (Selifonov & Starovoitov, 1991; Carrington *et al.*, 1994; Lloyd-Jones *et al.*, 1994). Recently, we identified and described Tn4371, a PCB catabolic transposable element in *Alcaligenes eutrophus*, which can be transferred by means of broad-host-range plasmids to other bacterial species (Springael *et al.*, 1993a, b).

Plasmids carrying transposons, such as RP4::Mu3A, R18.18 and R68.45, can mobilize large chromosomal DNA fragments and form prime plasmids (Haas & Holloway, 1976; Haas & Reimann, 1989; Lejeune *et al.*, 1983; Van Gijsegem & Toussaint, 1982). So far, this technique has mainly been used to complement mutations in the recipient strain (Haas & Reimann, 1989; Van Gijsegem *et al.*, 1987) and to do chromosomal mapping. Recently, it was used for *in vivo* cloning and transfer of aromatic catabolic genes. Zhang & Holloway (1992) used R68.45 to clone a chromosomal region of *P. aeruginosa* specifying the catechol *ortho*-cleavage pathway by complementation of a *catA* mutant of *P. aeruginosa*. Prime plasmid formation was also shown between plasmid R68.45 and a plasmid-encoded phenol catabolic pathway from *P. putida* (Herrmann *et al.*, 1988).

The aim of this study was to investigate whether chromosomally located catabolic pathways could be transferred by means of prime plasmid formation from a donor bacterium to a recipient, thereby introducing a completely new catabolic phenotype into the recipient strain. PCB catabolic genes have been found to be clustered and as such should be amenable to *in vivo* cloning. The degree of phylogenetic relationship between the donor (strain JB1) and the recipient (*A. eutrophus* strain CH34) was estimated by 16S rRNA sequence analysis.

METHODS

Bacterial strains, plasmids, media and culture conditions.

Strains and plasmids used in this study are listed in Table 1. Strain JB1 was originally isolated from garden soil by its ability to utilize BP, 2CBP, 3CBP and 4CBP as sole sources of carbon and energy (Parsons *et al.*, 1988). The CBPs are metabolized into their corresponding chlorinated benzoic acids following the BP *meta*-cleavage pathway.

Strain JB1 was able to grow on other aromatic compounds including *m*- and *p*-toluate, naphthalene, diphenylmethane, benzoate, and *m*- and *p*-hydroxybenzoate, and cometabolized higher chlorinated PCBs and chlorinated dioxins (Parsons *et al.*, 1988, 1990; Parsons & Storms, 1989). AE1185 is a derivative of strain JB1 into which the IncP1 plasmid RP4::Mu3A was introduced. RP4::Mu3A contains the deleted prophage Mu3A, which acts as a transposon and displays Cma⁺ (chromosome mobilizing activity) and the ability to form plasmid primes

(Lejeune *et al.*, 1983; Van Gijsegem & Toussaint, 1982; Van Gijsegem *et al.*, 1987).

Tris medium (Mergeay *et al.*, 1985) was used as a minimal medium, supplemented with the appropriate carbon source (0.5% glucose or lactate; 3 mM benzoate, *m*- and *p*-toluate or chlorinated benzoates). BP, CBPs and naphthalene were supplied as crystals. Heavy metals (1 mM NiCl₂ and 2 mM ZnSO₄) and the required amino acids were supplemented as described (Mergeay *et al.*, 1985). Chloride-free minimal medium was prepared as described by Dorn *et al.* (1974). LB was used as a rich medium. The antibiotics tetracycline, kanamycin and ampicillin were used at concentrations of 20, 50 and 50 µg ml⁻¹, respectively. All strains were grown at 30 °C.

Matings for plasmid transfer. Plate matings were done as described by Lejeune *et al.* (1983). All strains were grown on LB at 30 °C.

DNA-DNA hybridization. Southern blot hybridization (Dupont, NEN Research Products) was performed at 65 °C according to the recommendations of the manufacturer. The *bphCD* gene probe was a 1.4 kb *Pst*I-*Xba*I DNA fragment containing the complete *bphC* gene and part of the *bphD* gene of the PCB catabolic transposon Tn4371 of *A. eutrophus* A5 (De Wilde *et al.*, 1992; Springael *et al.*, 1993b). The *bphA1* gene probe was a 600 bp DNA fragment containing part of the *bphA1* gene (encoding biphenyl dioxygenase) of *Pseudomonas fluorescens* GS15. This 600 bp DNA fragment was generated by PCR using total DNA of the BP/4CBP-degrading strain *P. fluorescens* GS15 as template. The primers were designed on the basis of the *bphA1* sequence of *Pseudomonas pseudoalcaligenes* KF707 (Taira *et al.*, 1992). The sequences of the PCR primers were 5'-CAATCAAAGAAGTGCAGGGA-3', corresponding to nucleotides 511-530 of the KF707 sequence, and 5'-TGCACGGAATCACCCACTTC-3', corresponding to nucleotides 1153-1134. The PCR reaction was performed in 30 cycles, which consisted of a denaturation step of 1 min at 94 °C, a primer annealing step of 1 min at 60 °C and a primer extension step of 2 min at 70 °C. DNA fragments were purified from agarose using the Gene Clean II purification kit (BIO101). DNA labelling was carried out with a Multiprime labelling reagent kit (Amersham) with ³²P-labelled dCTP.

DNA preparations and gene cloning. Crude preparations of plasmid DNA were obtained as described by Kado & Liu (1989). Plasmid extractions for restriction enzyme digestion were done from *Escherichia coli* as described by Ish-Horowitz & Burke (1981). Total genomic DNA was isolated as described by Bron & Venema (1972). Restriction endonuclease analysis were done according to the manufacturer's recommendations (BRL). Cloning into plasmid vector pRK415 (Keen *et al.*, 1988) was performed by mixing 100 ng agarose-purified DNA fragment, 50 ng alkaline-phosphatase-treated *Pst*I-restricted pRK415 DNA, 1 U ligase enzyme (Pharmacia) and 2 µl 5 × ligase buffer in a total volume of 10 µl, and by incubating the mixture at 16 °C overnight. After phenol extraction and ethanol precipitation, the reaction mixture was dissolved in 2 µl water and electroporated into *E. coli* DH10β. The broad-host-range cosmid vector pLAFR3 was used to construct a gene cosmid library of R-*bph1*. The strategy used for cosmid cloning was as described by Staskavicz *et al.* (1987). The cosmid bank was introduced into *A. eutrophus* CH34 using pRK2013 as a helper plasmid and transconjugants carrying BphABC⁺ recombinant vectors were identified by production of the yellow *meta*-ring cleavage product, 2-hydroxy-6-oxo-phenylhexa-2,4-dienoate, on Tris/gluconate plates supplemented with BP.

Table 2. Transfer frequencies of catabolic pathways from strain JB1 to *A. entrophus* CH34 by means of RP4::Mu3A

Donor (relevant plasmid)	Recipient	Selected marker*	Transfer frequency (no. of transconjugants per transferred RP4::Mu3A or RP4)
AE1185 (RP4::Mu3A)	AE53	Lys ⁺	10 ⁻⁸
AE1236 (RP4)	AE53	Lys ⁺	< 10 ⁻⁹
AE1185 (RP4::Mu3A)	AE53	Mtol ⁺	< 10 ⁻⁹
AE1236 (RP4)	AE53	Mtol ⁺	< 10 ⁻⁹
AE1885 (RP4::Mu3A)	AE53	Bph ⁺	10 ⁻⁸
AE1236 (RP4)	AE53	Bph ⁺	< 10 ⁻⁹
AE1185 (RP4::Mu3A)	AE53	Mhb ⁺	10 ⁻⁹
AE1236 (RP4)	AE53	Mhb ⁺	< 10 ⁻⁹
AE1185 (RP4::Mu3A)	AE7	Tyu ⁺	10 ⁻⁷
AE1236 (RP4)	AE7	Tyu ⁺	< 10 ⁻⁹
AE1185 (RP4::Mu3A)	AE53	Nah ⁺	< 10 ⁻⁹
AE1236 (RP4)	AE53	Nah ⁺	< 10 ⁻⁹

* Phenotypes: Lys⁺, lysine biosynthesis ability; Mtol⁺, Bph⁺, Mhb⁺, Tyu⁺, Nah⁺, abilities to utilize *m*-toluate, biphenyl, *m*-hydroxybenzoate, tyrosine and naphthalene, respectively, as a carbon source.

Chemicals. BP, 3CBA, 4CBA, 2CBA, *m*-toluate, *p*-toluate, *m*-hydroxybenzoate and naphthalene were obtained from Janssen Chimica. 2CBP, 3CBP and 4CBP were obtained from Ventron. 2,3-Dihydroxybiphenyl was obtained from Sopar.

Growth curves and analysis of free chloride ions. Organisms were grown in 20 ml chloride-free minimal medium containing BP or CBPs as the sole carbon source, in 125 ml Wiame shake flasks at 30 °C, and growth was followed by measuring OD₆₆₀. Chloride levels were assayed by the colorimetric method of Bergmann & Sanik (1957). BP and CBPs were added as crystals in amounts corresponding to a concentration of 5 mM.

Phenotypic identification of strain JB1. Strain JB1 was identified using API 20NE and BIOLOG identification kits, extensive auxanography (API50CH, API500A and API50AA), and whole-cell fatty acid analysis (Midi, Inc.)

16S rRNA sequencing and analysis. Total genomic DNA of strain JB1 was digested with *Eco*RI, *Hind*III, *Bam*HI, *Pst*I, *Kpn*I, *Sst*I and *Sal*I and after electrophoresis blotted onto Hybond N (Amersham) membranes. The 16S rRNA gene of strain JB1 was localized on a 3 kb *Pst*I fragment by hybridization with a gene probe containing part of the JB1 16S rRNA gene. The gene probe was obtained on total DNA of strain JB1 by PCR with primers 1 and 16 (Wilmotte *et al.*, 1993). *Pst*I 3 kb fragments of the genomic DNA were cloned by ligation with vector pSK⁺ (Stratagene) and electroporation into *E. coli* DH5 α . Colony hybridization was performed to identify recombinant clones containing the 16S rRNA genes. Afterwards, 16 primers annealing to conserved areas (Wilmotte *et al.*, 1993) and the two M13 primer sites on the vector were used to sequence both strands of the 16S rRNA. The Sequenase kit version 2.0 (USB) and the T7 Polymerase kit (Pharmacia) were used following the manufacturers' instructions. The determined sequence was aligned by means of the program DCSE (De Rijk & De Wachter, 1993) with other known bacterial 16S rRNA sequences according to the primary and secondary structure features (Van de Peer *et al.*, 1994). Dissimilarity values were calculated without taking into account the indels (insertion/deletion events) and

were corrected for multiple mutations according to Jukes & Cantor (1969). All alignment positions were used in the analysis. The database containing the alignment of known bacterial 16S rRNA sequences can be consulted on-line and retrieved via WWW and anonymous ftp (Van de Peer *et al.*, 1996). A dendrogram was constructed from the distance matrix by the neighbour-joining method (Saitou & Nei, 1987), using the software package TREECON version 3.0 (Van de Peer & De Wachter, 1994). The statistical significance of the branches was assessed by bootstrap analysis, involving the construction of 500 re-sampled trees.

RESULTS

RP4::Mu3A-mediated mobilization of chromosomally located aromatic catabolic pathways

A. entrophus CH34 was unable to use BP, *m*-toluate, *m*-hydroxybenzoate or naphthalene as sole carbon sources. Matings were set up to transfer chromosomal DNA segments encoding catabolism of these aromatics, by means of RP4::Mu3A, from JB1 derivative AE1185 to *A. entrophus* AE53, a Lys⁻ derivative of *A. entrophus* CH34. In order to check the possibility of RP4::Mu3A-mediated transfer of chromosomal DNA from AE1185 to CH34, we looked at complementation of *lys-53* in AE53 and *tyuB7* in AE7. AE7 is a CH34 derivative unable to utilize tyrosine as a carbon source. AE1185 was counter-selected in all matings by including nickel or zinc in the selective medium.

Transfer of the ability to utilize BP (Bph⁺) and *m*-hydroxybenzoate (Mhb⁺) as a carbon source from strain AE1185 to *A. entrophus* AE53 occurred at frequencies of 10⁻⁸ and 10⁻⁹ per transferred plasmid, respectively (Table 2). No transconjugants able to grow on *m*-toluate (Mtol⁺) or naphthalene (Nah⁺) were obtained. Mutations *lys-53*

Fig. 1. (a) Plasmid patterns of Bph⁺ AE53 transconjugants. Lanes: 1, AE1185 carrying RP4::Mu3A (68 kb); 2–6, Bph⁺ AE53 transconjugants. (b) Plasmid patterns of Mhb⁺ AE53 transconjugants. Lanes: 1–3, Mhb⁺ AE53 transconjugants; 4, AE1185 carrying RP4::Mu3A; 5, AE53 carrying pMOL28 (168 kb) and pMOL30 (240 kb). (c) Plasmid patterns of Tyu⁺ AE7 transconjugants. Lanes: 1–3, Tyu⁺ AE7 transconjugants; 4, AE1185 carrying RP4::Mu3A; 5, AE7 carrying pMOL28 (168 kb) and pMOL30 (240 kb).

Fig. 2. (a) *Pst*I restriction patterns of Bph⁺ prime plasmids R-*bph*1, R-*bph*2 and R-*bph*3. (b–d) Southern blot hybridization using the following probes: (b) a 3.8 kb DNA fragment (see thick arrow in a) common to all Bph⁺ prime plasmids; (c) the *bphA1* gene of *P. fluorescens* GS15; (d) the *bphCD* gene cluster of *A. eutrophus* A5. Lanes: 1, RP4::Tn4371; 2, R-*bph*1; 3, R-*bph*2; 4, R-*bph*3; 5, RP4::Mu3A; 6, total genomic DNA of *A. denitrificans* JB1; 7, *Hind*III-digested λ . The thin arrow in (a) indicates the *Pst*I–*Pst*I fragment of Mu3A duplicated in the prime plasmids.

and *tyuB7* were both complemented at a frequency of 10^{-8} , the latter complementation being a third case of transfer of catabolic genes between strain JB1 and *A. eutrophus* CH34. No transconjugants were obtained in the absence

of the RP4::Mu3A plasmid or using RP4 as a mobilizing plasmid, which suggested the involvement of Mu3A in the mobilization of chromosomal DNA. Nineteen Bph⁺ *lys-53* transconjugants were retained and were designated

Fig. 3. For legend see facing page.

AE1216 to AE1235. The three Mhb⁺ *lys-53* transconjugants were designated AE1236, AE1237 and AE1238.

Identification and stability of Bph⁺ prime plasmids

Plasmid extraction from the Bph⁺ transconjugants demonstrated the presence of various rearranged RP4::Mu3A plasmids. Examples of RP4::Mu3A plasmids with insert are shown in Fig. 1(a), lanes 2, 3, 4 and 6. Other transconjugants had no insert or demonstrated deletions (lane 5). From the three Bph⁺ transconjugants carrying enlarged RP4::Mu3A plasmids shown in lanes 2, 3 and 4, the plasmids were transferred to *E. coli*, selecting for the antibiotic resistance markers, and then back to *A. eutrophus* with 100% co-transfer of the catabolic phenotype. This demonstrated the presence of plasmid primes containing the *bph* catabolic marker. The plasmid primes were designated R-*bph1*, R-*bph2* and R-*bph3*. Analysis of Bph⁺ AE53 transconjugants carrying RP4::Mu3A without inserts or with deletions demonstrated that in some cases the Tra functions were lost from the plasmids, as they were not able to transfer either the antibiotic resistance or the catabolic markers. From other transconjugant strains, transfer of RP4::Mu3A resistance markers occurred at a frequency of 10⁻² per recipient, whereas transfer of Bph⁺ occurred at a frequency of 10⁻⁷, suggesting that the Bph⁺ determinant had integrated in the chromosome and could be further mobilized by means of RP4::Mu3A.

Similar observations were found with the Mhb⁺ AE53 transconjugants (Fig. 1b). Only from the transconjugant carrying an enlarged plasmid (Fig. 1b, lane 2) could the plasmid be transferred to *E. coli*, selecting for the antibiotic resistance marker, and back to *A. eutrophus* with 100% co-transfer of the catabolic marker. The prime plasmid was designated R-*mhb1*.

The Tyu⁺ transconjugants all contained enlarged RP4::Mu3A plasmids. Examples are shown in Fig. 1(c). For all plasmids, the resistance markers and catabolic markers were 100% co-transferred.

Phenotypic analysis of *A. eutrophus* strains carrying Bph⁺ and Mhb⁺ RP4::Mu3A prime plasmids

A. eutrophus AE53 Bph⁺ and Mhb⁺ transconjugants were tested for growth on other aromatic compounds that the parental strain *A. eutrophus* CH34 is unable to utilize as a sole carbon source. Bph⁺ transconjugant strains were able to grow on 2-, 3-, and 4-CBP, and on diphenylmethane. No growth was observed with naphthalene, *m*-toluate, *m*-hydroxybenzoate or gentisate. All Mhb⁺ transconjugant strains also grew on gentisate but not on naphthalene, *m*-toluate, BP, diphenylmethane or CBPs.

Genetic analysis of Bph⁺ prime plasmids

R-*bph1*, R-*bph2* and R-*bph3* were isolated from *E. coli* and digested with *Pst*I (Fig. 2a). This enzyme cuts twice in the Mu3A element of RP4::Mu3A. As such, the presence of a duplicated copy of the Mu3A element in the prime plasmids can easily be visualized after *Pst*I digestion and gel electrophoresis (Van Gijssel *et al.*, 1987). The 2 kb *Pst*I-*Pst*I Mu3A internal fragment clearly showed a brightened band on the gel, demonstrating duplication of the element (Fig. 2a). The R-*bph* plasmids gave several common fragments, showing that in the three cases the same region of the JB1 chromosome had been mobilized with different flanking DNA sequences. The total lengths of the DNA inserts in R-*bph1*, R-*bph2* and R-*bph3* were estimated as 50, 52, and 47 kb, respectively. The JB1 chromosomal origin of the *in vivo* cloned DNA was demonstrated using a 3.8 kb DNA fragment, common to all prime plasmids, as a probe (see Fig. 2b). The *bphA1* and *bphCD* probes revealed on all Bph⁺ prime plasmids a 2.6 and a 4.4 kb fragment, respectively (Fig. 2c, d). The 4.4 kb fragment was cloned in the unique *Pst*I site of pRK415 and electroporated into *E. coli* DH10B. Tc^r colonies carrying the expected insert turned yellow after spraying with 0.1% 2,3-dihydroxybiphenyl/diethyl ether solution, demonstrating expression of the *meta*-cleavage enzyme, the *bphC* gene product. The cloned fragment complemented *bphD1* mutant *A. eutrophus* AE992, showing that both *bphC* and *bphD* were present. Partial *Sau*3A1-digested R-*bph1* was cloned into cosmid pLAFR3 and introduced into *A. eutrophus* CH34. Several clones carrying inserts of around 20 kb, able to convert BP into the yellow *meta*-cleavage product were obtained, showing the presence of *bphABC* genes on the prime plasmid.

Transfer of R-*bph1* into various soil bacteria: construction of a 3-chlorobiphenyl-mineralizing hybrid strain

Prime plasmid R-*bph1* was transferred from *E. coli* CM1073 to various α -, β - and γ -Proteobacteria. Expression of Bph⁺ was observed in the γ -Proteobacteria *Pseudomonas aeruginosa* 7NSK2 and *P. aeruginosa* JB2, in the β -Proteobacteria *Comamonas testosteroni* ATCC 11996, *Burkholderia cepacia* ATCC 17616, *B. cepacia* JH230, *A. eutrophus* ATCC 17697, *A. eutrophus* H16, *A. eutrophus* JMP222 and *A. denitrificans* ATCC 15749, and in *Pseudomonas* sp. B13 (subgroup unknown). Expression was not observed in the γ -Proteobacteria *E. coli*, *Pseudomonas fluorescens* V50, *Acinetobacter calcoaceticus* ATCC 10153, *Xanthomonas maltophilia* ATCC 13637, *Pseudomonas putida* PaW1 and *P. putida* ATCC 12633, in the β -Proteobacterium *Aquaspirillum autotrophicum* ATCC 29984, or in the α -Proteobacterium *Rhizobium meliloti* B23.

Fig. 3. Positions of donor strain JB1 and recipient strain CH34 (both arrowed) in the distance tree of β -Proteobacteria based on 16S rRNA sequence analysis. The γ -Proteobacterium *Pseudomonas putida* IFO14169T was used as outgroup. Bootstrap values are indicated at the corresponding nodes and expressed in %. The distance between two species is obtained by summing the lengths of the connecting horizontal branches using the scale at the top. T denotes a type strain.

Strains *P. aeruginosa* JB2, *B. cepacia* JH230 and *Pseudomonas* sp. B13 are able to utilize chlorinated benzoates, the end-products of CBP metabolism, as carbon sources. The corresponding *Pseudomonas* sp. B13 Bph⁺ transconjugants were able to grow on 5 mM 3CBP with stoichiometric release of chloride ions, demonstrating mineralization of 3CBP (data not shown). *B. cepacia* JH230 and *P. aeruginosa* JB2 Bph⁺ transconjugants on the other hand were able to utilize BP, 3CBP, 4CBP and 2CBP, as well as various mono- and di-chlorinated benzoates, but no release of chloride ions could be detected with the CBPs. A black product accumulated during the growth on 2CBP and 3CBP indicating accumulation of chlorinated catechols or phenylcatechols.

Evolutionary relationship between donor and recipient bacterium

To determine the degree of evolutionary relationship between donor and recipient bacterium, we performed identification tests of strain JB1 and sequenced its 16S rRNA. The recipient strain CH34 has been shown by 16S rRNA sequence analysis to belong to the *A. entrophus* cluster (H. Brim and others, unpublished results). Phenotypic identification methods allocated strain JB1 to the β -Proteobacteria, with tentative identifications as *Alcaligenes xylosoxidans* subsp. *denitrificans* (API20NE) and *Bordetella parapertussis* (BIOLOG). The auxanography pattern on the other hand did not conform to these descriptions. The fatty acid profile gave *Burkholderia cepacia* as a possible identification, although with a low score. Analysis of the 16S rRNA sequence demonstrated that strain JB1 is closer to *B. cepacia* than to the *Alcaligenes* lineage. The 16S rRNA gene of strain JB1 has a length of 1588 bp. Fig. 3 shows the distance tree constructed from all available 16S rRNA sequences of bacteria belonging to the β -Proteobacteria and includes strain JB1. It shows that strain JB1 belongs to a lineage containing *B. cepacia*, *Burkholderia gladioli*, *Burkholderia mallei* and *Burkholderia andropogonis*. *B. andropogonis* is the most closely related strain with 93.8% 16S rRNA similarity with JB1. The 16S rRNA sequence of *A. entrophus* shows 92.4% similarity with the 16S rRNA sequence of strain JB1. It can be concluded that the RP4::Mu3A-mediated *in vivo* transfer of the *bph*, *mhb* and *tyu* catabolic genes occurred between two different branches of the β -Proteobacteria.

DISCUSSION

This paper describes the *in vivo* cloning of chromosomally borne aromatic catabolic genes enabling the recipient organism to utilize a new carbon source by receiving a new set of catabolic genes. It shows that chromosomally located PCB catabolic pathways as suggested by other authors can be mobilized between bacteria and that broad-host-range plasmids and transposons may mediate transfer. In contrast with the PCB catabolic genes of *A. entrophus* A5, in which the mobilization was achieved by transposition of the catabolic genes contained within a transposon located in the chromosome (Springael *et al.*, 1993b), here, the catabolic genes were recruited due to the

interaction with a transposable element present on the plasmid. Transfer and expression was achieved between bacteria belonging to two different branches of the β -Proteobacteria, as shown by the distance tree based on 16S rRNA sequence analysis.

The transferred JB1 Bph⁺ chromosomal fragment also conferred the ability to catabolize CBPs and diphenylmethane upon *A. entrophus* CH34. In most cases, BP and CBPs are catabolized by the same set of enzymes (Ahmad *et al.*, 1990; Hayase *et al.*, 1990; Mondello, 1989). The hybridization experiments using *bphA1* and *bphC* as probes and the cloning of the *bphABCD* genes from the Bph⁺ prime plasmids indicate that only one set of genes was present in strain JB1. The presence of several different *bphC* genes was demonstrated in *P. putida* OU83 (Khan & Walia, 1989), in *Rhodococcus erythropolis* TA421 (Maeda *et al.*, 1995) and in *Rhodococcus globerulus* P6 (Asturias & Timmis, 1993). Furthermore, PCB-degrading bacteria differ in their ability to utilize diphenylmethane as a carbon source (Furukawa *et al.*, 1989). If the same set of genes is involved in metabolization of both diphenylmethane and the BP compounds, it shows once more the catabolic versatility of the BP catabolic enzymes and the interesting differences in the substrate selectivity patterns between BP catabolic pathways from different PCB degraders (Mondello, 1989; Erickson & Mondello, 1993). The Mhb⁺ prime plasmids also conferred degradation of gentisate upon *A. entrophus* CH34, suggesting that *m*-hydroxybenzoate is degraded via gentisate in strain JB1. A similar pathway is used by *P. putida* and by *Pseudomonas alcaligenes* (Hopper & Taylor, 1975; Poh & Bayly, 1980).

The *in vivo* cloned BP degradation pathway of strain JB1 did not confer the ability to grow on BP upon all bacterial strains into which the pathway was introduced. This could be due to the lack of suitable regulatory elements or other factors required to express the pathway genes in these hosts. Generally, the BP degradation pathway of strain JB1 seems to be well-expressed in bacteria of the β -group of the Proteobacteria. Only *A. autotrophicum* transconjugants did not grow on BP. From the γ -group, only *P. aeruginosa* expressed the pathway. On the other hand, its expression range seems to be larger than that of the BP degradation pathway present on Tn4371, which seemed to be well expressed only in *A. entrophus* and not in other β -Proteobacteria such as *B. cepacia* and *Chromobacterium* (Springael *et al.*, 1993b). Transfer of the Bph⁺ pathway into chlorobenzoate-degrading bacteria did lead to transconjugants able to completely mineralize CBP in *Pseudomonas* sp. B13, but not in *P. aeruginosa* JB2 and *B. cepacia* JH230. The activity of a *meta*-cleavage pathway and a chlorocatechol *ortho*-cleavage pathway in the same organism at the same time may result in toxic effects or repression of certain enzymes (Adams *et al.*, 1992; Sondossi *et al.*, 1992). Pathway assembly needs a synchronized and a regulated expression of both peripheral and lower pathway together in one organism.

Prime plasmid formation may be a mechanism by which catabolic genes can be acquired by plasmids. Although the frequency of gene transfer mediated by this mechanism might be expected to be relatively low in nature, the

process may play a role when conditions are favourable and selection pressure is high. The presence of mobilizing plasmids with broad-host-range transfer functions in natural populations has been described (McPherson & Gealt, 1986; Top *et al.*, 1994). Natural IncP α plasmids such as R68.45 and R18.18 isolated from clinical isolates are able to mobilize chromosomal DNA and to form prime plasmids in different bacterial genera (Haas & Holloway, 1976; Haas & Reimann, 1989). Little is known about the capability of catabolic and other environmental plasmids to transfer, mobilize or recruit genes. Some catabolic plasmids belong to the IncP β group (Burlage *et al.*, 1990), but their Cma⁺ and prime plasmid formation potential has yet to be demonstrated. The IncP-9 TOL plasmid is able to directly mobilize and retromobilize between *P. putida* strains chromosomal markers such as the camphor catabolic operon and a kanamycin resistance gene marker integrated at various locations in the chromosome. The mobilized DNA seems to re-insert into the chromosome of the recipient rather than remaining in the plasmid after conjugation (Maë *et al.*, 1991; Ramos-Gonzalez *et al.*, 1994). Prime plasmid formation would leave the catabolic pathway genes flanked by repeat elements, as is often found with catabolic plasmids (Eaton & Timmis, 1986; Layton *et al.*, 1992; Meulien *et al.*, 1981; Nakatsu *et al.*, 1991; van der Meer *et al.*, 1991). Many of these repeats have been shown to be IS elements, linked together with the catabolic genes to form composite transposons (Nakatsu *et al.*, 1991; van der Meer *et al.*, 1991). In *A. eutrophus* strain NH9, the 3CBA catabolic gene cluster present on an IncP α transmissible plasmid, is flanked by IS21-like sequences (Ogawa & Miyashita, 1995). On R68.45, IS21 is responsible for prime plasmid formation. Wyndham *et al.* (1994) suggested that the 3CBA catabolic plasmid IncP β pBRC60 evolved by prime plasmid formation leaving the catabolic genes flanked by IS1071.

In vivo cloning can be used for the construction of strains for xenobiotic degradation. It can be used for *in vivo* cloning of catabolic pathways from bacteria in which the mobilizing plasmid is able to replicate and whose expression may be expected in the recipient strain. The catabolic prime plasmids can easily be introduced into a variety of relevant bacteria to investigate the expression of the cloned catabolic genes (Haugland *et al.*, 1990; Kolenc *et al.*, 1988). Here, *in vivo* cloning led to new PCB-degrading heavy-metal-resistant bacteria with potential applications in the decontamination of mixed organic/inorganic pollutions (Springael *et al.*, 1993a, 1994) and to a hybrid chlorobiphenyl/chlorobenzoate-mineralizing pathway in strain *Pseudomonas* sp. B13. Moreover, the presence of the catabolic genes on broad-host-range plasmids may be of interest for the expansion of the catabolic versatility of indigenous populations of a polluted site (Barkay *et al.*, 1993; Fulthorpe & Wyndham, 1989, 1992; McClure *et al.*, 1989).

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