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Mutational Analysis of *pcpA* and Its Role in Pentachlorophenol Degradation by *Sphingomonas* (*Flavobacterium*) *chlorophenolica* ATCC 39723†

SUCHART CHANAMA‡ AND RONALD L. CRAWFORD*

University of Idaho Institute for Molecular and Agricultural Genetic Engineering, Moscow, Idaho 83844-1052

Received 22 July 1997/Accepted 5 October 1997

***Sphingomonas* (*Flavobacterium*) *chlorophenolica* ATCC 39723 degrades pentachlorophenol (PCP) through a catabolic pathway encoded by multiple genes. One gene required for PCP degradation is *pcpA*, which encodes information for a 30-kDa polypeptide, PcpA, found in the periplasm of the bacterium. The biological role of PcpA has remained unknown. We disrupted *pcpA* by replacing it with a defective copy through homologous recombination. The *pcpA* recombinant, mutant strains accumulated 2,6-dichlorohydroquinone (2,6-DiCH) as a metabolite of PCP. This work confirms that *pcpA* is essential for degradation of PCP by *S. chlorophenolica* ATCC 39723 and suggests that it encodes a protein involved in hydrolytic dehalogenation of 2,6-DiCH, an already established primary metabolite of the PCP catabolic pathway.**

Pentachlorophenol (PCP), an extensively used biocide which is severely toxic to humans and other organisms, is a serious environmental contaminant worldwide (13, 20, 21). Many aerobic microorganisms capable of degrading PCP have been isolated from contaminated sites. *Sphingomonas* (*Flavobacterium*) *chlorophenolica* ATCC 39723, a gram-negative bacillus isolated from a PCP-contaminated soil in Minnesota (22), can mineralize PCP at the high concentrations of 100 to 200 ppm (mg/liter). This organism has recently been renamed as a species of *Sphingomonas* on the basis of rRNA gene sequence analyses (5, 8, 15). It has been determined that PCP degradation by this bacterium proceeds via a primary hydroxylation of PCP by PCP-induced PCP 4-monooxygenase, encoded by a chromosomal *pcpB* gene (19, 26). The oxygenase-catalyzed reaction yields 2,3,5,6-tetrachloro-*p*-hydroquinone (TeCH), which is reductively dechlorinated to trichloro-*p*-hydroquinone (TrCH) and dichloro-*p*-hydroquinone (DiCH) by a second constitutive reductive dehalogenase, encoded by a chromosomal *pcpC* gene (18, 30, 31). DiCH is the most recently identified intermediate in PCP degradation by *S. chlorophenolica* ATCC 39723, although its isomeric form, i.e., 2,5-DiCH or 2,6-DiCH, was not distinguished in prior work. Lee and Xun (11) recently characterized a 2,6-DiCH chlorohydrolase from this strain that did not attack the 2,5 isomer. A third PCP-induced periplasmic protein (PcpA) has been characterized from the same bacterium, and the corresponding gene (*pcpA*) has been identified (27). The biological role of PcpA has remained unknown, yet it is obviously involved in the degradation of PCP, since the release of the protein from the periplasmic space of the cell with EDTA stops the PCP degradation activity of the cells (27). Thus, the lower pathway for PCP degradation beyond DiCH must still be investigated to fully understand the degradation of PCP.

The role of *pcpA* in the biodegradation pathway could be determined by disrupting the gene to block catabolism of PCP and simultaneously accumulating an intermediary metabolite that would be the likely product of the upstream PCP catabolic pathway preceding the catalysis by PcpA. Identifying such an intermediate should also allow the description of the substrate of the PcpA protein.

An effective way to disrupt a gene at a specific site is by gene targeting via homologous recombination, an approach often shown to be successful in creating site-specific mutations of genes in mammals, plants, bacteria, and other organisms (16, 17, 25). Gene targeting is specific to a particular gene of interest, and a selective marker, such as antibiotic resistance, can be introduced into the target gene as a part of the disrupting element, giving this method advantages over chemical, physical, and transposon mutagenesis procedures.

Here we describe the disruption of the genomic *pcpA* gene by gene targeting via homologous recombination, characterization of the mutated gene, and isolation and identification of PCP metabolites produced by mutant strains of *S.* (*Flavobacterium*) *chlorophenolica* ATCC 39723 containing the disrupted gene.

MATERIALS AND METHODS

Bacterial strains, plasmids, and primers. *S.* (*Flavobacterium*) *chlorophenolica* ATCC 39723 was isolated in our laboratory in 1985 (22, 24). PCR primers for *pcpA* amplification were synthesized by Integrated DNA Technologies, Inc. (Coralville, Iowa). Plasmid pRC002 was constructed by insertion of the *npt*II cassette into the *pcpA* open reading frame on plasmid pRC001 at the *Nru*I site. The sources of other bacterial strains and plasmids used in this work are listed in Table 1.

Culture media and conditions. Bacterial strains were grown in a mineral salts medium containing 0.82 g of $K_2HPO_4 \cdot 3H_2O$, 0.19 g of KH_2PO_4, 0.5 g of $NaNO_3$, 0.1 g of $MgSO_4 \cdot 7H_2O$, 4 g of L-sodium glutamate, 20 μmol of $FeSO_4$, and deionized water to a volume of one liter. For agar plates, 15 g of Bacto agar per liter was added to a mineral salts medium. For growing *pcpA* mutants of *Sphingomonas*, 15 mg of kanamycin per liter was added to the medium with and without 50 mg of PCP per liter. All *Sphingomonas* cultures, including the wild type and mutant strains, were grown at room temperature. For PCP degradation experiments, 50 mg of PCP per liter was added to cultures after the cell density reached 0.5 to 1.0 A_{560}. Cultures were incubated with shaking at 100 to 150 rpm.

Culture media for *Escherichia coli* RLC002 (*E. coli* GI724 carrying plasmid pRC002) were 10× M9 salts (6% Na_2HPO_4, 3% KH_2PO_4, 0.5% NaCl, 1% NH_4Cl), RM-Amp-Kan medium (1× M9 salts, 2% Casamino Acids, 1% glycerol, 1 mM $MgCl_2$, 100 μg of ampicillin per ml, 15 μg of kanamycin per ml), and

* Corresponding author. Mailing address: Food Research Center 103, University of Idaho Institute for Molecular and Agricultural Genetic Engineering, Moscow, ID 83844-1052.

† Publication no. 97504 of the Idaho Agricultural Experiment Station.

‡ Present address: Department of Biochemistry, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand.

TABLE 1. Strains and plasmids used in this work

Strain or plasmid (description)	Source
Strains	
S. (*Flavobacterium*) *chlorophenolica* ATCC 39723	University of Idaho, Moscow
E. coli GI724 (F^- lambda$^-$ *lacI*^{q} *lacPL8 ampC*::*PtrpCI*)	Invitrogen Corp., San Diego, Calif.
E. coli RLC001 (*E. coli* GI724 carrying plasmid pRC001)	S. Chanama (2a)
E. coli RLC002 (*E. coli* GI724 carrying plasmid pRC002)	S. Chanama (2a)
pcpA Sphingomonas mutants (*pcpA*::*npt*II in genomic DNA)	S. Chanama (2a)
Plasmids	
pKm+/− (sense and antisense of neomycin phosphotransferase II gene [*npt*II])	S. Chanama (2a)
pTrxFus (thioredoxin [*trxA*] fusion expression vector)	Invitrogen Corp.
pRC001 (*trxA-pcpA* fusion in pTrxFus)	S. Chanama (2a)
pRC002 (*pcpA*::*npt*II in pRC001)	S. Chanama (2a)

RMG-Amp-Kan plates (1× M9 salts, 2% Casamino Acids, 0.5% glucose, 1 mM $MgCl_2$, 100 μg of ampicillin per ml, 15 μg of kanamycin per ml, 1.5% agar). RLC002 cells were grown at 30°C.

Enzymes and chemicals. Restriction endonucleases, DNA modifying enzymes, and $Vent_R$ DNA polymerase were obtained from New England Biolabs, Inc. (Beverly, Mass.). PCP was obtained from Chemical Service (West Chester, Pa.). 2,3,5,6-Tetrachloro-*p*-benzoquinone, 2,3,5,6-TeCH, 2,5-dichloro-*p*-benzoquinone (2,5-DiCB), 2,5-DiCH, and 2,6-DiCB were purchased from Pfaltz & Bauer, Inc. (Waterbury, Conn.). 2,6-DiCH was prepared by Stefan Goszczynski (University of Idaho) by reduction of 2,6-DiCB with ascorbic acid, as described by others (9, 11, 29). 6-Chloro-*p*-hydroquinone (6-CH) was obtained from Aldrich Chemical Co. (Milwaukee, Wis.). All other chemicals were reagent grade, and all solvents were high-performance liquid chromatography (HPLC) or mass spectrometry (MS) grade.

Molecular techniques. Two 20-mer oligonucleotide primers, upper primer 5′-ATG GAA ACG AAC CAT ATC AC-3′ and lower primer 5′-ACA CCT GCA GGT CCA TGC CC-3′, were synthesized for PCR (6, 7) amplification of the coding sequence of the *pcpA* gene from chromosomal DNA of *Sphingomonas* with $Vent_R$ DNA polymerase. PCR was performed in a miniCycler (model PTC-150; MJ Research, Inc., Watertown, Mass.) with a temperature profile of 95°C for 3 min, 94°C for 1 min, 56°C for 30 s, 75°C for 55 s, and 75°C for 1 min; steps 2 to 4 were repeated for 35 cycles. Amplified *pcpA* sequences (PCR products) were separated by 1% agarose gel electrophoresis and isolated and purified by using a USBioclean MP kit (United States Biochemical Corp., Cleveland, Ohio).

Electrotransformation was performed with competent *Sphingomonas* cells. Cells were washed three times with sterile deionized water and once with sterile 10% glycerol in water and resuspended with sterile 10% glycerol and 30% polyethylene glycol 8000 in water. Aliquots (50 μl) of competent cells were prepared and kept at −80°C. Desalted pRC002 plasmid DNA (5 to 10 μg) was introduced with a gene pulser (Bio-Rad Laboratories, Hercules, Calif.) into 50 μl of competent *Sphingomonas* cells for electroporation in a 0.2-cm-electrode-gap electroporation cuvette under the following conditions: 2.5 kV, 600 O, 25 μF, and a pulse time of 13.2 to 13.7 ms. Then, 0.95 ml of SOC medium (2% tryptone, 0.5% yeast extract, 0.05% NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM glucose) was added immediately. The transformed cells were transferred to a polypropylene tube and incubated with shaking at room temperature for 20 h prior to being plated into selective medium (*Sphingomonas* mineral salts medium with kanamycin) and incubation at room temperature. Kanamycin-resistant clones were then identified. Their PCP catabolic defects were detected in the same selective medium with the addition of 50 mg of PCP per liter. By accumulation of PCP metabolites, mutants yielded colored products. As controls the competent cells were electroporated with pBluescript plasmid but without the addition of other DNA.

Total genomic DNA was isolated according to either the procedure of Ausubel et al. (2) or the protocol based on the DNAZOL guanidine-detergent lysis method (Gibco BRL, Life Technologies, Inc., Gaithersburg, Md.). Plasmids were isolated and purified by polyethylene glycol precipitation. DNA manipulations included restriction enzyme digestions, filling in the 5′ cohesive ends with Klenow fragment, ligation by T4 DNA polymerase, and electroporation as described by Ausubel et al. (2), Sambrook et al. (23), and Nickoloff (14).

DNA was denatured and transferred from the agarose gel to a Nytran nylon membrane by the Turboblotter alkaline transfer method (Schleicher & Schuell, Keene, N.H.). Southern hybridization was carried out by the Rad-Free nonisotopic detection method for probe labeling and detection (Schleicher & Schuell).

To construct the targeting vector for disruption of the genomic *pcpA* gene, a 2.7-kb neomycin phosphotransferase II (*npt*II) gene was released from plasmid pKm+/− as an *npt*II cassette by digestion with *Eco*RI and *Pvu*I. The 5′ overhanging ends of this fragment were filled with Klenow fragment DNA polymerase. The *npt*II cassette was then inserted into the *pcpA* open reading frame located on pRC001 at the *Nru*I site to create pRC002 (a 7.1-kb targeting vector). The construct was electrotransformed into *E. coli* GI724. Positive clones, designated RLC002, were identified by selection on RMG-Amp-Kan plates, by PCR with *pcpA* primers, and by size and restriction endonuclease mapping of purified plasmid DNA.

The plasmid pRC002 was introduced into competent *Sphingomonas* cells by electroporation. Positive clones containing a *pcpA*-disrupted allele were screened by kanamycin resistance and catabolic impairment, which were observed by the appearance of colored products on PCP plates.

PCP degradation and formation of intermediates by *pcpA* mutants. PCP degradation by whole cells of mutants and the wild-type *Sphingomonas chlorophenolica* was assayed by measuring the depletion of PCP in the cultures by HPLC. A 40-μl quantity of culture fluid taken at various time points was mixed with an equal volume of acetonitrile and centrifuged in a microcentrifuge for 10 min, and 40 μl of the supernatant was analyzed with a Hewlett-Packard (HP) HPLC (model 1090) equipped with a UV-visible (VIS) diode array detector set at a wavelength of 300 nm. A reverse-phase Supelcosil LC_{18} column (150 by 4.6 cm; Supelco, Inc., Bellefonte, Pa.) was used. The HPLC method involved the use of a 25 mM sodium acetate (pH 3.8) (A)-acetonitrile (B) gradient at a flow rate of 1 ml/min, as follows: 5 to 70% B (linear gradient) for 5 min, 70% B for 5 min, 100% B for 4 min, and 5% B for 0.5 min. A PCP standard was run under the same conditions.

The progression of intermediate production was detected and monitored by HPLC as described for the PCP degradation assay. Standard hydroquinones, including 2,3,5,6-TeCH, 2,5-DiCH, 2,6-DiCH, and 6-CH, were run under the same conditions for comparison. Cultures of *pcpA* mutants without added PCP were examined as controls.

To extract metabolites from culture supernatants, mutants were grown in 100 ml of glutamate minimal medium until the optical density at 560 nm reached 0.5 to 1.0. Then, 50 mg of PCP per liter was added to the cultures. After incubation at room temperature for 4 h, 50 ml of the culture medium was removed and extracted twice with 50 ml of ethyl acetate (neutral extract). The extracted solutions were then acidified to pH 2.0 and extracted twice with 50 ml of ethyl acetate (acid extract). The solvent in the extracts was removed under vacuum by rotary evaporation at 40°C. Finally, dried samples were dissolved in 0.5 ml of acetonitrile. Samples (2.5 μl) were analyzed by HPLC under the conditions described for PCP. Samples from acid extracts were also analyzed by HPLC-MS and gas chromatography (GC)-MS. Samples from neutral extracts were analyzed by GC-MS.

Spectrometric analysis. Absorption spectra of culture supernatants were measured at room temperature with a UV-VIS diode array spectrophotometer (model HP8452; Hewlett-Packard Co.).

An HP1050 HPLC equipped with a UV-VIS detector operating at 290 nm was used to deliver a sample to a particle beam vacuum desolvation interface connected to an MS quadruple detector (HP5989A MS controlled by HP59940A MS Chem-Station software, HP-UX series). The electron impact sample ionization mode was set as follows: repeller, 7 V; emission, 300 V; and electron energy, 70 eV. The source temperature was 250°C. A Supelcosil LC_{18} column was used for separation with a linear gradient from 5% acetonitrile (in 25 mM sodium acetate [pH 3.8]) to 70% acetonitrile in 5 min and isocratic from 5 to 20 min at a flow rate of 0.5 ml/min.

An HP series II model 5890 GC was equipped with a capillary-fused silica DB-5MS column (25 m by 0.21 mm by 0.33 μm; J&W Scientific, Folsom, Calif.) and an MS interface. The interface temperature was 280°C. The MS detector was set as it was for the HPLC-MS, except that the source temperature was 175 to 200°C. The linear gradient of the oven temperature was 100 to 300°C at 10°C/min. Products were identified by comparison of their retention times (RTs) with those of standards and/or their fragmentation patterns with those of standards as well as with the mass spectra of known compounds in the Wiley database stored in an MS ChemStation library. The library was searched by probability-based matching or parametric retrieval.

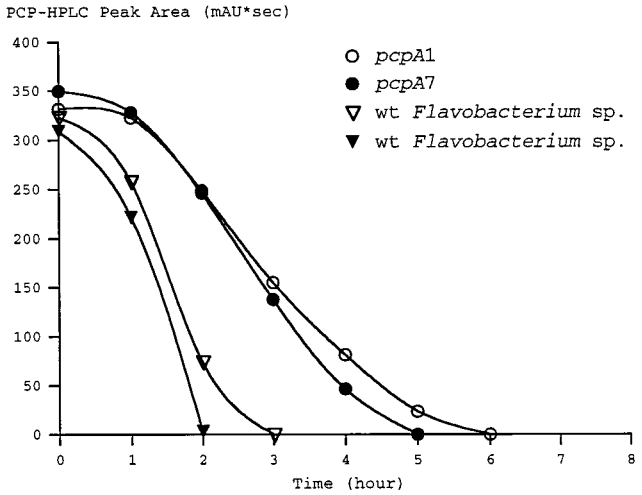

FIG. 1. Degradation of PCP (50 mg/liter) by whole cells of *pcpA* mutants and the corresponding *S. chlorophenolica* wild type (wt) replicates. mAu, milliabsorbance units.

RESULTS

Genetic disruption of *pcpA* in genomic DNA of *Sphingomonas chlorophenolica*. Actual catabolic mutants were identified by Southern blot hybridization of total genomic DNA isolated from each mutant. Eight kanamycin-resistant *Sphingomonas* clones, which produced a soluble purple color in PCP agar plates, were identified. Southern analyses of their genomic DNAs digested with *Eco*RI showed a single hybridizing band at 8.2 kb with *pcpA* and *npt*II probes, which indicated that allelic exchange had occurred via homologous recombination between *pcpA* flanking sequences of the targeting vector and the *pcpA* sequence of the host genome, resulting in an interrupted *pcpA* allele in the genome.

Characterization of the *Sphingomonas pcpA* catabolic mutants. In terms of morphology, pigment, and growth rate, the phenotypic characteristics of all *pcpA* mutants grown at room temperature (25°C) in solid medium containing mineral salts supplemented with 4 g of sodium glutamate per liter with or without 15 mg of kanamycin per liter did not differ from those of the wild-type *Sphingomonas*. All mutants formed visible, round, yellow, opaque colonies within 3 to 5 days on solid media. In liquid medium with the antibiotic, mutants grew more slowly than the wild-type strain and required at least 3 to 5 days at room temperature to show turbidity in the culture.

When mutants were grown on PCP agar plates containing 4 g of sodium glutamate per liter, they produced a soluble metabolite with a purple color after incubation for 2 days. The purple color was also observed after 2 to 3 h in liquid culture containing PCP (50 mg/liter) and with a cell density at A_{560} of 0.5 to 1.0. The purple metabolite turned brown with time, indicating its instability under aerobic conditions. In contrast, the parental strain did not produce colored metabolites under the same conditions of growth in solid and liquid culture media. A time course of UV-VIS spectrum changes of a culture supernatant of one *pcpA* mutant after incubation with PCP showed two peaks and one shoulder at 300, 350, and 520 nm, respectively. It appeared that a metabolite corresponding to the peaks at 300 and 520 nm was formed first and subsequently underwent a rapid transition to another product having a maximal peak at A_{350}. In the same culture, the color of the culture broth changed from colorless, to yellow, to purple, and finally to red-brown. The production of chromogenic compounds in the culture media by the mutants indicated their impairment in PCP catabolism at a point prior to ring fission.

To clearly establish that *pcpA* plays a significant role in the degradation of PCP and that disruption of this gene interferes with the degradative ability of the bacterium, whole cells of *pcpA* mutants and the wild-type strain were compared by PCP degradation assays (Fig. 1). As predicted, the PCP-degrading abilities of the *pcpA* mutants were decreased relative to those of the wild-type *Sphingomonas*. The *pcpA* mutant strains removed PCP completely after 5 to 6 h, while the parental isogenic strain required only 2 to 3 h. This result indicated that the mutants had not lost their ability to attack PCP, because the initial steps of the degradation pathway were not affected by this mutation, but that the lower part of the catabolism pathway was blocked due to malfunction or absence of the PcpA protein.

Isolation and identification of the metabolite produced by *pcpA* mutants. The development of color simultaneously with UV-VIS spectral changes in the culture of the *pcpA* mutant, which contained PCP under aerobic incubation, suggested that some intermediary metabolites accumulated as a result of the *pcpA* mutation. Ether and ethyl acetate extractions of metabolites from acidified culture supernatants after incubation in 50 ppm of PCP overnight produced colored extracts which were used to identify metabolites by GC-MS. Only a trace of DiCH was found in the extracts, implying that DiCH was a possible accumulated metabolite but that it might have become oxidized with time; hence, the yield and quality of the extracted metabolite might be time dependent. Therefore, the progression of metabolite formation in the culture was determined.

Time-course studies of intermediate formations by the *pcpA* mutants in the culture broths with 50 μg of PCP per ml were done directly with culture supernatants by HPLC (Fig. 2). Two types of intermediates were resolved. Examination of UV-VIS spectra of these intermediates showed two different spectral patterns, corresponding to spectra observed in the whole culture supernatants. Intermediate 1 at an RT of 6.2 min first appeared at 2 h after the addition of PCP and reached a maximum at 6 h. This was followed by another compound (intermediate 2) with an RT of 2.2 min. The decrease of the peak area of the first intermediate was followed by an increase of the peak area of the second intermediate, suggesting that the second metabolite was formed from the previous one. In addition, the first intermediate at an RT of 6.2 min and the

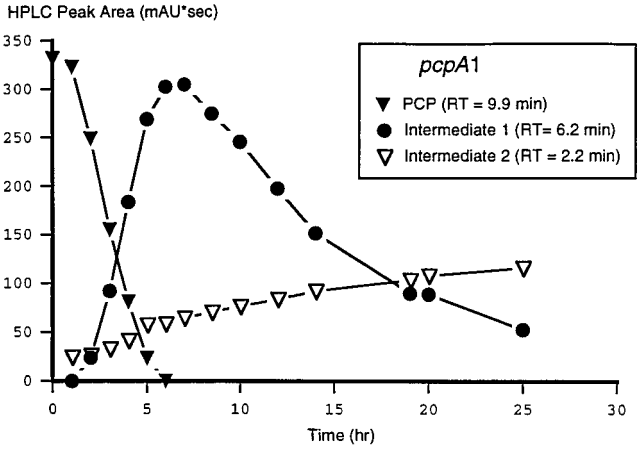

FIG. 2. Time course of metabolite production in cultures of *pcpA* mutants during PCP degradation (50 mg/liter). mAu, milliabsorbance units.

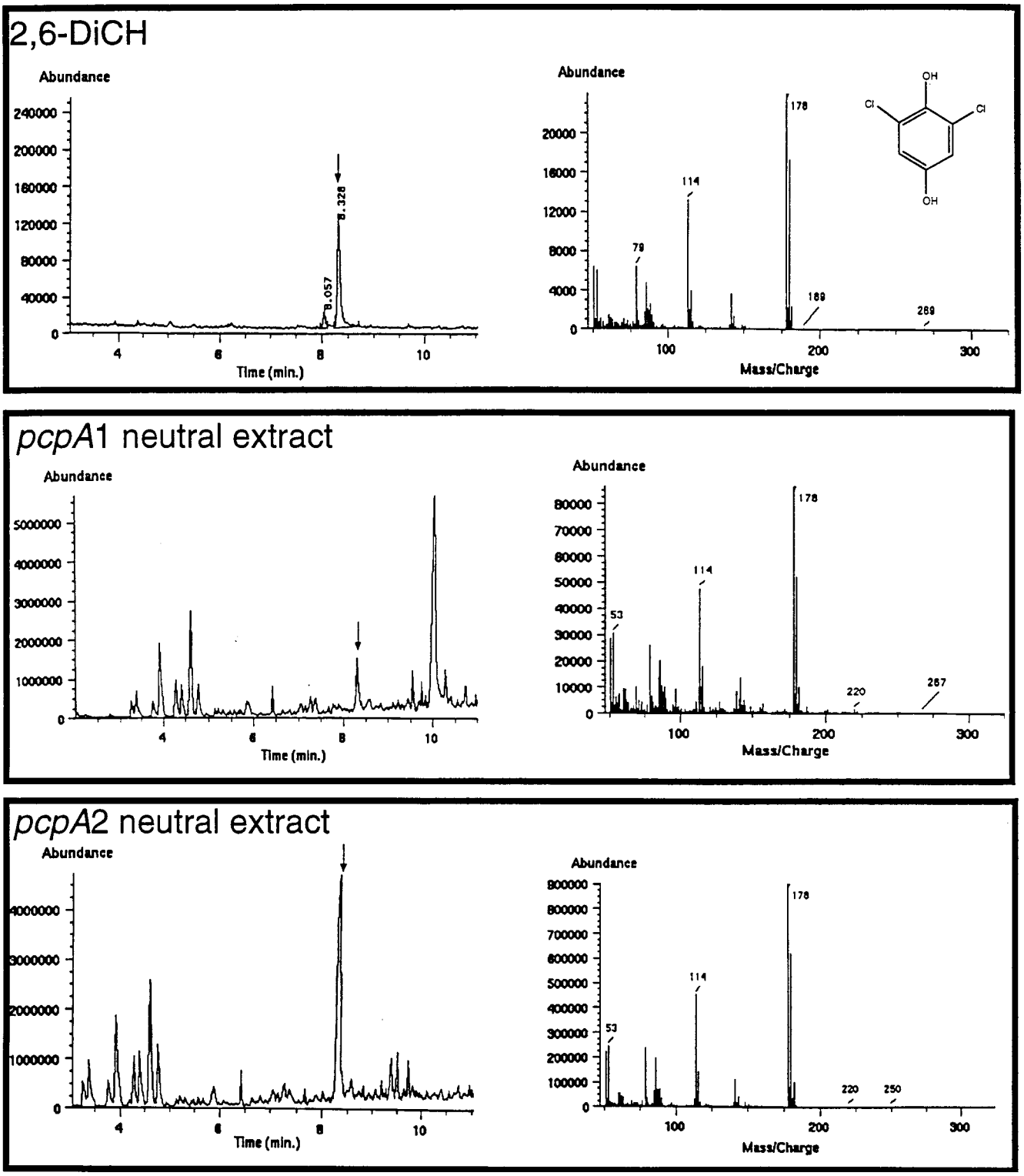

FIG. 3. GC-MS spectra of the primary metabolite in neutral ethyl acetate extracts obtained from cultures of two *pcpA* mutants (*pcpA*1 and *pcpA*2), which were incubated with 50 mg of PCP per liter for 4 h, and comparison with the standard. The arrows indicate the peaks that produced the associated mass spectrum.

second product at an RT of 2.2 min corresponded to the spectral characteristics of maximal peaks at 300 and 520 nm and 350 nm, respectively, in the prior time-dependent culture supernatants produced by the mutant. Thus, the sequence of intermediate formation could be determined. Intermediate 1 at an RT of 6.2 min, corresponding to a spectrum with a maximal peak at 300 nm with a shoulder at 520 nm, was produced first within 6 h and was then followed by the second metabolite

(intermediate 2) at an RT of 2.2 min, corresponding to a spectral peak at 350 nm.

To identify the intermediates accumulated, both neutral and acid extractions with ethyl acetate were done with 50 ml of the *pcpA* mutant cultures incubated with 50 mg of PCP per liter at 25°C for 4 h. HPLC analysis of acid extracts showed a major peak with an RT of 6.2 min that did not match any standard hydroquinone tested, whereas the neutral extracts showed a major peak with an RT of 6.9 min that perfectly matched the RT of 2,6-DiCH run as a standard. The identity of the compound as 2,6-DiCH was confirmed by HPLC-MS and GC-MS. HPLC-MS and GC-MS analyses of the acid extracts showed a small amount of compound from the main peak that was identified as 2,6-DiCH by comparison with the authentic compound. On the other hand, a single peak in the neutral extracts was found by GC-MS analyses to contain 2,6-DiCH at a high level and at high purity (80 to 90%), showing conclusively that the actual metabolite accumulated as a result of *pcpA* impairment was 2,6-DiCH (Fig. 3). First, 2,6-DiCH was produced from the early steps of PCP catabolism, which corresponded to the UV-VIS spectrum with a maximal peak at 290 to 300 nm. As soon as this compound accumulated in the culture, autooxidation occurred immediately, with transformation of 2,6-DiCH to 2,6-DiCB, which showed a maximal peak at 300 nm and a shoulder at 520 nm. This product corresponded to the yellow-purple color developed in the culture. Oxygen and a slightly alkaline environment promote dimerization and/or polymerization of quinones, forming colorless products (1, 3, 4) that may correspond to the spectrum with a maximal peak at 350 nm.

DISCUSSION

A variety of hydroxylated metabolites have been described in the degradation of PCP by bacteria grown on PCP as a sole source of carbon and energy. The degradation of PCP by *S.* (*Flavobacterium*) *chlorophenolica* ATCC 39723 begins with oxygenolytic *para* hydroxylation, yielding a chlorinated *para* hydroquinone (24, 26, 28). Specifically, PCP is converted to TeCH by PCP 4-monooxygenase (26). The subsequent reactions are the reductive dechlorinations that convert TeCH to TrCH and DiCH (31). Previous to the work described here, as well as the recent work of Lee and Xun (11), further catabolic pathway intermediates had not been identified, and the isomeric form of DiCH (2,5- or 2,6-DiCH) had not been clearly established.

In this study, we determined the intermediate beyond TrCH in the degradation sequence of PCP in *S.* (*Flavobacterium*) *chlorophenolica* ATCC 39723 by mutagenesis of the *pcpA* gene, which encodes a PCP-induced 30-kDa polypeptide found in the periplasmic space of the cells. By disruption of this gene with an antibiotic resistance gene (*npt*II) via homologous recombination, we produced mutants that lacked the ability to transform a PCP pathway intermediate, which we identified as 2,6-DiCH. This result shows that PcpA is essential for PCP degradation and is involved in the further transformation of 2,6-DiCH. This compound was also identified as an intermediate of PCP metabolism in a mutant of another PCP-degrading bacterium, strain KC-3, where the initial steps of PCP degradation are similar to those of *Sphingomonas* in our study (21). The possibility that 2,6-DiCH is a substrate for further catabolic reactions in PCP degradation was demonstrated when Lee and Xun (11) isolated and purified from the cytoplasm of the same *Sphingomonas* sp. an enzyme which converts 2,6-DiCH to 6-chloro-1,2,4-trihydroxybenzene via hydrolytic hydroxylation and dechlorination. The existence of this chlorohydrolase strongly supports the conclusion that 2,6-DiCH is a true substrate of the metabolic reactions of PCP degradation. *pcpA* encodes a polypeptide with a molecular mass of 30 kDa, which is smaller than the molecular mass of 42.5 kDa reported for the native chlorohydrolase. L. Xun and C. Mian (25a) recently overexpressed *pcpA* in *E. coli*, and the cell extracts showed 2,6-DiCH chlorohydrolase activity. The molecular mass of the overexpressed protein (PcpA) was about 30 kDa. Thus, it seems logical to assume that *pcpA* encodes 2,6-DiCH chlorohydrolase. The discrepancies in molecular weight and location of the functional protein (cytoplasm or periplasm) require further investigation.

The technique of gene targeting via homologous recombination, by which we demonstrated the genetic disruption of a *Sphingomonas pcpA* with *npt*II, has been shown to be useful for generating specific mutations in many organisms (16, 17, 25). All eight *pcpA* mutants that we selected carried the *pcpA-npt*II disruption, as determined by Southern analysis (23). All eight *pcpA-npt*II mutants were Amp^s, implying that the disruptions resulted from a double-crossover recombination and not by delivery plasmid cointegration (single crossover).

The identification of all biodegradation intermediates is a crucial step for outlining the mineralization pathway of PCP. Suitable extraction methods and selection of time points for sampling must be optimized. We examined two procedures for extracting PCP metabolites: neutral and acid extractions. Neutral extraction was suitable for extracting hydroxylated aromatic compounds, such as hydroquinones, while acid extraction was better suited for extracting benzoquinones and other organic compounds, as determined by HPLC and GC-MS analyses. Krumholz et al. (10) also saw this difference when they extracted polyhydroxylated benzenes from acidic aromatic and organic compounds by similar techniques. Since hydroquinones are autooxidized rapidly in air, time-dependent measurements of metabolite formation were made to determine an appropriate time point to obtain hydroquinone while minimizing oxidation. We separated hydroquinone in high quantity and quality from neutral extracts of cultures incubated with PCP for 4 h, while the product became rapidly oxidized in the acid extracts. A similar time-dependent extraction of the 2,6-DiCH metabolite during the degradation of 2,4,6-trichlorophenol by *Azotobacter* sp. strain GP1 has been reported previously (12).

Although the MS fragmentation patterns of 2,5-DiCH and 2,6-DiCH appear identical, we observed that the RTs of 2,5-DiCH and 2,6-DiCH are different. Under the conditions that we used, the RTs of 2,5-DiCH and 2,6-DiCH were 6.17 and 8.32 min, respectively. The metabolite extracted from cultures of our mutants by neutral extraction showed an RT and mass spectrum that perfectly matched those of authentic 2,6-DiCH. Thus, the actual intermediary metabolite accumulated during PCP degradation by the mutants with an impaired *pcpA* gene is a 2,6-DiCH. Furthermore, Lee and Xun (11) found that their chlorohydrolase dehalogenated 2,6-DiCH but not 2,5-DiCH. We therefore conclude that *pcpA* encodes information for 2,6-DiCH chlorohydrolase.

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