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REVIEW

The recent evolution of pentachlorophenol (PCP)-4-monooxygenase (PcpB) and associated pathways for bacterial degradation of PCP

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Abstract Man-made polychlorinated phenols such as pentachlorophenol (PCP) have been used extensively since the 1920s as preservatives to prevent fungal attack on wood. During this time, they have become serious environmental contaminants. Despite the recent introduction of PCP in the environment on an evolutionary time scale, PCP-degrading bacteria are present in soils worldwide. The initial enzyme in the PCP catabolic pathway of numerous sphingomonads, PCP-4-monooxygenase (PcpB), catalyzes the *para*-hydroxylation of PCP to tetrachlorohydroquinone and is encoded by the *pcpB* gene. This review examines the literature concerning *pcpB* and supports the suggestion that *pcpB*/PcpB should be considered a model system for the study of recent evolution of catabolic pathways among bacteria that degrade xenobiotic molecules introduced into the environment during the recent past.

Keywords Pentachlorophenol · PCP · *pcpB* · *Sphingomonas* · Lateral gene transfer · Gene recruitment

Abbreviations

PCP	Pentachlorophenol
LGT	Lateral gene transfer
MAAI	Maleylacetate isomerase
ORF	Open reading frame
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
DCHQ	Dichlorohydroquinone
TCP	Trichlorophenol
DCP	Dichlorophenol
TCBQ	Trichlorobenzoquinone
TCHQ	Tetrachlorohydroquinone
GSH	Glutathione

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Pentachlorophenol as a xenobiotic environmental contaminant

Polychlorinated phenols such as the trichlorophenols, the tetrachlorophenols, and pentachlorophenol (PCP) have been used extensively since the

1920s as preservatives to prevent fungal attack on wood (Colosio et al. 1993). During this time, they have become serious environmental contaminants. PCP-degrading bacteria are present in soils worldwide (Saber and Crawford 1985; Tiirola et al. 2002a; Kao et al. 2005; Yang et al. 2006; Mahmood et al. 2005). Although monochlorophenols and dichlorophenols are produced naturally by some fungi and insects (Gribble 1996), natural sources of PCP are not known; therefore, the degradation pathway(s) employed by bacteria to degrade PCP likely evolved during the approximately 60 years since the human introduction of PCP into the environment (Copley 2000). PCP-degrading pure cultures may be useful for bioaugmentation in the remediation of PCP-contaminated soils and waters (Saber and Crawford 1985; Bielefeldt and Cort 2005).

Pentachlorophenol degraders and the PCP biodegradation pathway

Numerous PCP degraders have been characterized well enough that information about these strains can be used to infer evolutionary relationships of enzymes and genes of the PCP catabolic pathway (Table 1). However, a number of interesting but lesser examined PCP-degrading bacteria have been isolated from various aquatic and soil environments. For example, numerous PCP degraders have been isolated from aquifers contaminated by polychlorophenols (Mannisto et al. 1999; Tiirola et al. 2002a, b; Nohynek et al. 1995). Such isolates typically degrade 2,4,6-trichlorophenol and 2,3,4,6-tetrachlorophenol, but only some degrade PCP. One isolate identified as *Novosphingobium* sp. strain MT1 (Tiirola et al. 2002a) was found to degrade 2,4,6-trichlorophenol, 2,3,4,6-tetrachlorophenol, and PCP at 8°C and carried a homolog of *pcpB*. Two gram-positive isolates obtained from these sites degraded PCP, but 2,3,4,6-tetrachlorophenol was required as an inducer. In another study a PCP-degrading bacterium was isolated from aquifer sediments and tentatively identified as *Pseudomonas mendocina* (strain NSYSU) *via* biochemical tests and 16S rRNA gene sequencing (Kao et al. 2004). Another strain tentatively identified by biochemical tests as *Pseudomonas fluorescens* was reported to degrade PCP and to harbor a PCP-4-monooxygenase with a molecular weight of 24,000 Da (Shah and Thakur 2003). Nam et al. (2003) reported isolation of a PCP-degrading *Pseudomonas veronii* strain PH-05 from a timber storage yard. This strain was identified by fatty acid analysis and 16S rRNA gene sequencing and was reported to produce tetrachlorocatechol from PCP. This transformation differs from those observed in other PCP-degrading bacteria, which universally metabolize PCP through a chlorinated hydroquinone intermediate. Further investigation of *P. veronii* strain PH-05 is needed to determine if its PCP catabolic pathway is truly different from other gram-negative bacteria examined thus far, or if the chlorocatechol intermediate is formed by a yet to be determined side reaction. The authors observed that the production of tetrachlorocatechol accounted for less than half of the consumed PCP and stated that alternative pathways might also be present. Thakur et al. (2002) isolated a strain identified as *Pseudomonas* sp. strain IST 103 (PCP103) that was capable of growth on PCP and produced a PCP-4-monooxygenase with a molecular size of 30 kDa. Evidence was obtained indicating that a gene encoding the monooxygenase was plasmid-encoded in this strain, an observation that would be novel if verified by follow-up studies. Yang et al. (2006) reported isolation of a PCP-degrading bacterium identified as *Sphingomonas chlorophenolica* by 16S rRNA gene analysis. It appears that this strain is similar to other sphingomonads studied previously. Martins et al. (1997) isolated three gram-negative bacterial strains capable of using PCP as a sole carbon and energy source. These strains were identified as members of the genera *Pseudomonas* (one strain) and *Acinetobacter* (two strains), though it is not clear from their report how these identifications were verified and little work was done to fully characterize their polychlorophenol degrading abilities.

Despite the isolation of many PCP degrading bacteria, a pathway for degradation of PCP is known in detail only for *Sphingobium* (formerly *Sphingomonas*) *chlorophenolica* ATCC 39723 (Lange et al. 1996; Orser et al. 1993, Orser and Lange 1994; Xun and Orser 1991a, b; Xun et al.

Table 1 Well-characterized bacteria that grow on pentachlorophenol

Strain name	Method of identification	Strain source	Other compounds degraded	*pcpB* Confirmed?	References
Sphingobium chlorophenolica ATCC 39723 (formerly *Flavobacterium* and *Sphingomonas*)	16S rRNA gene sequence	PCP-contaminated soil	3,5-dibromo-4-hydroxybenzonitrile), triiodophenol, tribromophenol, trichlorophenol, 2,4,6-trichlorophenol, and 2,3,5,6-tetrachlorophenol	Yes	Saber and Crawford (1985), Steiert and Crawford (1986), Steiert et al.(1987), Topp et al. (1992), Xun and Orser (1991)
Rhodococcus (*Mycobacterium*) *chlorophenolicus*	Chemotaxonomic and nutritional characteristics; 16S rRNA gene sequence	PCP-contaminated wood waste	3,4,5-, 2,3,4,6-, and 2,3,5,6-tetrachlorophenol, and 2,3,5- and 2,3,6-trichlorophenol; chloroguaiacols	No (not thought to be present)	Apajalahti et al. (1986), Apajalahti and Salkinoja-Salonen (1987), Haggblom et al. (1994), Briglia et al. (1994)
Sphingomonas spp. UG25 and UG30	16S rRNA gene sequence	PCP-contaminated soil	*p*-Nitrophenol (PNP), 2,4-dinitrophenol (2,4-DNP), *p*-nitrocatechol and 4,6-dinitro-*o*-cresol (DNOC).	Yes	Leung et al. (1997)
Arthrobacter ATCC 33790 (renamed *Sphingomonas chlorophenolica*)	16S rRNA gene sequencing	PCP-contaminated soil	Trichlorophenol	Yes	Stanlake and Finn (1982), Ederer et al. (1997)
Pseudomonas SR3 (renamed *Sphingomonas chlorophenolica*)	16S rRNA gene sequencing	PCP-contaminated soil from a lumber treatment waste site	2,3,5,6-; 2,3,6-; 2,4,6-; 2,4-; and 2,6-chloro-substituted phenols	Yes	Resnick and Chapman (1994), Ederer et al. (1997)
Pseudomonas RA2 (renamed *Sphingomonas chlorophenolica*)	16S rRNA gene sequencing	PCP-contaminated soil from wood treatment wastes	Non-ionic surfactant Tween 20	Yes	Radehaus and Schmidt (1992), Colores et al. (1995), Bielefeldt and Cort (2005), Ederer et al. (1997)

1992a, b; Dai et al. 2003). This strain was originally isolated by Saber and Crawford before the era of 16S rDNA phylogeny and was identified at the time as a strain of *Flavobacterium* (Saber and Crawford 1985). In the years since the original isolations of ATCC 39723 and other closely related PCP-degrading bacterial strains and adoption of 16S rDNA phylogeny for bacterial classification, the genus *Sphingomonas* has been divided into multiple genera (Takeuchi et al. 2001). *Sphingomonas chlorophenolica* (Karlson et al. 1996; Ederer et al. 1997) is now classified as a member of the genus *Sphingobium*. The rate-limiting step for PCP degradation in *Sphingobium chlorophenolicum* ATCC 39723 appears to be the *para*-hydroxylation of PCP to tetrachlorohydroquinone (McCarthy et al. 1997). This step is catalyzed by the enzyme PCP-4-monooxygenase, encoded by the *pcpB* gene (Orser et al. 1993) and works in concert with a tetrachlorobenzoquinone reductase encoded by *pcpD* (Dai et al. 2003). In addition to PCP, the PCP-4-monooxygenase can use trichlorophenols, tetrachlorophenols, and several other halogenated phenols as substrates (Xun et al. 1992a, b). The PCP catabolic pathway of *Sphingobium chlorophenolicum* ATCC 39723 is shown in Fig. 1.

In addition to PcpB, several of the other enzymes in the PCP pathway have now been characterized. Tetrachlorohydroquinone reductive dehalogenase (PcpC; Fig. 1) has been isolated and characterized from *Sphingobium chlorophenolicum* ATCC 39723 (Xun et al. 1992b; Anandarajah et al. 2000) and *Sphingomonas* sp. UG30 (Habash et al. 2002). PcpC is a member of the glutathione transferases that are widely found in both prokaryotes and eukaryotes. Though the UG30 PcpC shares 94% primary sequence identity with the PcpC from *S. chlorophenolicum* ATCC 39723, there are significant differences between the two enzymes in some of their functional and kinetic properties (Habash et al. 2002). 2,6-Dichloro-*p*-hydroquinone 1,2-dioxygenase (PcpA) has been purified from *Sphingobium* (formerly *Sphingomonas*) *chlorophenolica* ATCC 39723 (Xun and Orser 1991b; Xun et al. 1999; Ohtsubo et al. 1999). PcpA shows novel Fe^{2+} and O_2-dependent ring-cleavage dioxygenase activity against hydroquinone derivatives and within the PCP pathway converts 2,6-DCHQ to 2-chloromaleylacetate (Fig. 1).

Pentachlorophenol 4-monooxygenase (PcpB) and the *pcpB* gene

There have been numerous studies related to the DNA sequences of *pcpB* alleles from various PCP-degrading bacteria. Identical *pcpB* gene sequences have been found in three of four known PCP-degrading strains of *S. chlorophenolicum* (ATCC 39723, SR3, and RA2) (Karlson et al. 1996; Ederer et al. 1997). A *pcpB* gene characterized in *S. chlorophenolicum* ATCC 33790 differs by 10% from the other three sequences (Ederer et al. 1997; Crawford and Ederer 1999). A variant of *pcpB* was found in the sphingomonad strain UG30 (Cassidy et al. 1999; Leung et al. 1997, 1999). The sequence similarity between the UG30 gene and that of ATCC 39723 was 90%, while the similarity between the UG30 and ATCC 33790 genes was 89%. A *pcpB* gene homolog 98% similar to the gene of *S. chlorophenolicum* ATCC 39723 was observed in two proteobacterial strains isolated from soil samples from a PCP-contaminated wood treatment site, though the strains were not PCP degraders (Saboo and Gealt 1998).

Tiirola et al. (2002a) isolated from polychlorophenol-contaminated groundwater in Finland a strain identified as *Novosphingobium* sp. MT1 that degrades 2,4,6-trichlorophenol, 2,3,4,6-tetrachlorophenol, and PCP at 8°C. The strain carried a homolog of the *pcpB* gene originally observed in *Sphingobium chlorophenolicum*. The full-length *pcpB* allele had approximately 70% identity with the three *pcpB* genes previously sequenced from sphingomonads ATCC 39723, SR3, and RA2 (Karlson et al. 1996; Ederer et al. 1997) and was also closely related to the environmental clones obtained by Beaulieu et al. (2000) from chlorophenol-enriched soil samples. However, spontaneous deletion of *pcpB* from the MT1 genome resulted in the loss of chlorophenol

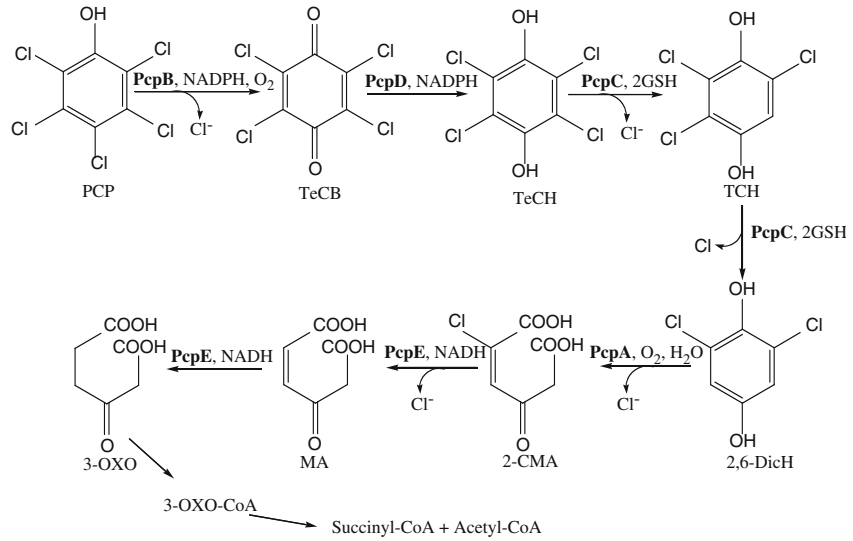

Fig. 1 Pentachlorophenol degradation pathway of *Sphingobium chlorophenolicum* ATCC 39723. PcpB, PCP hydroxylase; PcpD, TCBQ reductase; PcpC, TCHQ dehalogenase; GSH, glutathione; PcpA, 2,6-DCHQ dioxygenase; PcpE, MA reductase; PCP, pentachlorophenol; TeCB, tetrachloro-*p*-benzoquinone; TeCH, tetrachloro-*p*-hydroquinone; TCH, 2,3,6-trichloro-*p*-hydroquinone; 2,6-DiCH, 2,6-dichloro-*p*-hydroquinone; 2-ClMA, 2-chloromaleylacetic acid; MA, maleylacetic acid; 3-OXO, 3-oxoadipic acid (see Cai and Xun 2002; Dai and Copley 2004)

degradation abilities and the exact mechanism of this deletion was not reported.

The Finnish researchers also examined the distribution of *pcpB* in a phylogenetically diverse group of polychlorophenol-degrading sphingomonads isolated from their contaminated groundwater site (Tiirola et al. 2002b). All the isolates shared *pcpB* gene homologs with 98.9 to 100% sequence identity. An analysis of the 16S rRNA gene and *pcpB* phylogenetic trees of these strains suggested that a recent horizontal transfer of the *pcpB* gene was involved in the evolution of the catabolic pathways for degradation of polychlorinated phenols in these strains.

Yan and colleagues (2006) demonstrated that *Sphingobium chlorophenolicum* ATCC 39723 is quite capable of attaining the ability to degrade a xenobiotic molecule it could not previously degrade by gene acquisition. The gene cassette (*camA camB camC*) encoding a cytochrome P-450cam variant was integrated into a non-essential gene of this PCP degrader by homologous recombination. The recombinant strain could degrade hexachlorobenzene by conversion to PCP.

Lateral gene transfer and the origin of the pentachlorophenol pathway

Copley (2000) suggests that *pcpB* may have been recruited from another monooxygenase (e.g., 2,6-dichlorophenol 4-hydroxylase) and used by some bacteria for the degradation of the natural compound 2,6-dichlorophenol, which is produced as a sex pheromone by numerous tick species (Berger 1983; Borges et al. 2002). The PCP pathway enzymes of *S. chlorophenolicum* ATCC 39723, including *pcpB*, also function in the degradation of lesser-chlorinated phenols such as 2,6-dichlorophenol. There is no evidence thus far that another pathway containing a progenitor 2,6-dichlorophenol hydroxylase exists in this bacterium, so it would appear likely that *pcpB* (and probably *pcpA*) was obtained by lateral gene transfer (LGT). However, the whole genome of *S. chlorophenolicum* ATCC 39723 has not been sequenced, so the possibility of another, older dichlorophenol hydroxylase gene that may have been recruited for PCP catabolism in this organism cannot be ruled out.

Copley (2000) also provides evidence that another PCP pathway enzyme, PcpC (tetrachlorohydroquinone [TCHQ] dehalogenase), may have evolved from a glutathione (GSH)-dependent maleylacetate isomerase (MAAI) that is found in pathways for the biodegradation of tyrosine, phenylalanine, and phenylacetate. There appears to be another MAAI in *Sphingobium chlorophenolicum* that shows minimal TCHQ dehalogenase activity (Anandarajah et al. 2000). If this *S. chlorophenolicum* enzyme is the evolutionary precursor of PcpC, then acquisition of this enzyme activity would not be an example of LGT but instead an example of local recruitment of an existing enzyme through mutation(s) to perform a new function. However, it is also possible that an already evolved *pcpC* and perhaps other genes of the PCP pathway gene were obtained by *Sphingobium chlorophenolicum* ATCC 39723 through LGT from another organism. Though we have not done so here, a careful comparison of the genes and proteins within *S. chlorophenolicum* ATCC 39723 for TCHQ dehalogenase and MAAI could provide evidence for one of these origins of *pcpC* in this bacterium. It should be noted that evolutionary processes may not have been similar for other PCP-degrading bacteria, and studies of such processes in other bacteria probably will require evaluations of individual strains; however, *S. chlorophenolicum* ATCC 39723 remains the best model to date for in-depth study of PCP pathway evolution.

It may be possible to test Copley's (2000) hypothesis that *pcpB* and *pcpC* of *S. chlorophenolicum* ATCC 39723 evolved from a dichlorophenol hydroxylase and a maleylacetate isomerase, respectively. This could be done by ''evolving'' a bacterium that already degrades tyrosine (or phenylacetate) and/or a substrate such as 2,6-dichlorophenol or 2,4,6-trichlorophenol so that a new strain is selected that has attained the ability to degrade PCP. This might be done by exposing the original strain to the selective pressure of moderate to high levels of PCP and selecting over multiple generations a new strain that shows resistance to or ability to degrade this potent biocide. This might be accomplished by using an evolutionary algorithm to drive the selection process, as demonstrated by Boon et al. (2006), Heylen et al. (2006), Vandecasteele (2003), and Vandecasteele et al. (2003, 2006) for the optimization of processes carried out by mixed bacterial cultures. For example, a genetic algorithm (GA) could be used to optimize a mixed culture for degradation of PCP. The optimized culture would be developed using an initial pool of candidate organisms that degrade 2,6-DCP, 2,4,6-DCP, 3-hydroxy-benzoate, and tyrosine. Degraders of compounds such as 3-hydroxybenzoate should be included since it is quite possible that PcpB-like enzymes evolved from either dehalogenating or non-dehalogenating aromatic hydroxylases that share common ''hydroxylase'' motifs. Once PCP degradation had been optimized (e.g., after about 20 generations of selection pressure directed by use of a GA), the surviving strains could be examined for the evolved activity of PcpB- and PcpC-like enzymes.

Several known chlorophenol-degrading bacterial strains are potentially available for such an experiment, and these could be considered at *in vitro* evolution. *Ralstonia* sp. strain RK1 (Steinle et al. 1998) grows on 2,6-DCP; however, the involvement of a 4-hydroxylase in DCP degradation by this strain is not yet proven. The well-studied strain *Ralstonia eutropha* JMP134 (Louie et al. 2002) is another possibility. JMP134 grows on 2,4,6-trichlorophenol (TCP); involvement of a 4-hydoxylase has been shown, and the bacterium apparently does not grow on PCP. *Burkholderia cepacia* strain AC1100 (Martin-Le Garrec et al. 2001; Tomasi et al. 1995; Gisi and Xun 2003; Xun 1996; Danganan et al. 1994), recently proposed to be renamed as *Burkholderia phenoliruptrix* sp. *nov*. (Coenye et al. 2004), produces a monooxygenase that performs the dioxygen-dependent hydroxylation in the 4-position of 2,4,6-TCP. This enzyme does 4-hydroxylate PCP, but PCP is a poor substrate (Xun 1996); thus, AC1100 probably is not an ideal strain in which to study the evolution of *pcpB* by LGT. However, an oxygenase such as that of AC1100 could be a progenitor of *pcpB* via LGT to another bacterium such as *S. chlorophenolicum*. *Azotobacter* sp. strain GP1 (Wieser et al. 1997; Li et al. 1991) produces a single-component dehalogenating monooxygenase found in 2,4,6-TCP-grown cells that oxidizes 2,4,6-TCP or 2,6-dichlorophenol

(DCP) to 2,6-dichlorohydroquinone, but the enzyme does not oxidize PCP. It shows only 17% identity of amino acid sequence with PCP-4-monooxygenase of *S. chlorophenolicum* and thus is probably not the evolutionary progenitor of the latter enzyme. The study of this TCP-4-hydroxylase, which like *pcpB* hydroxylates the 4-position of 2,6-DCP even though no chlorine is present, indicates a close evolutionary relationship between dehalogenating and non-dehalogenating aromatic ring hydroxylases. It has not been established if any of these strains can grow on tyrosine, phenylalanine, or phenylacetate and thereby provide a progenitor maleylacetate isomerase for evolution of a PcpC-like protein; however, this function can be provided in the selection process by including known tyrosine degraders in the original mixture of gene donors for a GA-based optimization.

A question arises as to whether the PCP pathway as it exists in organisms such as *S. chlorophenolicum* ATCC 39723 evolved independently through an enzyme recruitment process at various places around the world after initial exposure to PCP (Tiirola et al. 2002b; Copley 2000), or whether once evolved, processes of LGT make the genes of the pathway available for rapid dissemination in PCP-exposed bacterial populations. Certainly once the PCP degradation pathway evolved, bacteria harboring these genes could become donors of the genes and thereby the source of the new pathway to other organisms in the local community. Whether LGT could result in dissemination of the PCP pathway worldwide in the sixty years since the first introduction of PCP into the environment is debatable but due to their small size and enormous abundance, unicellular organisms such as bacteria are thought to be able to rapidly disperse unhindered around the world (Fenchel 2003; Top and Springael 2003). Breitbart et al. (2004) observed that identical T7-like phage-encoded DNA polymerase genes from a unique clade were >99% conserved at the nucleotide level in multiple different environments, suggesting that phages move freely between biomes. Thus, it seems reasonable to hypothesize that proteins with *pcpB* activity could have evolved by recruitment (or LGT) of genes encoding a variety of monooxygenases, since these oxidases contain highly conserved ''monooxygenase'' domains that may adapt via mutation to recognize a substrate like PCP. This might be the case in particular for organisms outside the Proteobacteria, such as representatives of the Firmicutes and Actinobacteria (Takami et al. 2002; Ikeda et al. 2003) or even the Eukaryota (Schulte et al. unpublished data) whose whole genome sequences indicate the presence of PcpB-like proteins (Table 2).

To this point *pcpB* has been thoroughly described in a restricted number of bacterial genera. These include *Sphingobium* (Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae; *Sphingobium*); *Novosphingobium* (Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae; *Novosphingobium*); and *Pseudomonas* (Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; *Pseudomonas*). The identification of the latter organism as *Pseudomonas mendocina* (Kao et al. 2005) was originally reported in a thesis (Chai 2002) that is not readily accessible and thus needs confirmation. An actinobacterium (*Rhodococcus chlorophenolicus* PCP-I = *Mycobacterium chlorophenolicum* PCP-I) (Briglia et al. 1994) isolated in Finland for its ability to degrade polychlorinated phenols (Haggblom et al. 1988; Apajalahti and Salkinoja-Salonen 1987a, b) has been characterized to a lesser degree than *S. chlorophenolica* with regards to the mechanism it uses to degrade PCP and other polychlorinated phenols. As observed with the gram-negative sphingomonads (Fig. 1, first reaction), the gram-positive *Mycobacterium chlorophenolicum* PCP-I initiates metabolism of PCP and other polychlorinated phenols via *para*-hydroxylation to form tetrachloro-*p*-hydroquinones. However, there is some evidence that the *Rhodococcus*/*Mycobacterium* group may employ the cytochrome P450 for PCP dechlorination and hydroxylation rather than a monooxygenase like PcpB used by sphingomonads (Uotila et al. 1991, 1995). Also, Orser et al. (1993) performed DNA hybridizations with probes for *pcpB* using genomic DNAs from *Arthrobacter* sp. strain ATCC 33790 and *Pseudomonas* sp. strain SR3 (both now classified with the sphingomonads; Karlson et al. 1996; Ederer et al. 1997) finding a hybridizing fragment of the appropriate size, whereas there was no positive

Table 2 Comparison of translated *pcpB* loci reported for whole microbial genome sequences

Genome (accession number)	Taxonomic affiliation (phylum/class/order)	Accession number (PcpB-like sequence)	Expect value	Amino acid identities	Closest organism match	References
Oceanobacillus iheyensis HTE831 (NC_004193)	Firmicutes/Bacilli/Bacillales	NP_691559	1e–48	33% (137/412)	*Burkholderia mallei* ATCC 23344	Takami et al. (2002)
			3e–33	28% (102/353)	*Novosphingobium* sp. MT1	
			2e–32	28% (137/412)	*Sphingobium chlorophenolicum*	
Chlamydophila pneumoniae TW–183 (NC_005043)	Chlamydiae/Chlamydiae/Chlamydiales	NP_876428	0.001	25% (56/223)	*Novosphingobium* sp. MT1	Geng et al. (2003)
Streptomyces avermitilis MA-4680 (NC_003155)	Actinobacteria/Actinobacteria/Actinomycetales	NP_822087	2e–78	48% (187/383)	*Mesorhizobium loti* MAFF303099	Ikeda et al. (2003)
			2e–35	36% (104/287)	*Burkholderia mallei* ATCC 23344	
			2e–40	34% (135/391)	*Neurospora crassa*	
Mesorhizobium loti MAFF303099 (NC_002678)	Proteobacteria/Alpha-Proteobacteria/Rhizobiales	NP_104865	1e–89	48% (186/380)	*Streptomyces avermitilis* MA-4680	Kaneko et al. (2000)
			8e–42	33% (114/339)	*Sphingobium chlorophenolicum*	
			2e–41	33% (114/339)	*Flavobacterium* sp.	
			2e–42	31% (133/417)	*Neurospora crassa*	
Burkholderia mallei ATCC 23344 (NC_006848)	Proteobacteria/Beta-Proteobacteria/Burkholderiales	AAU48822	1e–51	34% (154/451)	*Sphingobium chlorophenolicum*	Nierman et al. (2004)
			1e–51	34% (155/452)	*Flavobacterium* sp.	
			4e–50	32% (173/534)	*Sphingomonas* sp. UG30	
			3e–51	31% (171/535)	*Novosphingobium* sp. MT1	
Neurospora crassa (BX294012.1)	Ascomycota/Sordariomycetes/Sordariales	CAD70786	3e–41	31% (121/389)	*Sphingobium chlorophenolicum*	Schulte et al. (unpublished)
			3e–41	31% (121/389)	*Flavobacterium* sp.	
			3e–41	31% (124/392)	*Sphingomonas* sp. UG30	
			6e–45	33% (140/412)	*Burkholderia mallei* ATCC 23344	

hybridization with genomic DNA from *Rhodococcus* (*Mycobacterium*) *chlorophenolicus*. Thus, the Actinobacteria that degrade PCP may employ similar chemistry but may use a different gene and protein for the initial *para*-hydroxylation of PCP. However, the work of Camus et al. (2002) in annotating the whole genome sequence of *Mycobacterium tuberculosis* H37Rv where a *pcpB*-like gene is observed indicates that more research on PCP degradation by mycobacteria is warranted. These authors found within the *M. tuberculosis* (Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Mycobacteriaceae; *Mycobacterium*; *Mycobacterium tuberculosis* complex) genome a gene similar to *pcpB* with an *E* value of 1.7e–07 and 23.3% identity in a 360 amino acid overlap. A similar gene has also been observed in the genome of *Mycobacterium bovis* (Garnier et al. 2003).

Genes representing putative *pcpB* loci have been reported for several other whole bacterial genome sequences (Table 2). It is not clear whether the putative *pcpB* genes described in Table 2 actually encode PCP hydroxylases since these activities have not been demonstrated in cell-free extracts prepared from the strains; these experiments need to be done. The methods used by most of the authors listed in Table 2 to identify these putative PCP-4-hydroxylase genes can also be questioned. These genes were elucidated from whole genome shotgun cloning and sequencing of the respective organisms. Open reading frame (ORF) predictions on translated amino acid sequences were made, and the ORFs were then annotated in SwissProt (BLASTP) and/or other protein databases. The best match was assigned as the putative identification for the protein in question. In all cases the match to PCP-4-hydroxylase was quite poor and indeed was not even the best match. For example, a BLAST search of the putative PCP-4-hydroxylase of *Oceanobacillus iheyensis* HTE831 revealed that this protein sequence shared 40% identity (201/501; expect value of 3e–112) to a putative flavoprotein monooxygenase (ZP_00526951) of *Solibacter usitatus* Ellin6076 (Altschul et al. 1997). Thus, annotations of at least some of these ORFs as *pcpB* alleles are not well supported.

Golovleva et al. (1991–1992) and Zaborina et al. (1997) reported that a strain of *Streptomyces rochei* can degrade polychlorinated phenols including PCP, using them as sole sources of carbon and energy. These authors detected chlorohydroquinols as intermediates during degradation of several polychlorophenols and observed activity of hydroxyquinol 1,2-dioxygenase that was induced by the presence of chlorophenols. These observations with this representative of the Actinobacteria are consistent with a PCP degradation pathway similar to that shown in Fig. 1. This at least lends support to the possibility of the occurrence of *pcpB* outside of the sphingomonads.

It is important to note that other known genes of the PCP pathway in the sphingomonads are not evident within the present genome annotations of the organisms described in Table 2. Several putative 3-oxoadipate enol-lactone hydrolase genes are found in the genome of *Streptomyces avermitilis* MA-4680, while the genome of *Burkholderia mallei* ATCC 23344 harbors putative 3-oxoadipate CoA-transferase (alpha subunit) and 3-oxoadipate enol-lactone genes; enzymes such as these could function in the later stages of a modified PCP pathway (Fig. 1), but their linkage to a sphingomonad-like PCP pathway is at best tenuous.

Pentachlorophenol pathway genes in environmental samples

There have been very few studies of the genes encoding PCP degradation within DNA isolated directly from the environment (metagenomic DNA). We are aware of only one such report. Beaulieu et al. (2000) cloned and sequenced PCR-amplified DNA related to the *pcpB* gene from soil slurries that had been acclimated with PCP or with a wood preservative mixture containing PCP and petroleum hydrocarbons. Of 16 clones examined, deduced amino acid sequences of 13 *pcpB*-like clones were highly related to *pcpB* of *Sphingomonas* strain UG30. This result suggested that enrichment with PCP resulted in selection of low phylogenetic diversity; however, sequences of an additional three clones were not closely related to sequences of any known *Sphingobium pcpB* genes.

We recently obtained several metagenomic clones of *pcpB* amplified by PCR directly from a soil that had been contaminated since the mid-1940s by PCP. The source site (Libby, Montana Ground Water Contamination Site) was added to the U.S. Environmental Protection Agency's (EPA) National Priorities List in 1983. The soil we employed came from treatment cells prepared by excavating and consolidating 45,000 cubic yards of contaminated soil and debris and treating it by enhanced natural bioremediation in lined treatment cells that will eventually be capped. PCR primers were designed against a consensus sequence derived using Vector NTI of whole *pcpB* genes available through the NCBI BLAST website. The resultant sequence was entered into Primer3 (http://www frodo.wi.mit.edu/cgi-bin/primer3), and the primer set giving the largest fragment (1358-bp product/1630-bp gene) with the best Tm and GC% match were employed for polymerase chain reaction (PCR) of the *pcpB* gene fragments; the forward primer was 184F (5′-TCG ATG GAG ATG ATG GAA CA-3′), and the reverse primer was 1541R (5′-ATG TAG TTG TCC GGC CTC AA-3′). *E. coli* clones CCL3 (University of Idaho) and CCL4 (courtesy of Dr. L. Xun, Washington State University) containing *pcpB* cloned from *S. chlorophenolicum* ATCC 33790 were used as positive controls. Control sequences were cloned into a TA cloning vector (Invitrogen, Carlsbad, CA), and two colonies were sequenced and verified by comparison against the BLAST database (Altschul et al. 1990) to be the appropriate *pcpB* gene fragments, confirming the efficacy of our primer set in amplifying known *pcpB* sequences. Metagenomic DNA isolated from the Libby, Montana soil was subjected to PCR amplification using both 16S rRNA gene bacterial primers 27F (Edwards et al. 1989) and 907R (Lane et al. 1985) (PCR controls) as well as our *pcpB* primer set; 16S RNA genes were amplified, but no obvious *pcpB* PCR product was evident in initial amplifications. When 1 μl of the initial PCR reaction was used as template for a subsequent *pcpB* PCR reaction, a product was obtained. The *pcpB* product was gel purified and cloned into a TA cloning vector, and 96 colonies were grown in a 96-well plate containing LB and 100 μg/ml ampicillin. Plasmids were isolated and the resultant plasmids were then digested with *Hin*PI and *Msp*I restriction endonucleases. The digested plasmids were electrophoresed on a 3.5% agarose gel as a means to group similar clone banding patterns through a standard restriction fragment length polymorphism (RFLP) analysis. Eight distinct patterns were observed and representatives of each group sequenced. The clone sequences were compared to one another and to known GenBank sequences at both the nucleotide (data not shown) and amino acid level (Fig. 2). The four metagenomic clones grouped into their own clade, most similar to the sequence of *S. chlorophenolicum* ATCC 39723. Interestingly, this is the strain for which the degradation pathway of PCP has been studied in detail. These observations support the hypothesis that lateral transfer of *pcpB* may be restricted to a narrow range of phylogenetic diversity encompassing primarily the Proteobacteria.

Conclusions

The gene *pcpB* and the unusual aromatic ring-dehalogenating enzyme PCP-4-monooxygenase (PcpB) represent an outstanding example of recent bacterial evolution toward the capability for metabolically productive degradation of anthropogenic chemicals introduced into the environment during the past 50–60 years. The gene is found worldwide within bacterial communities exposed to PCP, and it is likely that lateral transfer processes have been involved in dissemination of the gene, at least locally. It is quite possible that *pcpB* arises through processes of gene recruitment (Copley 2000) at specific geographic locations and then spreads by LGT in the local environment. PCP genes examined thus far appear not to be located on mobile genetic elements like catabolic plasmids, catabolic transposons, or conjugative transposons (though see Thakur et al. 2002), but the sphingomonads, where they have been most often observed, do appear to readily take up foreign DNA and/or accumulate natural point mutations in their genes. These would be excellent mechanisms to foster pathway evolution toward catabolism of molecules like PCP. In support of this hypothesis

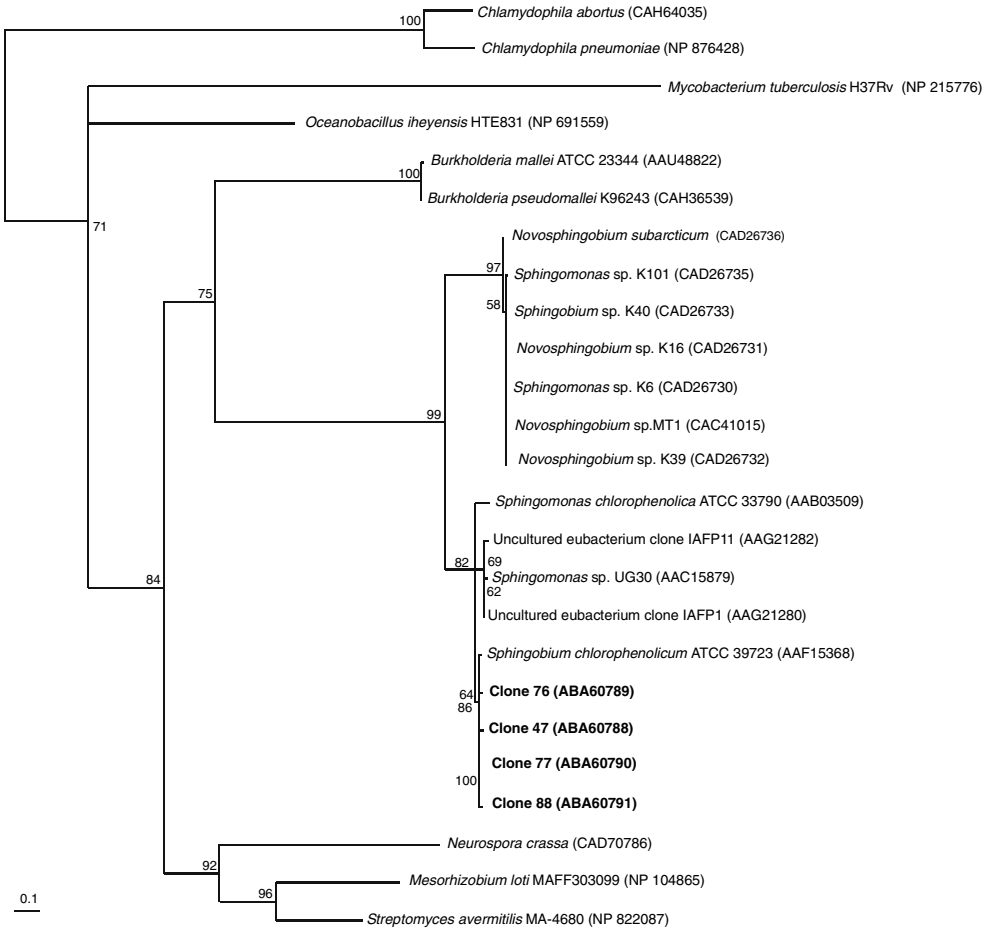

Fig. 2 Phylogenetic relatedness of known PcpB sequences (as calculated by the maximum likelihood method) and the translated sequences of those amplified from Libby, Montana PCP-contaminated soil using a consensus *pcpB* primer set. Metagenomic clones are indicated by numbers in bold. The bar represents 10% sequence divergence

is the observation that lateral transfer of *pcpB* may be restricted to a narrow range of phylogenetic diversity encompassing primarily the Proteobacteria. For example, the Actinobacteria such as *Mycobacterium chlorophenolicus* that *para*-hydroxylate PCP and other polychlorinated phenols may not as easily attain *pcpB* by lateral transfer and thus may have employed different enzymes (possibly cytochrome P450) for this purpose. This question requires further investigation with more mycobacterial strains since *pcpB*-like sequences have been observed in the genomes of several *Mycobacterium* spp. In this era of whole-genome sequencing, putative *pcpB* alleles are being annotated not only within the genomes of Proteobacteria but also in genomes of Firmicutes and Actinobacteria. Thus *pcpB*/PcpB should be considered a model system for the study of recent evolution among bacteria for catabolic pathways that facilitate the degradation of xenobiotic molecules. Since other genes involved in PCP catabolism are linked within bacteria such as *Sphingobium chlorophenolica* where they are found on two different gene fragments (Cai and Xun 2002; Dai and Copley 2004), it is likely that these also have been acquired by a combination of gene recruitment and lateral transfer events. Thus *pcpD* (trichlorobenzoquinone [TCBQ] reductase), *pcpC* (trichlorohydroquinone [TCHQ] dehalogenase), and *pcpA* (2,6-DCHQ dioxygenase) (Fig. 1) are candidates for further study of recent lateral gene

transfer in the evolution of the PCP catabolic pathway.

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