para-Nitrophenol (PNP), a priority environmental pollutant, enters the environment through industrial release and degradation of parathion-based pesticides. To date, several pure bacterial cultures have been isolated based on their ability to metabolize PNP (12, 14, 30), and two alternative oxidative pathways have been elucidated based on different intermediates that are present. The hydroquinone pathway, preferentially found in gram-negative bacteria, was proposed for a Moraxella sp. in which PNP monooxygenase activity in the membrane fraction was found to convert PNP to hydroquinone via the potential intermediate p-benzoquinone (30, 31), whereas in the hydroxquinol (1,2,4-trihydroxybenzene) pathway, preferentially found in gram-positive bacteria, PNP is converted to hydroquinol (12, 14, 16). A two-competent monooxygenase was partially purified from Arthrobacter sp. strain WBC-3. Furthermore, the pnpCDEF gene cluster next to pnpAB shares significant similarities with and has the same organization as a gene cluster responsible for hydroquinone degradation (hapCDEF) in Pseudomonas fluorescens ACB (M. J. Moonen, N. M. Kamerbeek, A. H. Westphal, S. A. Boeren, D. B. Janssen, M. W. Fraaije, and W. J. van Berkel, J. Bacteriol. 190:5190–5198, 2008), suggesting that the genes involved in PNP degradation are physically linked.

component PNP hydroxylase from Rhodococcus sp. strain PN1 that catalyzes the hydroxylation of PNP to form 4-nitrocatechol was characterized (32). Although the PNP catabolic genes for the hydroquinone pathway were reported to have been cloned from Pseudomonas sp. strain ENV2030 and Pseudomonas putida JS444 (43), there was no indication that these genes were functionally characterized, and their DNA sequences are unavailable in the databases.

Pseudomonas sp. strain WBC-3 utilizes methyl parathion (O,O-dimethyl O-p-nitrophenol phosphorothioate) and PNP as sole sources of carbon, nitrogen, and energy (5). The gene encoding methyl parathion hydrolase (EC 3.1.8.1), which catalyzes the hydrolyzation of methyl parathion to PNP, was functionally expressed (20). Here we report genetic and biochemical characterization of two enzymes involved in the initial steps of PNP degradation. p-Benzoquinone was found to be the product of the monooxygenation by purified PNP 4-monooxygenase, and it could be reduced to hydroquinone by purified p-benzoquinone reductase. This study should enhance our understanding of the genetic and biochemical diversity of microbial PNP degradation.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, primers, chemicals, media, and culture conditions.** The bacterial strains and plasmids used are listed in Table 1. Escherichia coli strains were grown and transformed as described previously (26). Strain WBC-3 was grown at 30°C in tryptone broth (LB) or minimal medium (19) with 1 mM PNP as the sole carbon and nitrogen source. The primer sequences used for PCR are available upon request. All reagents used in this study were purchased from Sigma Chemical Co. (St. Louis, MO) or Fluka Chemical Co. (Buchs, Switzerland).
TABLE 1. Bacterial strains and plasmids used in this study

<table>
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<th>Strain or plasmid</th>
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<td>PNP and methyl parathion utilizer, wild type</td>
<td>This study</td>
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<tr>
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<td>WBC-3 mutant with pnpA gene disrupted</td>
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</tr>
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<td>pZWJ1010</td>
<td>Ndel-EcoRI fragment containing pnpB inserted into pET28a</td>
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Cloning of PNP degradation genes and sequence analyses. Primers were designed based on a conserved region of benzoquinone reductases from *Phanerochaete chrysosporium* (accession no. AF106939) and *Triphysaria versicolor* (accession no. AF304642) in order to amplify a potential benzoquinone reductase gene from strain WBC-3. Genome walking was then conducted to clone the flanking regions of the reductase gene with methods described previously (28). The nucleotide sequence was determined by Invitrogen Technologies Co. (Shanghai, China). Open reading frames (ORFs) were identified using the ORFFinder program on the National Center for Biotechnology Information website. The deduced proteins were examined for sequence similarity with other proteins in the GenBank database using BLASTP (39).

Gene cloning and protein expression. *pnpA* and *pnpB* were PCR amplified from strain WBC-3 with the Pyrobest high-fidelity DNA polymerase (Takara, Dalian, China). The product containing *pnpA* was cloned into Ndel and Xhol sites of pET28a to produce pZWJ1009, and the product containing *pnpB* was cloned into Ndel and EcoRI sites of pET28a to produce pZWJ1010. Both procedures resulted in an N-terminal six-His-tagged fusion protein. The sequences were verified by DNA sequencing to ensure that no mutations had been introduced during the PCR. *E. coli* BL21(DE3) strains carrying the resulting plasmids were grown in LB at 37°C to an optical density at 600 nm (OD 600) of introduced during the PCR. sequencese were verified by DNA sequencing to ensure that no mutations had been procedures resulted in an N-terminal six-His-tagged fusion protein. The sequencese were verified by DNA sequencing to ensure that no mutations had been introduced during the PCR. *E. coli* BL21(DE3) strains carrying the resulting plasmids were grown in LB at 37°C to an optical density at 600 nm (OD 600) of introduced during the PCR.

Preparation of cell extracts. The cells were harvested by centrifugation and suspended in binding buffer (see below), and the cell extracts were prepared by sonication treatment as described previously (42).

Protein purification. His-tagged PnpA (His6-PnpA) and PnpB (His6-PnpB) were purified from *E. coli* cells overproducing the corresponding proteins, and all purification steps were performed at 4°C. The cell extracts from 400-mL cultures were ultracentrifuged at 177,000 × g for 1 h to remove membranes. Each supernatant was purified further by using nickel-nitriolactateic acid (Ni2+-NTA) agarose (Merck Biosciences, Darmstadt, Germany) according to the supplier’s recommendations. The binding buffer contained 300 mM NaCl, 50 mM sodium phosphate buffer, and 20 mM imidazole (pH 8.0). The elution buffer was the same as the binding buffer except that the final concentration of imidazole was 150 mM. The eluted fractions were aliquoted and stored at −70°C.

Enzyme assays. (i) PNP 4-monooxygenase. The PNP 4-monooxygenase assay was performed as described previously (31). The reaction mixtures (final volume, 1.0 mL) contained 0.05 mM substrate, 1.0 mM NADPH, 0.03 mM flavin adenine dinucleotide (FAD), 0.6 to 10 μg of protein, and 20 mM phosphate buffer (pH 7.0). The reference cuvette contained all of these compounds except the substrate, and the assay was initiated by addition of substrate. The molar extinction coefficient for NADP(+)H was 6,220 M−1 cm−1, and the molar extinction coefficient for PNP was 7,000 M−1 cm−1 at 420 nm and pH 7.0 (31). For time course assays, monooxygenase-catalyzed reactions were carried out using 30-ml reaction mixtures containing 75 μM PNP, 300 μM NADPH, 30 μM FAD, and 20 mM phosphate buffer (pH 7.0). The reaction was initiated by addition of 75 μg purified His6-PnpA. One-milliliter samples were withdrawn from the reaction mixture and extracted with equal volumes of ethyl acetate after acidification with HCl. The ethyl acetate layer was collected by centrifugation prior to high-performance liquid chromatography (HPLC) analysis. For gas chromatography (GC)-mass spectrometry (MS) analysis, the reaction mixtures were extracted with ether after acidification, and the extract was dried over sodium sulfate. When PNP 4-monooxygenase was assayed in the presence of PnpB, the 1-ml assay mixture contained 100 μM NADPH, 20 μM FAD, 20 μM flavin mononucleotide (FMN), 20 mM phosphate buffer (pH 7.0), and 0.5 μg His6-PnpA. Different amounts of His6-PnpB were added, while the amount of His6-PnpA was kept constant. The reaction was initiated by addition of 30 μg PNP, and the enzymatic rates were determined by measuring the decrease in absorbance at 420 nm due to PNP consumption.

(ii) p-Benzoquinone reductase. The p-benzoquinone reductase activity of cell extracts of strain WBC-3 was assayed by a method described previously (31). To eliminate the effect of nonenzymatic reduction of benzoquinone, a previously described method (24) was adopted for the assay of purified His6-PnpB. The reaction mixtures contained 50 mM morpholinepropanesulfonic acid (MOPS) (pH 7.2) along with 100 μM NADPH, 40 μM FMN, 40 μM p-benzoquinone, and variable amounts of His6-PnpB. The reaction was initiated by addition of the enzyme.

(iii) Hydroquinone ring cleavage enzyme. Hydroquinone oxidation by cell extracts of strain WBC-3 was assayed as described previously (30).

Where applicable, all assays were conducted with strains carrying only vectors as negative controls. One unit of enzyme activity was defined as the amount of activity required to catalyze the oxidation of 1 μmol of PNP (for PNP 4-monooxygenase) or NADPH (for p-benzoquinone reductase) per min at 30°C. Specific activities are expressed below in units per milligram of protein, and the values are expressed as means ± standard deviations calculated from triplicate assays. A kinetic analysis was done by measuring the rate of decrease of the substrate. Three independent sets of experiments were performed with at least six substrate concentrations ranging from 0.5 Km to 4 Km. Data were fitted with the Michaelis-Menten equation by using Grafit 6 software (R. J. Leatherbarrow, Erithicus Software Ltd., Horley, United Kingdom). The protein concentration was determined by the Bradford method (4) with bovine serum albumin as the standard. The nitrate concentration was determined by a previously described method (18).

Analytical methods. HPLC was performed using a column temperature of 30°C with an Agilent series 1200 system (Agilent Technologies, Palo Alto, CA) equipped with a C18 reversed-phase column (5 μm; 4.6 by 250 mm; Agilent Technologies). The mobile phase was 30% acetonitrile at a flow rate of 1 mL min−1. PNP and hydroquinone were quantified at 290 nm, while p-benzoquinone was quantified at 245 nm. Under these conditions, authentic PNP, and hydroquinone had retention times of 9.9, 4.5, and 3.1 min, respectively. Due to poor ionization efficiency for p-benzoquinone under electron spray ion-
Proposed pathway for methyl parathion and PNP catabolism in *Pseudomonas* sp. strain WBC-3, together with the catabolic reactions catalyzed by the *mph* and *pnp* gene products in vivo.

**A**

- **Methyl parathion**
  - Formed by the **p-Nitrophenol**
  - **Dimethylthiophosphoric acid**
  - **p-Nitrophenol**
  - **O2**
  - **H2O**
  - **NADPH**

**B**

- **tnpA orf1 orf2 tnpR tnpA**
- **pnpB orf3 orf4 orf5 pnpG pnpF pnpE pnpD pnpC pnpR**
- **1 kb**

**FIG. 1.** (A) Proposed pathway for methyl parathion and PNP catabolism in *Pseudomonas* sp. strain WBC-3, together with the catabolic reactions catalyzed by the *mph* and *pnp* gene products in vivo. (B) Organization of the *pnp* gene cluster of *Pseudomonas* sp. strain WBC-3. The large open arrows indicate the size and direction of transcription of each gene or ORF. The thin arrows under the gene cluster represent the small open arrows used in genome walking reactions. The star above large open arrows indicate the size and direction of transcription of each gene or ORF. The thin arrows under the gene cluster represent the small open arrows used in genome walking reactions.

**RESULTS AND DISCUSSION**

PNP degradation via the hydroquinone pathway by strain WBC-3. Although strain WBC-3 utilizes PNP as a sole source of carbon, nitrogen, and energy, for an LB-grown culture there was an induction period (3 to 4 h) prior to growth on PNP. Enzyme assays indicated that cell extracts of strain WBC-3 oxidized PNP at a specific activity of 0.0004 U mg^{-1} with nitrite released in the presence of NADPH. Activity of hydroquinone 1,2-dioxygenase, which catalyzed the conversion of hydroquinone to γ-hydroxymuconic semialdehyde (γ-HMSA), as well as γ-HMSA dehydrogenase, was also detected in the cell extracts by a method described elsewhere (30). All these biochemical characteristics of strain WBC-3 for PNP degradation were consistent with the report for *Moraxella* sp. (30), suggesting that strain WBC-3 degrades PNP via a typical hydroquinone pathway which involves the initial removal of a nitro group as nitrite and formation of hydroquinone, as shown in Fig. 1A.
Cloning and sequence analyses of the PNP catabolic gene cluster. A benzoquinone reductase was previously considered to be involved in the initial reactions of PNP catabolism in *Moraxella* sp. (31). Based on a conserved region of benzoquinone reductase genes from two fungi (1, 21), a pair of primers, ZP001 (5'-TAC GGACACGTGGAGAAGTACG-3') and ZP002 (5'-CACGT TCGCCGGAGACAGCGATC-3'), was initially employed to amplify a PCR product with an anticipated size of 500 bp from strain WBC-3. Subsequently, a 12,670-bp DNA fragment extending from this 500-bp region was obtained and sequenced after several cycles of genome walking, as outlined in Fig. 1B. Thirteen complete ORFs and two truncated ORFs (*pnpA* and *pnpR*) were deduced, as shown in Fig. 1B, and they were annotated on the basis of BLAST analysis.

The upstream region of the sequenced fragment (*pnpA to *tnpR*) contains genes encoding part of Tn5044, a thermosensitive mercury resistance transposon (15). *PnpA* is most similar (24% identity) to *MhaA*, the large component of a 3-hydroxy-phenylacetate hydroxylase from *P. putida* U (2). *PnpB* shares the highest sequence identity (58%) with *WrbA*, an NAD(P)H:quinone oxidoreductase from *E. coli* (24). Sequence alignment indicated that the motifs for FAD and NADPH binding, including GXXGXXG, DXXCSXHR, and GXHHLHGDAAH, are proposed to be the molecular masses of H6-PnpA and H6-PnpB as deduced from the hydroxylase activity (9), were conserved in *PnpA* compared with other FAD-dependent monoxygenases whose three-dimensional structures are available, such as phenol hydroxylase (8) and p-hydroxybenzoate hydroxylase (35).

Notably, the *pnpCDEFG* gene cluster of strain WBC-3 shares significant similarity with (between 61% and 82% identity at the amino acid level for the genes products) and has the same organization as the recently reported hydroquinone degradation cluster (*hapCDEFG*) involved in 4-hydroxyacetophenone catabolism by *Pseudomonas fluorescens* ACB (22, 23). The products of two of the ORFs in strain WBC-3, *PnpC* and *PnpD*, are proposed to be the α- and β-subunits of hydroquinone dioxygenase (corresponding to *HapC* and *HapD*) catalyzing ring cleavage of hydroquinone, yielding γ-HMSA (22, 23). *PnpE* and *PnpF* are likely to be a dehydrogenase and a reductase, respectively (corresponding to *HapE* and *HapF*), which convert γ-HMSA to β-ketoadipate via maleylacetate (22). The *pnpG* gene (corresponding to *hapG*) appears to encode a ring cleavage dioxygenase as the level of identity of *PnpG* with the hydroxyquinol 1,2-dioxygenase from *Burkholderia cepacia* R34 is 43% (13), but *HapG* is not directly involved in the degradation of hydroquinone (22).

Based on the sequence similarities and the proposed PNP degradation pathway, it can be tentatively concluded that the genes involved in PNP catabolism (*pnpABCD* and *pnpE*) are tightly clustered (Fig. 1B). One ORF (referred to as *pnpR*) may encode an LysR regulatory protein which contains a conserved helix-turn-helix DNA-binding motif, as well as an LysR substrate-binding domain.

Overexpression and purification of *PnpA* and *PnpB*. Recombinant *PnpA* and *PnpB* were overexpressed in *E. coli* BL21(DE3) as N-terminal His-tagged fusion proteins for easy purification. Large quantities of soluble and active H6-PnpA and H6-PnpB were produced and purified to apparent homogeneity by Ni2+-NTA affinity chromatography. When 69 mg of cell extract protein with a specific activity of 0.4 U mg⁻¹ was applied to 4 ml of Ni2+-NTA agarose beads, 1.4 mg of H6-PnpA was purified with a specific activity of 6.8 U mg⁻¹. For H6-PnpB purification, 1.4 mg of H6-PnpB with a specific activity of 21.9 U mg⁻¹ was obtained from 62 mg of cell extract protein with a specific activity of 2.3 U mg⁻¹. Single bands at apparent molecular masses of 47 kDa and 25 kDa were detected by SDS-PAGE, respectively (Fig. 2), corresponding to the molecular masses of H6-PnpA and H6-PnpB as deduced from the amino acid sequences. Both purified enzymes were colorless, indicating that there are no bound flavin prosthetic groups. As determined by gel filtration chromatography, H6-PnpA is considered a monomer and H6-PnpB is most likely a dimer.

*pnpA* catalyzes monoxygenation of PNP to *p*-benzoquinone. Cell extracts of *E. coli* BL21(pZWJJ009) containing *pnpA* were found to contain nitrite-forming PNP 4-monoxygenase. Neither PNP consumption nor nitrite release was detected in the negative controls when the expression vector contained no insert. Rapid degradation of PNP (λ<sub>max</sub> 400 nm) by the purified H6-PnpA occurred, as shown in Fig. 3, together with consumption of NADPH (λ<sub>max</sub> 340 nm). An isobestic point at 278 nm was also observed, indicating that there was transformation of PNP to *p*-benzoquinone (λ<sub>max</sub> 244 nm). Both benzoquinone and hydroquinone (λ<sub>max</sub> 289 nm) were identified as products of PNP monooxygenation in the system containing purified H6-PnpA by HPLC analysis by comparison with standards. Furthermore, the identification of *p*-benzoquinone was also confirmed by GC-MS by comparison with the mass spectra of authentic *p*-benzoquinone and the reference data for *p*-benzoquinone in the MS database (Fig. 4). The detection of hydroquinone may be due to benzoquinone reduction in the presence NADPH, and the same explanation was proposed previously for strain JS443 (25). In a time course
The assay of the monooxygenation reaction, the PNP consumption (75.6 μM) was equivalent to the total accumulation of both benzoquinone (59.2 μM) and hydroquinone (15.5 μM) (Fig. 5), indicating that the ratio of complete conversion of PNP to benzoquinone and hydroquinone is close to 1:1.

In general, quinones are formed from monooxygenase reactions after removal of an electron-withdrawing group, such as chlorine or a nitro group (10, 38). However, only 2-hydroxy-5-methylquinone, a relatively stable quinone, was directly captured as a product formed from 4-methyl-5-nitrocatechol by partially purified 4-methyl-5-nitrocatechol 5-monooxygenase of strain DNT4, and it was sequentially reduced to 2-hydroxy-5-methylquinol by a specific quinone reductase (10). On the other hand, both quinones resulting from pentachlorophenol monooxygenation by PcpB (6) and 2,4,6-trichlorophenol (2,4,6-TCP) monooxygenation by TcpA (3) were detected only after derivatization. Although for a long time benzoquinones (30, 31, 41) and 2-hydroxy-1,4-benzoquinone (14, 16, 25) have been suspected to be intermediates in the formation of corresponding quinols during PNP and ortho-nitrophenol (ONP) oxidative degradation, detection of quinone has not in fact been reported. Since the broad-specificity reductases NsfA (40) and WrbA (24) in E. coli were able to reduce benzoquinone to hydroquinone, only purified enzyme would make it possible to capture the previously proposed intermediate p-benzoquinone (providing it is also relatively stable) during nitrophenol monooxygenation, as demonstrated in this study for the first time.

**Biochemical properties of PnpA.** The Kₘ values of H₆-PnpA for NADPH and PNP were 137.4 ± 12.3 and 12.0 ± 1.6 μM, respectively. PNP monooxygenase activity was NADPH and FAD dependent. NADPH can be replaced by NADH with the same efficiency. Negligible activity was observed when FMN replaced FAD. Addition of magnesium or manganese ions had no effect on the activity. Purified H₆-PnpA was stable for several hours in the elution buffer on ice, but its activity was reduced to approximately 70% and 20% of the original activity after 12 and 40 h, respectively. Long-term storage of the purified enzyme had no effect on the activity. Purified H₆-PnpA was stable for several hours in the elution buffer on ice, but its activity was reduced to approximately 70% and 20% of the original activity after 12 and 40 h, respectively. Long-term storage of the purified enzyme seemed to be difficult, since 70% of the activity was lost after 7 days if the enzyme was kept in 50% glycerol at –70°C. Storage at –20°C without glycerol resulted in rapid inactivation of the enzyme. The following substrates were used to determine whether PnpA exhibited extended substrate specificity: 4-nitrocatechol, m-nitrophenol, p-nitrobenzoate, 2,6-dinitrobenzene, 2,4,6-trinitrophenol, p-chlorophenol. However, H₆-PnpA showed activity only with 4-nitrocatechol, and the relative activity was lower (1.9 ± 0.1 U mg⁻¹).

**Phylogenetic analysis reveals divergent origins of nitroarene monooxygenases.** To elucidate the phylogenetic relationships...
among the functionally identified nitroarene monooxygenases, PnpA from this study, the single-component 4-methyl-5-nitrocatechol monooxygenase (DntB) of Burkholderia sp. strain DNT (10), the oxygenase component (NpcA) of two-component PNP 2-monooxygenase of strain SAO101 (16), and the single-component ONP 2-monooxygenase (OnpA) of Alcaligenes sp. strain NyZ215 (37), together with the oxygenase component (NpdA2) of the two-component PNP monooxygenase of strain JS443 (25), were used to construct the distance neighbor-joining tree shown in Fig. 6 using the MEGA (version 3.1) package (17). The results indicated that these nitroarene monooxygenases are only distantly related and belong to phylogenetically distant taxa, suggesting that the nitroarene monooxygenases had divergent origins. PnpA is most closely related to the large component of 3-hydroxyphenylacetate hydroxylase (MhaA) (2), but it acts as a single-component monoxygenase. However, OnpA is located in an unrelated branch with salicylate 1-monooxygenase; NpcA and NpdA2 are clustered with chlorophenol 4-monooxygenase and 2,4,6-TCP monooxygenase; and DntB is located in a branch with 2,4-dichlorophenol hydroxylase. Thus, PnpA belongs to a different group of flavin monooxygenases.

PnpB catalyzes reduction of p-benzoquinone to hydroquinone. The product of the reaction catalyzed by H$_8$-PnpB was identified as hydroquinone by HPLC analysis. Quantitative analysis also indicated that 1.1 ± 0.3 mol hydroquinone was produced for each 1 mol of benzoquinone consumed. The $K_m$ values of H$_6$-PnpB for NADPH and benzoquinone were 181.6 ± 43.5 and 6.7 ± 2.8 μM, respectively. Furthermore, the H$_8$-PnpB activity was NADPH and FMN dependent. H$_7$-PnpB preferred FMN to FAD (at a ratio of 1:0.6) as its cofactor. In addition, the pure enzyme was fairly stable on ice for 12 h and did not show any apparent loss of activity after storage at −70°C in 50% glycerol for 1 month.

**FIG. 6.** Phylogenetic relationships of the nitroarene monooxygenases and their homologues. Five nitroarene monooxygenases (filled circles), as well as the homologues, were aligned using the Cluster W program (version 1.8) (33) with default settings. A distance neighbor-joining tree was then created using the Mega (version 3.1) package (17), and the bootstrap confidence limits (expressed as percentages) are indicated at the nodes. Among the functionally identified nitroarene monooxygenases, PnpA from this study, the single-component 4-methyl-5-nitrocatechol monooxygenase (DntB) of Burkholderia sp. strain DNT (10), the oxygenase component (NpcA) of two-component PNP 2-monooxygenase of strain SAO101 (16), and the single-component ONP 2-monooxygenase (OnpA) of Alcaligenes sp. strain NyZ215 (37), together with the oxygenase component (NpdA2) of the two-component PNP monooxygenase of strain JS443 (25), were used to construct the distance neighbor-joining tree shown in Fig. 6 using the MEGA (version 3.1) package (17). The results indicated that these nitroarene monooxygenases are only distantly related and belong to phylogenetically distant taxa, suggesting that the nitroarene monooxygenases had divergent origins. PnpA is most closely related to the large component of 3-hydroxyphenylacetate hydroxylase (MhaA) (2), but it acts as a single-component monoxygenase. However, OnpA is located in an unrelated branch with salicylate 1-monooxygenase; NpcA and NpdA2 are clustered with chlorophenol 4-monooxygenase and 2,4,6-TCP monooxygenase; and DntB is located in a branch with 2,4-dichlorophenol hydroxylase. Thus, PnpA belongs to a different group of flavin monooxygenases.

**FIG. 7.** PnpB enhanced the PNP degradation by PnpA. When H$_8$-PnpB was added to a reaction mixture containing H$_8$-PnpA, a clear increase in total PNP monooxygenase activity was observed (Fig. 7). A likely explanation for the enhanced activity was that H$_8$-PnpB reduced benzoquinone to hydroquinone, possibly preventing product inhibition of PnpA. Similarly, it was suggested that the quinone reductases could reduce quinones to the hydroquinone state to prevent interaction of the semiquinone with O$_2$ and production of superoxide (24). More recently, a quinone reductase for 6-chlorohydroxyquinone reductase was isolated from Pseudomonas putida U (41), which could reduce both quinones and semiquinones, and a quinone reductase for 2,4-dichlorophenol was isolated from Pseudomonas sp. (42). These findings suggest that quinone reductases play a role in the detoxification of 4-nitrotoluenes and 4-hydroxyphenylacetates, and they may be important for the degradation of these compounds by aerobic bacteria.
none reduction during 2,4,6-TCP degradation in Cupriavidus necator JMP134. TcpB, was also found to have enhanced TcpA monooxygenase activity that converts 2,4,6-TCP to 6-chlorohydroxyquinoline (3).

Genetic and functional analysis of pnp4B in strain WBC-3. To investigate the possible involvement of pnp genes in PNP degradation in vivo, derivatives of strain WBC-3 with deletions of pnpA and pnpB were individually constructed and functionally analyzed. Strain WBC-3△pnpA (with pnpA disrupted) was not able to grow on PNP. Although strain WBC-3△pnpB (with pnpB disrupted) was not completely unable to grow on PNP, the rates of PNP removal from a culture (0.05 mM h⁻¹ for PNP degradation, compared with 0.11 mM h⁻¹ for the wild-type strain) and the cell growth (a specific growth rate of 0.07 h⁻¹, compared with 0.13 h⁻¹ for the wild-type strain) were significantly reduced, apparently due to disruption of pnpB gene. The assay was done three separate times with similar results, and the results of a representative assay are shown in Fig. 8. The nonspecific enzymatic reduction of p-benzoquinone may partially complement the function of PnP in the mutant strain since p-benzoquinone reductase activity was still observed in wild-type strain WBC-3 (specific activity, 0.5 ± 0.02 U mg⁻¹) without PNP induction. The data presented here suggest that PnPB also plays a critical role in PNP degradation in strain WBC-3. A similar conclusion was reported for PcpD, which catalyzes both the hydroxylation of tetrachlorobenzoquinone (tetrachloroquinone) to tetrachlorohydroquinone in Sphingobium chlorophenolicum, as a mutant lacking functional PcpD was impaired for removal of pentachlorophenol from the medium (6).

Recently, the roles of two quinone reductases in the microbially degradation of substituted aromatic compounds were illustrated. OqPB from the ONP-utilizing strain NysZ215, reducing o-benzoquinone to catechol, was demonstrated to be a critical enzyme in ONP degradation in vivo when the pathway genes were transferred to P. putida PaW340 (37). Similarly, TcpB, acting as a quinone reductase for 6-chlorohydroxyquinone reduction, was also confirmed to play a critical role in 2,4,6-TCP degradation in strain JMP134, although tcpB inactivation mutants still oxidize 2,4,6-TCP (3).


