The catabolism of 2,4-xylenol and *p*-cresol share the enzymes for the oxidation of *para*-methyl group in *Pseudomonas putida* NCIMB 9866

Yan-Fei Chen · Hongjun Chao · Ning-Yi Zhou

Abstract *Pseudomonas putida* NCIMB 9866 utilizes *p*-cresol or 2,4-xylenol as a sole carbon and energy source. Enzymes catalyzing the oxidation of the *para*-methyl group of *p*-cresol have been studied in detail. However, those responsible for the oxidation of the *para*-methyl group in 2,4-xylenol catabolism are still not reported. In this study, real-time quantitative PCR analysis indicated *pchC*- and *pchF*-encoded *p*-cresol methylhydroxylase (PCMH) and *pchA*-encoded *p*-hydroxybenzaldehyde dehydrogenase (PHBDD) in *p*-cresol catabolism were also likely involved in the catabolism of 2,4-xylenol. Enzyme activity assays and intermediate identification indicated that the PCMH and PHBDD catalyzed the oxidations of 2,4-xylenol to 4-hydroxy-3-methylbenzaldehyde and 4-hydroxy-3-methylbenzaldehyde to 4-hydroxy-3-methylbenzoic acid, respectively. Furthermore, the PCMH-encoding gene *pchF* was found to be necessary for the catabolism of 2,4-xylenol, whereas the PHBDD-encoding gene *pchA* was not essential for the catabolism by gene knockout and complementation. Analyses of the maximum specific growth rate (μ_m) and specific activity of the gene-knockout strain to different intermediates revealed the presence of other enzyme(s) with PHBDD activity in strain 9866. However, PHBDD played a major role in the catabolism of 2,4-xylenol in contrast to the other enzyme(s).

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Introduction

Also known as 2,4-dimethylphenol, 2,4-xylenol is a byproduct of the coal gas industry. It has been listed in the Priority Pollutants List by the US Environmental Protection Agency for its environmental toxicity. To date, two bacterial strains, *Pseudomonas putida* NCIMB 9866 (Chapman and Hopper 1968) and *Paracoccus* sp. strain U120 (Rudolphi et al. 1991), were reported for their abilities to completely degrade such environmental pollutant. In *P. putida* NCIMB 9866, 2,4-xylenol catabolism is initiated by oxidation of the *para*-methyl group to a carboxyl group, forming 4-hydroxy-3-methylbenzoic acid via two putative intermediates of 4-hydroxy-3-methylbenzyl alcohol and 4-hydroxy-3-methylbenzaldehyde; the *ortho*-methyl group in 4-hydroxy-3-methylbenzoic is then also oxidized to a carboxyl group, via two putative intermediates of its corresponding alcohol and aldehyde, to produce 4-hydroxyisophthalic acid; 4-hydroxyisophthalic acid is converted to protocatechuic acid (PCA), entering the *ortho* ring-cleavage pathway for further metabolism (Fig. 1a) (Chapman and Hopper 1968). Two enzymes, 4-hydroxy-3-methylbenzoate hydroxylase and 4-hydroxyisophthalate hydroxylase, involved in 2,4-xylenol catabolism in this strain, were purified and characterized (El-Mansi and Hopper 1990; Elmorsi and Hopper 1977); their encoding genes, however, have not been reported.

Strain 9866 also utilizes *p*-cresol, and its catabolism is also initiated by oxidation of the *para*-methyl group to a carboxyl group, forming 4-hydroxybenzoic acid via two intermediates of 4-hydroxybenzyl alcohol and 4-hydroxybenzaldehyde, and finally enters the *ortho* ring-cleavage pathway of PCA after 4-hydroxybenzoic acid is directly converted to PCA (Fig. 1a) (Chapman and Hopper 1968). A 5,276-bp DNA sequence
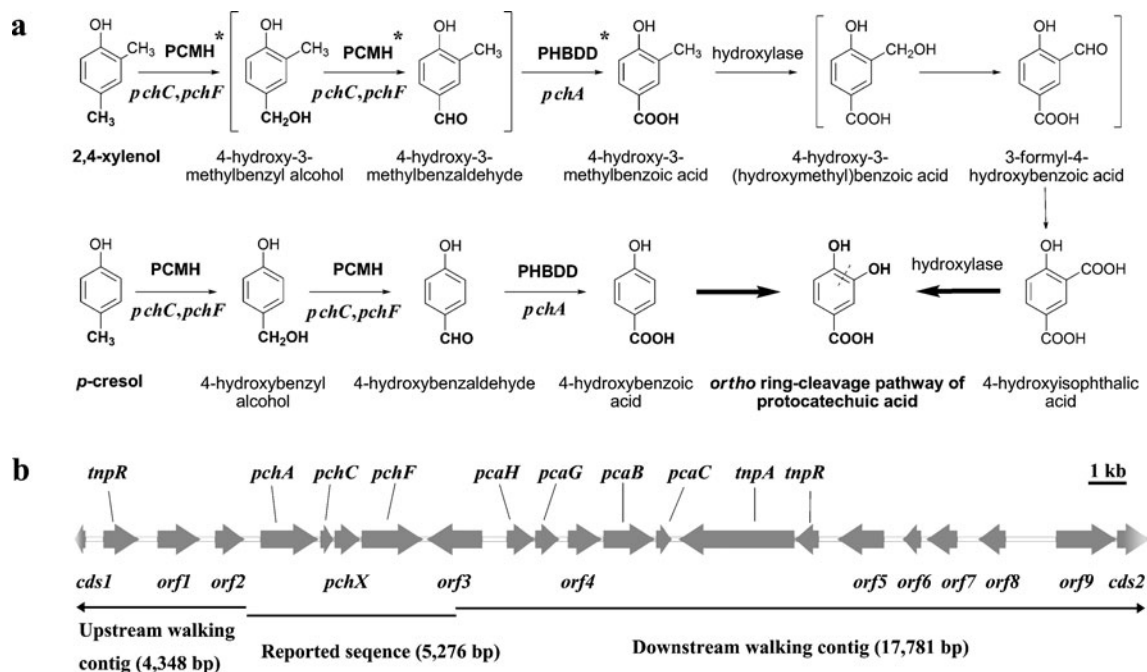
Fig. 1 a The proposed catabolic pathways of 2,4-xylenol and *p*-cresol in *Pseudomonas putida* NCIMB 9866 (Chapman and Hopper 1968; Cronin et al. 1999; El-Mansi and Hopper 1990; Elmorsi and Hopper 1977; Kim et al. 1994) and catabolic reactions catalyzed by the *pchC*- and *pchF*-encoded PCMH and *pchA*-encoded *p*-hydroxybenzaldehyde dehydrogenase (PHBDD) suggested in this study (marked by *asterisks*). **b** Organization of gene cluster obtained by genome walking in both directions from the reported sequence in plasmid pRA4000 (Dean et al. 1989; Kim et al. 1994). *pchA* encodes PHBDD. *pchC* and *pchF* encode the cytochrome and flavoprotein subunits of PCMH, respectively. *pchX* encodes a protein of unknown function (Cronin et al. 1999). Other open reading frames were annotated with a guide by BLAST results. *pcaH* and *pcaG*: protocatechuate-3,4-dioxygenase; *pcaB*: 3-carboxy-*cis*, *cis*-muconate cycloisomerase; *pcaC*: 4-carboxymuconolactone decarboxylase; *tnpA*: transposase; *tnpR*: resolvase

harboring *pchA*, *pchC*, and *pchF* genes from an 85-kb plasmid pRA4000 was determined (Fig. 1b) (Dean et al. 1989; Kim et al. 1994). Purified *pchC*- and *pchF*-encoded *p*-cresol methylhydroxylase (PCMH) was shown to catalyze two dehydrogenations of *p*-cresol to its corresponding alcohol and aldehyde (Hopper 1978; Hopper and Taylor 1977; Kim et al. 1994). The *pchA*-encoded *p*-hydroxybenzaldehyde dehydrogenase (PHBDD) was proved to catalyze the NADP⁺-dependent oxidation of 4-hydroxybenzaldehyde to 4-hydroxybenzoic acid (Cronin et al. 1999; Cronin and McIntire 1999). Although PCMH was also shown to have catalytic activity against 2,4-xylenol, its possible products were not identified, and its physiological role in the catabolism of 2,4-xylenol was not verified in vivo (Hopper and Taylor 1977). It would then be interesting to see whether the enzymes responsible for the initial oxidation of *p*-cresol are also involved in 2,4-xylenol oxidation. In addition, no enzyme has been reported to be responsible for the oxidation of 4-hydroxy-3-methylbenzaldehyde to 4-hydroxy-3-methylbenzoic acid in the initial oxidation of 2,4-xylenol. In this study, we report that the catabolism of 2,4-xylenol and *p*-cresol in strain 9866 shared the same enzymes in the oxidation of the *para*-methyl group.

Materials and methods

Bacterial strains, plasmids, primers, chemicals, media, and culture conditions

The bacterial strains and plasmids used are described in Table 1, primers in Table S1. The *Pseudomonas* strains were grown at 30 °C in minimal medium (Elmorsi and Hopper 1977) with different carbon sources. *Escherichia coli* strains were grown in lysogeny broth (LB) at 37 °C. The antibiotics were supplemented into the media with the concentration of 100 μg/ml of ampicillin sodium, 50 μg/ml of kanamycin sulfate, or 20 μg/ml of tetracycline hydrochloride, as necessary. All reagents used were purchased from Sigma Chemical Co. (St. Louis, MO, USA) or Fluka Chemical Co. (Buchs, Switzerland).

Real-time quantitative PCR

Strain 9866 was grown in minimal medium with 2 mM glucose as carbon source to an OD_{600} of 0.1, then induced by 2 mM 2,4-xylenol or *p*-cresol for 5 h. The total RNA was isolated with an RNAprep pure bacterial kit (Tiangen Biotech, Beijing, China) and reverse transcribed into cDNA

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristic(s)[a] or purpose	Reference or source
P. putida NCIMB		
9866	Wild-type strain with the phenotype of 2,4-xylenol[+] and *p*-cresol[+], Amp[R], Kan[S], Tc[S]	Chapman and Hopper (1968)
9866Δ*pchA*	NCIMB 9866 mutant with *pchA* gene replaced by kanamycin resistance gene from plasposon pTn*Mod*-Okm, Amp[R], Kan[R]	This study
9866Δ*pchA*[pRK415-*pchA*]	*pchA* gene was complemented by pRK415-*pchA* in 9866Δ*pchA*, Amp[R], Kan[R], Tc[R]	This study
9866Δ*pchF*	NCIMB 9866 mutant with *pchF* gene replaced by kanamycin resistance gene from plasposon pTn*Mod*-Okm, Amp[R], Kan[R]	This study
9866Δ*pchF*[pRK415-*pchF*]	*pchF* gene was complemented by pRK415-*pchF* in 9866Δ*pchF*, Amp[R], Kan[R], Tc[R]	This study
E. coli		
DH5α	F^{−} φ80(*lacZ*)ΔM15 Δ*lac*X74 *hsdR*(r$_k^-$m$_k^+$) Δ*recA*1398 *endA1 tonA*	TransGen Biotech
BL21(DE3)	F^{−} *ompT hsdS*(r$_B^-$m$_B^+$) *gal dcm*(DE3)	TransGen Biotech
WM3064	Donor strain for conjugation, 2,6-diaminopimelic acid auxotroph: *thrB*1004 *pro thi rpsL hsdS lacZ*ΔM15 RP4-1360 Δ(*araBAD*)567 Δ*dapA*1341::[*erm pir*(wt)]	Dehio and Meyer (1997), Saltikov and Newman (2003)
Plasmid		
pET-28a(+)	Expression vector, Kan[R], C/N-terminal His·Tag/thrombin/T7·Tag, T7 *lac* promoter, T7 transcription start, f1 origin, *lacI*	Novagen
pEX18Tc	Gene knockout vector, *oriT*[+], *sacB*[+], Tc[R]	Hoang et al. (1998)
pRK415	Broad host range vector, Tc[R]	Keen et al. (1988)
pTn*Mod*-Okm	Source of kanamycin resistance gene	Dennis and Zylstra (1998)
pET-28a(+)-*pchA*	Expression vector for PHBDD with C-terminal His·Tag by inserting *pchA* into the *Nco*I/*Hin*dIII restriction site	This study
pET-28a(+)-*pchFC*	Expression vector for PCMH with C- and N-terminal His·Tag by cloning *pchF* and *pchC* into the *Nde*I/*Hin*dIII restriction site	This study
pEX18Tc-*pchA*	*pchA* gene knockout vector containing two DNA fragments homologous to the upstream and downstream regions of the *pchA* and kanamycin resistance gene from pTn*Mod*-Okm	This study
pEX18Tc-*pchF*	*pchF* gene knockout vector containing two DNA fragments homologous to the upstream and downstream regions of the *pchF* and kanamycin resistance gene from pTn*Mod*-Okm	This study
pRK415-*pchA*	*pchA* gene complementation vector by fusing *pchA* into the *Hin*dIII/*Eco*RI restriction site of pRK415	This study
pRK415-*pchF*	*pchF* gene complementation vector by fusing *pchF* into the *Hin*dIII/*Eco*RI restriction site of pRK415	This study

[a] 2,4-xylenol[+], *p*-cresol, the ability to grow on 2,4-xylenol and *p*-cresol, respectively; Amp[R], Kan[R], Tc[R], resistant to ampicillin, kanamycin, and tetracycline, respectively; Kan[S], Tc[S], sensitive to kanamycin and tetracycline, respectively

using a PrimeScript RT Reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, Dalian, China). Real-time quantitative PCR (RT-qPCR) was performed on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA) using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) with primers in Table S1. The amount of target mRNA was normalized to that of 16S rRNA with the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001).

Gene cloning and expression, protein purification

pchA amplified by PCR from genome DNA of strain 9866 was digested by *Nco*I and *Hin*dIII, then cloned into pET-28a(+) to obtain expression construct of pET-28a(+)-*pchA*; *pchF* and *pchC* amplified were fused to the *Nde*I/*Hin*dIII restriction site

of pET-28a(+) with NovoRec® PCR one-step directional cloning kit (seamless cloning) (Novoprotein/Sinobio, Shanghai, China) to produce a construct pET-28a(+)-*pchFC*.

E. coli BL21(DE3) strains carrying the resulting expression constructs were grown at 37 °C to an OD_{600} of 0.5, then induced with 0.1 mM isopropyl-*β*-D-thiogalactopyranoside for 5 h at 30 °C. C-terminal His-tagged PchA (PchA-H$_6$), and N-terminal His-tagged PchF (H$_6$-PchF)/C-terminal His-tagged PchF (PchC-H$_6$) from corresponding cell extracts were purified by Ni^{2+}–NTA (nickel-nitrilotriacetic acid) agarose chromatography (Novagen, Madison, WI). The proteins binding to the column were washed and eluted with binding buffers (40 mM HEPES–KOH buffer (pH 8.0) for PchA-H$_6$, 50 mM glycine–NaOH buffer (pH 9.0) for H$_6$-PchF/PchC-H$_6$) containing 50 and 250 mM imidazole, respectively. The purified proteins were dialyzed against

binding buffers, analyzed by SDS-PAGE, and then stored at 4 °C.

Enzyme activity assay and intermediate identification

PCMH The enzyme activity assays of PCMH by H_6-PchF/PchC-H_6 against the substrates of *p*-cresol and 2,4-xylenol were followed as described (Hopper and Taylor 1977). For the identification of the products in H_6-PchF/PchC-H_6-catalyzed reactions from 2,4-xylenol, 1.5 ml of reaction mixture in 50 mM glycine–NaOH buffer (pH 9.0) containing 0.33 mM 2,4-xylenol, 0.67 mM phenazine methosulfate, 0.1 mM 2,6-dichlorophenolindophenol, and 147 μg of H_6-PchF/PchC-H_6 was set up. Samples withdrawn from the reaction were analyzed by high-performance liquid chromatography–diode array detector–mass spectrometry (HPLC-DAD/MS).

HPLC-DAD/MS analyses were performed on a Dionex UltiMate 3000 RS HPLC system with autosampler and a DAD detector, and an LCQ Fleet™ ion trap mass spectrometer equipped with an electrospray ionization source (Thermo Fisher Scientific, Waltham, MA, USA). Chromatographic separation was carried out on a Hypersil GOLD aQ column (150×2.1 mm, 3 μm particle size; Thermo Fisher Scientific) using gradient elution. The binary mobile phase consisted of solvents A (0.1 % acetic acid in water) and B (acetonitrile) as follows: 13 % B from 0 to 0.5 min; increasing to 20 %B from 0.5 to 0.6 min, increasing to 30 % B from 0.6 to 4.0 min, and increasing to 60 % B from 4.0 to 4.1 min, hold at 60 % B from 4.1 to 8.0 min, then back to 13 % B in 0.1 min and equilibrate for 1.9 min; the flow rate was 0.3 ml/min. The column temperature and sample tray were maintained at 30 °C. The injection volume was 15 μl, and the wavelength range of DAD for detection was 200–300 nm to monitor the UV absorption. The mass spectrometer was operated in the negative ion mode and full scan (50–300 m/z). The sample solutions were nebulized and evaporated using nitrogen as the sheath and auxiliary gas at the flow rate of 35 and 5 arb (1 arb=0.3 l/min), respectively. The ion spray voltage and transfer capillary voltage were set at 5.0 kV and −45 V, respectively. The transfer capillary temperature was maintained at 300 °C.

PHBDD The enzyme activity assays of PHBDD by PchA-H_6 to the substrates of 4-hydroxybenzaldehyde and 4-hydroxy-3-methylbenzaldehyde in the presence of cofactor of NAD^+ or $NADP^+$ (sodium salt) were conducted as described (Cronin and McIntire 1999) with modifications. The reaction mixtures (final volume, 1.0 ml) contained 40 mM HEPES–KOH buffer (pH 8.0), 50 μM of NAD^+ or $NADP^+$, 50 μM of 4-hydroxybenzaldehyde or 4-hydroxy-3-methylbenzaldehyde, and 1.3 μg enzyme with $NADP^+$ as cofactor or 104 μg enzyme with NAD^+ as cofactor. The

reference cuvette contained all these compounds except the substrate, and the assay was initiated by addition of substrate. Spectrophotometric changes at 340 nm during the transformation of 4-hydroxybenzaldehyde and 4-hydroxy-3-methylbenzaldehyde catalyzed by PchA-H_6 were monitored by a Lambda 25 UV/VIS Spectrometer (PerkinElmer, Waltham, MA). The substrates and products were confirmed with HPLC-DAD/MS by comparing the retention times, ultraviolet spectra, and mass spectra of standards with those of samples (see above). For the HPLC-DAD/MS analyses, the volume of reaction mixture was scaled up to 20 ml.

In the kinetics assays of PHBDD, four independent sets of experiments were performed with at least seven substrate concentrations ranging from 2 to 80 μM, and the concentration of cofactor was fixed at 100 μM. Data were fitted with the Michaelis–Menten equation by OriginPro 8 software (OriginLab, Northampton, MA). The protein concentration was determined by the Bradford method (Bradford 1976) with bovine serum albumin as the standard. One unit of enzyme activity was defined as the amount of enzyme required to catalyze the reduction of 1 μmol of $NADP^+$ or NAD^+ per min at 30 °C. Specific activities were expressed as units per milligram of protein.

Gene knockout and complementation

pEX18Tc-*pchA* and pEX18Tc-*pchF* for gene knockout were constructed by fusing PCR products of kanamycin resistance gene and two upstream and downstream fragments of the target genes, amplified with primers in Table S1, to *Sac*I/*Hin*dIII digested pEX18Tc with In-Fusion® HD Cloning Kit (TaKaRa, Dalian, China). These two resulting plasmids were transformed into *E. coli* WM3064 (2,6-diaminopimelic acid auxotroph) (Dehio and Meyer 1997; Saltikov and Newman 2003) before being conjugated to strain 9866 as described (Saltikov and Newman 2003). The double-crossover recombinants of 9866Δ*pchA* and 9866Δ*pchF* were screened on LB plates containing 10 % sucrose (*w/v*), ampicillin, and kanamycin.

pRK415-*pchA* and pRK415-*pchF* for gene complementation were constructed by fusing PCR products of *pchA* and *pchF*, amplified with primers in Table S1, to *Hin*dIII- and *Eco*RI-digested pRK415. They were transformed into *E. coli* WM3064 and then mated into the *pchA*- and *pchF*-deleted strain 9866 by conjugation to obtain 9866Δ*pchA*[pRK415-*pchA*] and 9866Δ*pchA*[pRK415-*pchF*], respectively.

Measurement of bacterial growth on different carbon sources

The bacterial growths of strain 9866 and its derivatives with 1.0 mM of each carbon source were measured at OD_{600} in

an automated turbidimeter, Bioscreen C (Labsystem, Helsinki, Finland). The data acquired were transformed to corresponding biomass of cell dry weight. Then, growth curves were fitted by the modified Gompertz equation (Zwietering et al. 1990) with OriginPro 8 software, and their maximum specific growth rates (μ_m, in per hour) were calculated.

Biotransformation of different substrates by strain 9866 and its mutants

Strain 9866 and its mutants were grown with 2 mM glucose to an OD_{600} of 0.1, then induced by 2 mM of 2,4-xylenol and p-cresol for 5 h before the biotransformation of 4-hydroxy-3-methylbenzaldehyde and 4-hydroxybenzaldehyde, respectively. Cells were harvested by centrifugation at 4,500×g for 1.0 min, washed twice, and diluted to OD_{600} of 0.3 with minimal medium, and then different substrates were added into each 10 ml of the suspending cells to a final concentration of 1 mM. One-half milliliter of sample was withdrawn from each reaction every 10 min, mixed with 0.5 ml of acetonitrile, and vortexed rigorously for 3 min to stop the reaction. The sample was then centrifuged at 14,500×g for 10 min before the supernatant was collected for quantitative analyses.

The analyses were perform on Agilent 1200 HPLC systems (Agilent Technologies, Palo Alto, CA) equipped with a variable wavelength detector and an Agilent ZORBAX 300SB-C18 column (250×4.6 mm, 5 μm particle size). The mobile phase consisted of solvents A (0.1 % acetic acid in water) and B (acetonitrile). The gradient program started with 10 % of B, followed by increasing to 30 % B from 0 to 11 min, increasing to 70 % B from 11 to 16 min, hold at 70 % B from 16 to 17 min, then back to 10 % B in 0.1 min and equilibrate for 2.9 min. The flow rate was 1.0 ml/min. The column temperature was 30 °C. The injection volume was 20 μl, and the detection wavelength was 250 nm. Under those conditions, the retention times of 4-hydroxy-3-methylbenzaldehyde, 4-hydroxy-3-methylbenzoic acid, 4-hydroxybenzaldehyde, and 4-hydroxybenzoic acid were 11.0, 8.7, 7.4, and 5.9 min, respectively. One unit of activity was defined as the amount of cell (milligram of cell dry weight) required to transform 1 μm of substrate per min at 30 °C.

Genome walking

Genome walking was conducted to clone the flanking regions of the 5,276-bp DNA sequence in plasmid pRA4000 with methods described previously (Zhang et al. 2009). The GenBank accession number for the nucleotide sequence obtained in this study is KC762703.

Results

$pchA$, $pchC$, and $pchF$ were highly transcribed in 2,4-xylenol-induced cell of strain 9866

$pchC$- and $pchF$-encoded PCMH and $pchA$-encoded PHBDD were reported to be responsible for the initial oxidation of p-cresol (Cronin et al. 1999; Cronin and McIntire 1999; Kim et al. 1994), and PCMH also had catalytic activity to 2,4-xylenol (Hopper and Taylor 1977). Therefore, transcriptional analyses of $pchA$, $pchC$, and $pchF$ under various induction conditions were conducted by RT-qPCR in order to investigate whether PCMH and PHBDD were also involved in the initial oxidation of 2,4-xylenol. The result indicated the relative transcription levels of $pchA$, $pchC$, and $pchF$ under 2,4-xylenol-induced condition were 6.5-, 8.3-, and 4.5-fold higher than those under uninduced condition, respectively. Similarly, the relative transcriptions of these three genes under p-cresol-induced condition were 4.6-, 4.5-, and 1.5-fold higher than those under uninduced condition (Fig. 2).

PCMH catalyzes the oxidation of 2,4-xylenol to 4-hydroxy-3-methylbenzaldehyde and its encoding gene is necessary for 2,4-xylenol catabolism

PCMH purified from strain 9866 has been previously proved to have a catalytic activity to 2,4-xylenol (Hopper and Taylor 1977). The products, however, were not identified. In this study, a total of 32 mg of recombinant PCMH with a specific activity of 0.18 U mg^{-1} was purified from 3,000 ml of culture. Compound 4-hydroxy-3-methylbenzaldehyde was identified as the product with a retention time at 6.0 min, λ_{max} of 286 nm, and a stable deprotonated ion of $[M-H]^-$ (m/z=134.99) in mass spectrum (Fig. 3). Thus, PCMH was confirmed to catalyze the oxidation of 2,4-xylenol to yield 4-hydroxy-3-methylbenzaldehyde. However, 4-hydroxy-3-methylbenzyl
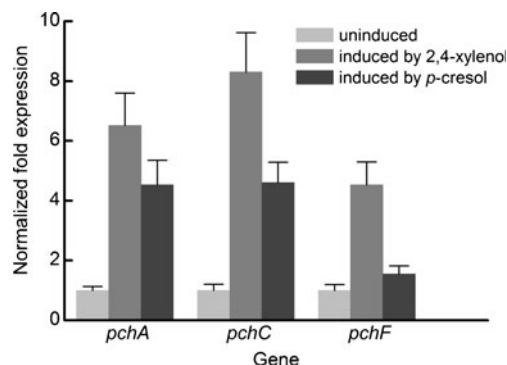
Fig. 2 Transcriptional analyses of $pchA$, $pchC$, and $pchF$ in strain 9866 under induced and uninduced conditions by RT-qPCR. All samples were run in triplicate, and *error bars* indicate standard deviations

alcohol, the putative transient intermediate, was not identified because its standard was commercially unavailable.

pchF encodes the flavoprotein subunit of PCMH and is essential for its enzyme activity as reported (Kim et al. 1994; Mcintire and Singer 1982). To verify the role of *pchF*- and *pchC*-encoded PCMH in 2,4-xylenol catabolism, *pchF* was knocked out. The results showed that 9866Δ*pchF* was no longer to grow with 2,4-xylenol or *p*-cresol as sole carbon and energy source. However, this mutant was still capable of growing on 4-hydroxy-3-methylbenzaldehyde or 4-hydroxybenzaldehyde, two products from 2,4-xylenol or *p*-cresol oxidation catalyzed by PCMH. On the other hand, *pchF*-complemented mutant 9866Δ*pchF*[pRK415-*pchF*] restored its ability to grow on 2,4-xylenol or *p*-cresol. This indicated PCMH encoded by *pchF* and *pchC* was necessary for the catabolism of 2,4-xylenol as well as *p*-cresol in strain 9866.

PHBDD catalyzes the NADP⁺-dependent oxidation of 4-hydroxy-3-methylbenzaldehyde to 4-hydroxy-3-methylbenzoic acid

It has been reported that PHBDD catalyzed the NADP⁺-dependent oxidation of 4-hydroxybenzaldehyde to 4-hydroxybenzoic acid in *p*-cresol catabolism (Cronin et al. 1999; Cronin and McIntire 1999). In this study, a total of 26 mg of recombinant PHBDD with a specific activity of 7.55 U mg^{-1} was purified from 200 ml of culture. PHBDD was found to be able to catalyze the dehydrogenation of 4-hydroxy-3-methylbenzaldehyde to 4-hydroxy-3-methylbenzoic acid in 2,4-xylenol catabolism, with

Fig. 3 HPLC-DAD/MS analysis of the products from the oxidation of 2,4-xylenol catalyzed by PCMH. **a** HPLC chromatogram of sample from the reaction detected by DAD at wavelength of 280 nm. Peaks at retention time of 6.0 and 7.6 min identify 4-hydroxy-3-methylbenzaldehyde and 2,4-xylenol, respectively. **b** Mass spectrum of peak at 6.0 min detected by MS at negative full scan mode

NADP⁺ or NAD⁺ as cofactor. Kinetics assays revealed that the K_m value of PHBDD to 4-hydroxy-3-methylbenzaldehyde with NADP⁺ (6.36±1.44 μM) was lower than that with NAD⁺ (20.55 ±1.91 μM) (Table 2). In terms of K_{cat}/K_m values, the catalytic efficiency of PHBDD to 4-hydroxy-3-methylbenzaldehyde with NADP⁺ was approximately three orders of magnitude greater than that with NAD⁺. Therefore, in addition to catalyzing 4-hydroxybenzaldehyde oxidation in *p*-cresol catabolism, PHBDD also catalyzes the NADP⁺-dependent oxidation of 4-hydroxy-3-methylbenzaldehyde to 4-hydroxy-3-methylbenzoic acid in 2,4-xylenol catabolism. On the other hand, the K_m value of PHBDD to 4-hydroxybenzaldehyde (3.57±0.32 μM) (in *p*-cresol catabolism) was evidently lower than that to 4-hydroxy-3-methylbenzaldehyde (6.36±1.44 μM) (in 2,4-xylenol catabolism), and its catalytic efficiency to 4-hydroxybenzaldehyde was sixfold higher than that to 4-hydroxy-3-methylbenzaldehyde.

Other PHBDDs may be involved in 2,4-xylenol and *p*-cresol catabolism

To examine the role of *pchA*-encoded PHBDD in 2,4-xylenol catabolism, *pchA* was knocked out and complemented. Surprisingly, strain 9866Δ*pchA* was still able to grow with 2,4-xylenol and *p*-cresol as well as their oxidation products of corresponding benzaldehydes and benzoic acids. In addition, 4-hydroxy-3-methylbenzoic acid and 4-hydroxybenzoic acid were detected simultaneously, during the biotransformation of 2,4-xylenol and *p*-cresol by strain 9866Δ*pchA*, respectively. This implied the presence of other enzyme(s) with PHBDD activity, in addition to *pchA*-encoded PHBDD.

Bacterial growth experiments also supported the above deduction. The maximum specific growth rates (μ_m, per hour) of strain 9866 were higher than those of strain 9866Δ*pchA*, to all four substrates tested as shown in Table 3. This indicated that *pchA* deletion resulted in the decline of catabolic rates of 2,4-xylenol, *p*-cresol, 4-hydroxy-3-methylbenzaldehyde, and 4-hydroxybenzaldehyde. In addition, strain 9866 exhibited 60 % higher specific activities to 4-hydroxy-3-methylbenzaldehyde (6.05±0.05 U mg^{-1}) and 4-hydroxybenzaldehyde (4.50±0.39 U mg^{-1}) than those from strain 9866Δ*pchA*, respectively. These results suggested that, despite the possible presence of PHBDD homologs, *pchA*-encoded PHBDD played a major role in the catabolism of 2,4-xylenol and *p*-cresol.
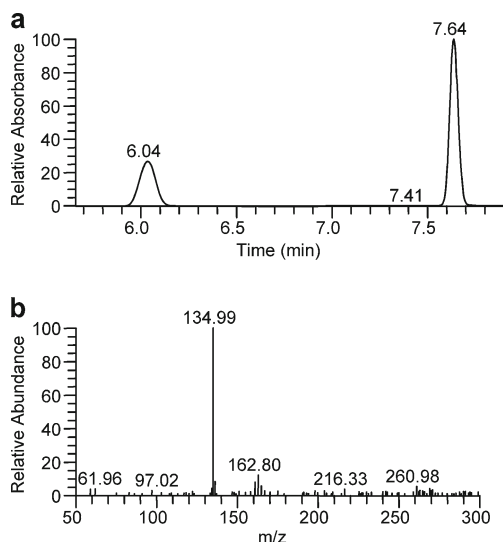
Discussion

Previously, *pchC*- and *pchF*-encoded PCMH and *pchA*-encoded PHBDD were reported to be responsible for the oxidation of the *para*-methyl group of *p*-cresol in the *p*-

Table 2 Kinetic parameters of the *pchA*-encoded PHBDD on 4-hydroxybenzaldehyde and 4-hydroxy-3-methylbenzaldehyde

Substrate	Cofactor	K_m (µM)	K_{cat} (min^{-1})	K_{cat}/K_m (µM^{-1} min^{-1})
4-Hydroxybenzaldehyde	NADP$^+$	3.57±0.32	15.82±0.36	4.4313±0.2992
	NAD$^+$	13.11±1.78	0.10±0.00	0.0076±0.1111
4-Hydroxy-3-methylbenzaldehyde	NADP$^+$	6.36±1.44	4.27±0.27	0.6714±0.1165
	NAD$^+$	20.55±1.91	0.10±0.00	0.0049±0.0005

The kinetic constants were calculated by nonlinear regression analysis, and the values are expressed as means ± standard deviations ($n=4$). k_{cat} values were calculated on the basis of a subunit M_r of 11,536.

cresol and 2,4-xylenol utilizer strain 9866 (Cronin et al. 1999; Kim et al. 1994). In this study, the high transcription of *pchC*, *pchF*, and *pchA* in the 2,4-xylenol-induced cell indicated that PCMH and PHBDD were likely also involved in the catabolism of 2,4-xylenol. *pchC* and *pchF* were proved to be located on two different operons (Kim et al. 1994), and *pchA* and *pchC* were found to be in the same operon in our study by reversed transcription PCR (data not shown). There were differences of relative transcription level between *pchAC* and *pchF* apparently because they were located on different operons and transcribed independently. Enzyme activity assay and intermediate identification have shown that the PCMH and PHBDD catalyzed the oxidations of 2,4-xylenol to 4-hydroxy-3-methylbenzaldehyde and 4-hydroxy-3-methylbenzaldehyde to 4-hydroxy-3-methylbenzoic acid, respectively. Nevertheless, the K_m value of PCMH to *p*-cresol was lower than that to 2,4-xylenol in a previous report (Hopper and Taylor 1977), and in this study, the K_m value of the PHBDD to 4-hydroxybenzaldehyde was found to be also lower than that to 4-hydroxy-3-methylbenzaldehyde. This indicated *p*-cresol and 4-hydroxybenzaldehyde in *p*-cresol catabolism were the probable physiological substrates for the PCMH and PHBDD in strain 9866, respectively.

On the other hand, the PCMH-encoding gene was found to be necessary for the catabolism of 2,4-xylenol and *p*-cresol, whereas the PHBDD-encoding gene was not essential for the catabolism. In addition to the *pchA*-encoded PHBDD, analyses of the maximum specific growth rate and specific activity of the 9866Δ*pchA* to different intermediates revealed the probable presence of other enzyme(s) with PHBDD activity. Indeed, several benzaldehyde dehydrogenase analogous to PHBDD were reported to exhibit activity to 4-hydroxybenzaldehyde in several strains, such as benzaldehyde dehydrogenase encoded by the *xylC* on plasmid pWW0 from *P. putida* (Inoue et al. 1995), benzaldehyde dehydrogenase II from *Acinetobacter calcoaceticus* (Mackintosh and Fewson 1988), and *areC*-encoded benzaldehyde dehydrogenase from *Acinetobacter* sp. strain ADP1 (Jones et al. 1999), though 4-hydroxy-3-methylbenzaldehyde was not tested for these enzymes.

In addition, the PCMH and PHBDD-encoding genes (*pchC*, *pchF*, and *pchA*) were also shown to be involved in the initial catabolism of 2,4-xylenol in this study and located on plasmid pRA4000 in strain 9866, rectifying the previous result that structural genes for 2,4-xylenol utilization were located on chromosome (Dean et al. 1989). Besides the PCMH and PHBDD, efforts to find the other genes and enzymes responsible for the lower catabolic pathway of 2,4-xylenol were also made by genome walking and enzyme activity assay. However, no genes encoding enzymes

Table 3 Maximum specific growth rates (μ_m) of bacteria to different substrates

Carbon source for growth[a]	Strain	Adjusted R^2	μ_m (h^{-1})[b]
2,4-Xylenol	9866	0.9731	0.16
	9866Δ*pchA*	0.9650	0.06
4-Hydroxy-3-methylbenzaldehyde	9866	0.9014	0.06
	9866Δ*pchA*	0.9713	0.04
p-Cresol	9866	0.9673	0.16
	9866Δ*pchA*	0.9647	0.09
4-Hydroxybenzaldehyde	9866	0.9557	0.32
	9866Δ*pchA*	0.9893	0.11

[a] Bacteria, grown with 2 mM glucose as carbon source to an OD$_{600}$ of 0.1, were induced by 2 mM of 2,4-xylenol and *p*-cresol for 5 h prior to inoculation for growth on 2,4-xylenol or 4-hydroxy-3-methylbenzaldehyde and *p*-cresol or 4-hydroxybenzaldehyde, respectively (2 % inoculum from culture with OD$_{600}$ of 0.2, v/v)

[b] μ_m was expressed with the average value ($n=5$)

responsible for the oxidation of *ortho*-methyl group of 2,4-xylenol and 4-hydroxyisophthalate hydroxylase were found in plasmid pRA4000 for 2,4-xylenol catabolism. It is possible that structural genes for 2,4-xylenol utilization were spread between plasmid and chromosome in strain 9866.

Interestingly, genes (*tnpA* and *tnpR*) encoding putative transposase and resolvase were found to flank the PCMH- and PHBDD-encoding genes (*pchC*, *pchF*, and *pchA*) and the putative structural genes identified for protocatechuate *ortho* cleavage pathway in pRA4000 (Fig. 1b). This implied transposition may have occurred in the evolution of catabolic pathways for 2,4-xylenol and *p*-cresol. In addition, 4-hydroxy-3-methylbenzoate hydroxylase catalyzing the initial hydroxylation of the *ortho*-methyl group of 2,4-xylenol was considered as monooxygenase, in contrast to the dehydrogenase of PCMH responsible for the initial hydroxylation of the *para*-methyl group (El-Mansi and Hopper 1990). It was suggested that there was a possibility that vanillate demethylase was recruited and evolved to 4-hydroxy-3-methylbenzoate hydroxylase as part of the 2,4-xylenol pathway (El-Mansi and Hopper 1990). Those speculations together with the results of this study suggested that genes for enzymes involved in the catabolism of 2,4-xylenol may be evolved from various origins.

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