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The role of the *Sphingomonas* species UG30 pentachlorophenol-4-monooxygenase in *p*-nitrophenol degradation

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Abstract

Pentachlorophenol-4-monooxygenase is an aromatic flavoprotein monooxygenase which hydroxylates pentachlorophenol and a wide range of polyhalogenated phenols at their *para* position. The PCP-degrading *Sphingomonas* species UG30 was recently shown to mineralize *p*-nitrophenol. In this study, the UG30 *pcpB* gene encoding the pentachlorophenol-4-monooxygenase gene was cloned for use to study its potential role in *p*-nitrophenol degradation. The UG30 *pcpB* gene consists of 1614 bp with a predicted translational product of 538 amino acids and a molecular mass of 59 933 Da. The primary sequence of pentachlorophenol-4-monooxygenase contained a highly conserved FAD binding site at its N-terminus associated with a βαβ fold. UG30 has been shown previously to convert *p*-nitrophenol to 4-nitrocatechol. We observed that pentachlorophenol-4-monooxygenase catalyzed the hydroxylation of 4-nitrocatechol to 1,2,4-benzenetriol. About 31.2% of the nitro substituent of 4-nitrocatechol (initial concentration of 200 μM) was cleaved to yield nitrite over 2 h, indicating that the enzyme may be involved in the second step of *p*-nitrophenol degradation. The enzyme also hydroxylated *p*-nitrophenol at the *para* position, but only to a very slight extent. Our results confirm that pentachlorophenol-4-monooxygenase is not the primary enzyme in the initial step of *p*-nitrophenol metabolism by UG30. The UG30 *pcpB* sequence has been deposited at the GenBank under accession number AF059680.

Keywords: Biodegradation; *p*-Nitrophenol; 4-Nitrocatechol; *pcpB*; Pentachlorophenol; *Sphingomonas* species

1. Introduction

Pentachlorophenol (PCP) and *p*-nitrophenol (PNP) are considered as priority pollutants by the USEPA [1,2] and can be found in various polluted environments. Several *Sphingomonas* strains are capable of mineralizing PCP [3–6]. Recently, some PCP-degrading *Sphingomonas* strains, such as *Sphingomonas* species UG30, *Sphingomonas chlorophenolica* RA2 and ATCC 39723 have also been shown to mineralize PNP [7].

Dechlorination of PCP by *S. chlorophenolica*, previously classified as *Flavobacterium* species ATCC 39723 [8], has been well studied. The bacterium con-

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tains a PCP-inducible *pcpB* gene which encodes PCP-4-monooxygenase, an enzyme that hydroxylates PCP to tetrachlorohydroquinone, TeCH [9]. PCP-4-monooxygenase possesses a broad substrate specificity and has been shown to catalyze the hydroxylation of the *para* position of a diverse range of polyhalogenated phenols with an *ortho* substituent [10].

Two pathways have been proposed for the initial aerobic biodegradation of PNP by bacteria. In the first pathway, represented by a *Moraxella* strain, PNP is converted to hydroquinone by a flavoprotein monooxygenase [11,12]. In the second pathway, represented by an *Arthrobacter* species and *Bacillus sphaericus* JS905, PNP is hydroxylated to 4-nitrocatechol [13,14]. We previously showed that several PCP-degrading *Sphingomonas* strains can mineralize both PCP and PNP. Initial TLC evidence showed that PNP was degraded by UG30 to form 4-nitrocatechol [7]. It is not known if PCP-4-monooxygenase was involved in subsequent steps of the 4-nitrocatechol metabolism. Information about the substrate specificity of the UG30 PCP-4-monooxygenase is required to establish the metabolic pathway of PNP in UG30. In this study, we cloned the *pcpB* gene from UG30 and examined the involvement of the gene product in PNP and 4-nitrocatechol degradation.

2. Materials and methods

2.1. Bacterial strains and plasmids

Sphingomonas species UG30 was isolated from a soil sample collected at a PCP-contaminated industrial site in Ontario, Canada [6]. Genomic DNA of *S. chlorophenolica* ATCC 39723 was used as a template to synthesize a *pcpB* gene. Plasmids pBluescript II KS$^+$ (Stratagene, La Jolla, CA, USA) and pET23a (Novagen, Madison, WI, USA) were used as cloning and expression vectors, respectively. *Escherichia coli* JM109 and BL21 (Novagen) were used as recombinant hosts of the cloning and expressing plasmids, respectively.

2.2. Cloning and sequencing of the UG30 pcpB gene

S. chlorophenolica ATCC 39723 and UG30 were cultured separately in a minimal salt-glutamate medium (pH 7) [6]. The culture was harvested during the late exponential phase and washed twice with TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Genomic DNA was extracted using the method of Marmur as described by Johnson [15]. Digoxigenin (DIG)-labelled *pcpB* gene probe was synthesized by PCR from genomic DNA of ATCC 39723 using a DIG labelling nucleotide mixture (Boehringer Mannheim Canada, Laval, Québec, Canada) as described by Liesack et al. [16]. The primer pair, PCPB1 (5′-CGC GCT GCA GCG GGA GAG AGA TTG TTA TTA TGT CGA CCT A) and PCPB2 (5′-CGC GAA GCT TTG TCA TCG CAC GGG TCT CCT CAG), were used to amplify the entire encoding region of the ATCC 39723 *pcpB* gene as described previously [6]. Genomic DNA of UG30 and ATCC 39723 was digested with *Eco*RI (Boehringer Mannheim). The resulting fragments were separated in a 1% agarose gel, transferred to a positively charged nylon membrane and hybridized with the DIG-labelled *pcpB* probe under high stringency conditions [17]. The UG30 DNA fragment which hybridized to the *pcpB* probe was located and excized. The DNA was extracted by a spinning protocol [18], purified by a phenol/chloroform procedure, ligated to *Eco*RI-digested pBluescript II KS$^+$ and electrotransformed into *E. coli* JM109. Transformants were screened by colony hybridization. A clone which hybridized to the *pcpB* probe was designated as pB44 and used for further analyses. The sequence of the insert in pB44 was determined in both directions using an Applied Biosystems Autosequencer or a T7 sequencing kit (Pharmacia Biotech, Baie D'Urfé, Québec, Canada).

2.3. Expression of the UG30 pcpB gene

The entire *pcpB* structural gene of UG30 was amplified by PCR using the high fidelity PWO DNA polymerase (Boehringer Mannheim Canada) and subcloned into pET23a. The PCR primers were pcpbx1 (5′-GGGAATTCCATATGATGTCGACC-TATCCAGTCAAC) and pcpbx2 (5′-CGCGGAAT-TCTCAACCGCGGCGCACAAGCAT). The *Nde*I and *Eco*RI sites (underlined) were incorporated into primers to facilitate directional cloning of the PCR product into pET23a. The resulting plasmid

was designated as pBX2 and electro-transformed into *E. coli* BL21.

Expression of PCP-4-monooxygenase was determined by SDS-PAGE. BL21(pBX2) and BL21(pET23a), an experimental control, were grown in 100 ml of LB broth (Difco) supplemented with 100 μg ml^{-1} ampicillin and 25 μg ml^{-1} chloramphenicol. IPTG was added to a final concentration of 0.4 mM when the OD_{600} of the cultures reached 0.6. The cultures were incubated for another 3 h, harvested, washed twice with 50 ml ice-cold 50 mM Tris-HCl buffer (pH 7.4) and resuspended in 5 ml of the same buffer. Cells were lysed by ten cycles of sonication (15 s blasting/60 s cooling) in an ice bath. The lysate was centrifuged at $12\,000 \times g$ for 10 min at 4°C. Supernatant was collected and the protein concentration was determined by the standard dye binding procedure of Bradford. Proteins in the cell extract were examined on a 10% SDS-PAGE gel.

2.4. PCP-4-monooxygenase activity determination

2.4.1. Colorimetric assays

The degradation of PNP and 4-nitrocatechol by the UG30 PCP-4-monooxygenase was studied by measuring the substrate disappearance and nitrite release. BL21(pBX2) and BL21(pET23a) cultures were grown, induced by IPTG, harvested and concentrated and lysed. The lysate was centrifuged at $5000 \times g$ for 30 min at 4°C. Cell extracts were collected and total cellular protein concentrations of the samples were determined as described earlier. Cofactor (NADPH) and substrate (PNP or 4-nitrocatechol) were added to the cell extract at final concentrations of 400 and 200 μM, respectively, and the samples were incubated at 30°C for 2 h. Samples were diluted with an equal volume of 0.5 M NaOH and measured at 420 and 520 nm for PNP and 4-nitrocatechol, respectively, using a Pye Unicam PU8610 UV/VIS spectrophotometer (Philips, Cambridge, UK). Nitrite concentrations were estimated spectrophotometrically using sulfanilamide and *N*-(1-naphthyl)-ethylenediamine hydrochloride reagent [7]. All assays were done in triplicate.

2.4.2. Gas chromatographic mass spectrometric (GC-MS) analysis

BL21(pBX2) and BL21(pET23a) cultures were grown, induced by IPTG, harvested and concentrated as described earlier. To prevent oxidation of the reaction product (i.e. tetrachlorohydroquinone), ascorbic acid was added to a final concentration of 1 mg ml^{-1}. The mixture was incubated at 30°C and the reaction terminated after 5 h by the addition of 1 ml of 1 M HCl. The reaction mixture was extracted three times with 2 ml of ethylacetate. The pooled extract was evaporated to dryness under a stream of nitrogen and 100 μl of bis(trimethylsilyl)trifluoroacetamide was added to the dry sample. The mixture was heated at 60°C for 30 min. The trimethylsilyl-derivatized sample was dried under nitrogen and redissolved in 100 μl heptane. The sample was analyzed with a HP 5890 series II GC equipped with a HP 5973 series mass selective detector and a HP-5 capillary column (Hewlett-Packard, Palo Alto, CA, USA). The carrier gas (helium) was set at 30 psi and the temperature program of the column was: 60°C for 2 min, 60°–150°C at 10°C min^{-1}, 150°–310°C at 3°C min^{-1} and 312°C for 34 min. Degradation products of PNP and 4-nitrocatechol generated by PCP-4-monooxygenase were also analyzed by GC-MS.

3. Results and discussion

3.1. Cloning and nucleotide sequence analysis of the UG30 pcpB gene

Southern hybridization of the *Eco*RI genomic digests of UG30 and ATCC 39723 revealed a single 2.5- and 3.0-kb DNA fragment, respectively. Ederer et al. [5] reported that the *pcpB* gene of *S. chlorophenolica* ATCC 39723, ATCC 33790, RA2, and SR3 were all located in a 3-kb *Eco*RI fragment of the respective bacterial genome. However, the *pcpB*-containing *Eco*RI fragment of UG30 was only 2.5 kb. This indicates sequence differences between UG30 and other PCP-degrading *Sphingomonas* species.

The *pcpB* positive fragment of UG30 was cloned into pBluescript II KS^+ and the insert was sequenced in both directions. The sequence has been deposited at the GenBank under accession number AF059680. The DNA fragment contained an open reading frame (ORF) of 1614 nucleotides with a predicted translational product of 538 amino acids and a mo-

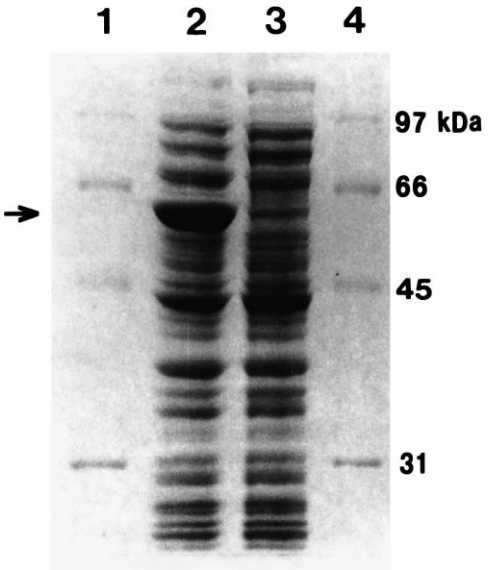

Fig. 1. Expression of UG30 *pcpB* by BL21(pBX2) cells. Coomassie blue-stained SDS-PAGE gel of whole cell protein extracts of BL21(pBX2), lane 2, and BL21(pET23a), lane 3. Lanes 1 and 4, protein molecular mass standards. An arrow indicates an overexpressed protein in BL21(pBX2) cells.

lecular mass of 59 933 Da. Expression of the *pcpB* gene in BL21(pBX2) revealed the synthesis of a protein of approximately 60 kDa (Fig. 1), which agreed with that predicted from the primary amino acid sequence. The predicted UG30 PcpB sequence showed a 93% and 90% similarity to the PCP-4-monooxygenases of *S. chlorophenolica* ATCC 39723 and ATCC 33790, respectively.

A FASTA search [19] showed 19–26% similarity between the UG30 PcpB and other aromatic flavoprotein monooxygenases listed in Table 1. A distance analysis of the aromatic flavoprotein monooxygenases, calculated by the Kimura's distance matrix formula [20] and the UPGMA (average linkage clustering) analysis of the phylogenetic inference package (PHYLIP) [21], showed a tight cluster between the UG30 PCP-4-monooxygenase and the other two *S. chlorophenolica* PCP-4-monooxygenases (Fig. 2). Representative protein sequences of dichlorophenol-6-monooxygenase, chlorophenol monooxygenase and phenol-2-monooxygensase formed another cluster and were more closely related to the PCP-4-monooxygenases than other aromatic monooxygenases. A BLAST search [22] revealed several highly conserved regions between PCP-4-monooxygenases and other aromatic monooxygenases. A highly conservative region (Val-17–Val-38), showing 41–55% similarity to other aromatic monooxygenases, was located at the amino-terminus of the UG30 PCP-4-monooxygenase. This region contained a FAD-binding motif, GXGXXGXXXAXXXXXXG, which is associated with a βαβ fold found in the FAD binding domain of many flavoprotein oxidoreductases [23]. The presence of the putative FAD binding site in the PCP-4-monooxygenases agrees with the study of Xun and Orser [24] that flavines play an important role in the dehalogenation of polyhalogenated compounds.

The *Eco*RI fragment also contained a second, incomplete ORF which starts 14 bp downstream of the *pcpB* gene. The sequence of this second partial ORF was 87% similar to the ATCC 39723 *pcpD* gene which encodes PCP 4-monooxygenase reductase [25]. Therefore, the arrangement of the *pcpB* and *pcpD* genes was the same in both ATCC 39723 and UG30.

Table 1
Nitrite released from 4-nitrocatechol (4-NC) and PNP catalyzed by the UG30 PCP-4-monooxygenase, expressed in BL21(pBX2) cells

Treatment[a]	Nitrate released		Substrate (4-NC or PNP) disappearance	
	nmol h^{-1} mg^{-1} cell protein	% Nitrate releasedin 2 h	nmol h^{-1} mg^{-1} cell protein	% Substrate disappeared in 2 h
BL21(pBX2)+4-NC	13.2 ± 1.3[b]	31.2	22.3 ± 0.7	52.6
BL21 (pET23a)+4-NC	0.5 ± 0.1	1.3	4.7 ± 0.9	12.0
BL21 (pBX2)+PNP	1.1 ± 0.1	2.6	1.3 ± 0.7	3.1
BL21(pET23a)+PNP	0 ± 0	0	0.1 ± 0.1	0.1

[a]Initial concentrations of 4-NC and PNP were 200 μM. Samples were incubated at 30°C for 2 h before being analyzed.
[b]Mean ± S.D. ($n = 3$).

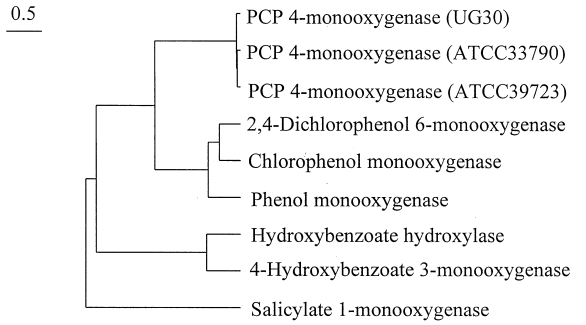

Fig. 2. Dendrogram based on a distance analysis of amino acid sequences of UG30 PCP-4-monooxygenase and other aromatic flavoprotein monooxygenases. Scale bar = 0.5 Kimura's distance unit. PCP-4-monooxygenase (UG30) from *Sphingomonas* species UG30, PCP-4-monooxygenase (ATCC 33790) from *Sphingomonas chlorophenolica* ATCC 33790, PCP-4-monooxygenase (ATCC 39723) from *S. chlorophenolica* ATCC 39723, 2,4-dichlorophenol-6-monooxygenase from *Alcaligenes eutrophus*, chlorophenol monooxygenase from *Ralstonia eutropha*, phenol monooxygenase from *Pseudomonas* species, hydroxybenzoate hydroxylase from *Acinetobacter calcoaceticus*, 4-hydroxybenzoate-3-monooxygenase from *Pseudomonas* species, salicylate-1-monooxygenase from *Pseudomonas putida*.

3.2. Degradative activities of UG30 PCP-4-monooxygenase

To assess the degradation of 4-nitrocatechol by the UG30 PCP-4-monooxygenase, both substrate release and product (nitrite) formation were followed. In the presence of the BL21(pBX2) cell extract, nitrite was released from 4-nitrocatechol at a rate of 13.2 nmol h^{-1} mg^{-1} protein and about 31% of the initial amount of 4-nitrocatechol was metabolized within 2 h to yield nitrite (Table 1). In control assays using extracts of BL21(pET23a) cells, only about 1.3% of the nitro group was released from 4-nitrocatechol. This shows that the UG30 PCP-4-monooxygenase effectively metabolized 4-nitrocatechol by eliminating the nitro group as nitrite ion. Colorimetric measurement revealed that 22.3 nmol of 4-nitrocatechol h^{-1} mg^{-1} disappeared during incubation. The discrepancy between the amount of NO_2^- detected and 4-nitrocatechol disappearance may be explained by the instability of 4-nitrocatechol. The UG30 PCP-4-monooxygenase exhibited a very low level of activity against PCP. Only 2.6% of the nitro group was released from PNP and only 3.1% of PNP disappeared from the reaction mixture after 2 h of incubation (Table 1).

GC-MS studies showed that only BL21(pBX2) cells exhibited PCP-4-monooxygenase activity. The experimental control with BL21(pET23a) did not hydroxylate PCP. At the beginning of the assay, only PCP was detected in the BL21(pBX2) extract. After 5 h incubation, most (> 80%) of the substrate was converted to TeCH and PCP disappeared completely after 16 h incubation (data not shown). The TeCH product had a retention time of 29.2 min which was identical to the retention time of an authentic TeCH standard. Its identity was further confirmed by mass spectrometric analysis (data not shown).

UG30 has been shown to degrade both PCP and PNP [7]. In some bacterial strains such as *Moraxella* species and *Pseudomonas putida*, a flavoprotein PNP-4-monooxygenase hydroxylates PNP to hydroquinone with the release of nitrite [12,26]. We previously showed that 4-nitrocatechol was the initial product of PNP degradation by UG30 [7]. This differs from the *Moraxella* species and *P. putida* above and suggests that UG30 may degrade PNP in the similar manner as *Arthrobacter* species JS 443 [13] and *Bacillus sphaericus* JS905 [14]. It was not known if PCP-4-monooxygenase is involved in the degradation of

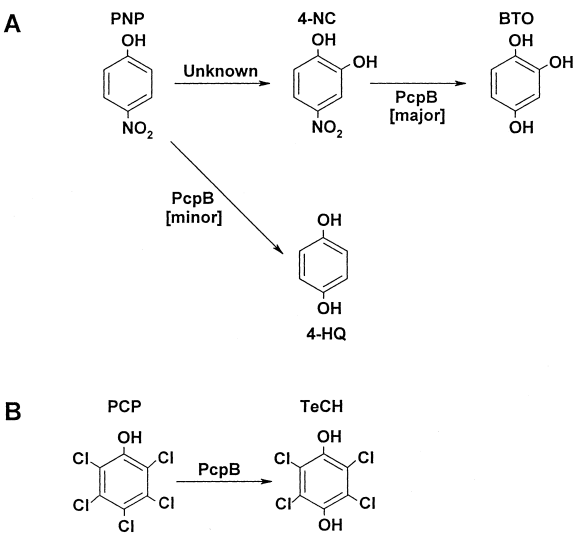

Fig. 3. Proposed involvement of PCP-4-monooxygenase in the degradation of PNP (A) and PCP (B) by UG30. PNP, *p*-nitrophenol; 4-NC, 4-nitrocatechol; BTO, 1,2,4-benzenetriol; 4-HQ, 4-hydroquinone; PCP, pentachlorophenol; TeCH, 2,3,5,6-tetrachlorohydroquinone.

4-nitrocatechol. Our experimental evidence did show that pre-growth of UG30 cells in PNP induces PCP degradation [7]. This may suggest the involvement of PCP-4-monooxygenase in PNP degradation. Furthermore, 4-nitrocatechol possesses a substituent *ortho* to the hydroxyl group and this has been shown to be the structural requirement for *para* hydroxylation of diverse aromatic phenols by the ATCC 39723 PCP-4-monooxygenase [10].

The UG30 PCP-4-monooxygenase was tested for the ability to degrade both PNP and 4-nitrocatechol. GC-MS studies on PNP degradation showed that only about 2% of the PNP was converted to *p*-hydroquinone after 5 h by the UG30 PCP-4-monooxygenase expressed in BL21(pBX2) cells (data not shown). In contrast, more than 85% of the 4-nitrocatechol was degraded by the PCP-4-monooxygenase. However, only a negligible amount of 1,2,4-benzenetriol was detected (data not shown). Since 1,2,4-benzenetriol is unstable in aqueous solutions, it is possible that this intermediate was auto-oxidized. Auto oxidation of 1,2,4-benzenetriol and 6-chloro-1,2,4-benzenetriol has also been reported previously [27,28].

Although the UG30 PCP-4-monooxygenase is capable of metabolizing 4-nitrocatechol like the PNP monooxygenase from *B. sphaericus* JS 905 [14], the two enzymes differ in several respects. First, PCP-4-monooxygenases appear to be single component monomeric enzymes ([9,24], this study), while PNP monooxygenase is a two component enzyme consisting of a flavoprotein reductase and an oxygenase [14]. Second, PCP-4-monooxygenases convert *p*-substituted polychlorophenols directly to chlorohydroquinones [10,25] instead of chlorobenzoquinones. While chlorobenzoquinone may be an intermediate, it has not been detected in any studies [10] and if it is formed, it is presumed to be reduced spontaneously to chlorohydroquinone. In contrast, the PNP monooxygenase first oxidizes 4-nitrocatechol to hydroxybenzoquinone, which is reduced enzymatically by the reductase component to 1,2,4-benzenetriol [14]. Third, PNP monooxygenase catalyzes both the hydroxylation of PNP and the oxidative release of nitrite from 4-nitrocatechol [14], while PCP-4-monooxygenase does not convert PNP to 4-nitrocatechol and its main action in the PNP pathway appears to be the oxidative removal of nitrite from 4-nitrocatechol. Finally, PNP monooxygenase has not been shown to dechlorinate polychlorophenols.

We propose that the UG30 PCP-4-monooxygenase is involved in PNP degradation through its action on 4-nitrocatechol (Fig. 3A). The product from this reaction is 1,2,4-benzenetriol, which can undergo ring fission. While hydroxybenzoquinone may be the transient intermediate in this reaction, we presently do not have any experimental evidence to show this. This reaction is consistent with our current understanding of the hydroxylation of PCP by PCP-4-monooxygenase to form tetrachlorohydroquinone (Fig. 3B) [10]. Although PCP-4-monooxygenase can also convert PNP to *p*-hydroquinone in vitro, it may be a minor PNP degradative pathway in *Sphingomonas* UG30.

Acknowledgments

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