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Molecular analysis of pentachlorophenol degradation

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Abstract

A limited number of microorganisms have been described for their ability to partially degrade pentachlorophenol (PCP), or to completely mineralize it. Several years ago we chose one of these microorganisms, *Flavobacterium* sp. strain ATCC 39723, for use in a detailed molecular analysis of the catabolism of PCP. This strain was chosen because it had previously been studied in great detail for its growth characteristics in relation to degradation of PCP. In this paper we provide an overview of the degradation pathway of PCP to 2,6-dichloro-*p*-hydroquinone by *Flavobacterium*. The specific biochemical reactions and the genes encoding the enzymes are reviewed. The successful transformation and site specific mutagenesis of *Flavobacterium*, as well as the discovery of two new *pcp* alleles is also presented.

Microbial degradation of PCP

Since its introduction in the 1930s, pentachlorophenol (PCP) has become one of the most versatile and widely used biocides on the market. Introduced primarily as a wood preservative for the wood products industry, it has acquired new uses in industry and agriculture as a long lived, broad spectrum biocide against bacteria, fungi, insects, lower and higher plants, and animals. The general toxicity of PCP is due to its ability to act as an uncoupler of oxidative phosphorylation. PCP is a polyhalogenated aromatic compound, and is unusually acidic with a pK_a of 4.70 in water, allowing it to be soluble in both aqueous and organic solvents (Crosby 1981).

A limited number of microorganisms have been described for their ability to partially degrade PCP, or to completely mineralize it. Thus far, only soil microorganisms have been described, which may be indicative of an adaptation to PCP through chronic exposure. Several fungi have been recognized for their ability to partially degrade PCP to less toxic intermediates, and are of interest because PCP resistant wood deteriorating fungi are a threat to PCP treated wood products.

Several isolates of PCP-degrading aerobic soil bacteria and anaerobic consortia from acclimated sewage sludge have been identified. Anaerobic microbial degradation of PCP is still quite undefined except for the isolation of the reductive dechlorination intermediates produced by these systems and the generation of CO_2 and CH_4 from the aromatic ring of PCP (Mikesell & Boyd 1986).

Several aerobic PCP-degrading soil bacteria have been isolated as pure cultures and described in the literature. A PCP-mineralizing *Arthrobacter* sp. strain KC-3 was isolated by Chu & Kirsch (1972) from a continuous-flow enrichment culture using low concentrations of PCP as the sole carbon source. This bacterium was later used in mutagenesis experiments to study the intermediate metabolites. Metabolites from mutants were identified as 2,3,5,6-tetrachloro-*p*-hydroquinone (TeCH), 2,6-dichloro-*p*-hydroquinone (DiCH), trace amounts of 2-chloro-*p*-hydroquinone (MoCH), and trichlorohydroxybenzoquinone.

Stanlake & Finn (1982) isolated a PCP-mineralizing *Arthrobacter* sp. strain ATCC 33790 by chemostat enrichment, and found it to be similar to the PCP-degrading *Arthrobacter* strain KC-3. Schenk et al. (1989) described the first dechlorination step of

PCP mineralization by *Arthrobacter* sp. strain ATCC 33790 as being oxygenolytic (requiring O_2) and requiring NADPH. TeCH was identified as the primary intermediate from the reaction of PCP with O_2 and NADPH in the presence of crude cell extracts. *Arthrobacter* sp. strain ATCC 33790 has been examined for its ability to degrade PCP in contained bioreactors as non-immobilized and immobilized cells, with the results being very favorable for use of the cells in removing PCP from aqueous environments (Schenk et al. 1989).

Two PCP-mineralizing strains of *Rhodococcus chlorophenolicus,* PCP-1 and CP-2, were isolated and described by Juha et al. (1986) and Häggblom et al. (1988a), respectively. Both strains produced TeCH as the primary intermediate. ^{18}O labelling experiments with whole cells of strain PCP-1 showed that $H_2{}^{18}O$ was the primary oxygen donor for *para* hydroxylation of PCP (Juha & Salkinoja-Salonen 1987a). A second study by Uotila et al. (1992) using strain PCP-1 demonstrated the role of a cytochrome-P450 enzyme in *para* hydroxylation of PCP by *Rhodococcus*. This study showed that under anaerobic conditions, $H_2{}^{18}O$ was preferred as the oxygen donor by the crude preparations of P450 enzyme, and that $^{18}O_2$ was preferred under aerobic conditions. The PCP degrading pathway for *Rhodococcus* sp. strain CP-2 was also shown to involve a second hydroxylation to form 3,5,6-trichloro-2-hydroxy-*p*-hydroquinone, with subsequent reductive dechlorinations to form dichloro-2-hydroxy-*p*-hydroquinone, monochloro-2-hydroxy-*p*-hydroquinone and finally just 2-hydroxy-*p*-hydroquinone (1,2,4-trihydroxybenzene) (Juha & Salkinoja-Salonen 1987b; Häggblom et al. 1989). Furthermore, strain CP-2 was found to O-methylate numerous chlorinated phenols, including PCP (Häggblom et al. 1988b). The chlorinated *p*-hydroquinone intermediates (Häggblom et al. 1988a) were also O-methylated and the corresponding anisols and methoxy phenols that were produced were also degraded.

Another well studied PCP-degrading bacterium is *Flavobacterium* sp. strain ATCC 39723. Isolated from PCP contaminated soils in Minnesota (Saber & Crawford 1985; Saber 1987), *Flavobacterium* has been shown to utilize PCP as a sole carbon source at high concentrations (100–200 ppm). The growth characteristics of *Flavobacterium* have been studied in great detail (Topp et al. 1988; Topp & Hanson 1990) and the cells have been shown to be most competent to degrade PCP in the presence of glutamate as a cosubstrate (Gonzalez & Hu 1991). The ability to degrade other chlorophenols has been studied (Steiert et. al. 1987), and the early steps in the catabolism of PCP described through the generation of mutants that could no longer grow on PCP (Steiert & Crawford 1986). TeCH and DiCH were identified as key intermediates from these PCP^- mutants, and the source of oxygen for *para*-hydroxylation appeared to be from H_2O as determined by ^{18}O labelling experiments.

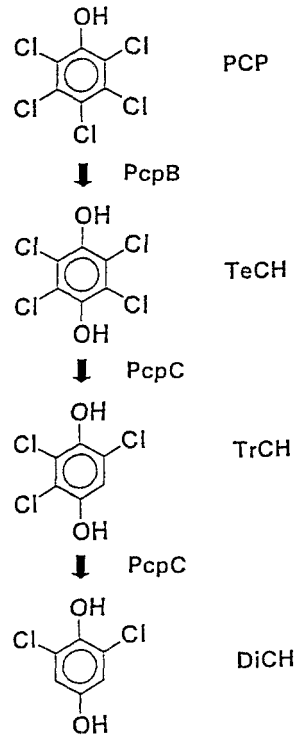

Fig. 1. Degradation pathway for bacterial catabolism of PCP as determined from identification of pathway intermediates. Abbreviations are TeCH: 2,3,5,6-tetrachloro-*p*-hydroquinone; TCH: 2,3,5-trichloro-*p*-hydroquinone; DiCH: 2,6-dichloro-*p*-hydroquinone, TrCOH: 2,5,6-trichloro-3-hydroxy-*p*-hydroquinone; DiCOH: 2,6-dichloro-3-hydroxy-*p*-hydroquinone; MCOH: monochloro-3-hydroxy-*p*-hydroquinone; TrOHB: 1,2,4-trihydroxybenzene.

Two pseudomonads have also been described in the literature to degrade PCP. The first, *Pseudomonas* sp. strain SR3, was isolated from PCP contaminated soils at the American Creosote Works, Pensacola, Florida (Resnick & Chapman unpublished). This microorganism was analyzed for use as a bioremediation tool in contained bioreactors, with > 88% of the PCP being removed from PCP contaminated ground water. The second, *Pseudomonas* sp. strain RA2, which was isolated from the PCP contaminat-

ed Broderick Wood Products site near Denver, Colorado. This bacterium has been reclassified as a *Sphingomonas* sp. and was shown to mineralize PCP at levels exceeding 160 ppm (Radehaus & Schmidt 1992). An overview of the degradation pathway to DiCH followed by all of the PCP mineralizing bacteria with the exception of *Rhodococcus chlorophenolicus* is presented in Fig. 1.

Biochemical analysis of PCP degradation

Until recently, the molecular biology of PCP degradation by the aforementioned bacteria was undefined, with the exception of the occasional reports involving crude extracts. Recently, it was shown that several proteins are induced by PCP in *Flavobacterium* sp. strain ATCC 39723 (Xun & Orser 1991a). One protein was purified and identified as a 30 kilodalton (kDa) PCP-induced periplasmic protein (PcpA). The purified protein was found to be missing in the F18 mutant of *Flavobacterium*, described by Steiert et al. (1987) as unable to degrade PCP beyond DiCH. The function of PcpA still remains to be elucidated.

Xun & Orser (1991b) also purified a 63 kDa PCP-induced protein from *Flavobacterium* and identified it as a PCP hydroxylase (PCP 4-monooxygenase), and reported on its reaction stoichiometries. This enzyme generates TeCH from PCP in the presence of O_2 and two NADPH (Xun et al. 1992c). PCP 4-monooxygenase has a diverse substrate range, with the ability to catalyze the *para* hydroxylation of numerous substituted phenols, removing hydrogen and halogen, cyano, and amino groups from the *para* position (Xun et al. 1992b). The gene for PCP 4-monooxygenase, *pcpB*, has been sequenced, and shown to be homologous to DNA from the PCP degrading *Arthrobacter* sp. strain ATCC 33790, *Pseudomonas* sp. strain SR3 based on Southern blot analysis (Orser et al. 1993a), and *Sphingomonas* (Orser unpublished). This correlates well with the reported production of TeCH from PCP in the presence of O_2 and NADPH by *Arthrobacter* cell extracts (Schenk et al. 1989). *Rhodococcus chlorophenolicus* genomic DNA does not hybridize with *pcpB* from *Flavobacterium*.

A nonredundant search of the National Center for Biotechnology Information (NCBI) databases, with the deduced amino acid sequence of PCP 4-monooxygenase as a query sequence, suggested similarity with numerous flavoprotein monooxygenases of bacterial origin involved in phenolic compound metabolism. The list, in descending order of similarity score, is shown in Table 1 with their corresponding alignment scores and NCBI database locations.

Multiple sequence alignment of PCP 4-monooxygenase with some of the highest scoring monooxygenases from NCBI, using the Genetic Computer Group (GCG) program PILEUP (Deveraux et al. 1984), revealed two highly conserved domains predicted to be involved with binding the flavin adenine dinucleotide (FAD) molecule (Fig. 2) (Lange et al. 1994). The first domain fits the consensus sequence of an ADP-binding $\beta\alpha\beta$-fold observed for many FAD-binding proteins (Bennett 1974; Eggink et al. 1990; Rossmann et al. 1974; Wierenga et al. 1986). The second domain is also involved in binding FAD, as predicted by a second conserved amino acid fingerprint (Bennett 1974). The similarity between the presumed FAD binding sites is further exemplified by the existence of shared epitopes. Tsuji et al. (1990) generated a monoclonal antibody that recognizes the FAD-binding site of 4-aminobenzoate hydroxylase, salicylate hydroxylase and D-amino acid oxidase, suggesting all contain similar structures. No observable NADPH-binding domain could be identified in the sequence based on the conserved $\beta\alpha\beta$-fold amino acid fingerprint.

The enzyme *p*-hydroxybenzoate hydroxylase from *P. fluorescens* is an external flavoprotein monooxygenase (Flashner & Massey 1974) that has become the model system for explaining the structures and catalytic mechanisms of many phenolic monooxygenases (Entsch et al. 1991; Flashner & Massey 1974; Schreuder et al. 1990). The NH_2-terminal 75% of PCP 4-monooxygenase shares 73% similarity with the amino acid sequence of *p*-hydroxybenzoate hydroxylase from *P. fluorescens*, and secondary structure predictions for the NH_2-terminal 75% of PCP 4-monooxygenase were similar to those predicted for *p*-hydroxybenzoate hydroxylase.

The multiple sequence alignment of the flavoprotein monooxygenases also revealed two regions abundant with tyrosine residues. These regions are positioned near the catalytically important tyrosine residues, Tyr-201, -221, and -385 of *p*-hydroxybenzoate hydroxylase (Entsch et al. 1991). Tyrosine residues at positions 216, 234, and 396 identified in PCP 4-monooxygenase may be involved in the formation and binding of a phenolate form of PCP, but this needs to be confirmed by site directed mutagenesis (Lange et al. 1994).

The structural domain implicated in dimerization of *p*-hydroxybenzoate hydroxylase, referred to as the

Table 1. Highest scoring monooxygenases from the BLAST search at the NCBI using PCP 4-monooxygenase as a query sequence. The monooxygenase substrates and the microbial sources of the monooxygenases are listed as well as their respective database locations at the NCBI.

Monoxygenase	Microorganism	Accession No.	Score
Pentachlorophenol	*Flavobacterium* sp.	(PIR) A40640	2833
Tetracenomycin	*Streptomyces glaucescens*	(PIR) S27689	211
Dichlorophenol	*Alcaligenes eutrophus*	(SP) P27138	207
Phenol	*Pseudomonas* sp.	(SP) P31020	200
Phenol	*Trichosporon cutaneum*	(GP) L04488	197
Phenol	*Pseudomonas pickettii*	(SP) Q91551	132
Aklavinone	*Streptomyces peucetius*	(GP) M73758	84
p-Hydroxybenzoate	*Pseudomonas fluorescens*	(GP) L13747	80
p-Hydroxybenzoate	*Pseudomonas aeruginosa*	(SP) P20586	80
p-Hydroxybenzoate	*Acinetobacter calcoaceticus*	(PIR) JU0142	80
p-Hydroxybenzoate	*Rhizobium leguminosarum*	(GP) L23969	79
Salicylate	*Pseudomonas putida*	(SP) P23262	77

GB = GenBank DNA sequence database; GP = GenPept (translated genbank); SP = SWISS-PROT protein database; PIR = PIR protein database.

interface domain in the model (Schreuder et al. 1990), corresponds to the COOH-terminal portion of PCP 4-monooxygenase. Because PCP 4-monooxygenase is suspected to form multimeric states (Xun & Orser 1991b), it is worth noting that the COOH-terminal region of PCP 4-monooxygenase may be involved with formation of multimeric states, or interactions with other proteins. Of particular interest to our lab is evidence for the existence of a PCP 4-monooxygenase reductase protein that may interact with PCP 4-monooxygenase (Lange & Orser unpublished data). Preliminary amino acid sequence analysis of an open reading frame in the region downstream of *pcpB* is highly similar to oxygenase reductases, cytochrome reductases and nitrate reductases from other microbial systems. Interactions with a reductase component could account for a larger interface domain at the COOH-terminal region of PCP 4-monooxygenase, as compared to that of *p*-hydroxybenzoate hydroxylase.

Further comparison between the *p*-hydroxybenzoate hydroxylase reaction mechanism described by Entsch et al. (1991) and the reactions catalyzed by PCP 4-monooxygense lends an explanation for the role of the second NADPH molecule required for oxidation of PCP but not required for the oxidation of tetrachlorophenol (TeCP) by the monooxygenase. We hypothesize that a very unstable chloroxide intermediate is formed at the C(4a) position of the isoalloxazine ring via a nucleophilic exchange reaction, and a 2,3,5,6-tetrachloro-semiquinone product is formed. The second NADPH would then be required for release of a chloride ion from the flavin and reduction of the semiquinone product to the hydroquinone form. The resulting hydroxyl at the C(4a) position would then condense with H^+ to form H_2O, and return the flavin ring to its original oxidized state. Replacement of the *para* chlorine with a hydrogen, such as in TeCP, would result in a similar process as described for *p*-hydroxybenzoate hydroxylase (Entsch et al. 1991), so that only one NADPH is required.

From whole cell studies of mutants derived from *Flavobacterium* strain ATCC 39723, TeCH was proposed to be reductively dehalogenated to DiCH (Steiert & Crawford 1986). We reported the detection of the reductive dehalogenation of TeCH to 2,3,6-trichloro-*p*-hydroquinone (TrCH) and then to DiCH by cell extracts of the *Flavobacterium* sp. strain ATCC 39723 with glutathione (GSH) as the reducing agent (Xun et al. 1992a). GSH is a common cofactor among eucaryotic detoxication enzymes classified as glutathione *S*-transferases (GSTs; EC 2.5.1.18) (Jakoby & Habig 1980). Although GSH has recently been reported as a cofactor for microbial dehalogenation (LaRoche & Leisinger 1990; Leisinger & Kohler-Staub 1990), the mechanism of halide removal was not reductive but rather by hydrolytic dechlorination of dichloromethane by *Hyphomicrobium* DM2.

A

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           1                                                        50
     TfdB  .......... .......... .......... .MALTIETDV LVVGTGPAGA
     PheH  MTTQRNDNLE QPGRSVIFDD GLSATDTPN. .ETNVVETEV LIVGSGPAGS
     TcmG  .......... .......... .MPVSDRPKG CILSTEEVPV LIVGGGLTGL
     PcpB  .......... .......... ...MSTYPIN APGQSADAAV LIVGGGPTGL
     PhhY  .......... .......... .......... .....MKTQV AIIGAGPSGL
Consensus  ---------- ---------- ---------- ------ET-V LIVG-G--GL

           51                                                       100
     TfdB  SAGALLARYG VRTMLINKYN ..WTAPTPRA HITNQRTMEI LRDLGLEAEA
     PheH  SAAMFLSTQG ISNIMITKYR ..WTANTPRA HITNQRTMEI LRDAGIEDQV
     TcmG  SAALFLSQHG VSCRLVEKHR ..GTTVLTRA SGISSRTMEL LRGVGLE.RT
     PcpB  IAANELLRRG VSCRMIDRLP ..VAHQTSKS CTIHARSMEM MEHIGIAARY
     PhhY  LLGQLLHKAG IDNVILERQT PDYVLGRIRA GVLEQGMVDL LREAGVDRRM
Consensus  SA---L-R-G V--R-IEK-- ---T--T-RA -----Q---E- LR-AG-E-R-
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B

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           351                                                      400
     TfdB  QGRVFCAGDA VHRHPPTNGL GSNTSIQDSF NLAWKIAMVL NGTADESLLD
     PheH  KGRVCCAGDA IHKHPPSHGL GSNTSIQDSY NLCWKLACVL KGQAGPELLE
     TcmG  SGRVFLAGDA AHVHPPAGAF GANGGIQDAH NLAWKLAAVL KGTAGDALLD
     PcpB  KGNVFLAGDA AHCHSPSGGS GMNVGMQDAF NLGWKIAMVE RGEAKPDLLD
     PhhY  HGRLFLAGDA AHIVPPTGAK GLNLAASDVS TLY.RLLLKA YREGRGELLE
Consensus  -GRV--AGDA AH---P---- GSN--IQD-- NL--KLA-VL ------ELLD

           401
     TfdB  TYTIERAPIA KQVVCRANKS
     PheH  TYSTERAPIA KQIVTRANGS
     TcmG  TYEQERLPIG AAVADQAWIR
     PcpB  TYHTERTPVA QQLLEGTHAM
     PhhY  RYSAICLRRI WKAERFS...
Consensus  TY-TE---I- -QVV--A---
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Fig. 2. Identification of the two FAD-binding domains in PCP 4-monooxygenase based on highly conserved regions of bacterial monooxygenases. The consensus sequences were acquired using the GCG program 'PRETTY'. Conserved amino acids in four out of five sequences are highlighted, and amino acid residue positions are given in parentheses. (A) The conserved ADP-binding ßαß-fold involved in binding FAD. (B) The second conserved domain involved in binding FAD. Abbreviations are: *TfdB*: 2,4-dichlorophenol monooxygenase from *Alcaligenes eutrophus*; *PheH*: phenol 2-monooxygenase from *Pseudomonas* sp.; *TcmG*: tetracenomycin monooxygenase from *Streptomyces glaucescens*; *PcpB*: PCP 4-monooxygenase from *Flavobacterium* sp.; *PhhY*: *p*-hydroxybenzoate 3-monooxygenase from *P. fluorescens*.

Reductive dehalogenation of aromatic compounds has long been recognized as a probable enzymatic mechanism, although no reductive dehalogenase has ever been purified from microorganisms (Reineke & Knackmuss 1988) prior to TeCH reductase from *Flavobacterium* (Xun & Orser 1992). The only reported reductive dehalogenase, a flavoprotein iodotyrosine deiodinase, was from bovine thyroid (Goswami & Rosenberg 1979). We purified the TeCH reductive dehalogenase by monitoring the conversion of TeCH to TrCH and DiCH (Xun & Orser 1992). Our data indicate that TeCH reductive dehalogenase utilizes GSH to reductively dechlorinate TeCH to TrCH with the production of GS-SG and the transient formation of an intermediate. TrCH as well as the intermediate formed appear to serve as subsequent substrates for the enzyme. Because the novel enzymic cascade reaction did not result in a homogeneous product, it was not possible to determine the stoichiometric basis of the reactions catalyzed by TeCH reductive dehalogenase. However, based on the observed enzymatic reactions, TeCH reductive dehalogenase should be classified as a GST. The catalytic mechanism of GSTs appears to depend on bringing the substrate and GSH into close proximity and on increasing the nucleophilicity of the thiol of GSH (Peterson & Guengerich 1988).

Eucaryotic GSTs have been studied extensively for their ability to bind both lipophilic compounds and GSH (Jakoby & Habig 1980). This dual binding capacity has resulted in very versatile proteins, albeit inefficient enzymes, for the organism confronted with various xenobiotics. This group of enzymes is capable of

recognizing a wide spectrum of substrates, but the catalytic attack is the same. GSTs activate the attack by GSH on an electrophilic center, usually electrophilic carbon sites, but electrophilic nitrogen or sulfur atoms may also be attacked (Douglas 1988). We did not determine if the activity of TeCH reductive dehalogenase is restricted to the attack of electrophilic carbons, or if it can attack electrophilic nitrogen or sulfur atoms as well. However, we do know that TeCH reductive dehalogenase does not utilize the GST 'test' substrate, 1-chloro-2,4-dinitrobenzene, as was reported for the dichloromethane dehalogenase isolated from the facultative methylotrophic bacteria, *Hyphomicrobium* and *Methylobacterium* (LaRoche & Leisinger 1990). In contrast, three GST isozymes isolated from *Proteus mirabilis* (Di Ilio et al. 1988; Mignogna et al. 1993) were reported to attack this substrate. The *Flavobacterium* enzyme appears to function as a 60,000 molecular weight dimer, a trait held in common among eucaryotic GSTs (Jakoby & Habig 1980) and the *Proteus mirabilis* GSTs, while the methylotrophic enzyme appears to function as a hexamer, but the monomer is still of similar size at 33 kDa (Leisinger & Kohler-Staub 1990). Two other bacterial GSTs have been reported, one for its involvement in degradation of the model lignin compound beta-etherase (Masai et al. 1993) and one for it role in fosfomycin resistance (Arca et al. 1990). Among reported GSTs, TeCH reductive dehalogenase appears to be unique.

Genetics of *flavobacterium*

Part of the reason for the slow acquisition of biochemical and molecular data for PCP degradation by *Flavobacterium* sp. strain ATCC 39723, and the other PCP degrading microorganisms, is the lack of a defined genetic system for these recalcitrant microbes. Previous attempts to mutagenize *Flavobacterium* had only slight success, generating a few chemically-induced mutants as described by Steiert and Crawford (1986). The generation of other PCP^- mutants, and attempts to transform *Flavobacterium* with exogenous plasmid DNA were unsuccessful (Saber 1987), and it was assumed that *Flavobacterium* could not be transformed.

The criteria for optimal transformation as described by Saunders & Saunders (1988) are: first, no endogenous plasmids should be present; second, the possibility of any existing restriction systems should be ruled out; third, a compatible replicating plasmid must be found; and fourth, a selectable marker must be known to work for the specific host. Assuming that *Flavobacterium* cells can be made competent, or survive the transformation protocols described in the literature, failure to meet any one of the above criteria can be enough to prevent transformation.

Flavobacterium sp. strain ATCC 39723 possesses at least one plasmid of approximately 100 kilobasepairs in size. Second, *Flavobacterium* DNA is not sensitive to certain restriction enzymes, and the sensitivity is independent of the GC content of the restriction enzyme recognition sequences. This indicates the presence of a DNA modification system whereby the DNA may be methylated at certain restriction enzyme recognition sequence, preventing their cleavage by the restriction enzyme. These systems are generally regarded as protection systems from foreign DNA, such as DNA from bacteriophages. Finally, a plasmid capable of replicating in *Flavobacterium* sp. strain ATCC 39723 has not been detected, and attempts to find one may be hindered if the bacterium is not first cured of a possible endogenous plasmid, and the restriction system is understood.

The gene encoding PCP 4-monooxygenase has been designated *pcpB* (Orser et al. 1993a). The open reading frame for *pcpB* is preceded by a typical *E. coli* ribosome binding site (GGAG) but there is little sequence similarity to a typical -10 or -35 promoter region upstream of the transcriptional start site. The N-terminal sequence deduced from the purified protein started with a serine residue. The alignment of the protein sequence with that of the predicted protein encoded by the gene shows the serine residue to be preceded by a methionine, which is apparently cleaved off in the mature protein. Serine is one of a small group of amino acids, when in the second amino acid position, that seems to permit the removal of the preceding fMet residue (Tsunasawa et al. 1985).

Fragments that hybridize to the *Flavobacterium pcpB* gene were found to be present in three other aerobic bacteria, *Arthrobacter* sp. strain 33790 (Schenk et al. 1989), *Pseudomonas* sp. strain SR3 (Resnick & Chapman personal communication), and *Sphingomonas* sp. (Radehaus & Schmidt 1992) all previously reported to dechlorinate PCP. We have cloned the hybridizing fragments of all three species for future sequence comparisons. No hybridization was detected with DNA from *Rhodococcus chlorophenolicus*. Furthermore, we did not expect nor observe a hybridizing fragment in the 4-chlorobenzoate degrading strain *Arthrobacter* sp. strain DSM 20407 (Müller et al.

1988). Whereas other microbial degradative pathway genes are typically clustered in operons and often on mobile elements, *pcpB* was not found to be part of an extensive operon or to be present on the 100 kb endogenous *Flavobacterium* plasmid. The isolated nature of the gene in relation to other dechlorinating functions could be attributed to its detoxication function in the cell. PCP 4-monooxygenase is characterized by having both a broad substrate specificity range (Xun et al. 1992b) and a broad catalytic range of reactions. These properties are also common for eucaryotic mixed function oxidases which have a general role in detoxication of foreign, lipophilic compounds (Jakoby 1980).

It is of interest that in *E. coli*, where the enzyme can be studied in isolation from the other *Flavobacterium* dechlorinating enzymes, PCP 4-monooxygenase performed as a detoxication enzyme, meaning that the dechlorination reaction occurred. The enteric membrane is not a barrier to the lipophilic compound PCP. It is curious that TeCH, the product of the enzymatic reaction, was found in the culture supernatant. *E. coli* probably has an inherent mechanism for eliminating toxic compounds like TeCH from its cytoplasm, or else the cells were dead and lysing. Furthermore, since *pcpB* was observed to be constitutively transcribed in *E. coli*, the specific regulatory components for *pcpB* are probably not present in *E. coli* (Orser et al. 1993a).

Results from our initial database searches with *pcpC,* the gene encoding TeCH dehalogenase, performed at the NCBI, revealed the existence of similarities between the *Flavobacterium* sp. TeCH reductase and the GSTs from carnation (Meyer et al. 1991), maize (Grove et al. 1988), and the bacterial GST from *Methylobacterium* sp. strain DM4 (La Roche & Leisinger 1990). In order to more thoroughly investigate the alignment of our GST with other GSTs, we chose two mammalian GSTs, one from human (Kikuchi et al. 1989) and one from guinea pig (Kamei et al. 1990) and the reported procaryotic GST from *Methylobacterium* sp. strain DM4 (La Roche & Leisinger 1990). Therefore, a total of six translated GST genes were aligned using the program PILEUP. Fig. 3 presents the most informative alignment of a subset of four of those sequences. Pairwise similarity scores between TeCH reductase and the maize GSTIII and the carnation GST were 0.46 and 0.43, respectively, whereas similarity scores with the other three GSTs ranged from 0.34 to 0.36 (Orser et al. 1993b).

The ORF for *pcpC* was preceded by a typical *E. coli* ribosome binding site (GAGG) with some sequence similarity of a typical promoter consensus sequence, having an AT rich region at −10 from the transcriptional start site. The native promoter for *pcpC* as cloned from *Flavobacterium* sp. strain ATCC 39723 did not direct transcription sufficiently to result in any detectable enzymatic activity in *E. coli*. However, placing *pcpC* under the p_{tac} promoter resulted in inducible enzymatic activity (Orser et al. 1993b). A region of inverted dyad symmetry which could be a potential Rho-independent terminator exists (+)28 to (+)68 nucleotides downstream of the stop codon. The N-terminal sequence of the purified protein initiated with a proline residue. The translated nucleotide sequence indicated that a methionine preceded the proline residue. We have previously reported (Orser et al. 1993a) that PCP 4-monooxygenase from *Flavobacterium* sp. strain ATCC 39723 also had a processed fMET, immediately followed by a serine residue. *pcpC*, in contrast to the two other genes we have characterized at the transcriptional level from *Flavobacterium* sp. strain ATCC 39723 (Orser et al. 1993a; Xun & Orser 1991a), was constitutively transcribed.

It is of interest that the *Flavobacterium* sp. GST held more similarity with two plant GSTs than with the two reported procaryotic GST sequences (La Roche & Leisinger 1990; Mignogna et al. 1993), whereas one reported procaryotic GST from *Methylobacterium* had equally high similarity scores with eucaryotic GSTs (La Roche & Leisinger 1990). However, in terms of the biochemistry this is not too surprising. The hydrolytic nature of the dechlorination reaction catalyzed by the GST from *Methylobacterium* sp. strain DM4 is very different from the reductive dechlorination catalyzed by the GST from the *Flavobacterium* sp. strain ATCC 39723. The maize GST III enzyme has been demonstrated to detoxify the herbicide ALACHLOR (Grove et al. 1988), whereas the GST from carnation was deduced from protein database searches of the predicted translational product of a nucleotide sequence of one senescence-related mRNA (Meyer et al. 1991). The actual role of the carnation GST during senescence is unknown.

More than one review has been written on the numerous electrophilic compounds, including many xenobiotics, that can serve as substrates for GSTs (Chasseaud 1979; Douglas 1988; Jakoby & Habig 1980). However, the natural substrates of GSTs are often not known. This is particularly true for the GST we reported from *Flavobacterium* sp. strain ATCC 39723. Even though we know that TeCH can serve as a substrate, we doubt that *pcpC* evolved for the PCP catabolism. Since *pcpC* is constitutively expressed and

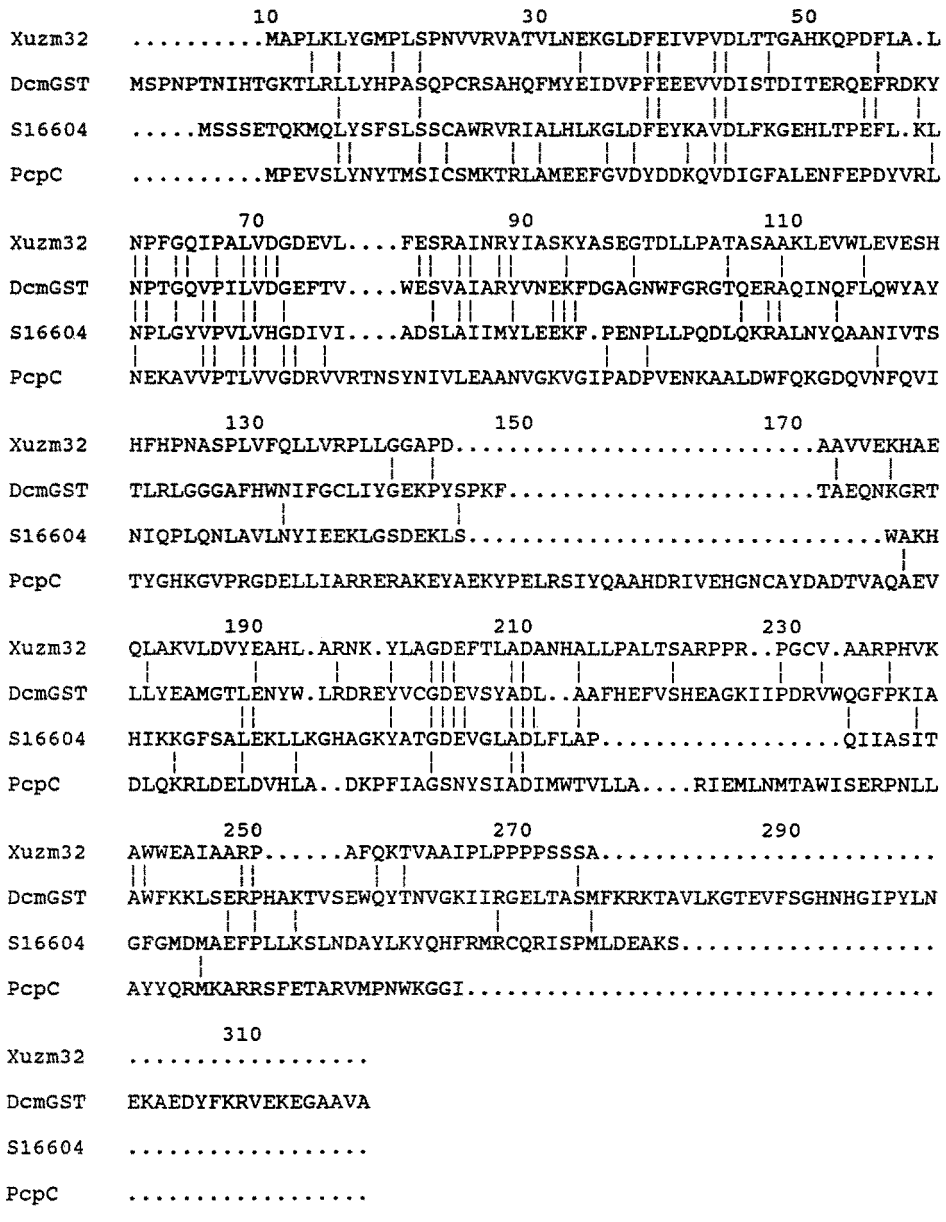

Fig. 3. Alignment of the amino acid sequences of *Flavobacterium* GST and GSTs from maize, *Methylobacterium*, and carnation. The alignment was accomplished using the GCG program 'PUBLISH'. Identical amino acids with no more than one mismatch are boxed. Abbreviations of the sequences used: Xuzm32, Maize GST III (9); Dcmgst, dichloromethane dehalogenase from *Methylobacterium* sp. strain DCM (16); S16604, GST from carnation (17).

increasing amounts of PCP did not influence the transcription, we suspect that this gene encodes an enzyme used in a general detoxication foray by this soil bacterium.

Sequence data acquired from the region downstream of *pcpB* have yielded two open reading frames (ORFs) (Fig. 4; Lange & Orser unpublished data). We believe these ORFs to be members of the PCP degradation pathway due to their high similarity to proteins from other metabolic pathways, including vanillate, phthalate, benzoate, toluate, and phenol catabolism in the case of the first ORF (*pcpD*) and naphthalene catabolism and nodulation formation in the case of the second ORF (*pcpR*).

The first ORF, *pcpD*, is directly downstream of *pcpB* and reads in the same direction as *pcpB*.

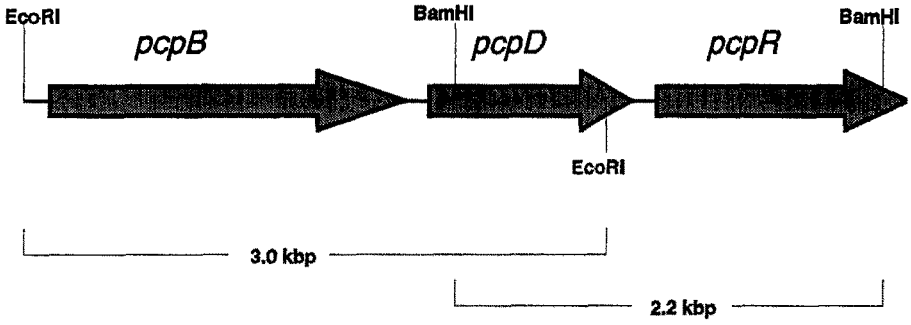

Fig. 4. Representation of the linear relationship between *pcpB* and two additional *pcp* alleles, *pcpD* and *pcpR*.

It has high similarity to the oxygenase reductases. This large group includes proteins such as VanB, a reductase component of vanillate demethylase (Brunel & Davison 1988); PDR, phthalate dioxygenase reductase; BenC, the benzoate dioxygenase reductase; XylZ, toluate dioxygenase reductase; MemC, methane monooxygenase component C; XylA, xylene monooxygenase reductase; phenol hydroxylase P5 protein; NADH ubiquinone oxidoreductase subunit; and nitrate reductase, all of which are members of the ferredoxin-$NADP^+$ reductase family that also includes cytochrome P-450 reductase, cytochrome b_5 reductase, and sulfite reductase (Karplus et al. 1991; Correll et al. 1992). If the sequence analysis is correct, then *pcpD* would encode a PCP 4-monooxygenase reductase protein involved in transferring electrons from NADPH, through the redox centers of PcpD, to the flavin adenine dinucleotide (FAD) prosthetic group of PCP 4-monooxygenase.

The oxygenase reductases are characteristic iron-sulfur proteins containing two domains involved in binding the prosthetic groups flavin mononucleotide (FMN) and the [2Fe-2S] center, and a third domain involved in binding nicotinamide adenine dinucleotide (NAD) cofactors. The oxygenase reductases are electron transport proteins involved in the transfer of electrons from reduced NAD to the FMN prosthetic group, then to the [2Fe-2S] center, and finally to the oxygenase component. One representative of this group of enzymes has been resolved by X-ray crystallography. Phthalate dioxygenase reductase (PDR) has been crystallized from *Pseudomonas cepacia* and examined in great detail, and presently serves as a model system. The PDR ferredoxin center is very similar to plant [2Fe-2S] ferredoxins, involving a CyS-X_4-CyS-X_2-CyS [2Fe-2S]-binding loop (Batie et al. 1987; Correll et al. 1992).

The second ORF, *pcpR*, that has been identified is found downstream from *pcpD*, and is read in the same direction as *pcpB* and *pcpD*. This ORF has been sequenced and has high amino acid sequence similarity to the activator protein from the naphthalene degradation regulon of *P. putida*, NahR (Schell & Sukordhaman 1989) and the activators for nodulation in *Rhizobium, Bradyrhizobium and Azorhizobium* species, NodD and SyrM (Barnett & Long 1990), as well as many other members of the LysR family of transcriptional activators (Lange & Orser unpublished data).

The members of the LysR family are typically 32–36 kilodaltons in size, and characterized as requiring an inducing compound for activation. Their similarity is highest at the NH_2-terminal region which is responsible for a helix-turn-helix DNA-binding motif that recognizes the DNA sequence termed the LysR motif (A-T-C-N9-G-A-T) they are often subject to negative autoregulation and their transcription frequently reads divergently from the genes which they control (Schlaman et al. 1992).

We have long suspected that PCP degradation is controlled by positive regulation due to the nature of enzyme induction in *Flavobacterium* in the presence of PCP, the presence of a putative LysR motif in the DNA sequence upstream of both the inducible *pcpA* and *pcpB* genes (Orser unpublished data), and the common occurrence of positive regulation in numerous systems involved in aromatic catabolism. Other positively regulated aromatic catabolic pathways include naphthalene and chlorocatechol (Coco et al. 1993; Rothmel et al. 1990; Van der Meer et al. 1991), phenol (Shingler et al. 1993), and 2,4-dichlorophenoxyacetic acid (Kaphammer & Olsen 1990). Surprisingly, the gene,

which we are calling *pcpR* would be transcribed in the same direction as *pcpB* and *pcpD* and not divergently as is the case for at least three other positive regulators including *nahR* for the naphthalene pathway and *nodD* involved in nodulation.

Transformation and gene disruption in *flavobacterium*

A missing link in biochemical and genetic analysis of the *Flavobacterium* dechlorination reactions had been the ability to conduct genetics in vivo. As we have now successfully transformed and site mutagenized *Flavobacterium*, this is no longer an obstacle to our continued progress. In particular we have recently demonstrated the ability to genetically manipulate the gene, *pcpB*, involved with PCP degradation in *Flavobacterium* sp. strain ATCC 39723 (Lange et al. 1994). To confirm the role of PCP 4-monooxygenase, encoded by *pcpB*, as the sole enzyme involved in the primary dechlorination of PCP by *Flavobacterium*, we sought to construct a genetic disruption of the gene. We successfully accomplished this by first physically disrupting the cloned *pcpB* gene with a DNA fragment containing the *nptII* gene from the plasmid pDM100 (Meletzus & Eichenlaub 1991), followed by transformation and chromosomal exchange in *Flavobacterium sp.* strain ATCC 39723 (Lange et al. 1994). Two groups of transformants resulted, cointegrates which contained both a wildtype copy of *pcpB* and a *nptII*-interrupted copy of *pcpB*; and resolved isogenic strains containing only the *nptII*-interrupted copy of *pcpB*. Two of the cointegrate strains were designated FCO313 and FCO314, and the two resolved strains FCO315 and FCO316. To establish the impact of the *pcpB* interruption on PCP 4-monooxygenase activity, whole cells of the cointegrates, FCO313 and FCO314, and of the isogenic mutant, FCO315, were used in PCP degradation assays and compared with the parental strain, *Flavobacterium* sp. strain ATCC 39723. As expected, the isogenic strain, FCO315, which contained a single disrupted copy of *pcpB* had lost its ability to degrade PCP, whereas the two cointegrates, FCO313 and FCO314, had retained some competence to degrade PCP. These data collectively demonstrate the primary oxygenolytic step in PCP degradation is mediated by PCP 4-monooxygenase in *Flavobacterium* sp. cells (Lange et al. 1994).

Relationships among bacterial PCP degraders

With every observation of microbial metabolism of a xenobiotic compound, one has to wonder where the genetic capacity came from. A true xenobiotic compound is one which is foreign to existing enzyme systems. This status may in fact be the case for many of the synthetic polyhalogenated compounds that have become recalcitrant pollutants in our environment. However, microorganisms evidently play a major role in the eventual breakdown of these pollutants (Alexander 1981). Very little is known about how microbial communities become 'adapted' to the presence of xenobiotic compounds. There is good reason to study these adaptive events as it may reveal the underlying principles of metabolic diversification in bacteria in general (Van der Meer et al. 1992). This type of information would be of value in predicting the outcome of bioremediation efforts at contaminated sites.

A longterm goal of our research is to eventually speculate on a progenote function of the enzymes which have evidently been co-opted for the purpose of PCP mineralization by a certain group of microbes. One approach to this enigma is through the evaluation of the interrelatedness and distribution of *pcp* gene homologues within the microbial community. Unfortunately conclusions made from sequence interrelatedness cannot be experimentally verified as we only have access to the final evolved microbes with no existing bacterial fossil record. Nonetheless, there is precedence for the proposed common evolutionary pathways of the different aromatic pathway enzymes, such as the β-ketoadipate pathway (Ornston & Neidle 1991) in *Acinetobacter* and *Pseudomonas*. The *pcp* genes should also prove useful in the identification of PCP-degrading competence in given soil and water samples, for tracking the release of PCP-degraders, as well as in the accumulation of a diverse group of PCP-degrading microorganisms for evolutionary analysis of dechlorination. We are interested in using the gene *pcpB* in a study of the evolution of the ability to dechlorinate PCP. *pcpB* may be a useful character for phylogenetic analysis of genetic similarities among aerobic dechlorinating microbes.

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