

para-Nitrophenol 4-monooxygenase and hydroxyquinol 1,2-dioxygenase catalyze sequential transformation of 4-nitrocatechol in *Pseudomonas* sp. strain WBC-3

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Abstract *Pseudomonas* sp. strain WBC-3 utilizes *para*-nitrophenol (PNP) as a sole source of carbon, nitrogen and energy. PnpA (PNP 4-monooxygenase) and PnpB (*para*-benzoquinone reductase) were shown to be involved in the initial steps of PNP catabolism via hydroquinone. We demonstrated here that PnpA also catalyzed monooxygenation of 4-nitrocatechol (4-NC) to hydroxyquinol, probably via hydroxyquinone. It was the first time that a single-component PNP monooxygenase has been shown to catalyze this conversion. PnpG encoded by a gene located in the PNP degradation cluster was purified as a His-tagged protein and identified as a hydroxyquinol dioxygenase catalyzing a ring-cleavage reaction of hydroxyquinol. Although all the genes necessary for 4-NC metabolism seemed to be present in the PNP degradation cluster in strain WBC-3, it was unable to grow on 4-NC as a sole source of carbon, nitrogen and energy. This was apparently due to the substrate's inability to trigger the expression of genes involved in degradation. Nevertheless, strain WBC-3 could completely degrade both PNP and 4-NC when PNP was used as the inducer, demonstrating its potential in bioremediation of the environment polluted by both 4-NC and PNP.

Keywords 4-Nitrocatechol · Hydroxyquinol 1,2-dioxygenase · *para*-Nitrophenol 4-monooxygenase · *Pseudomonas* · Transformation

Introduction

To date, two alternative pathways for *para*-nitrophenol (PNP) degradation have been identified. One is initiated by a two-component monooxygenase to produce hydroxyquinol as a ring-cleavage substrate, usually found in Gram-positive strains of bacteria (Kadiyala and Spain 1998; Kitagawa et al. 2004; Takeo et al. 2008). The other pathway is usually found in Gram-negative strains, with hydroquinone as the ring-cleavage substrate (Spain et al. 1979; Spain and Gibson 1991) and the degradation is initiated by a single-component monooxygenase (Zhang et al. 2009). During the identification of molecular determinants for the PNP catabolic pathway via hydroquinone in *Pseudomonas* sp. strain WBC-3, we cloned a 12.7 kb fragment containing all the genes necessary for PNP degradation (Zhang et al. 2009). Among the products encoded by these genes, PnpA is a single component PNP 4-monooxygenase converting PNP to *para*-benzoquinone. PnpB is a *para*-benzoquinone reductase that catalyzes the reduction of *para*-benzoquinone to hydroquinone. Additionally,

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the gene cluster *pnpCDEF* next to *pnpAB* shares significant similarities and the same organization with the *hapCDEF* gene cluster responsible for transforming hydroquinone to central metabolites of the tricarboxylic acid cycle by *Pseudomonas fluorescens* ACB (Moonen et al. 2008). Apart from the above genes in this cluster, the gene *pnpG* located between *pnpAB* and *pnpCDEF* seems unnecessary for PNP degradation. A BLAST search of Swiss-Prot suggested that PnpG exhibited 43% identity to hydroxyquinol 1,2-dioxygenase from *Burkholderia cepacia* R34 (Johnson et al. 2000). The presence of PnpG as a potential hydroxyquinol 1,2-dioxygenase in a strain with the hydroquinone pathway for PNP degradation has aroused our interest to investigate its role in the biodegradation capacity of strain WBC-3.

Materials and methods

Bacterial strains, growth conditions and chemicals

Escherichia coli strains were routinely cultivated at 37°C in lysogeny broth (LB) medium. *Pseudomonas* sp. strain WBC-3 was cultured at 30°C in LB medium with 50 µg ml⁻¹ ampicillin (Sun et al. 2004) or in minimal medium (MM) (Liu et al. 2005) supplemented with PNP or 4-nitrocatechol (4-NC) as the substrate. Due to their toxicity to the cells, PNP or 4-NC was supplied by regular addition at a low concentration (0.02–0.5 mM). Cell growth on PNP or 4-NC in liquid MM was monitored by measuring the optical density (OD) at 600 nm. PNP, 4-NC, hydroquinol and hydroxyquinol were purchased from the Sigma Chemical Company (St. Louis, MO, USA) or Fluka Chemical Company (Buchs, Switzerland).

Gene cloning and protein expression

The *pnpG* gene was amplified from strain WBC-3 by polymerase chain reaction (PCR) using the high-fidelity DNA polymerase *Pyrobest* (Takara Bio Inc., Dalian, China), and the product was cloned into the *NdeI* and *BamHI* sites of the plasmid pET28a to produce pZWJJ011. Plasmid pZWJJ009 containing *pnpA* in pET28a (Zhang et al. 2009) was used for PnpA expression. *E. coli* BL21 (DE3) strains carrying the recombinant plasmids were grown in LB supplemented with kanamycin (45 µg ml⁻¹) at 37°C to an

OD of 0.6 at 600 nm, and then induced for 4 h by the addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

Preparation of cell extracts and protein purification

All operations for enzyme purification were carried out at 0–4°C. *E. coli* BL21 cells over-expressing H₆-PnpG were harvested by centrifugation and suspended in binding buffer (300 mM NaCl, 50 mM sodium phosphate buffer and 10 mM imidazole, pH 7.4). Cell extracts were prepared by sonication (Zhou et al. 2001), and then centrifuged at 12,000×*g* for 1 h to remove cell debris and unbroken cells. The enzyme in the supernatant was further purified using Ni²⁺-NTA agarose (GE Healthcare, USA) according to the supplier's recommendations. H₆-PnpG was eluted using binding buffer supplemented with imidazole at a concentration of 80 mM. H₆-PnpA was purified as described previously (Zhang et al. 2009).

Enzyme assays

PNP 4-monooxygenase activity was assayed as described (Zhang et al. 2009). The activity against PNP was determined by measuring the decrease in absorbance at 420 nm due to PNP disappearance, the molar extinction coefficient of which was taken as 7000 M⁻¹ cm⁻¹ (Spain et al. 1979). PNP 4-monooxygenase activity against 4-NC was determined by measuring the decrease in absorbance at 340 nm due to NADPH consumption, the molar extinction coefficient of which was taken as 6220 M⁻¹ cm⁻¹. Hydroxyquinol 1,2-dioxygenase activity was determined by measuring the increase in absorbance at 243 nm due to the formation of maleylacetate. The molar extinction coefficient of maleylacetate was 42000 M⁻¹ cm⁻¹ (Duxbury et al. 1970). The standard enzyme assay mixture for all reactions, unless specified otherwise, contained 500 µl of 20 mM phosphate buffer (pH 7.4) supplemented with 0.2 mM of substrate for hydroxyquinol or 0.04 mM for PNP and 4-NC, and varying amounts of enzyme. Substrates were omitted from the reference cuvette, and reactions were initiated by the addition of substrates. One unit of PNP 4-monooxygenase activity was defined as the amount required to catalyze the oxidization of 1 µmol of PNP (against PNP) or

NADPH (against 4-NC) per min at 30°C. One unit of the dioxygenase activity is defined as the amount of enzyme required for the formation of 1 μmol of product. Their specific activities are expressed as U mg^{-1} protein. Protein concentrations were determined by the Bradford method (Bradford 1976) with bovine serum albumin as the standard. All values are expressed as means $\pm$ standard deviations from three independent experiments.

Biotransformation of 4-NC by *E. coli* cells expressing PNP monooxygenase

E. coli BL21[pZWJJ009] cells overproducing PnpA were harvested, washed and resuspended in phosphate buffer (pH 7.4). Biotransformation was initiated by the addition of 4-NC to a final concentration of about 200 μM into the concentrated cell suspensions (OD of 20 at 600 nm), with 0.5 μM sodium dithionite previously being added to inhibit hydroxyquinol auto-oxidation (Kadiyala and Spain 1998; Chauhan et al. 2000). Samples were taken at 1 min intervals followed by centrifugation at $12,000\times g$ for 10 min, and the supernatant was extracted with an equal volume of ethyl acetate. The organic phase was collected by centrifugation and subjected to HPLC analysis. The values are expressed as means $\pm$ standard deviations from three independent experiments.

Biotransformation of PNP and 4-NC by strain WBC-3 cells incubated with PNP or 4-NC

Strain WBC-3 was grown at 30°C for 48 h in MM supplemented with 10 mM succinate and 5% $(\text{NH}_4)_2\text{SO}_4$, in the presence of 0.5 mM PNP or 0.1 mM 4-NC as the inducer. After cells were washed and resuspended in MM with a final OD₆₀₀ of 0.2, cells incubated with PNP or 4-NC were determined for their ability to degrade PNP and 4-NC. PNP or 4-NC were added to final concentrations of 0.2 and 0.1 mM, respectively, into the resting cell suspensions with shaking. Samples were taken at certain time intervals and subjected to HPLC analysis. Experiments were performed with three independent repeats.

Analytical methods

HPLC analysis was performed at 30°C with an Agilent series 1200 system (Agilent Technologies,

Palo Alto, CA, USA) equipped with a C₁₈ reversed-phase column (5 μm ; 4.6×250 mm; Agilent Technologies). For analysis of the product from 4-NC biotransformation catalyzed by PNP monooxygenase, the mobile phase consisted of a linear gradient of acetonitrile/acetic acid (1%) ranging from 5/95 to 30/70 at a flow rate of 0.5 ml min^{-1} . Both the substrate and the product were quantitatively monitored at 290 nm. Under these conditions, authentic 4-NC and hydroxyquinol had retention times of 27.9 and 8.0 min, respectively. For quantitation of PNP and 4-NC in the biotransformation by PNP- and 4-NC-incubated cells of strain WBC-3, the mobile phase was 30% methanol at a flow rate of 1 ml min^{-1} and the substrates were quantified at 290 nm. Under these conditions, authentic PNP and 4-NC had retention times of 17.9 and 10.5 min, respectively.

Results

Over-expression and purification of H₆-PnpG

The N-terminal H₆-tagged PnpG was successfully expressed using *E. coli* BL21 (DE3) cells carrying pZWJJ011. The protein was present predominantly in the soluble fraction, exhibiting the expected molecular weight of 34.8 kDa as determined by SDS-PAGE in Fig. 1. H₆-PnpG was purified with an overall recovery of 10%.

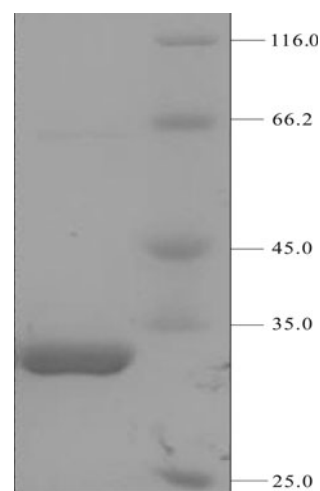


Fig. 1 SDS-PAGE with Coomassie Blue staining of H₆-PnpG. Lane 1 molecular mass standards (kDa), lane 2 purified H₆-PnpG

PnpG is a hydroxyquinol 1,2-dioxygenase

Among several types of ring cleavage substrates detected, H₆-PnpG was found to be active against both hydroxyquinol and catechol but not for protocatechuate and gentisate. During incubation with hydroxyquinol, the substrate peak with maximal absorbance at 278 nm gradually disappeared, giving rise to a new peak with maximal absorbance at 245 nm (Fig. 2), as described in an assay with

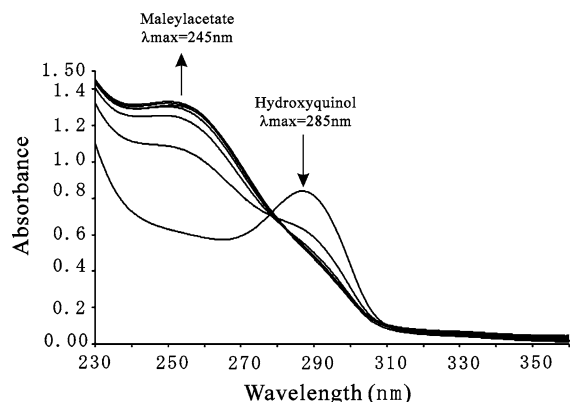
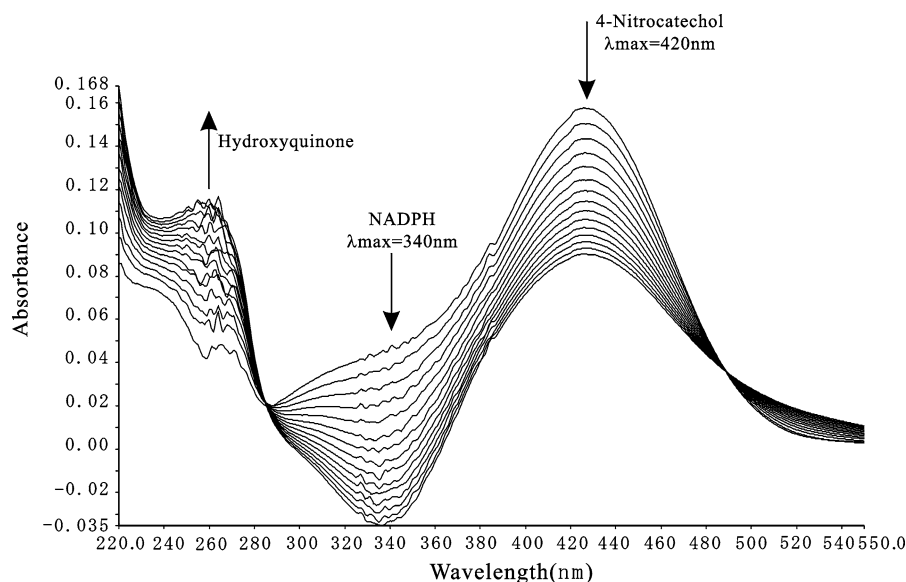


Fig. 2 Spectral changes during enzymatic oxidation of hydroxyquinol by purified H₆-PnpG. Hydroxyquinol (0.2 mM) was added to 500 μ l phosphate buffer (pH 7.4) containing 1.5 μ g purified H₆-PnpG to initiate the reaction. Scans were made at 30 s intervals against a control reaction lacking hydroxyquinol. The arrows indicate the directions of spectral changes

Fig. 3 Spectrophotometric changes during the transformation of 4-NC by purified H₆-PnpA. Sample and reference cuvettes contained 0.1 mM NADPH, 0.03 mM FAD, 20 mM phosphate buffer (pH 7.0) and 0.3 μ g H₆-PnpA in 0.5 ml volumes. The reaction was initiated by the addition of 4-NC to 40 μ M and the spectra were recorded every minute after the addition of 4-NC. Arrows indicate the direction of spectral changes



hydroxyquinol 1,2-dioxygenase against hydroxyquinol (Chapman and Ribbons 1976; Spain and Gibson 1991). This indicated that an *ortho*-ring cleavage of hydroxyquinol had occurred. The intradiol ring-cleavage activity of PnpG for hydroxyquinol was determined to be 26.9 ± 0.72 U mg⁻¹.

PnpA catalyzes monooxygenation of 4-NC to hydroxyquinol

In our previous study, PnpA was demonstrated to catalyze PNP monooxygenation to *para*-benzoquinone, and it was also active against 4-NC, although the product was unknown (Zhang et al. 2009). The purified H₆-PnpA in this study had similar specific activities towards PNP and 4-NC as those described previously (Zhang et al. 2009). In a 4-NC transformation catalyzed by purified H₆-PnpA, rapid degradation of 4-NC (λ_{max} = 420 nm) occurred, as shown in Fig. 3, together with consumption of NADPH (λ_{max} = 340 nm). There was also an increase in the absorbance at 260 nm, probably caused by the formation of hydroxyquinone (Chapman and Ribbons 1976; Bohuslavsek et al. 2005). Hydroxyquinone was generally thought to be reduced to hydroxyquinol in vivo by quinone reductases in a wide diversity of cells, such as broad-specific quinone reductase WrbA (Patridge and Ferry 2006) or NfsA (Zenno et al. 1996) from *E. coli*, or some unidentified quinone

reductases from the PNP utilizer strain SAO101 (Kitagawa et al. 2004).

Hydroxyquinol was identified as the product from 4-NC biotransformation by *E. coli* [pZWJ011] expressing PNP monooxygenase by HPLC analysis when compared with the standard. In a time course assay of the monooxygenation reaction, the 4-NC consumption (176.1 μM) was equivalent to the total accumulation of hydroxyquinol (176.5 μM), indicating that the ratio of complete conversion of 4-NC to hydroxyquinol approaches 1:1 (Fig. 4). This experiment was repeated three times, and similar results were obtained each time, with a standard deviation of 0.13.

Growth test of strain WBC-3 on 4-NC

To examine whether strain WBC-3 could utilize 4-NC as the sole source of carbon and nitrogen, different concentrations of 4-NC ranging from 0.02 to 0.5 mM was added to MM, but no growth could be observed 10 days post-inoculation. When PNP (0.5 mM) was used as the substrate, however, the growth of strain WBC-3 cells could be detected in less than 24 h.

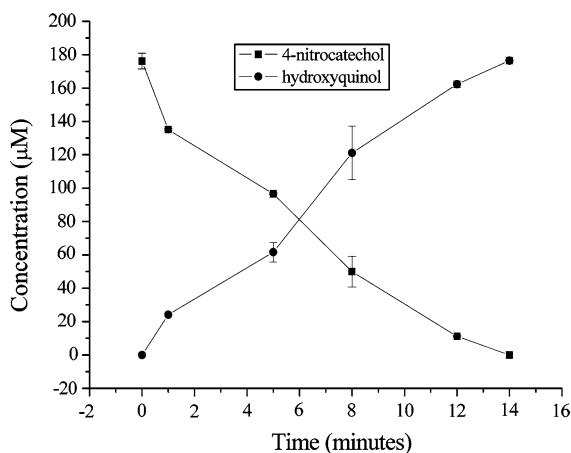


Fig. 4 Time course of 4-NC transformation by resting *E. coli* cells [pZWJ011] expressing PnpA. Samples were withdrawn at the indicated time points and treated immediately using the method described in the text. The consumption of 4-NC and hydroxyquinol production were quantified by HPLC. Similar results were obtained from three independent experiments, with a standard deviation of 0.13

4-NC does not serve as an inducer for the 4-NC or PNP degradation pathway

Strain WBC-3 cells incubated with PNP were able to completely degrade 0.24 mM PNP in 36 min and 0.1 mM 4-NC in 80 min. In contrast, strain WBC-3 cells incubated with 4-NC were unable to degrade either (Fig. 5), indicating that expression of PNP degradation genes could be induced by PNP but not 4-NC. However, if a small amount of PNP (0.02 mM) was added to 4-NC-incubated strain WBC-3 suspensions, both 4-NC and PNP could be completely

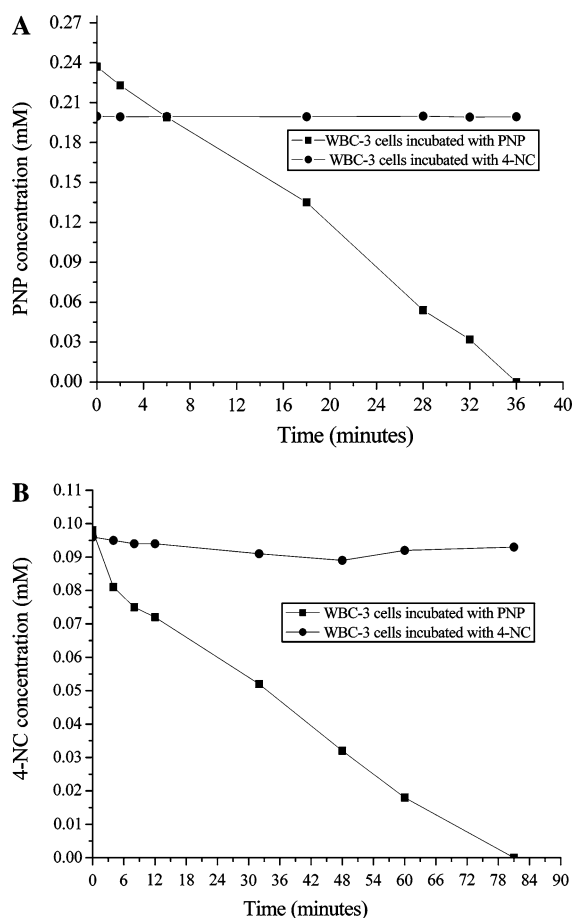
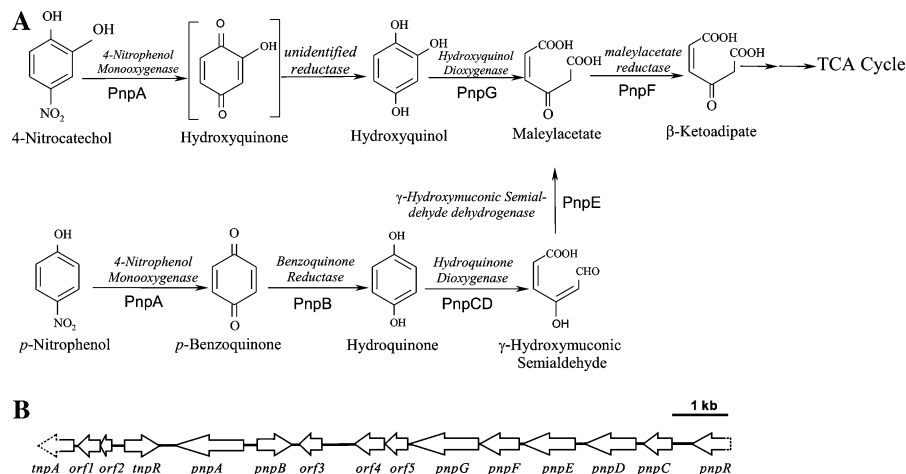


Fig. 5 **a** Degradation of PNP by *Pseudomonas* sp. strain WBC-3 cells incubated with PNP or 4-NC. **b** Degradation of 4-NC by *Pseudomonas* sp. strain WBC-3 cells incubated with PNP or 4-NC. The cells were incubated in MM (Liu et al. 2005) containing the substrate PNP or 4-NC. The inocula used were obtained from washed cells of strain WBC-3 grown on MM supplemented with 10 mM succinate and 5% $(\text{NH}_4)_2\text{SO}_4$ in the presence of PNP and 4-NC. The same degradation trend was observed in three independent repeats

Fig. 6 **a** Proposed 4-NC transformation steps in *Pseudomonas* sp. strain WBC-3, together with the reactions catalyzed by *pnp* genes products and the proposed pathway for PNP catabolism (Zhang et al. 2009). **b** Organization of the *pnp* gene cluster of *Pseudomonas* sp. strain WBC-3 (Zhang et al. 2009). The large open arrows indicate the size and direction of transposition of each gene or ORF



degraded in 2 days. The same degradation trend was observed over three independent repeats.

Discussion

In this study, we have identified PnpA and PnpG as the key enzymes involved in sequential 4-NC transformation steps in *Pseudomonas* sp. WBC-3, revealing the correlation between specific enzymes catalyzing reactions and their encoding genes. 4-NC was reported to be converted to hydroxyquinol as an intermediate in PNP degradation by two-component PNP monooxygenases in several Gram-positive PNP utilizers (Kadiyala and Spain 1998; Kitagawa et al. 2004; Takeo et al. 2008). In the Gram-negative PNP utilizer *Moraxella* sp., 4-NC was found to be oxidized by PNP-grown cells, but not by a partially purified particulate fraction containing PNP monooxygenase (Spain and Gibson 1991). No enzyme has been found to be involved in 4-NC monooxygenation in Gram-negative bacteria thus far. In this study, we demonstrated that purified single-component PNP 4-monooxygenase (PnpA) from strain WBC-3 was involved in the transformation of 4-NC to hydroxyquinol (Fig. 6a), the first case of a single-component PNP monooxygenase catalyzing 4-NC monooxygenation. Although *pnpG* located in the *pnp* cluster seems unnecessary for PNP degradation in this strain, it has been shown here to be a gene encoding a functional dioxygenase catalyzing the ring-cleavage reaction of hydroxyquinol to form maleylacetate (Fig. 6a). If this is the case in vivo, then 4-NC would

be completely degraded by strain WBC-3, as all genes necessary for 4-NC degradation were present in the *pnp* cluster (Fig. 6b). In fact, this strain was unable to grow on 4-NC. This is apparently due to the substrate's inability to trigger expression of genes involved in degradation as demonstrated in the study. Nevertheless, strain WBC-3 could completely degrade both PNP and 4-NC when PNP was used as the inducer, demonstrating its potential for bioremediation of environments polluted by both 4-NC and PNP.

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