World J Microbiol Biotechnol (2010) 26:665–673
DOI 10.1007/s11274-009-0221-1

ORIGINAL PAPER

A novel degradation pathway of chloroaniline in *Diaphorobacter* sp. PCA039 entails initial hydroxylation

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Received: 13 July 2009 / Accepted: 14 October 2009 / Published online: 12 December 2009

Abstract A novel degradation pathway of chloroaniline in *Diaphorobacter* sp. PCA039 entails initial hydroxylation. A novel gene cluster (*pca*), involved in the degradation of *p*-chloroaniline, was identified from *Diaphorobacter* sp. PCA039 capable of utilizing *p*-chloroaniline as sole carbon, nitrogen and energy source for growth. There were 29 ORFs in the *pca* cluster, in the order of *pcaTRKLMNOPR*$_1$*D*$_1$*C*$_1$*U*$_1$*R*$_2$*D*$_2$*C*$_2$*U*$_2$*EFGXJHYWZI*$_1$*I*$_2$*QS*. Based on sequence analysis, they were putatively identified as encoding a multicomponent phenol-hydroxylating oxygenase (*pcaKLMNOP*), two extradiol ring-cleavage dioxygenases, transcriptional regulatory proteins, enzymes mediating chlorocatechol degradation, and transportation functions. The genes *pcaKLMNOP* exhibited significant sequence identity (94%) to those of phenol hydroxylases (PH) from other bacteria, inferring that they might encode a multicomponent PH. This PH activity was also functionally characterized with the recombinant strain *E. coli* TOP10-S201 showing only PH activity, indicating that the degradation of *p*-chloroaniline by strain PCA039 was initiated by hydroxylation instead of normal dioxygenation. The other nineteen genes, *pcaR*$_1$*D*$_1$*C*$_1$*U*$_1$*R*$_2$*D*$_2$*C*$_2$*U*$_2$*EFGXJHYWZI*$_1$*I*$_2$, encode for further degradation of *p*-chloroaniline to intermediates of the TCA-cycle and transposases. RT-PCR revealed that the hydrolytic (*pcaF*) and dehydrogenetic pathways (*pcaE*, *pcaH*), the two degrading branches, are both necessary for complete degradation of aniline, *p*-chloroaniline, phenol and also 4-aminophenol; and of the two C23O sets, $PcaR_1D_1C_1U_1$ and $PcaR_2D_2C_2U_2$, PcaC1 is produced in the degradation of above four substrates, while PcaC2 is only expressed in *p*-chloroaniline degradation, suggesting that both C23O sets are needed for complete degradation of *p*-chloroaniline.

Keywords Chloroaniline · Biodegradation · *Diaphorobacter* sp. PCA039 · Gene cluster · Initial hydroxylation

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Introduction

Chloroanilines constitute a group of xenobiotics that have been in industrial use for a long time in the production of paints, pesticides, plastics, and pharmaceuticals (Meyer 1981). This group of compounds is considered as important environmental pollutants and accumulation in the long run may be detrimental to human health as a result of their persistence, toxicity, and transformation into hazardous intermediates (Pieper and Reineke 2004; Pizon et al. 2009). A number of bacterial species, capable of utilizing chloroanilines as sole source of nutrients, have been isolated from various environments such as a bioreactor (Kaminski et al. 1983), activated sludge (Boon et al. 2001; Ren et al. 2005) and soil (Vangnai and Petchkroh 2007). In bacteria, aerobic catabolic pathways for aromatic hydrocarbon degradation can schematically be divided into two major biochemical steps. First, early reactions, the so-called upper pathways or peripheral routes, channel the hydrocarbons towards the

formation of partially oxidized aromatic intermediates. Then, dihydroxylated aromatic molecules that can undergo the cleavage of the ring are produced and further processed to give compounds that can enter the tricarboxylic acid (TCA) cycle. Although a wide variety of very different peripheral routes for the oxidation of many different aromatic hydrocarbons exist, only a limited number of dihydroxylated compounds that can be cleaved and productively processed to enter the TCA cycle are known. However, until now, the peripheral routes for the bacterial aerobic degradation of aniline and substituted anilines have been found only to be initiated by the reaction catalyzed by aniline dioxygenase among those bacteria, such as *Frateuria* species ANA-18 (Murakami et al. 2003), *Delftia acidovorans* strain 7N (Urata et al. 2004), *D. tsuruhatensis* AD9 (Liang et al. 2005) and *Delftia* sp. AN3 (Zhang et al. 2008); and never by any other peripheral routes such as hydroxylation, which has normally been found in the degradation of phenol and toluene (Divari et al. 2003; Griva et al. 2003). The reactions for oxygenative ring fission of catechol and the subsequent conversion to TCA intermediates are limited to one of two metabolic alternatives: i.e., (1) intradiol (*ortho* and modified *ortho*)-cleavage, and (2) extradiol (*meta*)-cleavage. The use of these two cleavage routes is dependent upon the microbial species and/or the nature of the growth substrate. *Ortho*-cleavage is the dominant cleavage mechanism in the degradation of chlorinated compounds, as extradiol cleavage of halocatechols may produce dead-end or suicide metabolites (Klecka and Gibson 1981; Surovtseva et al. 1980).

Here we report gene cloning, DNA sequencing, and partial functional analysis of the complete chloroaniline-degrading cluster (*pca*), from *Diaphorobacter* sp. PCA039; and homology analysis and enzyme assays indicate that the degradation of *p*-chloroaniline by strain PCA039 is initiated by hydroxylation instead of dioxygenation.

Materials and methods

Chemicals

Aniline, *p*-aminophenol, phenol, and *p*-chloroaniline were from Sinopharm Chemical Reagent Beijing Co., Ltd. Catechol and 4-chlorocatechol were from Sigma–Aldrich® Co.

Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. All *Escherichia coli* strains were grown in Luria–Bertani (LB) medium (Sambrook and Russell 2001). *Diaphorobacter* sp. PCA039 was able to utilize *p*-chloroaniline as sole carbon, nitrogen and energy source for growth and was grown at 30°C in mineral salt medium (Ren et al. 2005).

DNA preparation and manipulation

Escherichia coli was grown aerobically in a Luria–Bertani medium, and chemically competent cells were transformed by heat shock. Extraction of genomic DNA was carried out following the procedures of Sambrook and Russell (2001). Recombinant plasmid DNA was isolated with a Tian-prep Mini kit (TianGen Biotech CO., Ltd). Restriction enzymes, T4 DNA ligase and calf intestinal alkaline phosphatases were from New England Biolabs (USA) or Takara (Tokyo, Japan).

Construction of genomic DNA library of strain PCA039

Genomic DNA of *Diahorobacter* sp. PCA039 was partially sheared with a Hamilton syringe to obtain approximately 40 kb-size fragments. The partially treated DNA fragments were end-repaired by End-repair enzyme mix of Copy-control™ Fosmid Library Production Kit (Cat. No. CCFOS059) and then ligated to the cloning-ready Copy-control pCC2FOS vector, and the recombinant molecules were packaged into λ phage followed by phage transfection to *E. coli* EPI300 strain by using protocols described in the MaxPlax™ Lambda packaging kit (Epicentre Biotechnologies, Madison, Wisconsin, USA).

Cloning of the catechol 2, 3-dioxygenase gene from strain PCA039 and library screening

DNA sequences for catechol 2, 3-dioxygenase (C23O) gene from *Acidovorax* sp. JS42 (accession number YP_984548) and other bacteria were used to design a pair of degenerate primers, CDf65: 5′-AACCAYGTDGCWTA CAAGGT-3′ and CDr235: 5′- GHCTTGAGBACRTCRT GCCA-3′ (S=C, G; Y=C, T; B=C, G, T; H=A, C, T; R=A, G; W=A, T; D=A, G, T) for PCR amplification of a part (510 bp) of C230 gene from genomic DNA of *Diahorobacter* sp. PCA039, and it was confirmed by sequencing and BLAST search. To screen the positive clones, colony PCR was performed using this pair of primers. Recombinant *E. coli* strains with PCR-positive were further confirmed by spraying catechol solution (10 mM) onto the colonies for detection of C23O activity.

DNA sequencing and sequence analysis

DNA sequencing of the recombinant Fosmid was performed by the shotgun method by SinoGenoMax Co., Ltd (Chinese National Human Genome Center, Beijing). The ORFs were analysed using DNAstar (Lynnon Biosoft) and

Table 1 Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
E. coli EPI300™-T1R	*[F-e14-(McrA-) D(mcrC-mrr) (TetR) hsdR514 supE44 supF58 lacY1 or D(lacIZY)6 galK2 galT22 metB1 trpR55 l-]*	Epicentre
E. coli TOP10	*La cX74 recA1 deoR F -mcrA Δ(mrr-hsdRMS-mcrBC) φ80 lacZΔM15ΔaraD139Δ(ara-leu) 7697 galU galK*	TianGen
Diaphorobacter sp. PCA039	Wide strain, using *p*-chloroaniline as sole carbon, nitrogen and energy source, isolated from activated sludge of Nanjing Chemical Plant, Nanjing, China	Ren et al. (2005)
E. coli EPI300-PCA1	Positive clone from Fosmid genomic library, containing FosB12	This study
E. coli TOP10-S201	Subclone containing plasmid S201	This study
E. coli TOP10-S202	Subclone containing plasmid S202	This study
E. coli TOP10-S203	Subclone containing plasmid S203	This study
Plasmids		
pUC118	Cloning vector, modified pUC18 vector with intergenic region from M13 (3,162 bp); Ap^r	Takara
pCC2FOS	Cloning vector; Cm^r	Epicentre
FosB12	pCC2FOS, carrying the complete *pca* cluster	This study
S201	pUC118, carrying *pcaKLMNOP* genes as a whole	This study
S202	pUC118, carrying *pcaC1* and *pcaC2*	This study
S203	pUC118, carrying *pcaC2*	This study

Ap^r ampicillin resistant, *Cm^r* chloramphenicol resistant

Vector NTI 10.0 software (Invitrogen, USA); homology search for protein sequences was carried out using the BLAST and FASTA programs (Altschul et al. 1990; Pearson 1990). The phylogenetic tree were generated using the neighbor joining method of Saitou and Nei (1987) with MEGA 4.0 software (Tamura et al. 2007), and multiple sequence alignment was done using Clustal_X (Thompson et al. 1997).

Preparation of intact cell suspension, crude enzymes and assays of enzyme activity

Recombinant strains (*E. coli* TOP10-S201, *E. coli* TOP10-S202 and *E. coli* TOP10-S203, Table 1) were grown to a density of A_{600} about 1.0–1.5, and then the cells were harvested by centrifugation at 10,000×*g* for 10 min. The cell pellets were washed twice in 50 mM sodium phosphate buffer (pH 7.6) containing 1 mM 2-mercaptoethanol, and resuspended in the same buffer. Cells were disrupted sonically by twenty 4-s 200-W bursts on ice with a Braun-Sonic 1510 apparatus. Cellular debris was removed by centrifugation at 120,000×*g* at 4 °C for 15 min, and the supernatant was used immediately for enzyme assays.

Phenol hydroxylase (EC 1.14.13.7) (PH) activity was assayed by measuring the decrease in A_{340}, using NADPH as the co-substrate (Cafaro et al. 2004). One unit of enzyme activity is defined as the amount of enzyme caused the oxidation of 1 μmol of NADPH per min.

Dioxygenase activity was determined with a Clark oxygen electrode (YSI, Ohio, USA), according to the method of Fukumori and Saint (1997). To estimate the endogenous respiratory rate, 0.3 ml of sterile distilled water instead of substrate solution was used in a parallel experiment.

Catechol 2,3-dioxygenase (EC 1.13.11.2) (C23O) activity was measured according to Nakazawa and Yokota (1973) using a DU-7 spectrophotometer (Beckman, USA). The absorption coefficient of 2-hydroxymuconic semialdehyde was 12,000 l mol^{-1} cm^{-1}. Enzyme specific activities are reported as μmol of 2-hydroxymuconic semialdehyde produced per minute per mg of protein.

Protein was determined according to the Bradford method with bovine serum albumin as the standard.

Transcription detection of genes in degradation of four substrates by RT-PCR

Diaphorobacter sp. PCA039 was grown on chloroaniline, aniline, phenol and 4-aminophenol for 72 h, respectively. Then the cells were harvested by centrifugation and frozen by immersion in liquid N_2, respectively. Total RNA was prepared by using TRIZOL kit (Invitrogen) according to the manufacturer's instructions, further purified by Axyprep™ Multi-source total RNA miniprep kit (Axygen) according to the manufacturer's instructions, and used as template for gene expression analysis by reverse transcriptase-PCR (RT-PCR)

according to the methods of Sambrook and Russell (2001) and the primers as listed in Table 2. The RT-PCR products were then authenticated by sequencing.

Nucleotide sequence accession number

The nucleotide sequence of the *p*-chloroaniline degradation gene cluster (*pca*) of *Diaphorobacter* sp. PCA039 has been deposited in the DDBJ/EMBL/GenBank under the accession number of FJ601374.

Results

Screening for C230 gene from genomic library of strain PCA039

A genomic library of strain PCA039 was constructed with more than 13, 000 recombinant strains of *E. coli* EPI300 harboring about 40 kb-size genomic DNA from *Diaphorobacter* sp. PCA039. From three of them, the specific fragment (510 bp) could be amplified, showing that they might carry the C23O gene from strain PCA039. A recombinant strain, *E. coli* EPI300-PCA1, contained a recombinant Fosmid, named FosB12, which carried a 28 kb DNA fragment from strain PCA039, was obtained (Table 1).

The *pca* cluster and the genes involved in *p*-chloroaniline degradation

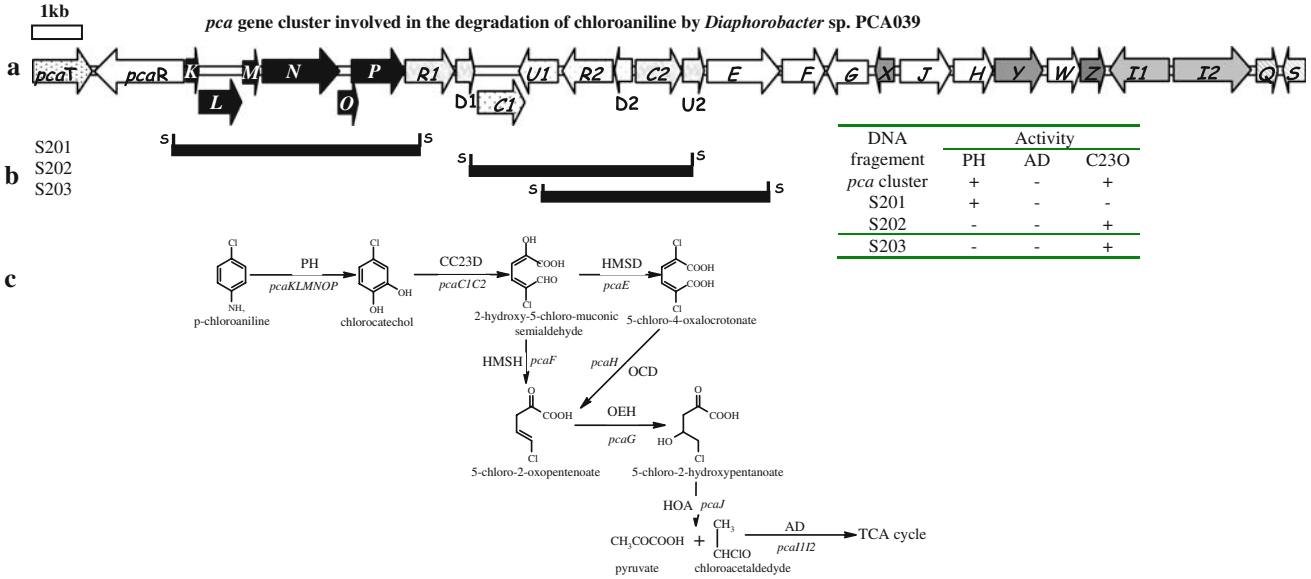
We obtained the DNA sequence of the ecombinant Fosmid, FosB12, identified 29 ORFs in a cluster spanning ~28 kb (Fig. 1) and named it as the *pca* cluster. Sequence and ORF analyses of this total 28 kb fragment indicated that there were 29 intact ORFs (Fig. 1a; Table 3), and the deduced products showed significant identity (73–100%, Table 3) to functionally identified proteins from other bacteria, especially to those from *Acidovorax* sp. JS42 (Copeland et al. 2006), a phenol-degrader. The high identity might explain the functions of these *orfs*. Among them, at least 20 (*pcaRKLMNOPR*$_1$*D*$_1$*C*$_1$*U*$_1$*R*$_2$*D*$_2$*C*$_2$*U*$_2$*EFGJI*$_1$*I*$_2$) were expected to contribute towards complete metabolism of *p*-chloroaniline to TCA-cycle intermediates via the *meta*-cleavage pathway as shown in Fig. 1b and summarized in Table 3. Homology analysis of the *pca* cluster revealed that it consists at least three parts as follows:

Degradative genes of the pca cluster

The products of *pcaKLMNOP*, PcaKLMNOP, showed significant sequence homology (73–94%) with a multi-component phenol hydroxylase (PH), present in phenol-degrading strains such as *Alcaligenes faecalis* IS-46 (Zhu et al. 2008) and *Acidovorax* sp. JS42 (Copeland et al. 2006), especially with those from *Acidovorax* sp. JS42. The remaining 14 gene products (PcaR$_1$D$_1$C$_I$U$_1$R$_2$D$_2$C$_2$U$_2$EFGJI$_1$I$_2$) exhibited considerable amino acid identity (73–100%) to enzymes of central catechol metabolism via the *meta*-cleavage pathway found in aniline-degrading and other aromatic-degrading bacteria. Interestingly, in the *pca* cluster, there were two C23O sets, PcaR$_1$D$_1$C$_I$U$_1$ and PcaR$_2$D$_2$C$_2$U$_2$. Phylogenetic analysis (data not shown) indicated that PcaC$_1$ and PcaC$_2$ belonged to two different branches in the phylogenetic tree, and shared only 41.9% identity and 57.5% similarity between themselves.

Table 2 Primers for RT-PCR

Primer	Sequence 5′-3′	Description	Fragment size (bp)
PHB-RT-5′	ACTGCCAGACCGAAACCC	Forward primer for *PcaL*	428
PHB-RT-3′	CATGTAGTCCTGCATCATCCC	Reverse primer for *PcaL*	
CD1-RT-5′	GCACGAAATGCGCCTGTA	Forward primer for *PcaC1*	482
CD1-RT-3′	AGGTCTCGTTGCGGTTGC	Reverse primer for *PcaC1*	
CD2-RT-5′	AGCTGTTCACCGAAGTGCTG	Forward primer for *PcaC2*	415
CD2-RT-3′	AGGAAGGCGTCGTTGAGTTT	Reverse primer for *PcaC2*	
HMSH-RT-5′	CCCCGTCGGTGGAGAACAT	Forward primer for *PcaF*	385
HMSH-RT-3′	GATGCCTCTGCCAGAAAGTCG	Reverse primer for *PcaF*	
HMSD-RT-5′	GACGTTCCAGGACATCAACCC	Forward primer for *PcaE*	401
HMSD-RT-3′	GGCAGACCACGGCAATCA	Reverse primer for *PcaE*	
OD-RT-5′	ATGACGCCTACGCCATCCAG	Forward primer for *PcaH*	461
OD-RT-3′	GCCGTTCTTCTCCAGCACCAG	Reverse primer for *PcaH*	
HOA-RT-5′	TCGGCACCGTCGATCACCT	Forward primer for *PcaJ*	372
HOA-RT-3′	CGATGGCGGCAATGGAATT	Reverse primer for *PcaJ*	



DNA fragement	Activity		
	PH	AD	C23O
pca cluster	+	-	+
S201	+	-	-
S202	-	-	+
S203	-	-	+

Fig. 1 Genetic organization of *pca* cluster (**a**) and putative chloroaniline degradation pathway (**c**) of *Diaphorobacter* sp. PCA039. The sequenced region of 28.4 kb is shown. *Black arrows* indicate PH genes, *open* and *grey arrows* indicate *meta*-cleavage pathway genes. **b** Details of plasmid construction are provided in Table 1. The function of other genes was explained in Table 3. Abbreviations: *PH* Phenol hydroxylase, *AD* aniline dioxygenase, *C23O* catechol 2, 3-dioxygenase, *HMSD* 2-hydroxymuconic semialdehyde dehydrogenase (EC), *HMSH* 2-hydroxymuconic semialdehyde hydrolase (EC 3.7.1.9), *OCD* 4-oxalocrotonate decarboxylase (EC 4.1.1.77), *OEH* 2-oxopent-4-dienoate hydratase, *HOA* 4-hydroxy-2-oxovalerate aldolase (EC 4.1.3.39), *ADA* acetaldehyde dehydrogenase. + Positive, − negative, *S Sau* 3AI site

Regulatory genes

There are at least three regulatory genes (*pcaR*, *pcaR*$_1$ and *pcaR*$_2$) and one potential candidate *orfZ* in the *pca* cluster. The deduced product (PcaR) of *pcaR* is a large protein with 596 amino acid residues. PcaR shows as high as 94% sequence identity (Table 3) to a Sigma 54 specific transcriptional regulator from *Acidovorax* sp. JS42 (Copeland et al. 2006). A conserved domain (CD) search reveals that a region of 180 residues at the C terminus homologous to the DNA-binding domains of various regulators (Calogero et al. 1994; Canellakis et al. 1993), in addition to a region corresponding to a proteobacterial transcriptional activator domain (PAD) at the N terminus and the V4R (vinyl 4 reductase) domain predicted to bind small molecules such as hydrocarbons. Sigma 54 specific regulator is a transcriptional activator found in the aromatic-compound-degrading cluster from bacteria such as *Acidovorax* sp. JS42. The predicted products of *pcaR*$_1$ and *pcaR*$_2$ show 98 and 93% sequence identity, respectively, to a LysR family transcriptional regulator found in aromatic compound degradation from *Comamonas* sp. JS765 and other homologous activators found in a variety of Proteobacteria. It is likely that PcaR$_1$ and PcaR$_2$ function by regulating two C23O systems. OrfZ shows high identity (55–100%) to the GntR family transcriptional regulator found in proteobacterial strains such as *Acidovorax* sp. JS42 (Copeland et al. 2006) and *R. eutropha* JMP134 (Kim et al. 1996).

Transposase genes and genes with unknown function

Besides the degradative genes and regulatory genes, in the *pca* cluster, *orfU1* and *orfU2* are located in the two C23O systems, which is a common phenomenon in the *meta*-cleavage pathway, though their exact function is unknown. Another gene, *orfY*, encodes a putative membrane transport protein similar to aromatic aminotransferase and transmembrane transport proteins found in other bacteria such as *Delftia* sp. AN3 (Zhang et al. 2008). Moreover, on the up- and down-stream of the *pca* cluster were located the transposases and hypothesized exodeoxyribonuclease III Xth, which were encoded by *pcaT* and *pcaQ*, respectively. These gene products are believed to function in horizontal gene transfer and protection among the strains. In addition, two *orfs* (*orfX*, *pcaQ*) also found with high similarity to those existed in the *meta* degradation pathway of an aniline degrader, *D. tsuruhatensis* AD9 (Liang et al. 2005) and a phenol degrader, *Acidovorax* sp. JS42 (Copeland et al. 2006), in addition to *P. putida* UCC22 (Fukumori and Saint 1997). However, the functions of their products have still not been identified.

Function of the genes *pcaKLMNOP*

As revealed in the homologous analysis, the products of genes *pcaKLMNOP* exhibited significant homology (Table 3) to those of multicomponent PH found in phenol

Table 3 Analytical data on *pca* cluster and related genes from other bacteria

ORF	Gene	Position in sequence (no. of nt)	Calculated mass	Putative function	Homologous protein (no. of residue, % identity)	Species harboring nearest neighbor	Accession No.
1	*pcaT*	1–1,188 (1,188)	44 Kd/396 aa	Transposase, IS4 family	Transposase, IS4 family (347/396, 87%)	*Polaromonas* sp. JS666	CP000316
2	*pcaR*	1,274–3,061 (1,788)	66 Kd/596 aa	Sigma 54 specific regulator	Sigma 54 specific, Fis family (559/593, 94%)	*Acidovorax* sp. JS42	CP000539
3	*pcaK*	3,226–3,480 (255)	10 Kd/85 aa	PH subunit	PH subunit (65/77, 85%)	*Acidovorax* sp. JS42	CP000316
4	*pcaL*	3,440–4,510 (1,071)	39 Kd/357 aa	PH subunit	PhcL (255/321, 79%)	*C. testosteroni* R5	AB024741
5	*pcaM*	4,495–4,818 (324)	12 Kd/108 aa	PH subunit	PH component (70/95, 73%)	*C. testosteroni* TA441	AB006479
6	*pcaN*	4,862–6,418(1,557)	60 Kd/519 aa	PH subunit	PH subunit (430/505, 85%)	*A. faecalis* IS-46	EF540866
7	*pcaO*	6,418–6,771 (354)	13 Kd/118 aa	PH subunit	MPT hydroxylase (110/118, 93%)	*Acidovorax* sp. JS42	CP000316
8	*pcaP*	6,782–7,840(1,059)	38 Kd/353 aa	PH subunit	MPT hydroxylase (346/353, 98%)	*Acidovorax* sp. JS42	CP000316
9	*pcaR1*	7,875–8,870(996)	37 Kd/332 aa	LysR regulator	LysR regulator (327/332, 98%)	*Acidovorax* sp. JS42	CP000539
10	*pcaD1*	8,877–9,245(369)	13 Kd/123 aa	Ferredoxin	Ferredoxin (123/123, 100%)	*Acidovorax* sp. JS42	CP000539
11	*pcaC1*	9,270–10,211 (942)	35 Kd/314 aa	C23O	C23O (300/314, 95%)	*Acidovorax* sp. JS42	CP000539
12	*orfU1*	10,208–10,990(783)	28 Kd/261 aa	Unknown product	Unknown product (173/184, 94%)	*Acidovorax* sp. JS42	CP000539
13	*pcaR2*	11,166–12,188 (1,023)	37 Kd/341 aa	LysR regulator	LysR family regulator (326/340, 95%)	*Acidovorax* sp. JS42	CP000539
14	*pcaD2*	12,231–12,581 (351)	13 Kd/117 aa	Ferredoxin	Ferredoxin (100/100, 100%)	*Acidovorax* sp. JS42	CP000539
15	*pcaC2*	12,698–13,621 (924)	35 Kd/308 aa	C23O	C23O (257/274, 93%)	*Acidovorax* sp. JS42	CP000539
16	*orfU2*	13,638–14,066 (429)	14 Kd/143 aa	Unknown product	DUF336 (113/118, 95%)	*Acidovorax* sp. JS42	CP000539
17	*pcaE*	14,132–15,589 (1,458)	52 Kd/486 aa	2-HMSD	2-HMSD (466/487, 95%)	*Acidovorax* sp. JS42	CP000539
18	*pcaF*	15,666–16,523 (858)	31 Kd/286 aa	2-HMSH	2-HMSH (286/286,100%)	*D. tsuruhatensis* AD9	AY940090
19	*pcaG*	16,526–17,359 (834)	29 Kd/278 aa	2-keto-4-pentenoate hydratase	4-OD (207/208, 99%)	*Acidovorax* sp. JS42	CP000539
20	*orfX*	17,613–18,059 (447)	16 Kd/149 aa	Acetaldehyde dehydrogenase	SD, NAD—binding (143/149, 95%)	*Acidovorax* sp. JS42	CP000539
21	*pcaJ*	18,300–19,325(1,026)	36 Kd/342 aa	HOD	HOD (199/207, 96%)	*P. putida* UCC22	D85415
22	*pcaH*	19,342–20,127(786)	28 Kd/262 aa	4-OD	4-OD (172/192, 89%)	*Acidovorax* sp. JS42	CP000539
23	*orfY*	20,151–21,131 (981)	34 Kd/327 aa	Exported protein	OrfJ (261/326, 80%)	*C. testosteroni* TA441	AB029044
24	*pcaW*	21,474–22,145 (672)	24 Kd/224 aa	Beta-lactamase domain like protein	Beta-lactamase domain protein (148/148, 100%)	*Acidovorax* sp. JS42	CP000539
25	*orfZ*	22,171–22,701 (531)	19 Kd/177 aa	Transcriptional regulator	Transcriptional regulator, GntR family (72/72,100%)	*Acidovorax* sp. JS42	CP000539
26	*pcaI1*	22,980–24,191 (1,212)	42 Kd/404 aa	Acyl-CoA DDP	Acyl-CoA DDP (381/386, 98%)	*Acidovorax* sp. JS42	CP000539
27	*pcaI2*	24,412–25,986(1,575)	57 Kd/525 aa	SDR	SDR (495/521, 95%)	*Acidovorax* sp. JS42	CP000539
28	*pcaQ*	26,427–26,855(429)	15 Kd/143 aa	Exodeoxyribonuclease III Xth	Exodeoxyribonuclease III Xth (143/143,100%)	*Acidovorax* sp. JS42	CP000539
29	*pcaS*	26,995–27,453 (459)	16 Kd/153 aa	Putitive orotate phosphoribosyltransferase	Orotate phosphoribosyltransferase (145/153, 94%)	*Acidovorax* sp. JS42	CP000539

The accession number for the nucleotide sequence of the *pca* cluster in GenBank is DQ661649. The amino acid sequences of the products of the genes are available in GenBank under the accession number FJ601374. Abbreviations: *PH* phenol hydroxylase, *MPT* methane/phenol/toluene hydroxylase, *C23O* catechol 2, 3 dioxygenase, *HMSD* 2-hydroxymuconic semialdehyde dehydrogenase, *HMSH* 2-hydroxymuconic semialdehyde hydrolase, *4-OD* 4-oxalocrotonate decarboxylase, *SD NAD–binding* semialdehyde dehydrogenase NAD-binding, *HOD* 4-hydroxy-2-oxovalerate aldolase, *Acyl-CoA DDP* acyl-CoA dehydrogenase domain protein, *SDR* short-chain dehydrogenase/reductase

degraders such as *Alcaligenes faecalis* IS-46 (Zhu et al., the *afp* products). However, they were not homologous to those of multicomponent aniline dioxygenase (AD) commonly found in aniline and substituted aniline degraders such as *D. tsuruhatensis* AD9 (Liang et al. 2005), *Delftia* sp. AN3 (Zhang et al. 2008) and *D. acidovorans* strain 7N(Urata et al. 2004). This might infer that the degradation of *p*-chloroaniline by strain PCA039 is unusually initiated by hydroxylation, as the degradation of phenol, not initiated by dioxygenation as the degradation of aniline and substituted anilines. To date, the degradation of aniline and substituted anilines was found only to be started with the peripheral reaction catalyzed by AD (Fujii et al. 1997; Liang et al. 2005; Murakami et al. 2003; Urata et al. 2004; Zhang et al. 2008); never started with any other peripheral steps. In order to confirm the function of the genes *pcaKLMNOP*, they, as a whole, were subcloned into plasmid pUC118 (Table 1) and transformed into *E. coli* TOP10. A recombinant strain, *E. coli* TOP10-S201, was obtained. Then recombinant strain *E. coli* TOP10-S201 was used for PH and AD assays.

As shown in Figs. 1 and 2, recombinant strain *E. coli* TOP10-S201 did only exhibit PH activity on substrates such as aniline, phenol, 4-aminophenol and chloroaniline, especially on aniline. However, it never exhibited AD activity on any substrates (Fig. 1). This result suggested that the degradation of *p*-chloroaniline by *Diaphorobacter* sp. PCA039 was initiated by hydroxylation instead of normal dioxygenation. After the regulatory gene *pcaR* knock-out, the mutant strain indeed lost the ability of

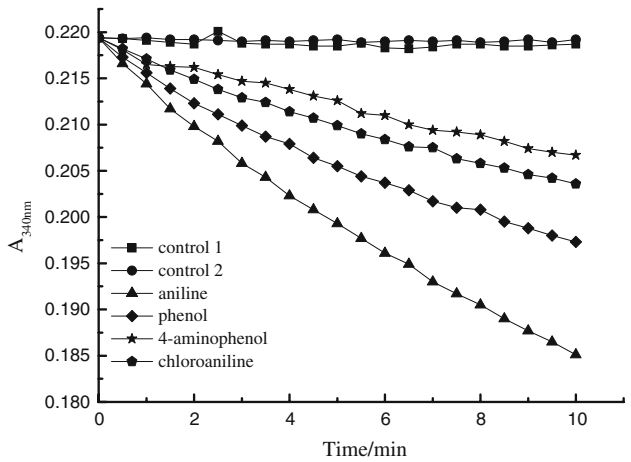


Fig. 2 Phenol hydroxylase assay of recombinant strain *E. coli* TOP10-S201 on various substrates, as expressed by absorption at 340 nm (A_{340}) due to the consumption of NADPH. A_{340} was recorded every 30 s and NADPH was added to all samples. —■— control l, *E. coli* TOP10/PH only; —●— control 2, *E coli* TOP10 + aniline; —▲— *E. coli* TOP10/PH + 4-aminophenol; —◆— *E. coli* TOP10/PH + chloroaniline; —★— *E. coli* TOP10/PH + phenol; —⬟— *E. coli* TOP10/PH + aniline

degrading chloroaniline (data not shown), also suggesting the products of genes *pcaKLMNOP* are necessary for chloroaniline degradation. This is a novel peripheral route for the degradation of chloroanilines that has never been reported before.

Prelude to gene expression and transcripts analysis of *pca* cluster when strain PCA039 grew on different substrates.

Transcripts of several genes of the *pca* cluster when strain PCA039 was grown on different substrates are represented in Fig. 3. This illustrates that all of the detected genes except *pcaC2* were expressed when strain PCA039 grew on aniline, phenol, 4-aminophenol and chloroaniline; however, the gene $pcaC_2$ was only expressed when strain PCA039 grew on chloroaniline. These results confirmed the degradation of all above four substrates to be initiated with the "hydroxylation" peripheral route instead of the "dioxygenation" peripheral route, then followed the *meta*-cleavage pathway in strain PCA039. Both the expression of the genes *pcaF*, and *pcaE* and *pcaH* means that the two degrading branches (hydrolysis and dehydrogenation) are both necessary for complete degradation of these substrates. Furthermore, the two C23O sets ($pcaC_1$ and $pcaC_2$) in *pca* cluster, $pcaC_1$ were expressed in the degradation of all above four substrates, while *pcaC2* was only expressed in the degradation of chloroaniline. It was strongly suggested that $pcaC_2$ might necessarily function in chlorocatechol cleavage, in turn $PcaC_1$ was enough for catechol metabolism. In addition, $PcaC_1$ and $PcaC_2$ have distinct substrate specificity, $PcaC_2$ showed relatively high activity on chlorocatechol (Catechol, 100%; chlorocatechol, 140%), while $TdnC_1$ showed less activity on chlorocatechol (Catechol, 100%; chlorocatechol, 7.5%). According to these results, a hypothetic pathway for the degradation of chloroaniline by strain PCA039 was proposed (Fig. 1c).

Discussion

Previous studies had pointed out that the first step in the degradation of aniline and substituted anilines was always dioxygenation, never degradation by any other peripheral routes (Fukumori and Saint 1997; Liang et al. 2005; Murakami et al. 2003; Urata et al. 2004; Zeyer et al. 1985; Zhang et al. 2008). Here, it can be concluded that the degradation of *p*-chloroaniline by *Diaphorobacter* sp. PCA039 is a novel peripheral route for the metabolism of anilines. Zeyer et al. deemed that the "normal" *ortho* ring fission pathway widely existed in the microbial degradation of aromatic compounds, chloroanilines, benzoate, phenol (Zeyer et al. 1985). And also, this led Janke et al. (1988, 1989) to propose that complete degradation of chloroaromatics should satisfy the following qualifications,

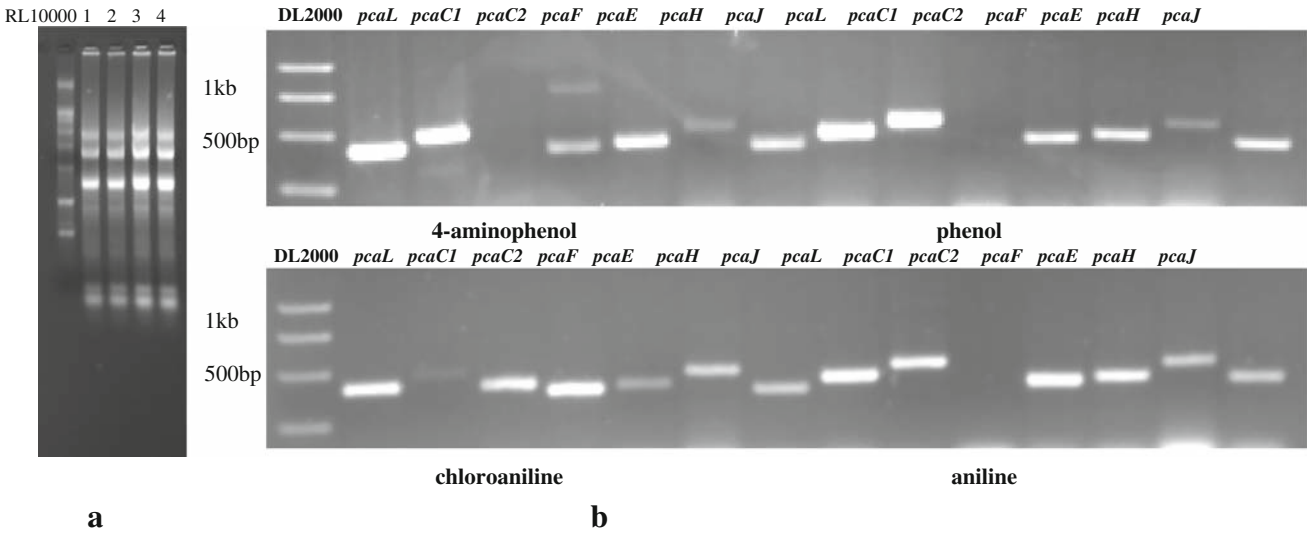


Fig. 3 RT-PCR detection of gene expression when strain PCA039 grew on different substrates. **a** RNA preparations after purification (RL10000, RNA markers, Takara) from cells grown on: *1* chloroaniline, *2* aniline, *3* phenol, *4* 4-aminophenol. **b** RT-PCR products on 1.5% agarose gel. DL2000, DNA marker (Takara). Four substrates used for growing strain PCA039 were indicated. *pcaL*, phenol hydroxylase subunit L; *pcaC1*, catechol 2, 3-dioxygenase (C1); *pcaC2*, catechol 2,3-dioxygense (C2); *pcaE*, HMSD; *pcaF*, HMSH; *pcaH*, 4-oxalocrotonate decarboxylase; and *pcaJ*, 4-hydroxy-2-oxovalerate aldolase gene

i.e., (1) it should be a low specificity oxygenase system; (2) be short of, or with a blocked *meta*-pathway; and (3) have a modified *ortho*-cleavage pathway. However, productive *meta*-cleavage pathways in several strains have recently been shown to exist, capable of degrading chloroaromatic compounds via *meta*-cleavage systems (Surovtseva et al. 1980; Arensdorf and Focht 1995; Ren et al. 2005). The different phylogenetic position and low identity of $PcaC_1$ and $PcaC_2$ inferred that they might have evolved from different ancestors. In addition, it was reported that TdnC and $TadC_1$ had relatively high activity on substituted catechols (methylcatechols), while $TdnC_2$ and $TadC_2$ have showed less activity on these substituted catechols (Fukumori and Saint 1997; Liang et al. 2005), and the authors assumed that it might be necessary for cells to acquire another C23O for these methylcatechols to expand the assimilation range for toluidines. In contrast, in strain PCA039, $PcaC_1$ was produced on all four substrates, while $PcaC_2$ was only produced on chloroaniline, as revealed by RT-PCR (Fig. 3). This might be due to only chloroaniline being able to induce the expression of $pcaC_2$. Furthermore, PcaC2 showed very high activity on chlorocatechol, while $PcaC_1$ had very low activity on chlorocatechol, suggesting that both $PcaC_1$ and $PcaC_2$ are necessary for the vigorous degradation of chloroaniline by strain PCA039.

Acknowledgments This work was supported by grants of Hi-Tech Research and Development Program of China ("863" program, No. 2006AA06Z316) and the Knowledge Innovation Program of the Chinese Academy of Sciences, No. KSCS2-YW-G-055-01.

References

Altschul SF, Gish W, Miller W et al (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Arensdorf JJ, Focht DD (1995) A *meta* cleavage pathway for 4-chlorobenzoate, an intermediate in the metabolism of 4-chlorobiphenyl by *Pseudomonas cepacia* P166. Appl Environ Microbiol 61:443–447

Boon N, Goris J, De Vos P et al (2001) Genetic diversity among 3-chloroaniline- and aniline-degrading strains of the Comamonadaceae. Appl Environ Microbiol 67:1107–1115

Cafaro V, Izzo V, Scognamiglio R et al (2004) Phenol hydroxylase and toluene/o-xylene monooxygenase from *Pseudomonas stutzeri* OX1: interplay between two enzymes. Appl Environ Microbiol 70:2211–2219

Calogero S, Gardan R, Glaser P et al (1994) RocR, a novel regulatory protein controlling arginine utilization in *Bacillus subtilis*, belongs to the NtrC/NifA family of transcriptional activators. J Bacteriol 176:1234–1241

Canellakis ES, Paterakis AA, Huang SC et al (1993) Identification, cloning, and nucleotide sequencing of the ornithine decarboxylase antizyme gene of *Escherichia coli*. Proc Natl Acad Sci USA 90:7129–7133

Copeland A, Lucas S, and Lapidus A (2006) Complete sequence of chromosome 1 of *Acidovorax* sp. Js42. Submitted to the embl/genbank/ddbj databases

Divari S, Valetti F, Caposio P et al (2003) The oxygenase component of phenol hydroxylase from *Acinetobacter radioresistens* S13. Eur J Biochem 270:2244–2253

Fujii T, Takeo M, Maeda Y (1997) Plasmid-encoded genes specifying aniline oxidation from Acinetobacter sp. strain YAA. Microbiology 143:93–97

Fukumori F, Saint CP (1997) Nucleotide sequences and regulational analysis of genes involved in conversion of aniline to catechol in *Pseudomonas putida* UCC22(pTDN1). J Bacteriol 179: 399–408

Griva E, Pessione E, Divari S et al (2003) Phenol hydroxylase from *Acinetobacter radioresistens* S13. Isolation and characterization of the regulatory component. Eur J Biochem 270:1434–1440

Janke D, Almofarji T, Straube G et al (1988) Critical steps in degradation of chloroaromatics by *Rhodococci*. 1. Initial enzyme-reactions involved in catabolism of aniline, phenol and benzoate by *Rhodococcus* sp. AN-117 and AN-213. J Basic Microbiol 28:509–518

Janke D, Ihn W, Tresselt D (1989) Critical steps in degradation of chloroaromatics by *Rhodococci*. 4. Detailed kinetics of substrate removal and product formation by resting pre-adapted cells. J Basic Microbiol 29:305–314

Kaminski U, Janke D, Prauser H et al (1983) Degradation of aniline and monochloroanilines by *Rhodococcus* sp. An 117 and a pseudomonad: a comparative study. Z Allg Mikrobiol 23: 235–246

Kim Y, Ayoubi P, Harker AR (1996) Constitutive expression of the cloned phenol hydroxylase gene(s) from *Alcaligenes eutrophus* JMP134 and concomitant trichloroethylene oxidation. Appl Environ Microbiol 62:3227–3233

Klecka GM, Gibson DT (1981) Inhibition of catechol 2, 3-dioxygenase from *Pseudomonas putida* by 3-chlorocatechol. Appl Environ Microbiol 41:1159–1165

Liang O, Takeo M, Chen M et al (2005) Chromosome-encoded gene cluster for the metabolic pathway that converts aniline to TCA-cycle intermediates in *Delftia tsuruhatensis* AD9. Microbiology 151:3435–3446

Meyer U (1981) Biodegradation of synthetic organic colorants. In: Leisinger T, Cook AM, Hutter R, Nuesch J (eds) Microbial degradation of xenobiotic and recalcitrant compounds, FEMS Symposium 12. Academic Press, London, pp 371–385

Murakami S, Hayashi T, Maeda T et al (2003) Cloning and functional analysis of aniline dioxygenase gene cluster, from *Frateuria* species ANA-18, that metabolizes aniline via an ortho-cleavage pathway of catechol. Biosci Biotechnol Biochem 67:2351–2358

Nakazawa T, Yokota T (1973) Benzoate metabolism in *Pseudomonas-putida* (arvilla) mt-2—demonstration of 2 benzoate pathways. J Bacteriol 115:262–267

Pearson WR (1990) Rapid and sensitive sequence comparison with Fastp and Fasta. Methods Enzymol 183:63–98

Pieper DH, Reineke W (2004) Degradation of chloroaromatics by Pseudomona(d)s. In: Ramos JL (ed) The pseudomonads. Kluwer Academic/Plenum, New York, pp 509–574

Pizon AF, Schwartz AR, Shum LM et al (2009) Toxicology laboratory analysis and human exposure to *p*-chloroaniline. Clin Toxicol 47:132–136

Ren HF, Li SQ, Liu SJ et al (2005) Isolation and characterization of a *p*-chloroaniline-degrading bacterial strain. Huan jing ke xue 26:154–158

Saitou N, Nei M (1987) The neighbor-joining method—a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp 8.46–8.53

Surovtseva EG, Vasilieva GK, Volnova AI et al (1980) Destruction of monochloroanilines in the *meta*-cleavage of *Alcaligenes faecalis*. Doklady Akademii Nauk Sssr 254:226–230

Tamura K, Dudley J, Nei M et al (2007) Mega4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599

Thompson JD, Gibson TJ, Plewniak F et al (1997) The Clustal_x windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882

Urata M, Uchida E, Nojiri H et al (2004) Genes involved in aniline degradation by *Delftia acidovorans* strain 7 N and its distribution in the natural environment. Biosci Biotechnol Biochem 68:2457–2465

Vangnai AS, Petchkroh W (2007) Biodegradation of 4-chloroaniline by bacteria enriched from soil. FEMS Microbiol Lett 268:209–216

Zeyer J, Wasserfallen A, Timmis KN (1985) Microbial mineralization of ring-substituted anilines through an *ortho*-cleavage pathway. Appl Environ Microbiol 50:447–453

Zhang T, Zhang J, Liu S et al (2008) A novel and complete gene cluster involved in the degradation of aniline by *Delftia* sp. An3. J Environ Sci (China) 20:717–724

Zhu CG, Zhang LY, Zhao LP (2008) Molecular cloning, genetic organization of gene cluster encoding phenol hydroxylase and catechol 2, 3-dioxygenase in *Alcaligenes faecalis* IS-46. World J Microbiol Biotechnol 24:1687–1695