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ORIGINAL PAPER

Characterization of a *para*-nitrophenol catabolic cluster in *Pseudomonas* sp. strain NyZ402 and construction of an engineered strain capable of simultaneously mineralizing both *para*- and *ortho*-nitrophenols

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Abstract *Pseudomonas* sp. strain NyZ402 was isolated for its ability to grow on *para*-nitrophenol (PNP) as a sole source of carbon, nitrogen, and energy, and was shown to degrade PNP via an oxidization pathway. This strain was also capable of growing on hydroquinone or catechol. A 15, 818 bp DNA fragment extending from a 800-bp DNA fragment of hydroxyquinol 1,2-dioxygenase gene (*pnpG*) was obtained by genome walking. Sequence analysis indicated that the PNP catabolic gene cluster (*pnpABCDEFG*) in this fragment shared significant similarities with a recently reported gene cluster responsible for PNP degradation from *Pseudomonas* sp. strain WBC-3. PnpA is PNP 4-monooxygenase converting PNP to hydroquinone via benzoquinone in the presence of NADPH, and genetic analysis indicated that *pnpA* plays a key role in PNP degradation. *pnpA1* present in the upstream of the cluster (absent in the cluster from strain WBC-3) encodes a protein sharing as high as 55% identity with PnpA, but was not involved in PNP degradation by either in vitro or in vivo analyses. Furthermore, an engineered strain capable of growing on PNP and *ortho*-nitrophenol (ONP) was constructed by introducing *onpAB* (encoding ONP monooxygenase and *ortho*-benzoquinone reductase which catalyzed the transformation of ONP to catechol) from *Alcaligenes* sp. strain NyZ215 into strain NyZ402.

Keywords Catabolism · Engineered strain · *ortho*-Nitrophenol · *Pseudomonas* · *para*-Nitrophenol · Simultaneous degradation

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Introduction

Nitrophenols, including *o*-nitrophenol (ONP), *m*-nitrophenol (MNP), and *p*-nitrophenol (PNP), are widely utilized in the synthesis of chemical products and are released into the environment in large quantities. Microbes play an important role in transforming these recalcitrant contaminants and the nitrogen recycling (Spain 1995). Several aerobic pure cultures of bacteria have been isolated with the ability to mineralize nitrophenols and different strategies were employed by them to convert these synthetic organic structures to central metabolites. Both reductive and oxidative pathways for the removal of nitro groups from nitrophenols have been described. The aerobic biodegradation of PNP can be divided into two pathways according to the intermediates involved.

The degradation pathway in which PNP is converted to maleylacetate via hydroquinone (hydroquinone pathway) was preferentially found in Gram-negative bacteria, as proposed in *Moraxella* sp. (Spain et al. 1979; Spain and Gibson 1991) and *Pseudomonas* sp. strain WBC-3 (Zhang et al. 2009). The degradation pathway in which PNP is converted to hydroxyquinol via 4-nitrocathechol (hydroxyquinol pathway), was illustrated in Gram-positive bacteria, such as *Bacillus sphaericus* (Kadiyala and Spain 1998) and *Arthrobacter* spp. (Jain et al. 1994).

Although many studies for nitrophenol degradation have been reported, genetic information related to nitrophenol degradation remains limited. Molecular characterization of ONP degradation has been reported in *Alcaligenes* sp. strain NyZ215, in which a single-component ONP 2-monooxygenase was involved in the initial steps of ONP catabolism (Xiao et al. 2007). The gene cluster involved in the hydroxyquinol pathways for PNP degradation was characterized in *Rhodococcus opacus* SAO101 (Kitagawa et al. 2004) and *Arthrobacter* sp. strain JS443 (Perry and Zylstra 2007) (Fig. 1). In both cases, a two-component PNP monooxygenase was involved in PNP mineralization. More recently, the molecular characterization for PNP degradation through hydroquinone pathway has been reported in *Pseudomonas* sp. strain WBC-3 (Fig. 1), in which a single-component PNP 4-monooxygenase was confirmed to convert PNP to benzoquinone during the degradation (Zhang et al. 2009). In parallel, PNP degradation was also studied in another *Pseudomonas* strain in our group. In this study, we report the cloning and sequencing of an overlapping fragment covering a continuous 15.8-kb region and the characterization of the gene encoding PNP 4-monooxygenase involved in the initial degradation step from this newly isolated PNP degrader *Pseudomonas* sp. strain NyZ402. On the other hand, an engineered strain capable of mineralizing both PNP and ONP was also successfully constructed, since nitrophenol isomers often co-exist in the chemical industry as well as in the environment, and isolates capable of degrading both PNP and ONP have not been described so far.

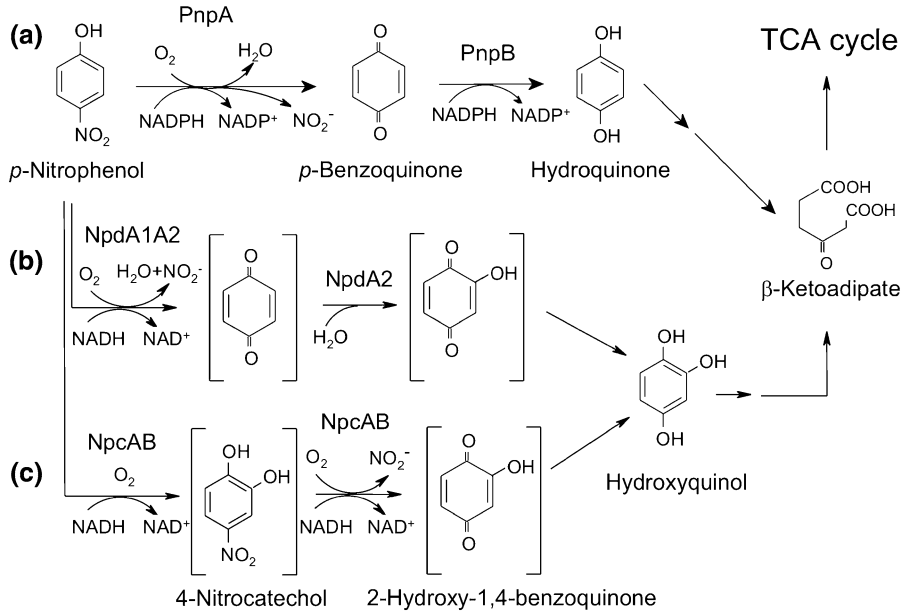

Fig. 1 Two alternative pathways of PNP degradation, together with the enzymes involved in the initial steps. **a** *Pseudomonas* sp. strain WBC-3; **b** *Arthrobacter* sp. strain JS443; **c** *Rhodococcus opacus* SAO101. TCA cycle, tricarboxylic acid cycle. PnpA and PnpB are single-component PNP monooxygenase and *p*-benzoquinone reductase, respectively, from strain WBC-3 (Zhang et al. 2009). NpdA1 and NpdA2 are reductase and oxygenase components, respectively, of PNP monooxygenase from strain JS443 (Perry and Zylstra 2007). NpcA and NpcB are the oxygenase and reductase components, respectively, of PNP monooxygenase from strain SAO101 (Kitagawa et al. 2004)

Materials and methods

Bacterial strains, plasmids, and growth conditions

All bacterial strains and plasmids used in this study are listed in Table 1. *Pseudomonas* sp. strain NyZ402 was deposited to the China Center for Type Culture Collection (CCTCC) and its CCTCC number is AB209245. *Pseudomonas* strains were grown at 30°C either on lysogeny broth (LB) medium or on minimal medium (MM) (Liu et al. 2005) with 0.7 mM PNP as a sole source of carbon, nitrogen, and energy. *Escherichia coli* strains were grown on LB at 37°C with 100 μg/ml ampicillin, 40 μg/ml kanamycin, or 100 μg/ml streptomycin when necessary.

Isolation and characterization of PNP degradation strain

Strain NyZ402 was isolated from activated sludge obtained from a nitrophenol-manufacturing factory in China by enrichment method. In brief, the samples were incubated in the carbon-free MM supplemented with PNP with shaking at 150 rpm. The culture was transferred (10% inoculum) to a fresh medium after PNP depletion. This process was carried out continuously for 1 month before cell suspensions were streaked onto LB agar plates. Developed colonies were incubated individually in liquid MM containing PNP (2 mM) for screening PNP-degrading strains by monitoring PNP depletion and nitrite release. The 27f and the 1492r universal primers (Lane 1991) were used to amplify its 16S rRNA gene by PCR.

Cloning of PNP degradation genes and sequence analyses

In order to isolate the catabolic cluster involved in PNP degradation in strain NyZ402, PCR-amplification of a putative hydroxyquinol 1,2-dioxygenase gene from strain NyZ402 was performed initially, followed by genome walking. A set of primers (primer a, 5′-GTCGACATATGACCGATCAAGAC AAAGCCATC, primer-3′ b, AAGCTTGGATTC

Table 1 Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant genotype or characteristic(s)	Reference or source
Alcaligenes sp. NyZ215	ONP degrader, wild type	Xiao et al. (2007)
Pseudomonas sp.		
NyZ402	PNP degrader, wild type	This study
NyZ402Δ *pnpA*	*pnpA* gene disrupted mutant of NyZ402	This study
NyZ402Δ *pnpA1*	*pnpA1* gene disrupted mutant of NyZ402	This study
NyZ402[pZWX33AB]	pZWX33AB transformed into strain NyZ402	This study
E. coli		
DH5α	*sup E44*, Δ*lac*U169 (Φ80*lac*ZΔM15), *rec*A1, *end*A1, *hsd*R17, thi^{-1}, *gyr*A96, *rel*A1	Woodcock et al. (1989)
BL21(DE3)	F^{-}*ompT hsdS*(r_B^- m_B^-) *gal dcm lacY1*(DE3)	Novagen Co
S17-1λpir	Tp^r Sm^r *rec*A, *thi*, *pro*, *hsd*R-M^+RP4: 2-Tc:Mu: Km Tn7 λpir	Simon et al. (1983)
Plasmids		
pET5a	Expression vector, Ap^r	Novagen Co
pEX18Tc	Tc^r, *sacB*$^+$, gene replacement vector with MCS from pUC18	Hoang et al. (1998)
pVLT33	Km^r, RSF1010-*lacI*^{q}/P_{tac} hybrid broad-host-range expression vector	de Lorenzo et al. (1993)
pTn*Mod*-OKm	Km^r, source of neomycin phosphotransferase II gene (*npt II*) gene	Zhang et al. (2009)
pZWX33AB	PCR fragment containing *onpAB* cloned into pVLT33	Xiao et al. (2007)
pZWA	*Nde*I-*Eco*RI fragment containing *pnpA* inserted into pET5a	This study
pZWA1	*Nde*I-*Eco*RI fragment containing *pnpA1* inserted into pET5a	This study
pZWAKO	*Pst*I- *Eco*RI fragment containg *pnpA* inserted into pEX18Tc where *pnpA* was disrupted by *npt II*	This study
pZWA1KO	*Hin*dIII- *Eco*RI fragment containg *pnpA* inserted into pEX18Tc where *pnpA1* was disrupted by *npt II*	This study

ATGACTCACTCTGCCTCCATGACGA) designed according to the nucleotide sequence of *pnpG*, a putative hydroxyquinol 1,2-dioxygenase gene from *Pseudomonas* sp. strains WBC-3 (Zhang et al. 2009), was used to amplify its homolog from strain NyZ402. PCRs were carried out in a final volume of 50 μl, and the reaction mixtures contained 5 μl of 10× buffer [100 mM Tris HCl (pH 8.3), 500 mM KCl] (TakaRa, Dalian, China), 0.2 μM (each) primer, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates (dNTPs), 1 U Taq polymerase (TakaRa, Dalian, China), and 10 ng of DNA template for each reaction. PCR conditions were as follows: (1) 94°C for 5 min; (2) 30 cycles, with 1 cycle consisting of denaturation (45 s at 94°C), annealing (45 s at 55°C), and extension (1 min at 72°C); and (3) 72°C for 10 min. Genome walking was then conducted to clone the flanking regions of this gene with the method described (Siebert et al. 1995). ORFfinder and Blast programs at National Center for the Biotechnology Information (NCBI) website were used to identify open reading frames (ORFs) and to analyze amino acid sequence and nucleotide sequence identities with other proteins and genes in the GenBank database.

Cloning and expression of *pnpA* and *pnpA1* in *E. coli*

The in vitro expression of *pnpA* and *pnpA1* was to obtain large quantity of recombinant PnpA and PnpA1 for their functional identification. Both *pnpA* and *pnpA1* were amplified from genomic DNA of strain NyZ402 by Pyrobest DNA Polymerase (TakaRa, Dalian, China) before they were digested with *Nde*I and *Eco*RI. The cycling protocol was 95°C for 5 min, followed by 30 cycles of 95°C for 45 s, 54°C for 45 s, and 72°C for 1 min, and 1 cycle of 72°C for 10 min. The obtained fragments containing *pnpA* and *pnpAl* were then ligated into the expression vector pET5a to produce plasmids pZWA and pZWA1 respectively. The inserts of the clones were sequenced to ensure that no mutation had been incorporated during the PCR. The pET5a constructs were then transformed into *E. coli* BL21(DE3) for expression with induction of 0.1 mM of isopropyl thiogalactoside (IPTG) at 30°C for 4 h after it was grown in LB at 37°C to an optical density at 600 nm (OD_{600}) of 0.6. The expressed proteins were identified by standard sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on 12% acrylamide gels and stained with standard Coomassie blue (Sambrook et al. 1989).

Preparation of cell extracts and enzyme assays

The cells over-expressing PnpA and PnpA1 were harvested for their functional identification via enzyme assays. The method used for preparation of cell extracts was the same as described by sonication treatment (Liu et al. 2005). The PNP 4-monooxygenase activity was determined as described previously (Spain et al. 1979). The molar extinction coefficient for NAD(P)H was 6,220 M^{-1} cm^{-1} at 340 nm and PNP was 7,000 M^{-1} cm^{-1} at 420 nm, pH 7.0, respectively, (Spain et al. 1979). Catechol 1,2-dioxygenase assay was performed as previously explained and a molar extinction coefficient for the production of *cis*, *cis*-muconate was 16,900 M^{-1} cm^{-1} at 260 nm (Zeyer et al. 1986). All assays were also conducted with strains carrying vectors only as controls. One unit of enzyme activity was defined as the amount required for the disappearance (or production) of 1 μmol of substrate (or product) per min at 30°C. Specific activities are expressed as units per milligram of protein.

Gene knockout experiments

The gene knockout experiments were carried out for genetic analysis of *pnpA* and *pnpA1* to identify their physiological roles in PNP degradation in vivo. In general, target genes were cloned into the gene replacement vector pEX18Tc (Hoang et al. 1998) with insertion of a kanamycin resistance gene (*nptII*) from plasposon pTn*Mod*-Okm (Dennis and Zylstra 1998), as a selectable marker. For *pnpA* knockout, the upstream or downstream of *pnpA* were amplified by primer pairs AKOuarmL-AKOuarmR (with *Eco*RI and *Sal*I sites respectively) or AKOdarmL-AKOdarmR (with *Sal*I and *Pst*I sites respectively). These two PCR fragments were cloned into *Eco*RI and *Pst*I sites of pEX18Tc by three-fragment ligation and the kanamycin cassette was then inserted into the *Sal*I site of this fragment. For *pnpA1* disruption, *pnpA1* (with an internal *Sal*I site) was PCR-amplified with primer pairs A1KOuarmL-A1KOdarmR (with *Eco*RI and *Hin*dIII sites respectively). The PCR product was cloned into the *Eco*RI and *Hin*dIII sites of pEX18Tc and the kanamycin cassette was subsequently inserted into the *Sal*I site of *pnpA1*. Both constructs

(pZWAKO and pZWA1KO) were transformed into mobilizer strain *E. coli* S17-1 (Simon et al. 1983) before being conjugated into strain NyZ402 by biparental matings as described (Schweizer 1992). Finally, double crossover recombinants were screened on sucrose plates (Schweizer 1992) and mutated strains were confirmed by their resistance to kanamycin and PCR analysis. The specific growth rates for wild-type strain NyZ402 and its derivatives were calculated by using the $\ln(X/X_0)/T$, where X is the maximum OD_{600}, X_0 is the OD_{600} at time zero, and T is the time that it took to reach the maximum OD_{600} (in hours).

Construction and characterization of an engineered strain mineralizing both PNP and ONP

An engineered strain with the ability to degrade both PNP and ONP was constructed by incorporation of ONP catabolic genes, *onpAB*, into target strain NyZ402. For this purpose, plasmid pZWX33AB (Table 1) was transformed into strain NyZ402 from *E. coli* S17-1 by biparental conjugation. The resulting transconjugants were selected on LB plates with streptomycin and kanamycin. MM was used for the growth test by supplementing with 0.1 mM PNP, 0.1 mM ONP, 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and kanamycin (20 μg/ml). Strain NyZ215, as a control, was inoculated into the same medium without IPTG and antibiotics. Cell growth was determined spectrophotometrically by measuring the absorbance at 600 nm. In the biotransformation experiment, strains were grown in MM with 0.1 mM ONP, 0.1 mM PNP, 0.1 mM IPTG and 50 μg/ml kanamycin with the addition of 0.5% yeast extract to enhance the biomass. The induced cells were harvested by centrifugation, washed twice by MM and suspended in a minimal volume of MM to give an OD_{600} of 5.0. Biotransformations were initiated by the addition of 0.1 mM ONP and 0.1 mM PNP and samples were collected at appropriate intervals to monitor the progress of the reaction. Wild-type strain NyZ402 was taken as a control.

Plasmid stability test

The stability of plasmid pZWX33AB in the engineered strains was investigated by a previously described method (Gavigan et al. 1997). Single colonies of strain NyZ402 [pZWX33AB] were streaked on kanamycin and streptomycin agar plates and incubated for a day. Single colonies were then inoculated in 4 ml of liquid medium with kanamycin and grown to log phase. Cultures were diluted 1:100 in antibiotic-free LB or MM with 0.1 mM ONP as the sole carbon source, and grown for 24 h. This process was repeated daily for 4 days. The appropriate dilutions were plated out on LB agar plates with and without kanamycin in order to ascertain the proportion of colonies resistant to kanamycin compared with the total number of cells. Colonies were scored overnight, and the results represent the average of three independent experiments.

Analytical methods

Quantity and quality analysis of chemicals as well as nitrite were performed to monitor the processes of PNP or ONP biodegradation. Specific chemicals were measured by High performance liquid chromatography (HPLC) with a Gilson 715 system using a C18 reversed-phase column (25 cm × 4.6 mm, 5 μm particle size; Supelco) at 30°C using a Gilson 119 UV/VIS detector. PNP and hydroquinone were detected at 290 nm with methanol:ammonium acetate (0.1 M, pH 4.2) (70:30 v/v) as the mobile phase at a flow rate of 0.8 ml per min. Under these conditions, authentic PNP and hydroquinone had a retention time of 5.1 and 4.3 min respectively. The mixture of PNP and ONP was detected at 278 nm with methanol:ammonium acetate (0.1 M, pH 4.2) (50:50 v/v) at a flow rate of 0.8 ml per min. Under these conditions, the authentic PNP and ONP had retention times of 7.9 and 10.2 min respectively. Nitrite was assayed by a published method (Lessner et al. 2003), and protein concentrations were determined by the Bradford method (Bradford 1976) with bovine serum albumin as the standard.

Nucleotide sequence accession number

The sequences of the 1,308-bp 16S rRNA gene and the 15,818-bp PNP degradation gene cluster from strain NyZ402 have been deposited in the GenBank database under accession numbers GU126734 and GU123925 respectively.

Results and discussion

Isolation and characterization of PNP utililizer *Pseudomona* sp. strain NyZ402

After several weeks of enrichment, a PNP-degrading bacterial strain, designated NyZ402, was isolated using PNP as a sole source of carbon, nitrogen, and energy. The characteristics of this bacterium demonstrated that it was Gram-negative and was classified as a *Pseudomonas* strain based on the sequence analysis of its 16S rRNA gene.

Accumulation of nitrite in the media during the PNP degradation by strain NyZ402 indicated that it had an oxidative pathway for PNP degradation, which may be similar to the pathway in *Pseudomonas* sp. strain WBC-3 (Zhang et al. 2009) and *Moraxella* sp. (Spain and Gibson 1991). The PNP 4-monooxygenase activity was detected in cell extracts of strain NyZ402 grown on MM with PNP but it was not detected in those grown on LB, suggesting that the expression of this enzyme was induced by PNP. Strain NyZ404 was also capable of growing on hydroquinone and catechol but not on *o*-nitrophenol, *m*-nitrophenol, 4-nitrocatechol or 1,2,4-benzenetriol. 4-nitrocatechol, however, can only be transformed by PNP induced cells.

Cloning and sequence analyses of the PNP catabolic gene cluster

An 800-bp DNA fragment, obtained using primers mentioned in the materials and methods, showed 74% identity to *pnpG* gene from strain WBC-3. Subsequently, a DNA fragment of 15,818 bp extending from this 800-bp region was obtained and sequenced after several cycles of genome walking. Fourteen complete ORFs and one incomplete ORF were deduced and their annotations were done on the basis of BLAST analysis (Fig. 2). The *pnp* catabolic cluster, *pnpABCDEFGR*, shares significant similarities with a recently reported gene cluster responsible for PNP degradation from strain WBC-3 (Zhang et al. 2009), suggesting the genes involved in PNP degradation in this strain are also physically linked. Among these genes, *pnpA* and *pnpB* were proposed to encode PNP 4-monooxygenase and benzoquinone reductase respectively. The genes encoding the lower pathway for PNP degradation formed a compact operon, which encode the two-component hydroquinone 1,2-dioxygenase (PnpCD), γ-hydroxymuconic semialdehyde dehydrogenase (PnpE) and maleylacetic acid reductase (PnpF) respectively. Different to strain WBC-3, an additional potential PNP monooxygenase gene *pnpA1*, was located upstream of *pnpA* in strain NyZ402, which encodes a protein with a 55% identity with PnpA. *orf2* and *orf3* flanking *pnpA1* could have encoded another two putative regulatory proteins, sharing 28% and 41% identities to transcriptional regulator PtxR from *Pseudomonas aeruginosa* (Hamood et al. 1996) and SrpS from *Pseudomonas putida* S12 (Wery et al. 2001) respectively.

Up to now, the genetic determinants for PNP degradation have been identified for both hydroxyquinol and hydroquinone pathways in several geographically dispersed isolates including *Rhodococcus opacus* SAO101 (Kitagawa et al. 2004), *Arthrobacter* sp. JS443 (Perry and Zylstra 2007), *Pseudomonas* sp. strain WBC-3 (Zhang et al. 2009), and strain NyZ402 in this study. On the one hand, the genes' sequences are conserved for each pathway, since high sequence similarities were observed between the degradation clusters from strains SAO101 and JS443, as well as between strains WBC-3 and NyZ402. On the other hand, both the genes' sequences and their organizations were significantly different between the hydroxyquinol and hydroquinone pathways, implying divergent originations of these two pathways.

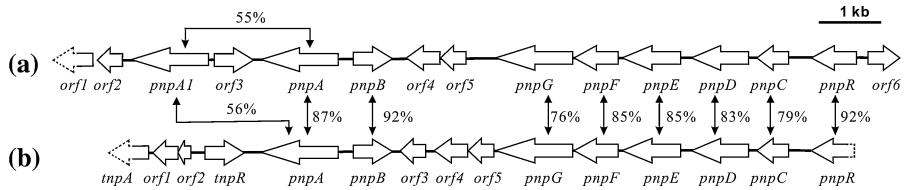

Fig. 2 Comparison of *pnp* gene clusters between *Pseudomonas* sp. strains NyZ402 (**a**) and WBC-3 (**b**). The *double-headed arrows* demonstrated the alignment of the catabolic genes with significant similarities. *Percentage* identities at the amino acid level are indicated adjacent

Furthermore, PNP monooxygenases, the key enzyme in the initial PNP degradation, have also shown significant diversity in their genetic determinants and biochemical characters. In the hydroxyquinol pathway, a two-component monooxygenase catalyzes the monooxygenation of PNP to hydroxyquinol (Kitagawa et al. 2004; Perry and Zylstra 2007), whereas it is a single component enzyme that catalyzes the monooxygenation of PNP to benzoquinone in the hydroquinone pathway (Zhang et al. 2009). The disparate origins of the two different PNP degradation cluster and associated genes described in this study interestingly illustrated how bacterial evolved independently to achieve two different catabolic pathway for the same synthetic compound PNP. Moreover, in the hydroquinone pathway, genetic difference was also present in PNP degradation clusters between strains NyZ402 and WBC-3. Gene *pnpA1*, a close homolog of *pnpA*, and another two putative regulators-encoding genes (*orf2* and *orf3*) present in strain NyZ402 were absent in strain WBC-3.

Functional expression and biochemical characterization of PnpA and PnpA1 in *E. coli*

After induction with IPTG, cell extracts of *E. coli* BL21 (DE3) [pZWA] were found to contain PNP 4-monooxygenase with a specific activity of 0.05 U/mg against PNP. Neither PNP consumption nor nitrite release was detected in the controls where the expression vector contained no insert. SDS–PAGE revealed elevated level of a 45-kDa polypeptide (lane 5 in Fig. 3), corresponding to the molecular masses of PnpA from the amino acid sequences. Following substrates were also used to determine whether PnpA exhibited an extended substrate specificity: 4-nitrocatechol, *m*-nitrophenol, *o*-nitrophenol, *p*-nitrobenzoate, nitrobenzene, *m*-dinitrobenzene, 2,6-dinitrotoluene, 3-hydroxy-4-nitrobenzoate and 2,4,6-trinitrophenol. Of these substrates, however, PnpA only showed activity against 4-nitrocatechol but with a lower relative activity (73% lower than that with PNP as the substrate). PNP monooxygenase activity was NAD(P)H and FAD dependent. NADPH is the preferred electron donor to NADH for PnpA, with a ratio of 3:1 in terms of relative activity. Spectrophotometric analysis revealed that 2.2 mol of NADPH were oxidized when each mol of PNP consumed,similar to the oxidation of PNP by cell extracts from *Moraxella* sp. (Spain et al. 1979). This

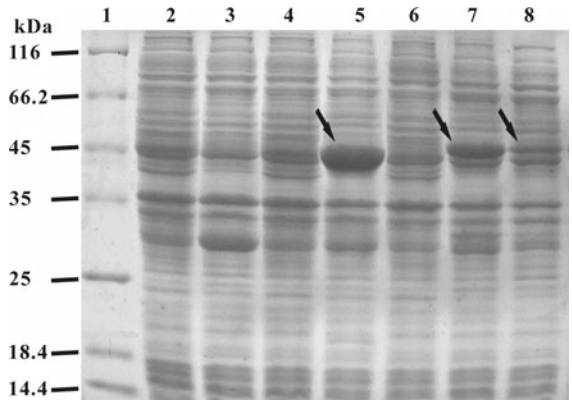

Fig. 3 SDS–PAGE of PnpA and PnpA1 expression in *E. coli*. *Lane1*, molecular mass standards; *lane2*, uninduced cell extracts of *E. coli* BL21[pET5a]; *Lane3*, IPTG induced cell extracts of *E. coli* BL21[pET5a]; *lane4*, uninduced cell extracts of *E. coli* BL21[pZWA]; *lane5*, IPTG induced cell extracts of *E. coli* BL21 [pZWA]; *Lane6*, uninduced cell extracts of *E. coli* BL21[pZWA1]; *lane7*, IPTG induced cell extracts of *E. coli* BL21[pZWA1]; *lane8*, the supernatant of IPTG induced cell extract *E. coli* BL21 [pZWA1]

indicated that the reaction occurred via an intermediate. The product of PNP monooxygenation was confirmed to be hydroquinone by HPLC analysis by comparison with the retention time of authentic hydroquinone.

SDS–PAGE of the cell extracts from *E. coli* BL21(DE3) carrying *pnpA1*, after induction with IPTG, showed elevated level of polypeptides of 45-kDa, fitting to the molecular masses of PnpA1 as deduced from the amino acid sequences (lane 7 in Fig. 3). However, neither PNP monooxygenase activity nor nitrite release was detected from the cell extracts of *E. coli* BL21 [pZWA1] carrying *pnpA1* with PNP or 4-nitrocatechol as substrate, although soluble protein was obtained for this recombinant enzyme, as shown in lane 8 of Fig 3.

Genetic analyses of *pnpA* and *pnpA1* in strain NyZ402

To investigate the physiological roles of *pnpA* and *pnpA1* genes in the PNP degradation in vivo, derivatives of strain NyZ402 with deletions of *pnpA* and *pnpA1* were individually constructed and functionally analyzed. The mutants were screened by kanamycin resistance and confirmed by diagnostic

PCR. Strain NyZ402Δ*pnpA* (with *pnpA* disrupted) completely lost the ability to grow on PNP (Fig. 4). However, no evident difference was observed in the rates of PNP removal and the cell growth between mutant NyZ402Δ*pnpA1* (with *pnpA1* disrupted) and the wild-type strain. The latter exhibited a rate of 33 μM h^{-1} for PNP degradation and a specific growth rate of 0.17 h^{-1} for its growth. The results suggested that the *pnpA* gene was functionally active and absolutely essential for PNP mineralization in strain NyZ402, while gene *pnpA1* was not directly involved in the degradation. One plausible explain is that it might be a result of *pnpA* reduplication during the processes of bacteria acquiring *pnp* catabolic cluster via a horizontal gene transfer event, but became a pseudogene at later evolution process. However, other possible roles of *pnpA1* in this strain can not be ruled out, although no physiological role was identified for PnpA1 in PNP degradation.

Construction of an engineered strain degrading both PNP and ONP

All the three nitrophenol isomers are important environmental pollutants and often co-exist in the chemical industry as well as in the environment. Although *Pseudomonas putida* strain B2 was reported to degrade both ONP and MNP (Zeyer and Kearney 1984), isolates capable of degrading both PNP and ONP have not been described so far. The cell extracts of strain NyZ402 grown on catechol showed catechol 1,2-dioxygenase activity with a specific activity of 0.01 U/mg. However, this activity was not detected in cell extracts from the strain grown on LB. This indicated that the expression of catechol 1,2-dioxygenase in strain NyZ402 was induced by catechol. Due to its ability to grow on catechol through the *ortho*-cleavage pathway, strain NyZ402 was chosen as a host stain for the construction of an ONP catabolic pathway by introducing the upper pathway of ONP degradation from strain NyZ215, allowing the transformation of ONP to catechol.

The plasmid pZWX33AB containing *onpA* (ONP 2-monooxygenase gene) and *onpB* (benzoquinone reductase gene) (Xiao et al. 2007) was transformed into strain NyZ402 by biparental mating. The resulting transconjugant NyZ402[pZWX33AB] containing *onpAB* was able to grow on ONP (0.1 mM) within 5 days, without losing its ability to grow on PNP. In the biotransformation experiment shown in Fig. 5, NyZ402[pZWX33AB] expressing OnpAB can transform ONP and PNP at rates of 8.6 and 7.5 μM h^{-1}

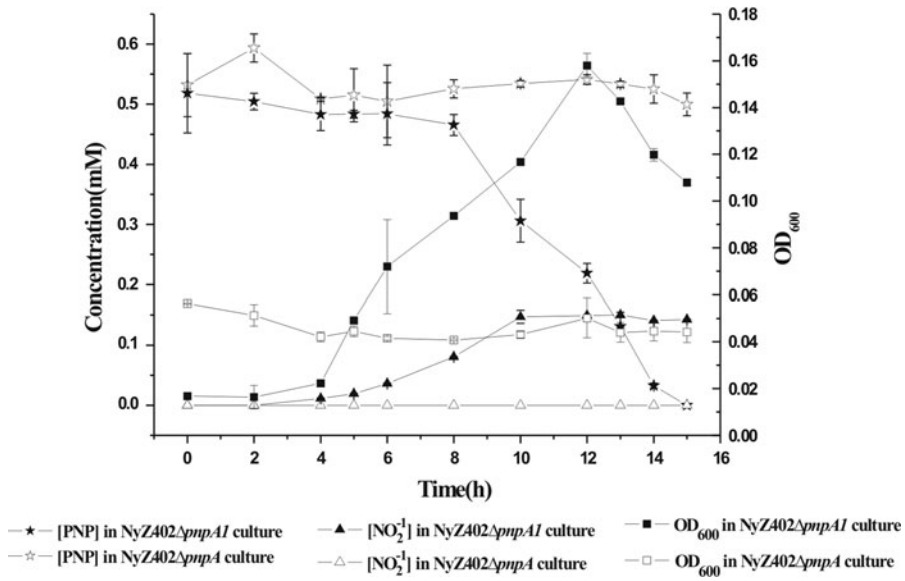

Fig. 4 Degradation of PNP by *Pseudomonas* sp. strain NyZ402Δ*pnpA* and NyZ402Δ *pnpA1*. PNP is degraded with concomitant release of nitrite by NyZ402Δ *pnpA1* but could not be degraded by NyZ402Δ *pnpA*. The inoculum used was obtained from washed cells of NyZ402Δ *pnpA* or NyZ402Δ *pnpA1* grown overnight on LB. Means of triplicates are shown with standard deviations (*error bars*). No evident difference was observed in the rates of PNP removal and the cell growth between mutant NyZ402Δ *pnpA1* and the wide-type strain NyZ402

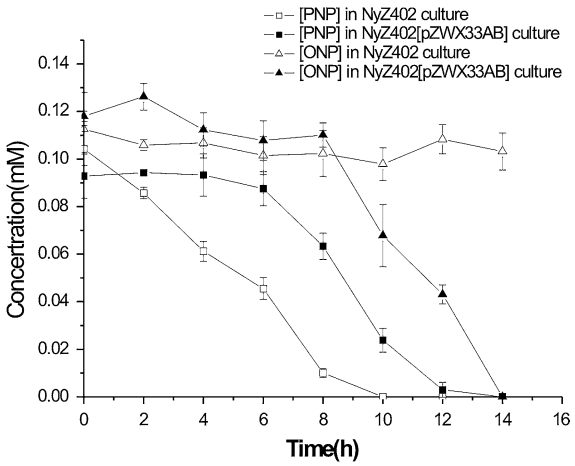

Fig. 5 Simultaneous degradation of *o*-nitrophenol and *p*-nitrophenol by *Pseudomonas* sp. strains NyZ402[pZWX33AB]. Means of triplicates are shown with standard deviations (*error bars*). The reactions were initiated by addition of 0.1 mM ONP and 0.1 mM PNP and samples were collected at appropriate intervals to monitor the progress of the reactions. Wild-type strain NyZ402 was taken as a control

respectively, whereas NyZ402, in the same medium, can only degrade PNP at a rate of 10 μM h^{-1}. This is a good example to demonstrate that multiple metabolic pathways from different organisms could be assembled into a single heterogenous host, creating a genetically modified organism capable of degrading two target compounds.

The slower growth of the engineered *Pseudomonas* strain on PNP and ONP as the sole carbon source could be attributed to the toxicity of ONP to the cells, since the rate of PNP removal was also decreased to 10 μM h^{-1} in the presence of ONP, in comparison with 33 μM h^{-1} for the wild type strain in the absent of ONP. In fact, the ONP as substrate was suggested to be supplied gradually to the medium for its toxicity, as proposed in the research of ONP degrader NyZ215 (Xiao et al. 2007).

Stability of pZWX33AB in engineered strain

The stability of plasmid pZWX33AB in the engineered strains was also investigated as described in "Materials and methods". The results indicated, among 100 colonies tested, that strain NyZ402[pZWX33AB] completely lost plasmid pZWX33AB when grown in LB after 4 days' repetitively culturing without antibiotic pressure. However, 42% of single colonies of NyZ402[pZWX33AB] grew in MM with ONP still kept plasmid pZWX33AB without antibiotic selective pressure in 100 colonies tested. Therefore, the instability of plasmid pZWX33AB (originated from the broad host range vector pVLT33) in the host strain NyZ402, was also a possible reason for the slower growth of the recombinant strain, although other possibilities, such as the unidentified rate-limiting steps and the substrate uptake, can not be ruled out. Nevertheless, this study has provided us a firm base for the construction of more efficient and rational engineered strains for their application in the simultaneous mineralization of pollutants isomers.

Conclusions

A 15, 818 bp *para*-nitrophenol (PNP) catabolic gene cluster (*pnpABCDEFG*) was obtained from newly isolated *Pseudomonas* sp. strain NyZ402. PnpA was confirmed to be a PNP 4-monooxygenase and genetic analysis indicated that *pnpA* plays a key role in the PNP degradation. Furthermore, an engineered strain capable of growing on PNP and *ortho*-nitrophenol (ONP) was constructed.

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