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Verification of the Role of PCP 4-Monooxygenase in Chlorine Elimination from Pentachlorophenol by *Flavobacterium* sp. Strain ATCC 39723

Cleston C. Lange,* B. John Schneider,† and Cindy S. Orser[1]

*Cooperative Institute for Research in the Environmental Sciences, University of Colorado, Boulder, Colorado 80309, *Department of Biochemistry, University of Minnesota, St. Paul, Minnesota 55108; and †Department of Microbiology, University of Colorado Health Science Center, Denver, Colorado 80262*

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The bacterial enzyme PCP 4-monooxygenase from *Flavobacterium* sp. strain ATCC 39723 catalyzes the oxygenolytic removal of the first chlorine from pentachlorophenol. PCP 4-monooxygenase is an FAD binding, NADPH requiring oxygenase, with similar functional domains as other bacterial flavoprotein monooxygenases specific for phenolic substrates. However, the definitive proof for the singular role of an oxygenolytic elimination of the primary chlorine from pentachlorophenol by *Flavobacterium* sp. has awaited the development of a genetic system whereby targeted mutagenesis via allelic exchange could be carried out with the corresponding gene from PCP 4-monoxygenase, *pcpB.* We report the development of a genetic system for *Flavobacterium* sp. strain ATCC 39723, and its application in targeted mutagenesis of the *pcpB* allele for elimination of PCP 4-monooxygenase activity. © 1996 Academic Press, Inc.

Pentachlorophenol (PCP) is a polychlorinated aromatic compound widely used as a wood preservative and general purpose, broad spectrum biocide. The potential for PCP degrading microorganisms and immobilized enzymes as bioremediation tools has prompted interest in elucidation of the PCP degradation pathways. *Flavobacterium* sp. strain ATCC 39723 has been recognized for its competence in PCP degradation for quite some time (9), although the biochemistry and genetic determinants for such activities have only recently begun to be elucidated in our laboratory (6,7,13,14,16). Much of the reason for the gap from physiological to molecular genetic studies has been the recalcitrant nature of the microorganism. Previous attempts at transforming and mutagenizing strain ATCC 39723 were unsuccessful in our hands (3).

Our laboratory has been immersed in the molecular analysis of the dechlorination of PCP by *Flavobacterium* sp., and we previously demonstrated that PCP induces the presence of several proteins in the bacterium (12). In particular, we reported the first chlorine elimination from the benzene ring to be oxygenolytic through the action of PCP 4-monooxygenase (15), encoded by the gene *pcpB* (6). The enzyme converts PCP to 2,3,5,6-tetrachloro-*p*-hydroquinone (TCHQ) in the presence of oxygen and NADPH. The enzyme has been purified to homogeneity from strain ATCC 39723 (13) its diverse substrate range reported (14), and the corresponding genetic determinant described (6).

Whereas other microbial degradative pathway genes are typically clustered in operons and often on mobile elements (8), *pcpB* was not found to be part of a much larger 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 a general detoxication function in the cell. The enzyme is further characterized as having a broad substrate range and a broad catalytic reaction group. These properties are also common for eucaryotic mixed function oxidases which have a general role in detoxication of foreign, lipophilic compounds (4).

In this report, we describe the successful transformation of *Flavobacterium* sp. strain ATCC 39723, the demonstration of homologous recombination via allelic exchange, a targeted mutagen-

[1] Corresponding author: Cindy S. Orser, CIRES, University of Colorado, Boulder, CO 80309. Fax: (303) 492–1149.

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esis event, and verification of the explicit role of PCP 4-monooxygenase, encoded by *pcpB*, in the degradation of PCP.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Escherichia coli HB 101 and JM105 were used as recombinant hosts. *Flavobacterium* sp. strain ATCC 39723 was kindly provided by R. Crawford (University of Idaho). The *Flavobacterium* strain was cultured at 26°C in glutamate mineral media. Cells were induced for PCP degradation by addition of PCP to 50 μM at an $O.D._{600}$ of 0.40 (early log phase). Plasmid pCO222, containing the intact *pcpB* gene, was described previously (6). The plasmid pDM100 (5) was used as a source for *nptII*, the intervening DNA used to construct the interrupted *pcpB* gene in pCO309. The plasmid pBluescript II was obtained from Stratagene (La Jolla, Calif.).

Chemicals, enzymes, and stock solutions. All chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.) or Aldrich Chemical Co. (Milwaukee, Wis.). PCP was prepared as a 37.5 mM solution in 0.1M NaOH. [α-P^{32}]dCTP was purchased from New England Nuclear Corp. (Boston, Mass.). Restriction endonucleases and DNA modifying enzymes were purchased from United States Biochemical Corp. (Cleveland, Ohio). DNA radiolabeling was performed using the T7 Quick Prime kit from Pharmacia Inc. (Piscataway, New Jersey).

DNA extraction and Southern analysis. Plasmid DNA for restriction enzyme analysis was extracted from *E. coli* hosts by the hexadecyltrimethylammonium bromide (CTAB) DNA extraction method (2). Total genomic DNA was isolated from *Flavobacterium* sp. by previously described methods (1). Southern analysis and the isolation of supercoiled plasmid DNA by cesium chloride-ethidium bromide density gradients was performed as described by Sambrook *et al.* (10).

Transformation of Flavobacterium sp. strain ATCC 39723. Flavobacterium sp. was made competent for transformation by washing three times with sterile distilled water, one time with sterile 10% glycerol, and resuspending in sterile 10% glycerol, 30% polyethylene glycol (MW 8000) at 0.6% of the original volume. Fifty μl of this suspension was used per electroporation event with 5 μg of plasmid DNA in a volume of up to 2 μl. Electroporation settings of 2.5 kV and 600 Ohms on a BioRad Gene Pulser with pulse controller typically resulted in a 13.4 msec pulse length using 0.2 cm gapped electroporation cuvettes. Following electroporation, 1 ml of SOC (10) was added and the cells were allowed to recover with shaking at room temperature for 20 h before plating on Luria broth agar medium containing 15 μg/ml kanamycin. Plates were incubated at room temperature for 4 to 5 days before colonies became visible. Colonies were picked at 7 days and streaked to fresh plates. Controls included cells electroporated without any transforming DNA and cells electroporated with pBluescript II DNA as the vector only control.

Whole cell PCP degradation assays. The PCP concentration in *Flavobacterium* cultures were measured using high-pressure liquid chromatography (HPLC) by addition of 20 μl of acetonitrile to 20 μl timed aliquots of cultures. Samples were then centrifuged for 3 minutes in a microcentrifuge and 35 μl of supernatant loaded onto a Waters HPLC system (Medford, Mass.) with a C18 reverse phase column using an acetonitrile gradient and acetate buffer as described previously (13). PCP was monitored with a Waters programmable multiwavelength detector set at 318 nm absorbance.

RESULTS AND DISCUSSION

The plasmid pC0322, containing the intact *pcpB* gene on a 2.3 kb *Bam*HI to *Eco*RI fragment in pBluescript, was cut with *Sph*I restriction endonuclease to interrupt the open reading frame of *pcpB*, and blunt ended with T4 DNA polymerase. This fragment was then ligated to the Klenow filled-in, *npt*II containing *Hind*III to *Nde*I fragment from pDM100 (Fig. 1). Positive clones containing the *pcpB* interrupted gene were identified by kanamycin resistance and restriction enzyme analysis. One of the positive clones was picked and designated CO309. Purified plasmid DNA from CO309 was used to transform electrocompetent *Flavobacterium* sp. cells. Twenty-four kanamycin resistant *Flavobacterium* colonies were identified and total genomic DNA isolated from each. Southern analysis of the genomic DNA samples restricted with *Eco*RI, and probed with a ^{32}P-labeled *pcpB* fragment, revealed twenty-two of the 24 kanamycin resistant *Flavobacterium* to have two hybridizing bands, one corresponding to the 3.0 kb wild type *pcpB* fragment, and one at 6.9 kb, corresponding to a cointegrate whereby pBluescript containing the *nptII* interrupted *pcpB* gene had inserted at the *pcpB* allele in the genome (Fig. 2). Two of the 24 genomic DNA samples had only one hybridizing signal at 4.6 kb, indicating that the cointegrates had resolved to leave an interrupted *pcpB* allele in the genome (Fig. 2). Southern analysis using a radiolabeled *nptII* gene fragment confirmed the presence of *nptII* at 6.9 and 4.6 kb for the cointegrates and resolved strains, respectively (Fig. 2). Two of the cointegrate strains were designated FCO313 and FCO314 and the two resolved strains, FCO315 and FCO316.

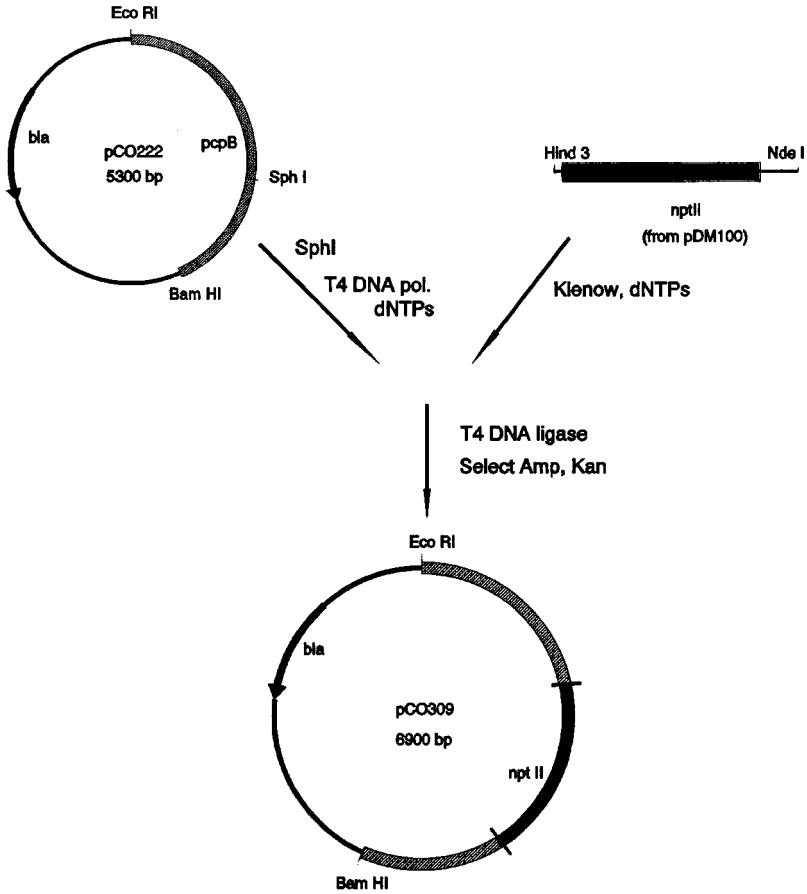

FIG. 1. Construction of pCO309 for allelic exchange in *Flavobacterium* sp. strain ATCC 39723.

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 in comparison with the parental strain, *Flavobacterium* sp. strain ATCC 39723 (Fig. 3). 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.

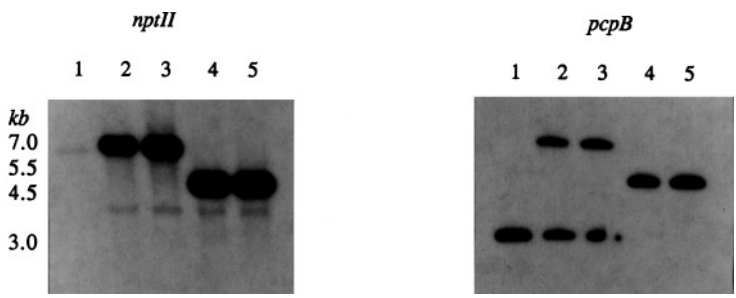

FIG. 2. Southern analysis of *Eco*RI-digested total genomic DNA from *Flavobacterium* transformants. The filter on the left is the result from probing with *nptll* and on the right, the same filter was stripped and reprobed with *pcpB.* Lane 1, *Flavobacterium* parental strain; lane 2, transformant FCO313; lane 3, transformant FCO314; lane 4, transformant FCO315; lane 5, transformant FCO316.

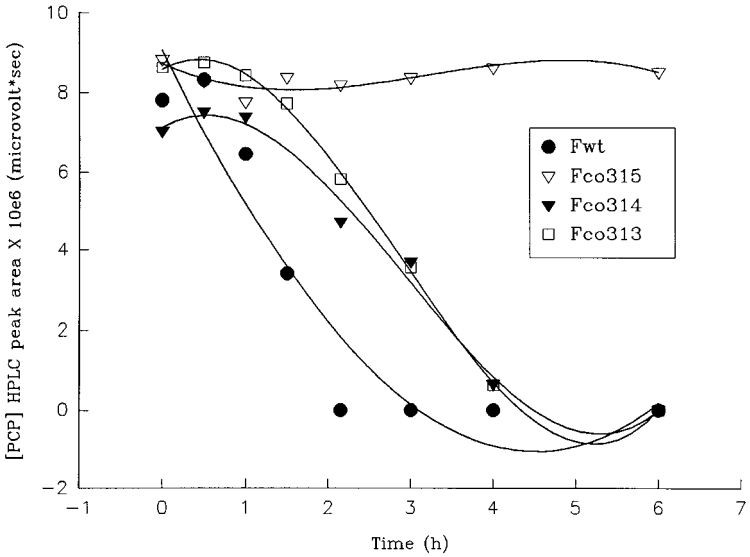

FIG. 3. Representation of PCP concentration versus time from HPLC analysis of acetonitrile extracted cultures as labeled. Fwt is the parental *Flavobacterium* sp. strain ATCC 39723.

We have demonstrated the ability to genetically manipulate the gene, *pcpB*, involved with PCP degradation in *Flavobacterium* sp. strain ATCC 39723. The isogenic mutant, FCO315, has been shown to be altered in its ability to degrade PCP, whereas in the cointegrate strains, FCO313 and FCO314, are still able to display a PCP^+ phenotype similar to that of wild type *Flavobacterium* sp. strain ATCC 39723. The slight lag observed for the two cointegrates, compared to the parental *Flavobacterium* sp., may be a reflection of the regulation of the PCP metabolic pathway. These data collectively show the solitary oxygenolytic pathway for removal of the primary chlorine during PCP degradation, in contrast to a previous report that *Flavobacterium* sp. cells utilized a hydrolytic pathway for the primary chlorine elimination (11).

ACKNOWLEDGMENT

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