JOURNAL OF BACTERIOLOGY, Jan. 1993, p. 411–416
0021-9193/93/020411-06$02.00/0

Vol. 175, No. 2

Cloning, Sequence Analysis, and Expression of the *Flavobacterium* Pentachlorophenol-4-Monooxygenase Gene in *Escherichia coli*†

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Received 27 July 1992/Accepted 2 November 1992

The *pcpB* gene of *Flavobacterium* sp. strain ATCC 39723 was cloned by using a degenerate primer designed from the N-terminal sequence of the purified enzyme. The nucleotide sequence of *pcpB* was determined and found to encode an open reading frame of 1,614 nucleotides, yielding a predicted translation product of 538 amino acids, in agreement with the estimated size of the purified protein analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The transcriptional start of *pcpB* was found to be 80 bp upstream of the translational start, and the transcript was found to be induced in *Flavobacterium* sp. strain ATCC 39723 by the presence of pentachlorophenol but to be constitutive in the *Escherichia coli pcpB* clone. DNA hybridizations with genomic DNAs from *Arthrobacter* sp. strain ATCC 33790 and *Pseudomonas* sp. strain SR3 revealed a similar-size 3.0-kb *Eco*RI fragment, whereas there was no positive hybridization with genomic DNA from *Rhodococcus chlorophenolicus*. Cell extracts from an *E. coli pcpB* overexpression strain, as well as the whole cells, were proficient in the dechlorination of pentachlorophenol to tetrachlorohydroquinone. Protein data base comparisons of the predicted translation products revealed regions of homology with other microbial monooxygenases, including phenol-2-monooxygenase and tryptophan-2-monooxygenase.

Pentachlorophenol (PCP) is a polychlorinated aromatic compound widely used as a preservative in wood industries. Several microorganisms have been isolated for their abilities to degrade PCP (1, 14, 17, 20, 22). Our laboratory has been characterizing the biochemistry and genetics of the dechlorination of PCP by the gram-negative *Flavobacterium* sp. strain ATCC 39723. We have previously demonstrated that PCP induces the presence of several proteins in *Flavobacterium* sp. strain ATCC 39723 (23) and reported the purification of PCP hydroxylase (24), heretofore referred to by its more specific name of PCP-4-monooxygenase (26). PCP-4-monooxygenase converts PCP to 2,3,5,6-tetrachloro-*p*-hydroquinone (TeCH) in the presence of oxygen and NADPH (24, 26). This enzyme not only catalyzes dehalogenation but also removes hydrogen and nitro, amino, and cyano groups from the benzene ring at the *para* position in relation to the hydroxyl of phenol (25). Here, we report the cloning, sequencing, and expression of the corresponding genetic determinant, *pcpB*, for PCP-4-monooxygenase.

The long-range goal of our research is to understand the molecular mechanism of dehalogenation that has evolved among diverse microorganisms to inactivate the oxidative phosphorylation uncoupler, PCP. We are interested in using gene *pcpB*, reported here, 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.

(A preliminary account of this work has previously appeared as an abstract [11]).

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MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *Escherichia coli* HB101 and JM105 were used as recombinant hosts. *Flavobacterium* sp. strain ATCC 39723 (14), provided by R. Crawford (University of Idaho), *Pseudomonas* sp. strain SR3, provided by S. Resnick (University of Iowa), *Arthrobacter* sp. strain ATCC 33790 (17), obtained from the American Type Culture Collection, and *Rhodococcus chlorophenolicus* (1), obtained from M. Salkinoja-Salonen (University of Helsinki, Helsinki, Finland), have previously been reported to degrade PCP. *Arthrobacter* sp. strain DSM 20407 (10) was originally described for its ability to degrade 4-chlorobenzoate. *Flavobacterium* sp. strain ATCC 39723 was cultured in mineral medium and induced for PCP degradation as previously described (14). Plasmid pBluescript II was obtained from Stratagene (La Jolla, Calif.). Expression vector pKK223-3 was obtained from Pharmacia Inc. (Piscataway, N.J.).

Enzymes and chemicals. Restriction endonucleases, modifying enzymes, and isopropyl-β-D-thiogalactopyranoside (IPTG) were purchased from U.S. Biochemical Corp. (Cleveland, Ohio). Protein standards were from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). [α-^{32}P]dCTP, [γ-^{32}P]dATP, and [α-^{35}S]dATP were from New England Nuclear Corp. Goat anti-rabbit conjugate alkaline phosphatase was purchased from Tago, Inc. (Burlingame, Calif.). 5-Bromo-4-chloro-3-indolyl phosphate and *p*-nitroblue tetrazolium chloride were purchased from Kirkegaard & Perry Laboratories (Gaithersburg, Md.). All other chemicals were purchased from Sigma.

Assay for polypeptides with PCP-4-monooxygenase antibody. *E. coli* CCL3, harboring a *pcpB* overexpression plasmid, and the vector-alone control, *E. coli* CCL5, were evaluated for their reactions to a PCP-4 monooxygenase-specific antibody. *E. coli* CCL3 and CCL5 were grown in

Luria broth with the appropriate selection with or without IPTG induction and assayed for the presence of proteinaceous material cross-reacting with the PCP-4-monooxygenase antibody. The cells were pelleted, lysed with lysis buffer (2% β-mercaptoethanol, 100 mM Tris hydrochloride [pH 7.0], 2% sodium dodecyl sulfate [SDS], 10 mM EDTA, 20% glycerol, 1 mM phenylmethylsulfonyl fluoride), and resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (8) with 12% acrylamide. The proteins resolved in the polyacrylamide gel were subsequently transferred to a nitrocellulose membrane by using a Bio-Rad (Richmond, Calif.) minitransfer cell, and immunoblots were performed as described below in "Analytical methods."

Nucleic acid extraction, cloning, and transcript mapping. Plasmid DNA was extracted by the CTAB method of Del Sal et al. (5). Preparative isolation of genomic and plasmid DNAs was via cesium chloride-ethidium bromide density gradients.

RNA isolation and transcript mapping were done as described by Summers (19) and Jones et al. (7). A 33-mer designated PcpB19, complementary to the *pcpB* sequence at nucleotide positions 473 to 505, was end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Following annealing to total RNA, primer extension was carried out by avian myeloblastosis virus reverse transcriptase. The products were resolved on a 6% polyacrylamide gel containing 7 M urea and visualized by autoradiography. Northern (RNA) analysis was done essentially as described by Sambrook et al. (15).

DNA sequence analysis. The PCP-4-monooxygenase structural gene from *Flavobacterium* sp. strain ATCC 39723 was sequenced by the dideoxy chain termination method of Sanger et al. (16) by using [α-^{35}S]dATP and either commercial primers or primers designed for the sequence. Single-stranded DNA templates were recovered from pBluescript phagemid subclones by using the helper phage, VCSM13, as recommended by Stratagene. Compilation of sequence data and DNA sequence analysis were accomplished by using PCGene software, version 6.01 (Intelligenetics, Mountain View, Calif.).

Preparation of cell extracts and whole-cell assay. *E. coli* CCL3 was cultured in 1 liter of Luria broth containing 50 mg of ampicillin per ml. At an optical density at 600 nm of 0.7, IPTG was added to a final concentration of 1 mM. After 2 to 3 h of incubation at room temperature with shaking, the cells were pelleted and used in the preparation of cell extracts as described previously (24). Ammonium sulfate fractions (40 to 60%) were dialyzed for 4 h against 1 liter of 20 mM Tris–5 mM EDTA buffer (pH 8.0) at 4°C and then reprecipitated with 60% ammonium sulfate and stored at −20°C in 50% glycerol with 2 mM dithiothreitol. The PCP-4-monooxygenase enzyme assay was run as previously described for *Flavobacterium* sp. strain ATCC 39723 (24). Spectrophotometric whole-cell assays were run as previously described (14), with all cells, including *E. coli*, cultured at room temperature. Verification of the production of TeCH from PCP was confirmed by high-pressure liquid chromatography (HPLC) retention time.

Analytical methods. Proteins were assayed by SDS-PAGE (10% acrylamide) using a Bio-Rad protein apparatus and quantified by the Lowry assay (9). Immunoblotting was performed with a slight modification of the procedure described by Bollag and Edelstein (3) by using a Bio-Rad transblot electrophoretic transfer cell. Nonspecific binding of antibody was blocked by a 1-h incubation in 3% gelatin at 37°C with gentle agitation. A 1:1,000 dilution of rabbit polyclonal antibody, prepared by Berkeley Antibody Co. (Richmond, Calif.), was used for detection of PCP-4-monooxygenase. Labeling with goat anti-rabbit conjugate alkaline phosphatase and detection were performed as recommended by the manufacturers. Laser densitometry of Western blots (immunoblots) was performed with a Zeineh soft-laser scanning densitometer (model SLR-1D/2D; Biomed Instruments Inc., Fullerton, Calif.). Enzyme assays were performed as previously described by using a Waters HPLC (24). The N-terminal amino acid sequence of the purified protein was determined on an Applied Biosystems automated protein sequenator (Applied Biosystems, Foster City, Calif.). Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer.

Nucleotide sequence accession number. The nucleotide sequence of the *Flavobacterium* sp. strain ATCC 39723 *pcpB* gene has been assigned GenBank accession number M98557.

RESULTS

N-terminal sequence of PCP-4-monooxygenase and cloning of *pcpB*. The N-terminal sequence (residues 1 to 13) of PCP-4-monooxygenase was determined to be Ser-Thr-Tyr-Pro-Ile-Asn-Ala-Pro-Gly-Gln-Ser-Ala-Asp. From the N-terminal sequence, a 37-mer degenerate primer corresponding to amino acid residues 2 to 13 was designed for use as a hybridization probe to identify the corresponding gene, *pcpB*. The primer was designed to account for our previous knowledge of codon preference in *Flavobacterium* strain ATCC 39723 (23). The primer was designated LX-6 and had the following 5′-to-3′ sequence: AC(C/G)TA(T/C)CC(C/G)AT(C/T)AA(T/C)GC(G/C)CC(C/G)GG(G/C)CA(G/A)(A/T)(G/C)(C/G)GC(G/C)GA(C/T)I. DNA hybridization of randomly ^{32}P-labeled primer LX-6 to *Eco*RI-digested total genomic DNA from *Flavobacterium* sp. strain ATCC 39723, separated on a 0.8% agarose gel, revealed a single 3.0-kb fragment. *Eco*RI-digested total genomic DNA was separated on a preparative gel, the region spanning 3 kb was excised, and the DNA was electroeluted. The eluted *Eco*RI DNA fragments were ligated to *Eco*RI-digested pBluescript KS$^-$ DNA, which encodes ampicillin resistance. Amp^r *E. coli* transformants containing the specific fragment were identified by colony hybridization. One such positive clone, designated CO221, and the corresponding hybrid construct, pCO221, were isolated. The *Eco*RI insert of pCO221 was subcloned into the same vector as two *Eco*RI-*Bam*HI fragments, 2.3 and 0.7 kb, and designated pCO222 and pCO223, respectively.

We found that *Flavobacterium* sp. strain ATCC 39723 contained one visible plasmid of approximately 100 kb. Isolated plasmid DNA was banded twice in CsCl-EtBr gradients to eliminate contaminating genomic DNA and evaluated by Southern analysis using the 2.3-kb *Eco*RI-*Bam*HI fragment as a probe. In two separate experiments, the probe did not hybridize with the plasmid DNA.

Genomic DNAs were isolated from other microorganisms reported to dechlorinate PCP, as well as from *Arthrobacter* sp. strain DSM 20407, a 4-chlorobenzoate degrader, and from *E. coli*. Using the 2.3-kb *Eco*RI-*Bam*HI fragment containing *pcpB*, Southern analysis revealed the presence of a hybridizing 3.0-kb *Eco*RI fragment in *Flavobacterium* sp. strain ATCC 39723, *Pseudomonas* sp. strain SR3, and *Arthrobacter* sp. strain ATCC 33790 but not in *R. chlorophenolicus*, *Arthrobacter* sp. strain DSM 20407, or the *E. coli* control (Fig. 1). We have cloned the hybridizing fragments

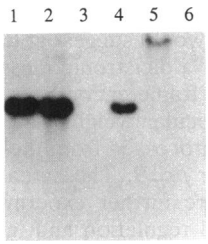

FIG. 1. Southern analysis of *Eco*RI-digested genomic DNAs probed with the 2.3-kb *Eco*RI-*Bam*HI fragment from *pcpB*. Lane 1, *Pseudomonas* sp. strain SR3; lane 2, *Flavobacterium* sp. strain ATCC 39723; lane 3, *Arthrobacter* sp. strain DSM 20407; lane 4, *Arthrobacter* sp. strain ATCC 33790; lane 5, *E. coli* JM105; lane 6, *R. chlorophenolicus*.

from both *Pseudomonas* sp. strain SR3 and *Arthrobacter* sp. strain ATCC 33790 for future sequence comparisons.

Nucleotide sequence and transcript mapping of *pcpB*. The entire coding sequence and promoter region for *pcpB* were found to reside within the 3.0-kb *Eco*RI fragment of pCO221. The 3.0-kb *Eco*RI fragment was subcloned, and a 2.3-kb *Eco*RI-*Bam*HI fragment was sequenced in both orientations. Within this sequence, there was an open reading frame of 1,614 nucleotides in length, which we designated *pcpB* (Fig. 2). The predicted translation of the nucleotide sequence of *pcpB* was identical to the N-terminal sequence of the purified protein. The predicted translational product of *pcpB* was 538 amino acids in length with a predicted molecular weight of 59,932, which is comparable to 63,000, the estimated molecular weight of the purified protein analyzed by SDS-PAGE (24).

Nucleotide data base searches revealed no sequences with greater than 56% similarity, but that degree of similarity did include both *tfdB*, which encodes 2,4-dichlorophenol hy-

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   CCTTATG CGGCCGGCAC GCAGGCCTGG TCTGTGCGTG AACCGGGCGG CTTATAGGCT   57

CCCATGGTTT AACTTTGCAA CCATAATGTC GCCGGGCGTT ACCTTTGCGC GATCGCCCGC  117

CCGCAAGACC GATTCCGCCG CAAGCCGCGC TCATTTTCCG GCGCAGGTTA TTCAGGTTCC  177

GAATGCCCCA ATTCGACCGC ACCGCCTATC GACGTTAACA TCCGCGCCGG GCTTTGATAC  237

CCGGCAAAAG CAAATGACGG CCAAGTTCAT TATGAACAGG CACGTTATTT GGCAGAATCC  297

AAAGTATTCA TCGGGAGAGA GATTGTTATT  ATG TCG ACC TAT CCA ATC AAT GCG  351
                                  Met Ser Thr Tyr Pro Ile Asn Ala
                                   1               5

CCG GGC CAA TCC GCC GAT GCC GCG GTT TTG ATC GTC GGC GGC GGG CCG  399
Pro Gly Gln Ser Ala Asp Ala Ala Val Leu Ile Val Gly Gly Gly Pro
     10                  15                  20

ACG GGG CTG ATT GCG GCC AAT GAA TTG CTG CGC CGC GGC GTA TCG TGC  447
Thr Gly Leu Ile Ala Ala Asn Glu Leu Leu Arg Arg Gly Val Ser Cys
 25                  30                  35                  40

CGC ATG ATC GAT CGC CTG CCG GTC GCT CAC CAG ACG TCC AAA TCC TGC  495
Arg Met Ile Asp Arg Leu Pro Val Ala His Gln Thr Ser Lys Ser Cys
                 45                  50                  55

ACC ATC CAT GCA AGA TCG ATG GAG ATG ATG GAA CAT ATC GGC ATC GCC  543
Thr Ile His Ala Arg Ser Met Glu Met Met Glu His Ile Gly Ile Ala
             60                  65                  70

GCC CGC TAC ATA GAA ACG GGC GTC AGG AGC AAC GGG TTC ACG TTC AAC  591
Ala Arg Tyr Ile Glu Thr Gly Val Arg Ser Asn Gly Phe Thr Phe Asn
         75                  80                  85

TTC GAG AAT ACG GAT GCG AAC GCG CTG CTC GAC TTT TCC GTC CTG CCG  639
Phe Gly Asn Thr Asp Ala Asn Ala Leu Leu Asp Phe Ser Val Leu Pro
     90                  95                 100

GGC AGA TAT CCG TTC ATC ACC ATC TAT AAC CAG AAT GAA ACC GAA CGG  687
Gly Arg Tyr Pro Phe Ile Thr Ile Tyr Asn Gln Asn Glu Thr Glu Arg
105                 110                 115                 120

GTG CTG CGG CAC GAT CTG GAG GCG ACC TAC AGC TTC CAG CCG GAA TGG  735
Val Leu Arg His Asp Leu Glu Ala Thr Tyr Ser Phe Gln Pro Glu Trp
                125                 130                 135

GGC ACG CAG TTG CTG GCG CTC AAT CAG GAT GAA AAC GGC ATC CGG GCT  783
Gly Thr Gln Leu Leu Ala Leu Asn Gln Asp Glu Asn Gly Ile Arg Ala
            140                 145                 150

GAT CTG AGG CTG AAG GAC GGG ACG AAG CAG ACG ATC TCC CCG CGC TGG  831
Asp Leu Arg Leu Lys Asp Gly Thr Lys Gln Thr Ile Ser Pro Arg Trp
        155                 160                 165

GTG ATC GGC GCG GAC GGC GTG CGC AGC CGC GTC CGC GAA TGC CTG GGC  879
Val Ile Gly Ala Asp Gly Val Arg Ser Arg Val Arg Glu Cys Leu Gly
    170                 175                 180

ATC GCC TAT GAA GGC GAG GAT TAT GAA GAA AAT GTC CTT CAG ATG ATG  927
Ile Ala Tyr Glu Gly Glu Asp Tyr Glu Glu Asn Val Leu Gln Met Met
185                 190                 195                 200

GAC GTC GGC ATC CAG GAT TTC GAA GCG GGC GAC GAC TGG ATT CAC TAT  975
Asp Val Gly Ile Gln Asp Phe Glu Ala Gly Asp Asp Trp Ile His Tyr
                205                 210                 215

TTC ATC GGT CAG GAC AAA TTC GTC TTC GTG ACG AAG CTG CCG GGT TCC 1023
Phe Ile Gly Gln Asp Lys Phe Val Phe Val Thr Lys Leu Pro Gly Ser
            220                 225                 230

AAT TAT CGC GTG ATT ATC AGC GAC CTT GGC GGC GCC AAC AAA TCG AAT 1071
Asn Tyr Arg Val Ile Ile Ser Asp Leu Gly Gly Ala Asn Lys Ser Asn
        235                 240                 245

CTG GAA GAA ACG CGG GAA GCC TTC CAG GGC TAT CTC AGT TCC TTC GAC 1119
Leu Glu Glu Thr Arg Glu Ala Phe Gln Gly Tyr Leu Ser Ser Phe Asp
    250                 255                 260

GAT CAT GCG ACG CTC GAC GAG CCG CGT TGG GCG ACC AAA TGG CGG GTG 1167
Asp His Ala Thr Leu Asp Glu Pro Arg Trp Ala Thr Lys Trp Arg Val
265                 270                 275                 280

TGG AAG CGG ATG GCG ACG GCC TAT CGC AAG GGC AAC GTC TTC CTG GCA 1215
Trp Lys Arg Met Ala Thr Ala Tyr Arg Lys Gly Asn Val Phe Leu Ala
                285                 290                 295

GGC GAC GCG GCG CAT TGC CAT TCG CCG TCG GGC GGC AGC GGC ATG AAC 1263
Gly Asp Ala Ala His Cys His Ser Pro Ser Gly Gly Ser Gly Met Asn
            300                 305                 310

GTC GGC ATG CAG GAC GCC TTC AAC CTG GGC TGG AAG ATC GCC ATG GTG 1311
Val Gly Met Gln Asp Ala Phe Asn Leu Gly Trp Lys Ile Ala Met Val
        315                 320                 325

GAA CGC GGC GAA GCC AAG CCC GAC CTG CTC GAC ACC TAT CAT ACC GAA 1359
Glu Arg Gly Glu Ala Lys Pro Asp Leu Leu Asp Thr Tyr His Thr Glu
    330                 335                 340

CGG ACG CCC GTC GCC CAG CAG TTG CTG GAA GGC ACG CAC GCC ATG CAT 1407
Arg Thr Pro Val Ala Gln Gln Leu Leu Glu Gly Thr His Ala Met His
345                 350                 355                 360

GAG ATC ATC ATG GGG CAT GGC AAG GGC CTG ACC GAC CGC ATC GAA TTG 1455
Glu Ile Ile Met Gly His Gly Lys Gly Leu Thr Asp Arg Ile Glu Leu
                365                 370                 375

ACG CAG GCG CCC GGT TGG CAT GAC GCC GCC ACC TAC CGC GTG TCG GGC 1503
Thr Gln Ala Pro Gly Trp His Asp Ala Ala Thr Tyr Arg Val Ser Gly
            380                 385                 390

ATG TCC TAT AAT TAT CGC GAC CAG CTC GTC AGC TTC AAC GAC GAC CGG 1551
Met Ser Tyr Asn Tyr Arg Asp Gln Leu Val Ser Phe Asn Asp Asp Arg
        395                 400                 405

CTG GCC GGA CCC AGC GCT GGC GAC CGC ATT CCC GAC GCG GAA CTG GCG 1599
Leu Ala Gly Pro Ser Ala Gly Asp Arg Ile Pro Asp Ala Glu Leu Ala
    410                 415                 420

CCC CGC ATC CGG TTG TTC GAC CTG GTC CGC AAC ACC CGG CCG ACG CTG 1647
Pro Arg Ile Arg Leu Phe Asp Leu Val Arg Asn Thr Arg Pro Thr Leu
425                 430                 435                 440

CTC GTG GCG CCC GCG ACC GAA GCG GAA GTG GCG GAA GCG GAG AAG CTG 1695
Leu Val Ala Pro Ala Thr Glu Ala Glu Val Ala Glu Ala Glu Lys Leu
                445                 450                 455

CGC GAC CTG ATC CGC GAG CAG TGG CCG CTG GTG AAG CCC GTC CTC GTC 1743
Arg Asp Leu Ile Arg Glu Gln Trp Pro Leu Val Lys Pro Val Leu Val
            460                 465                 470

CGT CCG CAG GGA AGC GAG GAA TCC ATC GAG GGC GAC GTC CAT GTC GAC 1791
Arg Pro Gln Gly Ser Glu Glu Ser Ile Glu Gly Asp Val His Val Asp
        475                 480                 485

AGC TAT GGC CAG CTC AAG CGC GAA TGG GGC GAC AAT GCG AAG GGA TGG 1838
Ser Tyr Gly Gln Leu Lys Arg Glu Trp Gly Asp Asn Ala Lys Gly Trp
    490                 495                 500

GCG GCG CTG TTG AGG CCG GAC AAC TAC ATC CAT GCG CGG GCG GGC CTG 1887
Ala Ala Leu Leu Arg Pro Asp Asn Tyr Ile His Ala Arg Ala Gly Leu
505                 510                 515                 520

GAT CGC GGC GAT CTT CTG GTC CAG GCG ATC GAC GCG ATG CTT GTG CCG 1935
Asp Arg Gly Asp Leu Leu Val Gln Ala Ile Asp Ala Met Leu Val Pro
                525                 530                 535

TGC GCC TGA          GGAGACCCGT GCGATGACAA ACCCCGTTTC GACAATCGAC 1984
Cys Ala ---
        539

ATGACGGTCA CGCAGATCAC CCGCGTGGCC AAGGACATCA ACTCTTACGA ACTTCGCCCG 2044

GAACCCGGCG TGATATTGCC GGAGTTCACC GCGGGGGCGC ATATCGGCGT TTCGCTTCCC 2104

AACGGGATCC AGCGCACGTA TTCGCTCGTC AACCCGCAGG CGAGAGGGAC CGTTACGTGA 2164

TCACGGTCAA CCTCGACCGC AACAGCCGGG GCGGTCGCGC TACCTCCACG AGCAGTTGCG 2224
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FIG. 2. Nucleotide sequence of the *Flavobacterium* sp. strain ATCC 39723 *pcpB* gene, including upstream and downstream flanking regions and the predicted translation product. The transcriptional start (−80) is underlined, as is the putative ribosome binding site.

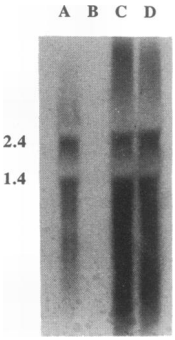

FIG. 3. Northern analysis of *pcpB*. The 2.3-kb *Eco*RI-*Bam*HI fragment containing *pcpB* was hybridized to total RNA from either *Flavobacterium* sp. strain ATCC 39723 or CO221. Lanes contain RNAs as follows: A, PCP-induced, ATCC 39723; B, uninduced, ATCC 39723; C, PCP-induced, CO221; D, uninduced, CO221. Molecular sizes in kilobases are shown on the left.

droxylase (12), and *vanAB*, which encodes vanillate demethylase (4). Protein data base searches with the predicted translational product from *pcpB* revealed regions of identity with phenol-2-monooxygenase from *Trichosporon cutaneum* (18) and tryptophan-2-monooxygenase from *Pseudomonas syringae* pv. savastanoi (27). Analysis of the 538-residue translational product from *pcpB* predicted a transmembrane helix from position 15 to position 30 according to the method of Rao and Argos (13).

Primer extension of a radiolabeled 33-mer oligonucleotide fragment, complementary to nucleotides 473 to 505 of the reported sequence and hydridized to total RNA isolated from exponentially growing ATCC 39723 cells induced with PCP 1 h prior to harvesting, gave a predominant product from which the 5′ end of the *pcpB* transcript could be assigned to a C residue at position −80 in relation to the translational start site (data not shown). Northern analysis using the 2.3-kb *Eco*RI-*Bam*HI fragment as a probe revealed two potential transcripts, 2,400 and 1,400 nucleotides in length (Fig. 3). This would suggest at least two possibilities: *pcpB* may be part of a dicistronic message, with the downstream open reading frame encoding a protein of 260 amino acids or with a molecular weight of 29,000, or the smaller transcript could be processed from the larger transcript and be monocistronic for *pcpB*. The actual nature of the *pcpB* transcript will require further experimentation. *pcpB* was under transcriptional regulation and inducible by the presence of PCP in *Flavobacterium* sp. strain ATCC 39723 (Fig. 3), as we previously observed for another *Flavobacterium* gene, *pcpA* (23), whereas the same two genes in *E. coli* were regulated constitutively.

Expression of *pcpB* by *E. coli*. The expression plasmid, pCL3, was constructed by digesting pCO221 with *Hpa*I, which cleaves just 5′ to the ATG, and *Hin*dIII, which cleaves outside of the open reading frame, ligating with *Sma*I-*Hin*dIII-digested pKK223-3 vector DNA, and electroporating into competent JM105 cells (Fig. 4). An Amp^r *pcpB* positive clone was identified by colony hybridization and designated CCL3. pKK223-3 was electroporated into JM105, and the strain was designated CCL5. CCL5 served as the vector-only control in expression experiments. CCL3, which contained *pcpB* under the direction of the hybrid p_{tac} promoter (Fig. 4), was inducible for the production of PCP-4-monooxygenase as visualized by Western blot analysis of total protein extracts (Fig. 5). Furthermore, CCL3 produced an enzymatically active PCP-4-monooxygenase as assayed from cell extracts as well as by whole cells, whereas control strain CCL5 was unable to degrade PCP to TeCH.

In whole-cell studies, CCL3 and CCL5 were both induced with IPTG and monitored spectrophotometrically for the disappearance of PCP from the medium. Even though both cultures removed PCP from the medium, only the *pcpB*-containing clone acquired the dark-yellow color characteristic of quinones. To confirm the conversion product, 20-μl aliquots from overnight-PCP-treated cells were mixed with the same volume of acetonitrile and the resulting supernatant

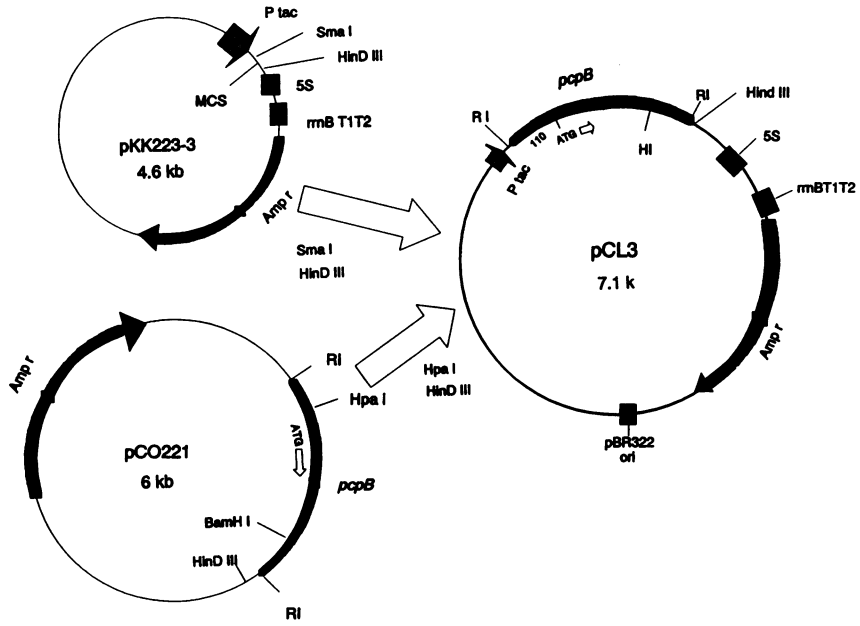

FIG. 4. Construction of the *pcpB* expression clone.

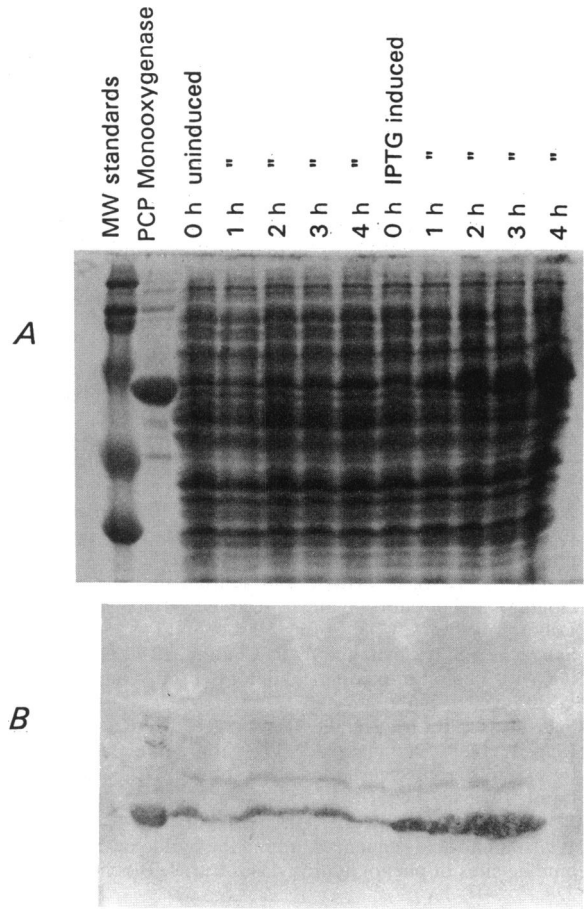

FIG. 5. Expression of PCP-4-monooxygenase by *E. coli*. (A) SDS-PAGE of total cellular proteins from CCL3 following induction with IPTG and staining with Coomassie blue. Protein molecular weight (MW) standards in descending order are as follows: 205, 116, 97, 66, 45, and 29 kDa. (B) Western analysis of a duplicate gel detecting the presence of PCP-4-monooxygenase with polyclonal antibody.

from the lysed-cell pellet was analyzed by HPLC. HPLC chromatograms showed the presence of TeCH in CCL3 but not in CCL5 (Fig. 6). Further analysis revealed that TeCH was present in the culture supernatant of CCL3 and not found within the CCL3 cell pellet (data not shown).

Cell extracts prepared from CCL3 and CCL5 were fractionated with ammonium sulfate as previously described (24). HPLC chromatograms in Fig. 7 show the production of TeCH from PCP by fractionated cell extracts from CCL3 but not by extracts from CCL5. The activity level for CCL3 cell extracts was comparable to the activity level for *Flavobacterium* sp. strain ATCC 39723 cell extracts.

DISCUSSION

The open reading frame for *pcpB* was preceded by a typical *E. coli* ribosome binding site (GGAG), but little resemblance to a typical −10 or −35 promoter region exists upstream of the transcriptional start site. The N-terminal sequence deduced from the purified protein began with a Ser. The alignment of the protein sequence with that of the predicted translation product of the gene shows the Ser residue to be preceded by a Met, which must be cleaved off

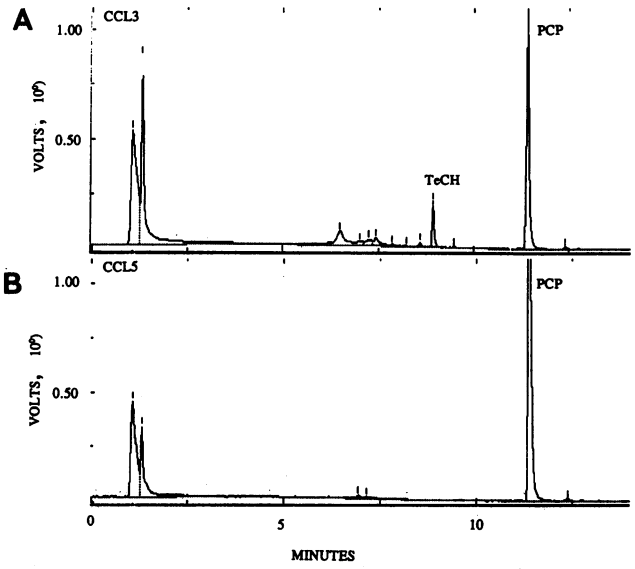

FIG. 6. HPLC chromatograms of the supernatants from acetonitrile-extracted whole-cell assays. (A) CCL3; (B) CCL5.

in the mature protein. Ser is one of a small group of amino acids that, when in the second amino acid position, seem to permit the removal of the preceding fMet residue (21).

The *Flavobacterium* gene, *pcpB*, encoding PCP-4-monooxygenase was found to be present in two other aerobic bacteria, *Arthrobacter* sp. strain ATCC 33790 and *Pseudomonas* sp. strain SR3, previously reported to dechlorinate PCP (17). The absence of the gene from *R. chlorophenolicus* is actually confirmatory of the nonoxygenolytic PCP-degradative pathway proposed for that organism (2). Furthermore, we neither expected nor observed a hybridizing fragment in the 4-chlorobenzoate-degrading *Arthrobacter* sp. strain DSM 20407 (10). Data base comparisons revealed partial identities with other reported monooxygenases that either have affinity for aromatics or are flavoproteins utiliz-

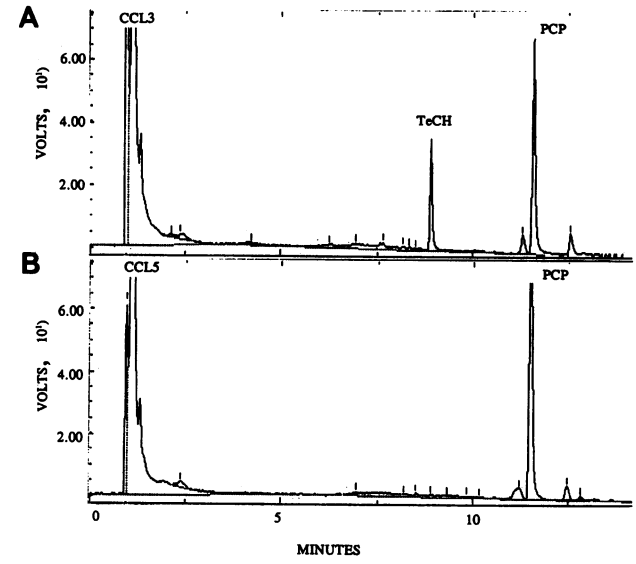

FIG. 7. HPLC chromatograms of the reaction mixtures from cell extracts. (A) CCL3 extracts; (B) CCL5 extracts.

ing NADPH (4, 18, 27). Unfortunately, there are no suitable comparisons to be drawn with *pcpB*, as there have been no reports of other monooxygenases which dechlorinate polychlorinated aromatic rings. 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 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 (25) 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 (6).

It is of interest that in *E. coli*, in which the enzyme can be evaluated in isolation from the other *Flavobacterium* dechlorinating enzymes, PCP-4-monooxygenase performed as a detoxication enzyme. The enteric membrane is not a barrier to the lipophilic compound, PCP, so the substrate was apparently transported. 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 regulated in *E. coli*, the specific regulatory components for *pcpB* are probably not present in *E. coli*.

This gene should prove to have utility 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.

ACKNOWLEDGMENTS

We acknowledge support from USDA grant 91-34214-6051.

We thank C. Bohach and J. Dutton for critical comments in the review of the manuscript.

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