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Transfer and expression of PCB-degradative genes into heavy metal resistant *Alcaligenes eutrophus* strains

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Abstract

Sites polluted with organic compounds frequently contain inorganic pollutants such as heavy metals. The latter might inhibit the biodegradation of the organics and impair bioremediation. Chromosomally located polychlorinated biphenyl (PCB) catabolic genes of *Alcaligenes eutrophus* A5, *Achromobacter* sp. LBS1C1 and *Alcaligenes denitrificans* JB1 were introduced into the heavy metal resistant *Alcaligenes eutrophus* strain CH34 and related strains by means of natural conjugation. Mobile elements containing the PCB catabolic genes were transferred from *A. eutrophus* A5 and *Achromobacter* sp. LB51C1 into *A. eutrophus* CH34 after transposition onto their endogenous IncP plasmids pSS50 and pSS60, respectively. The PCB catabolic genes of *A. denitrificans* JB1 were transferred into *A. eutrophus* CH34 by means of RP4::Mu3A mediated prime plasmid formation. The *A. eutrophus* CH34 transconjugant strains expressed both catabolic and metal resistance markers. Such constructs may be useful for the decontamination of sites polluted by both organics and heavy metals.

Introduction

There is a growing interest in the use of selected bacterial strains specialized in degradation of specific hazardous compounds for the treatment of industrial waste and the bioremediation of polluted sites (Finn 1983; Jain & Sayler 1987; Morgan & Watkinson 1989). In many cases, the genes involved in the degradation of the compound by these strains have been characterized even up to the DNA sequence level. Bacteria seem to harbour an enormous potential of genetic tools to build up new catabolic pathways (van der Meer et al. 1992).

The potential bioremediation applications of some of these specialized strains have been tested in laboratory conditions simulating polluted sites. Both successful (Crawford & Mohn 1985; Edgehill & Finn 1983; Golovleva et al. 1988; Havel & Reineke 1992; Havel & Reineke 1993; Hickey et al. 1993; Kilbane et al. 1983; Ramos et al. 1991; Valo & Salkinoja-Salonen 1986; van der Meer et al. 1987) and unsuccessful (Harkness et al. 1993; Brunner et al. 1985; Focht & Brunner 1985; Focht & Shelton 1987) experiments involving inoculation of selected xenobiotic degraders to remove the pollutant have been described. However, most work was done with sterile contaminated material, and/or employing only the one compound, the introduced bacterium is capable of degrading. In reality, polluted sites contain a mixture of different xenobiotics which may interfere with the degradation of the contaminant of interest by the added bacterial strains (Ramos et al. 1991).

Potential co-contaminants are heavy metals. In the United States, 37% of sites polluted with organic compounds also contain inorganic contaminants such as heavy metals (Kovalick et al. 1991). When heavy metals are available to the microbial cells, they may impair the biodegradation of xenobiotic organics. Heavy metals are known to influence organic matter decomposition by the natural bacterial flora (Babich & Stotzky 1983) and are considered as important inhibitors of activated sludge processes (Tyagi et al. 1986). Low metal concentrations may cause a significant inhibition of biodegradation of the herbicide

2,4-dichlorophenoxyacetic acid in soil systems (Said & Lewis 1991). Although some xenobiotic-degrading bacteria carry mercury resistance genes, frequently associated with a catabolic plasmid (Burlage et al. 1990; Chaudry & Huang 1983; Don et al. 1985; Harder & Kunz 1986; Tardiff et al. 1991), resistance to other toxic heavy metals, such as zinc, nickel, cadmium, cobalt, copper or chromium, is usually low. The growth of well-characterized strains specialized in catabolism of xenobiotics such as *Pseudomonas putida* PaW1, *P. putida* AC858, *P. putida* PpG7 and *Alcaligenes sp.* BR60 is totally inhibited at low heavy metal concentrations (Springael et al. 1993a). A commonly used wood preservative containing chromate, copper and arsenate was found to be toxic for a pentachlorophenol-degrading *Flavobacterium* strain in soil slurries (Topp & Hanson 1990). Copper also (1 μM) prevented growth of the carbon tetrachloride degrading *Pseudomonas* sp. strain KC at neutral pH (Criddle et al. 1991). The majority of 100 different naphthalene, phenanthrene, house fuel oil and toluene degrading bacteria, isolated from a coaltar contaminated environment, were sensitive to heavy metals (D. Springael, unpubl.).

Other bacterial strains have been isolated which show high resistance to the heavy metals zinc, copper, nickel, cadmium, mercury, chromium, lead and cobalt. These strains originate from a variety of biotopes strongly contaminated with heavy metals and they share various characteristics (Diels & Mergeay 1990). They belong to the genus *Alcaligenes* and carry large plasmids governing multiple resistance to heavy metals (Diels et al. 1989). These strains are now generally referred to as metallotolerant *Alcaligenes eutrophus* strains (Diels et al. 1989).

The expression of genes involved in the catabolism of xenobiotics in these metal resistant bacteria may be interesting from the point of view of decontamination of sites polluted by both organics and heavy metals. Several strains able to degrade organic xenobiotics also belong to the genus *Alcaligenes* (Miguez et al. 1986; Don et al. 1985; Bedard et al. 1987; Fulthorpe & Wyndham 1989; Shields et al. 1985; Furukawa et al. 1978; Furukawa et al. 1989; Parsons et al. 1988; Schraa et al. 1986). Moreover, the 2,4-D catabolic genes of plasmid pJP4 of *A. eutrophus* JMP134 were expressed very well in the metallotolerant *A. eutrophus* strain CH34, indicating that *A. eutrophus* shows good expression properties for catabolic genes (Friedlich et al. 1983). We introduced PCB catabolic genes into the heavy metal resistant *A. eutrophus* strains by means of natural conjugation in order to construct bacteria capable of degrading PCBs in the presence of various heavy metals. In addition, this approach gave us the opportunity to study the potential of natural transfer of PCB degradative genes between bacteria and their expression in different hosts.

Many xenobiotic degradative pathways are carried on plasmids and/or transposable elements (Sayler et al. 1990; van der Meer et al. 1992). However, few data exist on mobilization of PCB degradative genes. There is some evidence that PCB degradative pathways can be spread among bacteria. Furukawa et al. (1989) demonstrated by DNA-DNA hybridization the occurrence of similar gene cassettes carrying PCB degradative genes in various PCB degraders. Loss of the ability to degrade PCBs by *Rhodococcus globerulus* P6 was associated with small deletions in its plasmid, pKF1, suggesting the plasmid was involved in PCB degradation (Furukawa & Chakrabarty 1982). Further evidence for the pKF1/PCB degradation association was not presented, but recently transfer of the PCB catabolic pathways was demonstrated to *Pseudomonas* sp. CB15 (Adams et al. 1992). On the other hand, genes involved in PCB metabolism in *R. globerulus* P6 were cloned from the chromosome (Asturias & Timmis 1993). Transfer of PCB catabolic pathways was further demonstrated from *P. putida* JHR to *Burkholderia cepacia* JH230 (Havel & Reineke 1991) and from *P. putida* BN10 to *Pseudomonas* sp. B13 (Mokross et al. 1990). The genetic events involved are not known. Conjugative genetic elements associated with PCB degradation were only reported by Selifonov & Starovoitov (1991) who described two conjugative plasmids involved in PCB degradation, pBS241 of *P. putida* BS893 and pBS311 in *P. putida* U83. Here, we review and report recent results on the construction of PCB degrading heavy metal resistant *A. eutrophus* strains.

PCB degrading donor strains

Three different bacterial strains (Table 1) were used as donor strains for the PCB degradative pathway, i.e. *A. eutrophus* A5 (Shields et al. 1985), *Achromobacter* sp. LBS1C1 (Pettigrew et al. 1991; Layton et al. 1992), and *Alcaligenes denitrificans* JB1 (Parsons et al. 1988). All strains utilize biphenyl (BP) and 4-chlorobiphenyl (4CBP) as sole source of carbon and energy and can cometabolize several other chlorinated biphenyls; 4-chlorobenzoate is accumulated from 4CBP. *A. eutrophus* A5 and *A. denitrificans* JB1 were initially chosen

Table 1. PCB degrading bacterial strains used as donors of PCB degradation genes.

Strain	BP/CBP metabolism	End product	Plasmids (size) (Markers)[a]	Maximum tolerated (mM) concentrations of heavy metals	Isolation environment	References
A. eutrophus A5	BP 4CBP	CO_2 4CBA	pSS50 (51 kb) IncP (Mer^+)	Co^{2+} (< 0.1) Zn^{2+} (1.0) CrO_4^{2-} (< 0.5) Ni^{2+} (0.2) Cu^{2+} (0.3) Cd^{2+} (0.2)	PCB contaminated sediment of Fort Loundon Reservoir Lake, Knoxville, Tennessee, USA	Shields et al. 1985 Springael et al. 1993a Layton et al. 1992
Achromobacter sp. LBS1C1	4CBP	Cl^-, 4-hydroxybenzoate	pSS60 (60 kb) IncP1 (Mer^+) (Fcb^+)	Co^{2+} (ND) Zn^{2+} (< 1.0) Ni^{2+} (< 0.8) CrO_4^{2-} (ND) Cu^{2+} (ND) Cd^{2+} (ND)	PCB contaminated lake water of Fort Loundon Reservoir Lake, Knoxville, Tennessee, USA	Pettigrew et al. 1990 Layton et al. 1992 Burlage et al. 1990
A. dentrificans sp. JB1	BP 4CBP 3CBP 2CBP	CO_2 4-chlorobenzoate 3-chlorobenzoate 2-chlorobenzoate	pAVAD1V (2.8 kb) (Ap^r)	Co^{2+} (< 0.1) Zn^{2+} (0.2) CrO_4^{2+} (< 0.5) Ni^{2+} (0.2) Cu^{2+} (< 0.1) Cd^{2+} (0.2)	Non-contaminated garden soil from Amsterdam, The Netherlands	Parsons et al. 1989 Springael et al. 1993a J. van Thor, pers. commun.

[a] Abbreviations: Mer^+, Ap^r: resistance to mercury and ampicillin respectively; Fcb^+: ability to dehalogenate 4CBA; ND: not done.

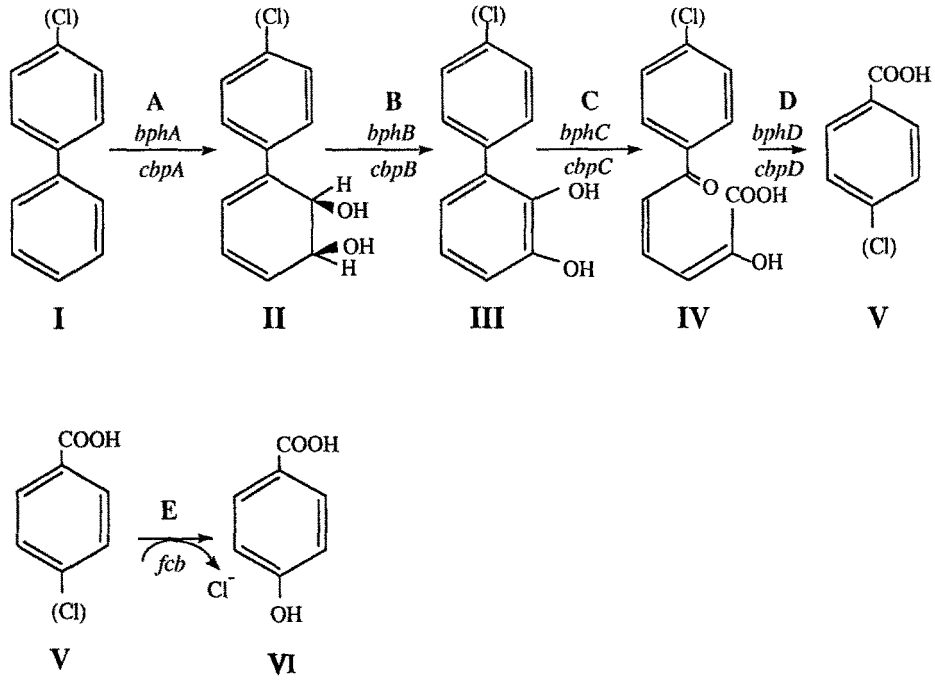

Fig. 1. Compounds: I, 4-chlorobiphenyl (4CBP); II, 2,3-dihydroxy-1-phenyl-cyclohexa-4,6-diene (dihydrodiol); III, 4'-chloro-2,3-dihydroxybiphenyl; IV, 2-hydroxy-6-oxo-6-(4-chlorophenyl)hexa-2,4-dienoate (HOCPDA); V, 4-chlorobenzoate (4CBA); VI, p-hydroxybenzoate. Enzymes: A, 4CBP dioxygenase; B, chlorodihydrodiol dehydrogenase; C, 4'-chloro-2,3-dihydroxybiphenyl dioxygenase; D, HOCPDA hydrolase; E, 4CBA dehalogenase. In most strains biphenyl and 4-chlorobiphenyl are degraded through the same enzymes. The corresponding genes for biphenyl (*bph*), 4-chlorobiphenyl (*cbp*) and 4-chlorobenzoate (*fcb*) degradation are indicated. *bphA* encodes a multicomponent enzyme and consists of at least 4 genes (Taira et al. 1992).

as donor strains because the strains belong to the same genus as the metal resistant recipient strains that were used. Like *A. eutrophus*, *Achromobacter sp.* LBS1C1 belongs to the β-Proteobacteria. In addition, PCB-degradative enzymes in *A. denitrificans* JB1 seem to have an extended substrate range as the bacterium is also able to grow on 3-chlorobiphenyl (3CBP) and 2-chlorobiphenyl (2CBP) without elimination of chloride.

BP and the CBPs are metabolized through the major pathway for BP/CBP degradation (Catelani et al. 1973), which includes *meta*-cleavage of a 2,3-dihydroxybiphenyl compound (Fig. 1). This pathway converts BP to benzoate and the monochlorinated biphenyls to their corresponding monochlorobenzoates. Benzoate is further metabolized via a catechol *ortho*-cleavage pathway in *A. eutrophus* A5 and *Achromobacter sp.* LBS1C1 and via a catechol *meta*-cleavage pathway in *A. denitrificans* JB1. The chlorobenzoic acids are end-products except for *Achromobacter sp.* LBS1C1 which contains a dehalogenase converting 4-chlorobenzoate into *p*-hydroxybenzoate (Layton et al. 1992) (Fig. 2).

In these strains, the PCB-degradative pathway is chromosomally encoded. *A. eutrophus* A5 contains a 51 kb plasmid, pSS50, which is dispensable for PCB degradation in A5 and other strains (Springael et al. 1993a). *Achromobacter sp.* LBS1C1 contains a 60 kb plasmid, pSS60, which carries the 4-chlorobenzoate dehalogenase genes (Layton et al. 1992). pSS50 and pSS60 are very similar except for the presence of the dehalogenase determinant on pSS60, which is not present on pSS50. Both plasmids were assigned to the IncP plasmid incompatibility group. They display a plasmid core with the same genetic structure as described for IncPβ antibiotic resistance plasmids, such as R751, found in clinical isolates. This plasmid group shares a mercury resistance determinant, which is expressed in *Escherichia coli* (Burlage et al. 1990; Springael 1992) and includes also other catabolic plasmids such as pJP4 (Don et al. 1985) and pBRC60 (Burlage et al. 1990). pSS50 and pSS60 are broad host range plasmids and are able to capture mobilizable

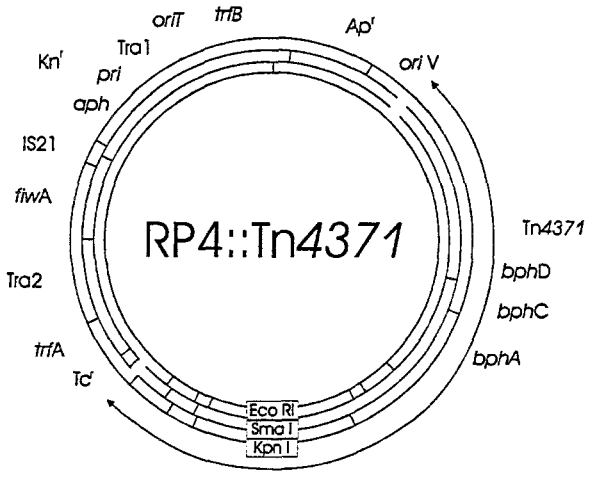

Fig. 2. Physical and genetic map of plasmid RP4::Tn*4371*. The borders of the PCB catabolic genes are indicated. *bph*C-*bph*D were identified by gene cloning, *bph*A was identified by DNA-DNA hybridization using a *bph*A probe generated by the polymerase chain reaction (PCR) based on the published sequence of the *bph*A1 gene of *Pseudomonas pseudoalcaligenes* KF707 (Taira et al. 1992). The outer circle denotes KpnI restriction sites, the centre circle SmaI restriction sites and the inner circle EcoRI restriction sites.

plasmids by retrotransfer. Retrotransfer is the ability of conjugative plasmids, to mobilize genes into the cell containing the conjugative plasmid (Mergeay et al. 1987; Springael et al. 1993a).

Heavy metal resistant it A. eutrophus recipient strains

The two metalloresistant *A. eutrophus* strains CH34 and SV661 were used as recipients for PCB degradative pathways. *A. eutrophus* CH34 is considered as the type strain for a large number of heavy metal resistant *A. eutrophus* isolates from different sites highly polluted with heavy metals (Table 2) (Diels et al. 1989; Diels & Mergeay 1990). These metalloresistant strains may constitute a separate group inside the *A. eutrophus* genus (this group includes also a group of isolates known as CDC group IVc2, K. Kersters, pers. comm.) and may display features such as the ability to grow autotrophically, the presence of large plasmids governing heavy metal resistance (Diels et al. 1990) and a very peculiar property of temperature induced mutagenesis and mortality (Dong et al. 1992; Van der Lelie et al. 1992; Sadouk et Mergeay 1993). Unlike non metal-resistant *A. eutrophus* strains, CH34-like strains are unable to utilize fructose and *m*-hydroxybenzoate (Diels et al. 1989; Springael 1992). Furthermore, chemolithotrophy in *A. eutrophus* CH34 is encoded by chromosomal determinants, whereas chemolithotrophy in non metal-resistant strains is plasmid borne (Mergeay et al. 1985).

A. eutrophus CH34 is the most studied metalloresistant *A. eutrophus* strain from the point of view of genetics, physiology and biotechnology (Mergeay et al. 1985; Mergeay 1990; Sadouk & Mergeay 1993). It was isolated from the sediment of a zinc decantation tank of a zinc factory (Mergeay et al. 1978). The strain carries two megaplasmids, governing multiple heavy metal resistances. Plasmid pMOL28 (165 kb) confers resistance to Co^{2+}, Ni^{2+}, CrO_4^{2-}, Hg^{2+} and Tl^{+}; pMOL30 (240 kb) confers resistance to Cd^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, Hg^{2+} and Tl^{+}. Both plasmids are poorly self-transferable in intra-species matings but can be mobilized by IncP helper plasmids through mercury transposon mediated formation of cointegrates (Mergeay et al. 1985).

Cd^{2+}, Zn^{2+} and Co^{2+} resistance on pMOL30 and Ni^{2+} and Co^{2+} resistance on pMOL28 are encoded by the *czcNICBAD* (Nies et al. 1989; Dong Q, pers. comm.) and *cnrYRHCBA* gene clusters (Liesegang et al. 1993; Siddiqui et al. 1989), respectively. Both resistances involve a cation/proton efflux antiporter system via a protein complex associated with the bacterial membrane (Nies & Silver 1989; Sensfuss & Schlegel 1989). *cnrCBA* and *czcCBA*, encoding the heavy metal resistance structural genes, share a high degree of DNA sequence homology, indicating a common ancestry of the two metal resistant operons (Liesegang et al. 1993). The regulatory genes *czcI/czcD* and *cnrYRH* are completely different (Liesegang et al. 1993; Collard et al. 1993). Chromium resistance on pMOL28 is encoded by the *chr* operon which lies adjacent to the *cnr* operon (Nies A et al. 1990). Mercury resistance is carried on the transposable elements, Tn*4380* in pMOL30 and Tn*4378* in pMOL28, respectively (Diels et al. 1985).

A. eutrophus CH34 appears to be very amenable to genetic analysis and a circular genetic map of the chromosome is available (Sadouk & Mergeay 1993). The strain is a good recipient in intra- and intergeneric matings mediated by plasmid RP4::Mu3A (pULB113) and was found to accept and express foreign genes including catabolic ones, very well (Friedrich et al. 1983; Lejeune et al. 1983; Waelkens et al. 1987). These features together with its high resistance to various

Table 2. Some representative heavy metal resistant strains isolated in our laboratory from sites highly polluted with heavy metals.

Strain	Isolation environment	Plasmid (size) (kb)	Heavy metal resistance[a]	Aromatic carbon source[b]					Reference
				1	2	3	4	5	
A. eutrophus CH34	Zinc factory (Belgium)	pMOL28 (165) pMOL30 (240)	Ni, Co, Hg, Tl, Cr Cd, Zn, Co, Cu, Hg, Tl	+	+	-	-	+	Mergeay et al. 1985
A. eutrophus DS185	Zinc desert (Belgium)	pMOL90 (319) pMOL85 (189) pMOL80 (4)	Zn, Cu Zn, Co, Cd, Cu, Pb	-	+	-	-	-	Diels et al. 1989
A. eutrophus SV661	Zinc factory (Belgium)	pMOL284 (165) pMOL304 (240)	Ni, Co, Hg, Cr Cd, Zn, Co, Cu, Hg	+	+	-	-	+	Diels et al. 1990
A. eutrophus AS2	Mining region (Zaire)	pMOL286 (27) pMOL306 (240)	Ni, Co, Cr, Hg Zn, Cd, Co, Cu, Hg	+	+	-	ND	ND	Diels et al. 1990
A. eutrophus ER121	Zinc factory (Belgium)	pMOL68 (138) pMOL69 (149) pMOL70 (162)	 Cd, Zn, Hg	+	+	+	-	ND	Diels et al. 1990
				ND	+	-	ND	ND	L. Diels, unpubl.
A. eutrophus AB2	Mining region (Zaire)	pMOL299 (190) pMOL230 (320) pMOL231 (5)	 10 mM Tl (chromosome)						
Sphingomonas WS1 janokuyae	Zinc desert (Belgium)	pMOL219 (170) pMOL220 (245)	Zn, Cu, Ni (plasmid?)	ND	ND	ND	ND	ND	L. Diels, unpubl.
Arthrobacter ilicis WS14	Zinc desert (Belgium)	–	Ni, Zn, Cr, Pb	ND	ND	ND	ND	ND	L. Diels, unpubl.
Arthrobacter ilicis RM1	Metal-recycling factory (Austria)	–	Ni, Zn, Co, Cr, Pb	ND	ND	ND	ND	ND	R. Margesin, unpubl.

[a] Heavy metal resistances are plasmid borne except where indicated.

[b] Aromatic carbon sources: 1, benzoate; 2, *p*-hydroxybenzoate; 3, *m*-hydroxybenzoate; 4, salicylate; 5, phenol; ND = not done.

heavy metals, made *A. eutrophus* CH34 very suitable for our purposes.

A. eutrophus SV661 contains two non-selftransferable megaplasmids, pMOL284 (164 kb) and pMOL304 (240 kb), carrying Ni^{2+} and Zn^{2+} resistance, respectively, and showing cross-hybridization with the *cnr* and *czc* operons of pMOL28 and pMOL30 of CH34 (Diels & Mergeay 1990).

Transfer and expression of PCB catabolic genes in the metallo resistant *A. eutrophus* strains

Different matings were set up to transfer BP/4CBP catabolism ($Bph^+/4Cbp^+$) from the PCB degrading donor strains into the metal resistant recipient strains. Selection for the combined phenotype was easily achieved by selection on Tris minimal medium plates (Schlegel et al. 1961) containing 1 mM Ni^{2+} or 2 mM Zn^{2+}, concentrations of heavy metals which inhibit growth of the donor strains, and using BP or 4CBP as sole carbon source (Table 3).

Transfer and expression of the PCB catabolic genes of A. eutrophus A5 and Achromobacter sp. LBS1C1 into metalloresistant A. eutrophus strains: evidence for the existence of PCB catabolic transposons

Transfer of $Bph^+/4Cbp^+$ from *A. eutrophus* A5 into *A. eutrophus* strains CH34 and SV661 occurred at a frequency of 10^{-6} per recipient strain (Table 3), enabling the transconjugants to utilize both compounds as new carbon sources in the presence of various heavy metals and to metabolize 4CBP into 4-chlorobenzoate. Transconjugants carried enlarged pSS50 plasmids, all of the same size. In resting cell assays, AE707 (Table 4), an *A. eutrophus* CH34 $Bph^+/4Cbp^+$ transconjugant degraded several PCBs of Aroclor 1242 in the presence of heavy metals (Table 5) (Springael et al. 1993a).

The nature of the inserted DNA segment was analyzed in more detail. The DNA segment was found to be a 59 kb large transposable element, originating from the chromosome of *A. eutrophus* and was designated as Tn*4371*. The element translocated as a single contiguous piece of DNA between different replicons, chromosome as well as plasmids, and at different locations of the same replicon (Springael et al. 1993b). The PCB degradative genes were mapped in the central part of the transposon by means of sub-cloning and hybridization (Fig. 2).

A *bphC-bphD* gene cluster was cloned (De Wilde et al. 1992) and preliminary DNA sequence data indicate that the *bphC* gene of Tn*4371* is nearly identical at the DNA level to the *bphC* gene of *Pseudomonas* sp. KKS102 (Kimbara et al. 1989). Gene sequences showing cross-hybridization with Tn*4371 bphC* were found in several PCB degradative bacteria of various origin and genera. DNA of other PCB degradative bacteria did not hybridize (De Wilde et al. 1992). It demonstrates that different gene classes of *bph* genes exist. Similar results were obtained using the *bphABC* genes of *P. pseudoalcaligenes* as a probe (Furukawa et al. 1989) and the *bphABCD* gene clusters of *Pseudomonas* sp. LB400 (Yates & Mondello 1989) and *Comamonas testosteroni* B356 A5 probes (Ahmad et al. 1990). *bph* genes are generally found to be clustered (Kimbara et al. 1989; Furukawa et al. 1986; Kahn & Walia 1989; Mondello 1989; Hayase et al. 1990; Ahmad et al. 1990). *bphA* and *bphCD* are also located on the same 10 kb *Eco*RI-*Sma*I fragment of Tn*4371*, and both *bphA* and *bphC* are activated by BP, indicating that the *bph* genes are also clustered and may form one operon.

The introduction of the catabolic genes into several bacterial genera by means of an RP4::Tn*4371* plasmid, conferred the ability to grow on BP on both metal resistant and non-resistant *A. eutrophus* species, but not on other bacteria such as *P. fluorescens* X0150, *P. putida* KT2440, *P. aeruginosa* 7NSK2, a *Chromobacterium sp., Acinetobacter calcoaceticus* ATCC10153 and *Burkholderia cepacia* V250 (Springael et al. 1993b). The expression of the PCB catabolic genes outside *A. eutrophus* and related strains appeared to be very poor. Recently, however, the catabolic genes of Tn*4371* were shown to be expressed in different fluorescent pseudomonads of the natural endogenous population of a soil microcosm (H. de Rore & E. Top, pers. comm.).

The *bphC* gene of Tn*4371* showed also strong cross-hybridization with total genomic DNA of *Achromobacter sp.* strain LBS1C1. The fact that *Achromobacter sp.* LBS1C1 was isolated from the same environment as A5 (Pettigrew et al. 1990) and carries the pSS50 related plasmid, pSS60 (Burlage et al. 1990), prompted us to set up experiments to transfer the PCB degradative genes of LBS1C1 to *A. eutrophus* CH34, using pSS60 as a mobilizing plasmid. $Bph^+/4Cbp^+$ transferred to *A. eutrophus* CH34 at a frequency of 10^{-6} per recipient (Table 3). As with *A. eutrophus* A5 as a donor strain, all transconjugants contained an enlarged pSS60 plasmid, designated as pSSD60. This indicates that *Achromobacter sp.* LBS1C1 carries a PCB mobile element as well which was shown

Table 3. Conjugative transfer of PCB catabolic pathways into heavy metal resistant *A. eutrophus* strains.

Donor (Relevant plasmid/size)	Recipient (Relevant plasmid)	Selection medium	Transfer frequency Number of transconjugants/ Number of recipients	Transfer frequency Number of transconjugants/ Number of transferred IncP plasmid	Size plasmid in transconjugants (kb)
A5 (pSS50/51 kb)	CH34 (pMOL28, pMOL30)	MM, BP, Zn^{2+} 2 mM	10^{-6}	10^{-5}	110
A5 (pSS50/51 kb)	SV661 (pMOL284, pMOL304)	MM, BP, Zn^{2+} 2 mM	10^{-6}	10^{-5}	110
A5 (pSS50/51 kb)	CH34 (pMOL28, pMOL30)	MM, 4CBP, Zn^{2+} 2 mM)	10^{-6}	10^{-5}	110
A5 (RP4/60 kb)	CH34 (pMOL28, pMOL30)	MM, BP, Zn^{2+} 2 mM	10^{-6}	10^{-5}	110
LBS1C1 (pSS60/60 kb)	CH34 (pMOL28, pMOL30)	MM, BP, Ni^{2+} 1 mM	10^{-6}	10^{-5}	119
LBS1C1(pSS60/60 kb)	CH34 (pMOL28, pMOL30)	MM, 4CBP, Ni^{2+} 1 mM	10^{-6}	10^{-5}	119
JB1	CH34 (pMOL28, pMOL30)	MM, BP, Ni^{2+} 1 mM	$\leq 10^{-9}$	–	–
JB1 (RP4/60 kb)	CH34 (pMOL28, pMOL30)	MM, BP, Ni^{2+} 1 mM	$\leq 10^{-9}$	–	–
JB1 (pULB113/68 kb)	CH34 (pMOL28, pMOL30)	MM, BP, Ni^{2+} 1 mM	10^{-9}	10^{-8}	115–170

Abbreviations: MM: minimal medium.

Table 4. Characteristics of representative constructed heavy metal resistant PCB degrading *A. eutrophus* strains.

Strain	Origin	Selected markers[a]	Relevant plasmids	Heavy metal resistance phenotype[a]	Xenobiotic degrading phenotype[a]
A. eutrophus AE707	*A. eutrophus* CH34 × *A. eutrophus* A5	Bph^+ Zin^+	pSSD51[b], pMOL28, pMOL30	Nic^+, Chr^+, Cob^+, Mer^+, Cad^+, Zin^+, Plu^+, Cup^+	Bph^+, $4Cbp^+$
A. eutrophus AE860	*A. eutrophus* SV661 × *A. eutrophus* A5	Bph^+ Zin^+	pSSD54[b], pMOL284, pMOL304	Nic^+, Chr^+, Cob^+, Mer^+, Cad^+, Zin^+, Plu^+, Cup^+	Bph^+, $4Cbp^+$
A. eutrophus AE1459	*A. eutrophus* CH34 × *Achromobacter sp.* LSB1C1	Bph^+ Nic^+ $4Cbp^+$ Nic^+ $4Cba^+$	pSSD60[b], pMOL28, pMOL30	Nic^+, Chr^+, Cob^+, Mer^+, Cad^+, Zin^+, Plu^+, Cup^+	Bph^+, $4Cbp^+$, $4Cba^+$
A. eutrophus AE1216	*A. eutrophus* CH34 × *A. denitrificans* JB1 (RP4::Mu3A)	Bph^+ Nic^+	Bph^+ R prime pMOL28, pMOL30	Nic^+, Chr^+, Cob^+, Mer^+, Cad^+, Zin^+, Plu^+, Cup^+	Bph^+, $4Cbp^+$, $3Cbp^+$, $2Cbp^+$

[a] Abbreviations: Nic^+, Chr^+, Cob^+, Cad^+, Zin^+, Plu^+, Cup^+: Ability to grow in presence of Ni^+ (1 mM), CrO_4^{2+} (0,5 mM), Co^{2+} (0.5 mM); Cd^{2+} (0.8 mM); Zn^{2+} (2 mM); Pb^{2+} (0.5 mM) and Cu^{2+} (0,8 mM) respectively; Bph^+, $4Cbp^+$, $3Cbp^+$, $2Cbp^+$, $4Cba^+$: ability to utilize BP, 4CBP, 3CBP, 2CBP 4CBA, respectively, as a carbon source.

[b] pSSD51, pSSD54 are pSS50::Tn*4371* plasmids; pSSD60 is a pSS60 plasmid carrying the PCB degradation genes of *Achromobacter sp.* LBS1C1.

by restriction enzyme analysis to differ from Tn*4371*. The transconjugants were also able to grow on 4-chlorobenzoate and to mineralize 4CBP with elimination of chloride. Both markers $4Cbp^+$ and Fcb^+ were transferred together with pSSD60 (Springael, unpublished). The combination of a chromosomal 4CBP catabolic pathway with 4CBA dehalogenase genes on the pSS60 plasmid constituted a conjugative plasmid encoding conversion of CBP to a dechlorinated compound which can be utilized as a carbon source by various soil bacteria.

Tn*4371* belongs to a list of other transposons involved in degradation of organic xenobiotics. Tn*4651* (56 kb) and Tn*4653* (70 kb) carry the toluene/xylene catabolic genes of the TOL plasmid, pWWO (Tsuda et al. 1989), and Tn*4655* carries the naphthalene catabolic genes of the NAH7 plasmid (Tsuda & Iino 1990). These three transposons were all classified as class II transposable elements. On the other hand, Tn*5280* involved in chlorobenzene degradation (van der Meer et al. 1991) and Tn*5271* involved in chlorobenzoate degradation (Nakatsu et al. 1991) are class I composite transposable elements and are flanked by IS elements. Recently, another catabolic mobile element carrying genes involved in dehalogenation of dehalogenated alkanoic acids was identified. The corresponding element (DEH) does not appear to be a conventional transposon because inserts in plasmid targets varied in size between 6 and 13 kb (Thomas et al. 1992).

Transfer and expression of the PCB catabolic genes of A. denitrificans JB1 into metallo resistant A. eutrophus strains: selection of prime plasmids as a way to clone PCB chromosomal genes in vivo

No transconjugants were obtained after mating *A. denitrificans* JB1 with *A. eutrophus* CH34 to introduce the PCB catabolic genes into CH34 (Table 3). Therefore, IncP plasmid RP4::Mu3A (pULB113) was introduced into *A. denitrificans* JB1. RP4::Mu3A is an RP4 derivative which contains the effective transposon Mu3A, a mini-Mu derivative of phage Mu, deleted for the lytic phage functions while retaining the sites required for transposition. Due to Mu3A, the plasmid is able to recruit genomic DNA and to form prime plasmids (van Gijsegem & Toussaint 1982). This technique of in vivo cloning has been used mainly to complement auxotrophic mutations in a recipient strain and to do chromosome mapping (Haas & Holloway 1976; Lejeune et al. 1983; Haas & Reimann 1989). Recently, this technique was used for the in vivo cloning of catabolic pathways. Plasmid R68.45 was used to clone a plasmid encoded catabolic pathway for phenol degradation from *P. putida* (Herrmann et al. 1988). Zhang & Holloway (1992) used a R68.45 derivative to complement a *catA* mutant of *P. aeruginosa* PAO. The recruited chromomal DNA segment contained the *catA* gene but also all catabolic genes necessary for benzoate mineralization via catechol ortho-cleavage. PCB catabolic genes have always been found to be clustered and as such should be amenable to in vivo cloning (Ahmad et al. 1990; Furukawa & Miyazaki 1986; Hayase et al. 1990; Khan & Walia 1989; Kimbara et al. 1989; Mondello 1989) (Fig. 3).

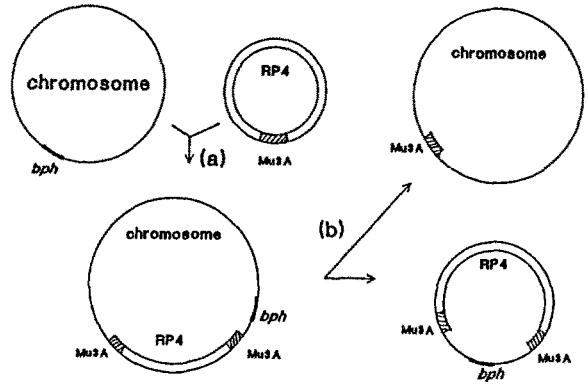

Fig. 3. Model of in vivo cloning and prime plasmid formation of PCB catabolic chromosomal genes using RP4::Mu3A (pULB113). (a) Plasmid RP4::Mu3A fuses with the bacterial chromosome near the *bph* locus; (b) R-prime formation: an RP4 carrying the *bph* locus flanked by two Mu3A elements in the same orientation is formed by deletion mediated by the Mu3A element distal from the *bph* locus on the chromosome.

In our experiments, chromosomal DNA segments encoding the catabolism of BP to BA in *A. denitrificans* JB1 were transferred and expressed into *A. eutrophus* CH34 at a frequency of 10^{-8} by means of RP4::Mu3A mediated prime plasmid formation (Table 3). Transconjugants were able to utilize BP as a new carbon source and harboured prime plasmids with recruited *A. denitrificans* JB1 chromosomal DNA segments ranging in size from 40 to 100 kb (Springael et al. 1992). Many prime plasmids turned out to be unstable. However, some of them could be transferred to a Rec^- *E. coli* strain and back to *A. eutrophus* without deletions or loss of the catabolic phenotype.

Integration of the catabolic genes in the *A. eutrophus* CH34 chromosome also seems to occur. *A. eutrophus* CH34 transconjugants also utilized 4CBP, 2CBP,

Table 5. Cometabolization of di- and tri-chlorinated isomers of Aroclor 1242 by *A. eutrophus* A5 and metal resistant PCB degrading *A. eutrophus* CH34 transconjugant AE707 (Springael et al. 1993a).

Strain	Plasmid	Incubation medium	% degradation[a]				
			diCBPs[b]			triCBPs[b]	
			1	2	3	1	2
A. eutrophus A5	pSS50	MM	92	92	92	29	60
A. eutrophus A5	pSS50	MM + Zn^{2+} (2 mM)	0	12	16	5	1
A. eutrophus A5	pSS50	MM + Ni^{2+} (1 mM)	40	15	17	0	0
A. eutrophus AE707	pSSD51, pMOL28, pMOL30	MM + Zn^{2+} (2 mM)	95	91	93	57	64
A. eutrophus AE707	pSSD51, pMOL28 pMOL30	MM + Ni^{2+} (1 mM)	50	27	31	5	10

Abbreviations: MM: Minimal medium.
[a] Degradation is expressed in % in comparison with CH34 as a control (0%). The indicated CBP isomers of Aroclor 1242 were not identified.
[b] The number indicates unidentified di- and tri-CBPs of Aroclor 1242.

3CBP and methanediphenyl in the presence of heavy metals (Table 4) (Springael et al. 1992). In addition, several more highly chlorinated PCB's and 2-chlorodibenzo(p)dioxine were cometabolized (JR Parsons, pers. comm.). Furukawa et al. (1989) demonstrated degradation of methanediphenyl by bacteria carrying cloned BP degradative genes, suggesting a broad substrate specificity of PCB catabolic genes. The prime catabolic plasmids were transferred with expression of the Bph^+ catabolic genes into different bacterial recipients as *B. cepacia, A. eutrophus* and *P. aeruginosa.* When the Bph^+ prime plasmids were transferred to the 3CBA mineralizing strain *Pseudomonas sp.* B13, the resulting transconjugants were able to mineralize 3CBP completely showing a synergy between the two degradative pathways in B13 (Springael, unpublished).

The results demonstrate that in vivo cloning by means of prime plasmid formation may be a way to clone in a simple way chromosomal catabolic regions of a wide variety of bacterial genera which are difficult to manipulate using in vitro techniques (Haas & Reimann 1989, van Gijsegem et al. 1987). The recently reported capability of gene capture by IncP plasmids may even be a way to clone genes from bacteria in which the plasmid is not able to replicate (Mergeay et al. 1987; Powell et al. 1987) or to isolate new catabolic pathways from the environment including non-culturable bacteria (Hill et al. 1992).

Conclusions

Chromosomal PCB catabolic genes of three different PCB degrading bacteria were transferred by conjugation into the heavy metal resistant *Alcaligenes eutrophus* strain CH34 and related strains and expressed therein. This was achieved either by selftransposition of a genetic element containing the catabolic genes from the chromosome onto a conjugative plasmid or by recruiting a chromosomal segment carrying the catabolic genes using a plasmid equipped with an efficient transposon. Both endogenous and introduced IncP plasmids functioned as mobilization vectors and recruiting elements of the catabolic DNA. These observations show that chromosomally located PCB catabolic pathways, as already suggested by other authors (Furukawa et al. 1981; Yates & Mondello 1989) can be mobilized between bacteria and that in particular broad host range plasmids and transposons function as transfer mechanisms.

The results are highly significant and have wide range implications. From a molecular ecological point of view, they emphasize the role of broad host range plasmids such as IncP in stressed environments. IncP plasmids were initially identified in clinical isolates of *P. aeruginosa* as natural antibiotic resistance vectors. They are considered to play an important role in the spread of resistance genes among bacteria and the adaptation of bacteria to antibiotics. This incompatibility group exhibits a wide replication host range. As such, other plasmids and even chromosomal genes can be mobilized by means of the IncP helper system

to a wide range of bacteria (Haas & Reimann 1989; Smith & Thomas 1989). In the last decade, it has become evident that these plasmids also play a role in gene exchange in natural environments (Mergeay et al. 1990; Sayler et al. 1990). Plasmids for catabolism of man-made chemicals such as pJP4, pBRC60 and the pSS50/pSS60 related plasmids were found to belong to the IncP family of plasmids. They exhibit a plasmid core gene organization almost identical to that of the well-known IncP resistance plasmids (Burlage et al. 1990). They may not only represent a form of highly mobile DNA which can be transferred into and impart novel phenotypes to recipient organisms in the environment but may also function as recruiters of DNA to constitute new catabolic phenotypes by means of the two genetic systems mentioned here. For example, the 2,4-D catabolic plasmid pJP4 seems to consist of different DNA modules which are thought to have been separately recruited to finally constitute a complete pathway (Perkins et al. 1990; van der Meer et al. 1992).

It would be of interest to study the mobilization, recruiting and retrotransfer capabilities of these environmental IncP plasmids in more detail. pSS50/pSS60 related plasmids were identified in several other bacteria of the Fort Loundon Reservoir Lake environment. All these bacteria are able to degrade 4CBP (Pettigrew & Dayler 1986). Thus, although pSS50/pSS60 does not specify conversion of BP/4CBP into benzoate/4-chlorobenzoate, there seems to be a relationship between the presence of the pathway in these strains and the occurrence of pSS50/pSS60 related plasmids. On the other hand, Pettigrew et al. (1990) demonstrated the occurrence of PCB assimilating bacteria in the lake which did not carry a pSS50 related plasmid. It would be of interest to check if they contain Tn*4371* related sequences. The availability of individual gene probes for the pSS50 plasmid, the Tn*4371* transposon, the 4-chlorobenzoate dehalogenase gene and the PCB degradative genes may help in understanding the ecological role of pSS50 related plasmids and of the transposon in that particular environment.

There are several interesting implications from a biotechnological point of view. Because of their wide host range, natural IncP plasmids equipped with catabolic genes can be introduced into a wide number of bacterial genera and strains useful in environmental biotechnology. Catabolic hybrid strains may be constructed by introducing the plasmid catabolic pathway into xenobiotic degraders exhibiting complementing pathways, as shown by introducing Bph^+ prime plasmids in the 3CBA mineralizing *Pseudomonas sp.* B13 to constitute a 3CBP mineralizing hybrid strain (Springael, unpublished). Introduction of the catabolic pathways into strains able to metabolize other xenobiotic organics can be useful in the construction of strains which can degrade different co-contaminating organics (Haugland et al. 1990). They can be introduced into psychotrophic bacteria to constitute bacteria able to degrade compounds in cold environments (Kolenc et al. 1988) or into bacteria which show good adhesion properties for immobilization on bacterial carriers in bioreactors etc. Furthermore, such constructions may be of particular interest for the expansion of the catabolic versatility of indigenous biodegrading populations (Barkay et al. 1993; Fulthorpe & Wyndham 1991; Fulthorpe & Wyndham 1992). Laboratory-selected bacteria may exhibit poor survival once they are introduced into a natural environment due to various obstacles. Directed transmission of genes involved in catabolism of hazardous organic compounds to members of the indigenous microbial population of the contaminated site may be a superior alternative to the introduction of specialized strains. In this context, degradation of 3-chlorobenzoate in a lake microcosm was correlated with the transfer of the degradative genes of the IncP catabolic plasmid pBRC60 into members of the natural microbial community rather than with the activity and survival of the initially introduced host of the plasmid, *Alcaligenes sp.* BR60 (Fulthorpe & Wyndham 1989).

In this paper, we concentrated on the transmission and expression of PCB catabolic genes into the heavy metal resistant strain, *A. eutrophus* CH34, a bacterium able to survive in harsh environments (Diels & Mergeay 1990). Previously, 2,4-D degrading heavy metal resistant *A. eutrophus* strain has been constructed by introducing the 2,4-D catabolic plasmid pJP4 into *A. eutrophus* CH34 (Friedrich et al. 1983; Springael et al. 1993a). Recently, we showed that also the chlorocatechol degradation pathway of *Pseudomonas* sp. B13 was well-expressed in *A. eutrophus* CH34 (Springael, unpublished results).

Metalloresistant strains related to *A. eutrophus* CH34 were found also to be taxonomically undistinguishable from unnamed isolates registrated as CDC group IV C2 (K Kersters, pers. comm.). Interesting enough, two reports were published about 3-chlorobenzoate-degrading organisms that belong to this CDC group IV c2: McClure et al. (1991) isolated the strain AS2 from a laboratory scale activated sludge unit. Strain AS2 contained a plasmid, pQM300,

which transferred 3- chlorobenzoate degradation to a *P. putida* isolate. In a groundwater bioremediation system at a chemical landfill site, Wyndham et al. (1994) found various Tn*5271* probe-positive isolates. Tn*5271* is a transposon that contains genes for degradation of 3-chlorobenzoate. These two observations confirm the capacity of strains related to *A. eutrophus* CH34 to express plasmid or transposon genes involved in the degradation of chloroaromatic compounds.

More generally, all these observations show the remarkable ability of strains related to *Alcaligenes eutrophus* CH34 to express genes of environmental concern. Other recent isolates from our laboratory which were found to be highly resistant to heavy metals belonged to the genera *Arthrobacter* (Gram positive), *Sphingomonas* (α-Proteobacteria) and *P. aeruginosa* (Table 2). These genera contain organic xenobiotic degraders and may also be used for the construction of hybrid strains expressing heavy metal resistance and catabolic properties simultaneously. Especially *Arthrobacter* is interesting because it constitutes a gram-positive background for the expression of 'gram-positive genes'.

Strains with catabolic and resistance genes are of particular interest in clean-up of organic waste of soil, sludge and waters polluted by both heavy metals and organics. Moreover, *A. eutrophus* CH34 was shown to be effective in the treatment of industrial waste waters and soils contaminated with high concentrations of heavy metals, in a membrane bioreactor and in slurry reactors, respectively. Zn^{2+}, Cd^{2+} and Cu^{2+} could be removed with 99% efficiency from the solution (Diels et al. 1993), whereas between 70 and 90% of Cd^{2+} could be removed from a sandy garden soil (Diels et al. 1991). In the future, we will look at the behaviour of the constructed strains in soil microcosms and in waste water bioreactors contaminated with both organics and heavy metals and at their potential to remove both types of xenobiotics simultaneously.

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