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Characterization of the Terephthalate Degradation Genes of *Comamonas* sp. Strain E6

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We isolated *Comamonas* sp. strain E6, which utilizes terephthalate (TPA) as the sole carbon and energy source via the protocatechuate (PCA) 4,5-cleavage pathway. Two almost identical TPA degradation gene clusters, *tphR*$_{I}$*C*$_{I}$*A2*$_{I}$*A3*$_{I}$*B*$_{I}$*A1*$_{I}$ and *tphR*$_{II}$*C*$_{II}$*A2*$_{II}$*A3*$_{II}$*B*$_{II}$*A1*$_{II}$, were isolated from this strain. Based on amino acid sequence similarity, the genes *tphR*, *tphC*, *tphA2*, *tphA3*, *tphB*, and *tphA1* were predicted to code, respectively, for an IclR-type transcriptional regulator, a periplasmic TPA binding receptor, the large subunit of the oxygenase component of TPA 1,2-dioxygenase (TPADO), the small subunit of the oxygenase component of TPADO, a 1,2-dihydroxy-3,5-cyclohexadiene-1,4-dicarboxylate (DCD) dehydrogenase, and a reductase component of TPADO. The growth of E6 on TPA was not affected by disruption of either *tphA2*$_{I}$ or *tphA2*$_{II}$ singly; however, the *tphA2*$_{I}$ *tphA2*$_{II}$ double mutant no longer grew on TPA, suggesting that both TPADO genes are involved in TPA degradation. Introduction of a plasmid carrying *tphR*$_{II}$*C*$_{II}$*A2*$_{II}$*A3*$_{II}$*B*$_{II}$*A1*$_{II}$ conferred the TPA utilization phenotype on *Comamonas testosteroni* IAM 1152, which is able to grow on PCA but not on TPA. Disruption of either *tphR*$_{II}$ or *tphC*$_{II}$ on this plasmid resulted in the loss of the growth of IAM 1152 on TPA, suggesting that these genes are essential to convert TPA to PCA in E6. The genes *tphA1*$_{II}$, *tphA2*$_{II}$, *tphA3*$_{II}$, and *tphB*$_{II}$ were expressed in *Escherichia coli*, and the resultant cell extracts containing TphA1$_{II}$, TphA2$_{II}$, and TphA3$_{II}$ converted TPA in the presence of NADPH into a product which was transformed to PCA by TphB$_{II}$. On the basis of these results, TPADO was strongly suggested to be a two-component dioxygenase which consists of the terminal oxygenase component (TphA2 and TphA3) and the reductase (TphA1), and *tphB* codes for the DCD dehydrogenase.

Protocatechuate (PCA) is an important intermediate metabolite in the bacterial degradation of various aromatic compounds, including vanillate, hydroxybenzoates, and phthalates. There are three recognized catabolic pathways for the degradation of PCA: the PCA 2,3-cleavage (9), PCA 3,4-cleavage (16), and PCA 4,5-cleavage (19, 20) pathways. Previously, we characterized the genes and enzymes involved in the PCA 4,5-cleavage pathway of *Sphingomonas paucimobilis* SYK-6, a well-characterized degrader of lignin-derived compounds (14, 15, 21, 22). During the course of the study on the PCA 4,5-cleavage pathway genes, one of the intermediate metabolites, 2-pyrone-4,6-dicarboxylate (PDC), was found to be useful as a starting material for the production of biodegradable PDC polyamides and polyesters (29, 30). PDC production from lignin is expected to serve as a new process to exploit lignin as an abundant aromatic bioresource. In addition, PDC production via PCA from various petrochemical aromatic compounds, including phthalate isomers, may also be valuable for the production of biodegradable polymers from petrochemical materials. Terephthalate (TPA) is largely used for the production of polyethylene terephthalate and may be a good starting compound for PDC production. To develop the conversion of TPA to PDC, we need to understand the enzymatic systems and the corresponding genes for TPA degradation. However, little is known about the TPA degradation genes.

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Comamonas testosteroni T-2 is reported to degrade TPA to 1,2-dihydroxy-3,5-cyclohexadiene-1,4-dicarboxylic acid (DCD) by using TPA 1,2-dioxygenase (TPADO), and the resulting DCD is converted to PCA (28) (Fig. 1). Schläfli et al. first reported the purification of the oxygenase component of TPADO from strain T-2, which consists of large and small subunits of 49,000 and 18,000 Da, respectively; however, the reductase component was not identified (28). Shigematsu et al. purified the oxygenase component of TPADO from *Delftia tsuruhatensis* T7, and the nucleotide sequences of the oxygenase *terZ*α large-subunit gene and the partial sequence of the oxygenase small-subunit gene were determined (31). The characterization of the TPA degradation *tph* gene clusters of *C. testosteroni* YZW-D (36) and *Rhodococcus* sp. strain DK17 (7) were reported; however, detailed results on the characterization of the *tph* genes of these strains were not presented.

In this study, we isolated a TPA degrader, *Comamonas* sp. strain E6, and found two DNA regions in E6 containing DNA segments which are similar to the *terZ*α gene; they were isolated, sequenced, and characterized. Disruption of the cloned genes in E6 and their expression in *Escherichia coli* and *Comamonas* hosts revealed the function of the genes for TPA degradation.

MATERIALS AND METHODS

Isolation of bacteria. Strain E6 was isolated from soil in Nagaoka, Niigata, Japan. Two grams of soil was inoculated in 10 ml of W minimal salt medium (24) containing 10 mM TPA as the sole carbon and energy source, and the cultures were incubated at 28°C with shaking at 150 rpm. After growth was observed, 400 μl of culture was transferred to 10 ml of fresh medium. After nine serial sub-

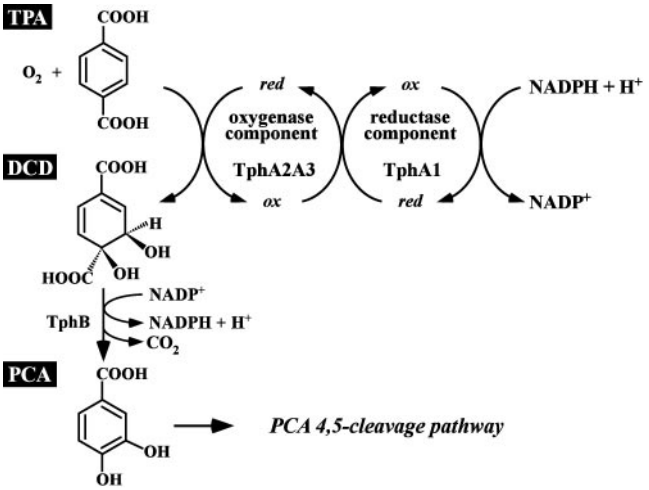

FIG. 1. Conversion of TPA to PCA by *Comamonas* sp. strain E6. The following gene products are involved in the conversion of TPA to PCA: $TphA2_I$ and $TphA2_{II}$, oxygenase large subunit of TPADO; $TphA3_I$ and $TphA3_{II}$, oxygenase small subunit of TPADO; $TphA1_I$ and $TphA1_{II}$, reductase component of TPADO; and $TphB_I$ and $TphB_{II}$, DCD dehydrogenase.

cultures, a sample was taken from the final enrichment tube, serially diluted, and plated on W medium agar plates containing 10 mM TPA. Single colonies were obtained after incubation for 1 day at 30°C. After three serial single-colony isolations on W medium plates containing 10 mM TPA, a single colony was designated strain E6.

Strains and plasmids. The strains and plasmids used for this study are listed in Table 1. *Comamonas* sp. strain E6 was grown in W minimal salt medium containing 10 mM TPA or in Luria-Bertani (LB) medium at 30°C. The E6 mutants were grown in LB medium. If necessary, 50 mg of kanamycin/liter and 30 mg chloramphenicol/liter were added to the cultures. *C. testosteroni* IAM 1152 transformants harboring plasmids carrying the tph_{II} genes were grown in W medium containing 10 mM TPA or in LB medium. *Escherichia coli* strains were grown on LB medium at 37°C. For cultures of cells carrying antibiotic resistance markers, the medium for *E. coli* transformants was supplemented with 100 mg of ampicillin/liter or 25 mg of kanamycin/liter, and the media for IAM 1152 transformants was supplemented with 50 mg of tetracycline/liter or 100 mg of kanamycin/liter.

DNA manipulations and DNA sequencing. DNA manipulations, including total DNA isolation, construction of deletion derivatives, and DNA sequencing, were performed as described in a previous study (1). The 16S rRNA gene was amplified by PCR with the universal primers 8F and 1492R (Table 2). The PCR products were purified from a 0.8% agarose gel and were sequenced using primers r1L, r2L, r3L, r4L, and f3L. Sequence analysis was performed using the GeneWorks program (Intelligenetics, Inc., Mountain View, CA.). Homology searches were performed using the DDBJ database with the BLAST program. A pairwise alignment was performed with the EMBOSS alignment tool (http://www.ebi.ac.uk/emboss/align).

Cloning of *tph* genes. The TPA-F and TPA-R primer set (Table 2) was designed based on the conserved sequence in the *terZ*α gene from *Delftia tsuruhatensis* T7 and the *bphA1c* and *bphA1d* genes from *Novosphingobium aromaticivorans* DSM 12444 and were used to amplify the *tphA2* gene sequence in strain E6. A 305-bp PCR-amplified fragment was used for colony hybridization as a probe to isolate the TPA degradation genes from E6 gene libraries which were constructed using charomid 9-36 (26) with HindIII or SacI digests of the E6 total DNA. Colony and Southern hybridization analyses were performed using the digoxigenin system (Roche, Mannheim, Germany).

Expression of $tphA1_{II}$, $tphA2_{II}$, $tphA3_{II}$, and $tphB_{II}$ in *E. coli* and preparation of the cell extract. The 0.4-kb DNA fragment carrying the 5′ terminus of the $tphA2_{II}$ gene was amplified by PCR using Ex*Taq* polymerase (Takara Bio Inc., Kyoto, Japan) with pSK63 as the template and the TphA2-F and TphA2-R primer set. The 0.4-kb PCR product was cloned into pT7Blue and sequenced. The 0.3-kb NdeI-BglII fragment of the resultant plasmid was inserted into pETDuet-1, and the 1.5-kb BglII-XhoI fragment carrying the 3′ terminus of $tphA2_{II}$ and $tphA3_{II}$ was inserted into the same restriction sites to generate pETtpa23. The coding sequence of $tphA1_{II}$ was amplified by PCR using pSK63 as the template with the TphA1-F and TphA1-R primer set. The 1.1-kb PCR product was cloned into pGEM-T and sequenced. The 1.1-kb NdeI-XhoI fragment of the resulting plasmid was inserted into pRSFDuet-1 to generate pRSFtpa1. The 0.7-kb DNA fragment carrying the 5′ terminus of the $tphB_{II}$ gene was amplified by PCR using pSK63 as the template with the TphB-F and TphB-R primer set. The 0.7-kb PCR product was cloned into pT7Blue and sequenced. The 1.1-kb XhoI-SacI fragment of pSK63 was inserted into the same sites in the resultant plasmid to generate pT7tpbc. The 1.2-kb NdeI-SacI fragment of pT7tpbc was cloned into pETDuet-1 to construct pETtpb. *E. coli* BL21(DE3) cells harboring pETtpa23, pRSFtpa1, both pETtpa23 and pRSFtpa1, or pETtpb were grown in LB medium containing ampicillin, kanamycin, ampicillin and kanamycin, or ampicillin, respectively, at 30°C. Expression of the genes was induced for 3 h by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when the optical density of the culture at 600 nm reached 0.5. The cells were harvested by centrifugation and suspended in FE2 buffer (50 mM Tris-HCl buffer [pH 7.0], 10% glycerol, 0.1 mM ferrous ammonium sulfate, and 2 mM L-cysteine hydrochloride) for the BL21(DE3) cells harboring pETtpa23 and pRSFtpa1 and in 50 mM Tris-HCl (pH 7.0) for the BL21(DE3) cells harboring pETtpb. Cells suspended in the buffer were sonicated, and the cell lysate was centrifuged at 15,000 × *g* for 10 min. The resulting supernatant was used as the cell extract. The protein concentration was determined using the method of Bradford (6). The expression of the gene was confirmed using sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis. The gels were stained with Coomassie brilliant blue.

Enzyme assays. The activities of transformation of the crude TPADO to TPA and other substrates were determined by measuring the decrease in the amount of the substrates by high-pressure liquid chromatography (HPLC) (Alliance 2690 separations module; Waters, Milford, MA) with a TSKgel ODS-80 column (6 by 150 mm; Tosoh, Tokyo, Japan). The 1-ml assay mixture contained FE2 buffer, 100 μM substrate, 1 mM NADPH, and either a mixture of cell extracts of *E. coli* BL21(DE3) harboring pETtpa23 and BL21(DE3) harboring pRSFtpa1 (100 μg of protein each) or the cell extract of BL21(DE3) harboring both pETtpa23 and pRSFtpa1 (100 μg of protein). A portion of the reaction mixture was removed at various sampling times and analyzed using HPLC. For analysis of the conversion of substrates, the mobile phase was a mixture of water (74.5%), acetonitrile (24.5%), and phosphoric acid (1%) at a flow rate of 1 ml/min. Compounds were detected using the following wavelengths: for TPA, benzoate, and *p*-hydroxybenzoate, 280 nm; and for phthalate and isophthalate, 240 nm. The retention times of TPA, benzoate, *p*-hydroxybenzoate, phthalate, and isophthalate were 6.2, 14.1, 6.2, 6.3, and 6.8 min, respectively.

Identification of the reaction product. To identify the reaction product of TPA catalyzed by TPADO, 1 mM TPA was incubated for 30 min at 30°C with the cell extract of *E. coli* BL21(DE3) harboring pETtpa23 and pRSFtpa1 (1 mg protein) in a 1-ml reaction mixture containing FE2 buffer and 5 mM NADPH. The reaction was stopped by adding methanol (25% final concentration). The reaction products were analyzed using HPLC-mass spectrometry (HP1100 series LC-MSD; Agilent Technologies Co., Palo Alto, CA). The mobile phase was a mixture of water (74.5%), acetonitrile (24.5%), and acetic acid (1%), using a flow rate of 1 ml/min. The compounds were detected at 280 nm, where the mass spectra were obtained using the method described previously (1). The 100-μl reaction mixture containing the product from TPA catalyzed by TPADO was prepared as described above and incubated for 60 min at 30°C with the cell extract of *E. coli* BL21(DE3) harboring pETtpb (100 μg protein) in a 1-ml reaction mixture containing 50 mM Tris-HCl buffer (pH 7.0) and 100 μM $NADP^+$. The reaction mixture was analyzed using HPLC as described for the enzyme assay. The compounds were detected at 280 nm.

Construction and analysis of mutants of *Comamonas* sp. strain E6. A 2.0-kb HindIII-Eco47III fragment carrying $tphA2_I$ was cloned into the HindIII-SmaI site of pBluescript II SK(+) to generate pDSR431, and a 0.19-kb PstI fragment was deleted for $tphA2_I$ disruption. A 1.2-kb PstI fragment carrying the kanamycin resistance gene (*kan*) from pUC4K was inserted into the PstI site to construct pDSR432. A 3.1-kb SacI-KpnI fragment of pDSR432 was blunt ended and cloned into the SmaI site of pK19*mobsacB* to generate pDSR43. A 1.7-kb HindIII-Eco47III fragment carrying $tphA2_{II}$ of pSK63 was cloned into SK(+) to generate pDSR631, and a 0.5-kb SphI-PstI fragment was deleted for $tphA2_{II}$ disruption. A 1.0-kb SmaI-PstI fragment carrying the chloramphenicol resistance gene (*cat*) from pUC6C was inserted into the blunt-ended SphI-PstI sites to construct pDSR632. A 2.3-kb SacI-XhoI fragment was cloned into the SmaI site of pK19*mobsacB* to generate pDSR63.

pDSR43 and pDSR63 were introduced into E6 cells by electroporation, and the candidates for the $tphA2_I$ and $tphA2_{II}$ mutants were screened using the method described previously (1). The $tphA2_I$ $tphA2_{II}$ double mutant was ob-

TABLE 1. Strains and plasmids used in this study

Species and strain or plasmid	Relevant characteristic(s)[a]	Reference or source
Comamonas sp.		
E6	Wild type; TPA^+	This study
DE61	E6 derivative, *tphA2*$_I$::*kan* Km^r	This study
DE62	E6 derivative, *tphA2*$_{II}$::*cat* Cm^r	This study
DE612	DE61 derivative, *tphA2*$_I$::*kan tphA2*$_{II}$::*cat* Km^r Cm^r	This study
C. testosteroni IAM 1152	TPA^- PCA^+	34
E. coli		
DH5α	*supE44* Δ*lacU169* (ϕ80 *lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	13
JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*) F′[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	38
BL21(DE3)	F^- *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3); T7 RNA polymerase gene under the control of the *lacUV* promoter	33
Plasmids		
pBluescript II SK(+)	Cloning vector; Ap^r	32
charomid 9-36	pTBE-PL9 derivative, Ap^r	26
pUC4K	Ap^r Km^r	35
pUC6C	pUC1918 carrying *cat*; Ap^r Cm^r	T. Iwasaki
pT7Blue	PCR fragment cloning vector; Ap^r	Novagen
pGEM-T	PCR fragment cloning vector; Ap^r	Invitrogen
pK19*mobsacB*	*oriT sacB* Km^r	27
pKT230	IncQ broad-host-range cloning vector, Km^r	3
pKT230MC	pKT230 with multicloning sites of pUC119	23
pJB866	RK2 broad-host-range expression vector; Tc^r P_m *xylS*	5
pETDuet-1	Expression vector; ColE1 replicon, T7 promoters, Ap^r	Novagen
pRSFDuet-1	Expression vector; RSF1030-derived replicon, T7 promoters, Km^r	Novagen
pVAS4	Charomid 9-36 with a 4.3-kb HindIII fragment carrying *tphA2*$_I$*A3*$_I$*B*$_I$*A1*$_I$	This study
pVAS8	Charomid 9-36 with a 6.3-kb HindIII fragment carrying *tphA2*$_{II}$*A3*$_{II}$*B*$_{II}$*A1*$_{II}$	This study
pSCH1	Charomid 9-36 with a 9.2-kb SacI fragment carrying *tphR*$_I$*C*$_I$*A2*$_I$*A3*$_I$*B*$_I$*A1*$_I$	This study
pSCH14	Charomid 9-36 with a 7.8-kb SacI fragment carrying *tphR*$_{II}$*C*$_{II}$*A2*$_{II}$*A3*$_{II}$*B*$_{II}$*A1*$_{II}$	This study
pSK43	SK(+) with a 4.3-kb HindIII fragment carrying *tphA2*$_I$*A3*$_I$*B*$_I$*A1*$_I$	This study
pSK63	SK(+) with a 6.3-kb HindIII fragment carrying *tphA2*$_{II}$*A3*$_{II}$*B*$_{II}$*A1*$_{II}$	This study
pETtpa23	pETDuet-1 with a 1.8-kb NdeI-XhoI fragment carrying *tphA2*$_{II}$*A3*$_{II}$	This study
pRSFtpa1	pRSFDuet-1 with a 1.1-kb NdeI-XhoI fragment carrying *tphA1*$_{II}$	This study
pT7tpbc	pT7Blue with a 1.2-kb NdeI-SacI fragment carrying *tphB*$_{II}$	This study
pETtpb	pETDuet-1 with a 1.2-kb NdeI-SacI fragment carrying *tphB*$_{II}$	This study
pDSR431	SK(+) with a 2.0-kb HindIII-Eco47III fragment carrying *tphA2*$_I$ of pSK43	This study
pDSR432	pDSR431 with an insertion of the 1.2-kb PstI fragment carrying *kan* from pUC4K replacing the 0.19-kb PstI fragment	This study
pDSR43	pK19*mobsacB* with a 3.1-kb SacI-KpnI fragment of pDSR432	This study
pDSR631	SK(+) with a 1.7-kb HindIII-Eco47III fragment carrying *tphA2*$_{II}$ of pSK63	This study
pDSR632	pDSR631 with an insertion of the 1.0-kb SmaI-PstI fragment carrying *cat* from pUC6C replacing the 0.5-kb SphI-PstI fragment	This study
pDSR63	pK19*mobsacB* with a 2.3-kb SacI-XhoI fragment of pDSR632	This study
pJSK432	pJB866 with a 2.0-kb HindIII-Eco47III fragment carrying *tphA2*$_I$*A3*$_I$ of pSK43	This study
pJSK63	pJB866 with a 6.3-kb HindIII fragment carrying *tphA2*$_{II}$*A3*$_{II}$*B*$_{II}$*A1*$_{II}$ of pSK63	This study
pEJ89	pJB866 with a 8.9-kb EcoRI fragment carrying *tphR*$_{II}$*C*$_{II}$*A2*$_{II}$*A3*$_{II}$*B*$_{II}$*A1*$_{II}$	This study
pEJ80	pEJ89 with a deletion of a 0.5-kb NheI-HindIII fragment carrying part of *tphC*$_{II}$	This study
pEJ84	pEJ89 with a deletion of a 0.6-kb MunI-Tth111I fragment carrying part of *tphR*$_{II}$	This study
pKTC	pKT230 with a 1.6-kb XbaI-BglII fragment carrying *tphC*$_{II}$	This study
pKTR	pKT230MC with a 1.8-kb KpnI fragment carrying *tphR*$_{II}$	This study

[a] Km^r, Cm^r, Ap^r, and Tc^r, resistance to kanamycin, chloramphenicol, ampicillin, and tetracycline, respectively.

tained by the introduction of pDSR63 into the *tphA2*$_I$ mutant cells. Southern hybridization analysis was performed to examine the disruption of *tphA2*$_I$ and *tphA2*$_{II}$, using a digoxigenin system. The total DNA of the *tphA2*$_I$ mutant was digested with SphI, and the 0.7-kb SalI fragment carrying *tphA2*$_I$ and the 1.2-kb PstI fragment carrying *kan* were used as the probes. The total DNAs of the *tphA2*$_{II}$ mutant and the *tphA2*$_I$ *tphA2*$_{II}$ double mutant were digested using SmaI-XhoI and EcoRI-XhoI, and the 1.0-kb PstI fragment carrying *cat* was used as the probe. The resulting strains, i.e., the *tphA2*$_I$ mutant, the *tphA2*$_{II}$ mutant, and the *tphA2*$_I$ *tphA2*$_{II}$ mutant, were designated DE61, DE62, and DE612, respectively. A complementary plasmid, pJSK432, carrying *tphA2*$_I$*A3*$_I$ was introduced into DE612 cells by electroporation.

Expression of *tph*$_{II}$ genes in *C. testosteroni* IAM 1152. pEJ89 carrying *tphR*$_{II}$*C*$_{II}$*A2*$_{II}$*A3*$_{II}$*B*$_{II}$*A1*$_{II}$ was introduced into *C. testosteroni* IAM 1152 by electroporation. The deletion derivatives of pEJ89 were constructed as shown in Table 1 and Fig. 2. The IAM 1152 transformants harboring the pEJ89 derivatives were preincubated in LB medium containing tetracycline, washed with W medium, and then transferred to 10 ml W medium containing 10 mM TPA and tetracycline at 30°C. The growth of the transformants on TPA was examined by monitoring the optical density at 600 nm of the cultures. Complementary plasmids, pKTC and pKTR, were introduced into the IAM 1152 cells harboring pEJ80 and pEJ84, respectively, by electroporation. These cells were cultured in W medium containing 10 mM TPA, tetracycline, and kanamycin.

TABLE 2. PCR primers used in this study

Primer	Sequence
8F	5′-AGAGTTTGATCCTGGCTCAG-3′
1492R	5′-CGGTTACCTTGTTACGACTT-3′
r1L	5′-GTATTACCGCGGCTGCTGG-3′
r2L	5′-CATCGTTTACGGCGTGGAC-3′
r3L	5′-TTGCGCTCGTTGCGGGACT-3′
r4L	5′-ACGGGCGGTGTGTACAAG-3′
f3L	5′-GTCCCGCAACGAGCGCAAC-3′
TPA-F	5′-TGCGTCTAYCACSMVTGG-3′
TPA-R	5′-TCSRMRTAGAGCTTCCAGTT-3′
TphA2-F	5′-GGAACGCATATGCAAGAATCC-3′
TphA2-R	5′-TTGACACCTATCTCGAAGG-3′
TphA1-F	5′-GAGGACTCCATATGAACCACC-3′
TphA1-R	5′-TATTACTGCCTCGAGTCCGC-3′
TphB-F	5′-CCATATGACAATAGTGCACC-3′
TphB-R	5′-TGACAGAACCATGTCATCTC-3′

Nucleotide sequence accession numbers. The nucleotide sequences reported in this paper were deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession numbers AB238678, AB238679, and AB238680.

RESULTS AND DISCUSSION

Isolation of a TPA degrader, *Comamonas* sp. strain E6. The TPA degrader E6 was isolated from the enrichment culture by using selection on W medium containing 10 mM TPA as the sole source of carbon and energy. The nucleotide sequence of the 1,481 bp carrying the 16S rRNA gene of strain E6 showed >99% identity with the 16S rRNA gene of the type strain of *Comamonas testosteroni* ATCC 11996. E6 was therefore classified as a *Comamonas* sp.

E6 grew on phthalate, isophthalate, vanillate, syringate, and PCA as well as TPA. When E6 was grown on TPA, PCA 4,5-dioxygenase and 2-pyrone-4,6-dicarboxylate hydrolase activities were detected (data not shown), suggesting that TPA is degraded through the PCA 4,5-cleavage pathway in E6.

Cloning and nucleotide sequences of the two TPA degradation gene clusters. The deduced amino acid sequence of the product of the *terZ*α gene from *D. tsuruhatensis* T7, coding for the oxygenase large subunit of TPADO (31), showed 42 to 46% identity with those of the products of putative oxygenase large-subunit genes, *bphA1c* and *bphA1d*, from *Novosphingobium aromaticivorans* DSM 12444 (25). Based on the amino acid sequence similarity of these enzymes, a degenerate primer set for the amplification of DNA containing a motif for a Rieske-type [2Fe-2S] iron-sulfur cluster was designed and used to generate a 305-bp fragment from E6. The nucleotide sequence of this fragment showed 99% identity with the T7 *terZ*α sequence. Southern hybridization analysis of the E6 total DNA using the 305-bp amplified product as a probe showed two band patterns (data not shown), suggesting the presence of the two TPADO genes. Using colony hybridization with the amplified 305-bp fragment as the probe, pVAS4 carrying the 4.3-kb HindIII fragment and pVAS8 carrying the 6.3-kb HindIII fragment were isolated from the E6 charomid libraries, and pSCH1 carrying the 9.2-kb SacI fragment and pSCH14 carrying the 7.8-kb SacI fragment that overlaps the 4.3-kb HindIII fragment and 6.3-kb HindIII fragment were

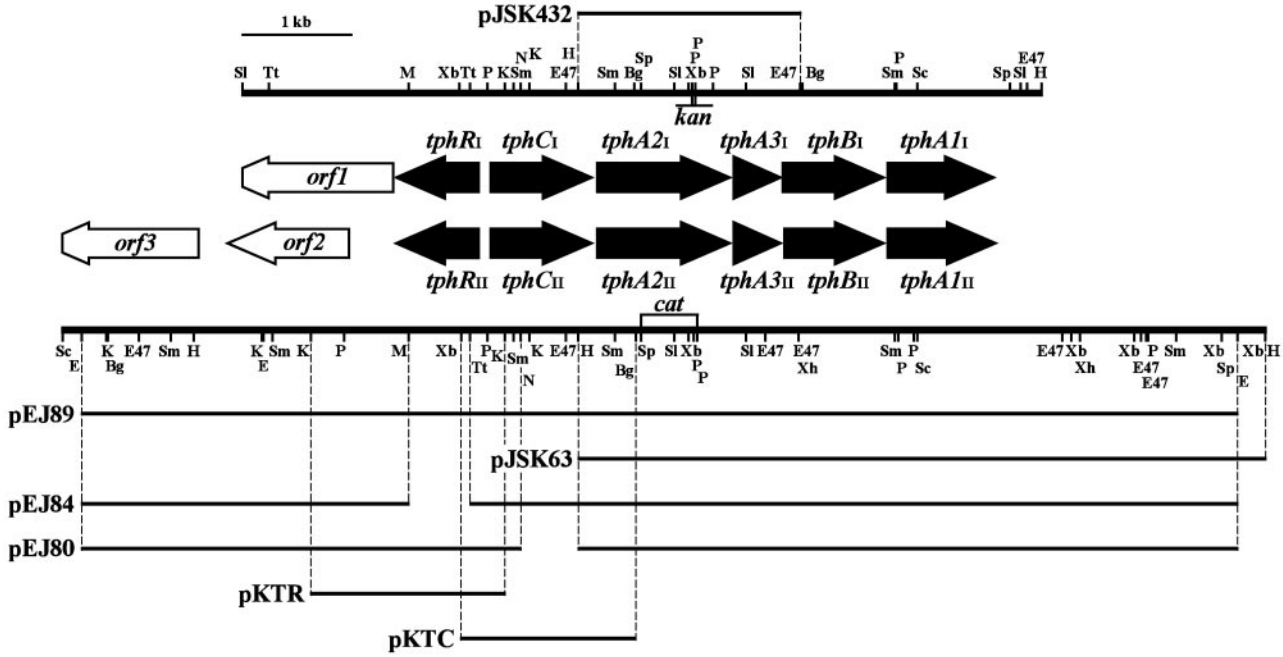

FIG. 2. Organization of the two TPA degradation gene clusters in *Comamonas* sp. strain E6 and deletion analysis. Restriction maps of the 7.3-kb SalI-HindIII fragment carrying the tph_1 genes and the 11-kb SacI-HindIII fragment carrying the tph_{II} genes are shown. The lines above and below the maps indicate the region included in each plasmid. These plasmids (except pJSK432, which was used as a complementary plasmid for the $tphA2_I$ $tphA2_{II}$ double mutant) were introduced into *C. testosteroni* IAM 1152 to examine role of each *tph* gene. *kan* and *cat* indicate the positions of the *kan* and *cat* gene insertions into the $tphA2_I$ mutant, the $tphA2_{II}$ mutant, and the $tphA2_I$ $tphA2_{II}$ double mutant. Abbreviations for restriction enzymes: Bg, BglII; E, EcoRI; E47, Eco47III; H, HindIII; K, KpnI; M, MunI; N, NheI; P, PstI; Sc, SacI; Sl, SalI; Sm, SmaI; Sp, SphI; Tt, Tth111I; Xb, XbaI; Xh, XhoI.

TABLE 3. *Comamonas* sp. strain E6 *tph* genes and predicted products

Gene[a]	Representative homolog	Identity (%)[b]		Organism	Accession no.
		tph_I	tph_{II}		
tphR (99.2)	Transcriptional regulator (*tphR*)	97.6	97.3	*C. testosteroni* YZW-D	AAX18939
	IclR-type transcriptional regulator (*pcaR*)	38.9	38.9	*R. opacus* 1CP	AAC38247
tphC (100)	Terephthalate permease (*tphC*)	96.3	96.3	*C. testosteroni* YZW-D	AAX18940
	Extracytoplasmic solute receptor (*bugT*)	34.7	34.7	*B. pertussis*	AAO61652
tphA2 (99.5)	Terephthalate 1,2-dioxygenase oxygenase component large subunit (*terZ*α)	96.6	96.4	*D. tsuruhatensis* T7	BAC15591
	Terephthalate 1,2-dioxygenase oxygenase component large subunit (*tphA2*)	99.5	99.5	*C. testosteroni* YZW-D	AAX18941
	Terephthalate 1,2-dioxygenase oxygenase component large subunit (*tphA1*)	65.4	65.4	*Rhodococcus* sp. DK17	AAR90187
	Large-subunit aromatic oxygenase (*bphA1c*)	45.3	45.5	*N. aromaticivorans* DSM 12444	AAD04009
	Large-subunit aromatic oxygenase (*bphA1d*)	41.6	41.4	*N. aromaticivorans* DSM 12444	AAD04002
	Naphthalene 1,2-dioxygenase iron sulfur protein component large subunit (*nahAc*)	24.2	24.4	*P. putida* NCIB 9816-4	AAO64274
tphA3 (98.1)	Terephthalate 1,2-dioxygenase oxygenase component small subunit (*tphA3*)	95.5	97.4	*C. testosteroni* YZW-D	AAX18942
	Terephthalate 1,2-dioxygenase oxygenase component small subunit (*tphA2*)	50.0	50.0	*Rhodococcus* sp. DK17	AAR90188
	Small-subunit aromatic oxygenase (*bphA2c*)	33.3	34.0	*N. aromaticivorans* DSM 12444	AAD04008
	Small-subunit aromatic oxygenase (*bphA2d*)	37.0	36.4	*N. aromaticivorans* DSM 12444	AAD04001
	Naphthalene 1,2-dioxygenase iron sulfur protein component small subunit (*nahAd*)	19.2	19.1	*P. putida* NCIB 9816-4	AAO64275
tphB (94.3)	Terephthalate dihydrodiol dehydrogenase (*tphB*)	98.4	94.0	*C. testosteroni* YZW-D	AAX18943
	Terephthalate dihydrodiol dehydrogenase (*tphB*)	40.1	40.7	*Rhodococcus* sp. DK17	AAR90189
	4-Hydroxythreonine-4-phosphate dehydrogenase (*pdxA*)	35.8	35.2	*Rhizobium* sp. NGR234	AAQ87151
tphA1 (95.8)	Terephthalate 1,2-dioxygenase reductase (*tphA1*)	94.9	93.2	*C. testosteroni* YZW-D	AAX18944
	Terephthalate 1,2-dioxygenase reductase (*tphA4*)	42.9	42.6	*Rhodococcus* sp. DK17	AAR90190
	Naphthalene 1,2-dioxygenase reductase component (*nahAa*)	26.6	26.0	*P. putida* NCIB 9816-4	AAA25904

[a] The numbers in parentheses indicate the percent identity between the deduced amino acid sequences of tph_I and tph_{II} of E6.
[b] Percentages of identity were obtained by aligning the deduced amino acid sequence by use of EMBOSS alignment tool.

also isolated. The nucleotide sequences of these fragments showed two sets of six open reading frames (ORFs). Each of these ORFs were more than 90% similar to the TPA degradation genes (*tph* genes) of *C. testosteroni* YZW-D, whose nucleotide sequences have been recently deposited in the database (accession number AY923836). The E6 genes were designated $tphR_I$ and $tphR_{II}$, $tphC_I$ and $tphC_{II}$, $tphA2_I$ and $tphA2_{II}$, $tphA3_I$ and $tphA3_{II}$, $tphB_I$ and $tphB_{II}$, and $tphA1_I$ and $tphA1_{II}$, as shown in Fig. 2. Similarities between E6 *tph* genes and representative homologs are summarized in Table 3.

Based on the sequence similarities, $TphA2_{I/II}$ and $TphA3_{I/II}$ appear to be the oxygenase large and small subunits, respectively, of TPADO. $TphA2_I$ and $TphA2_{II}$ have Cys-X_1-His-X_{17}-Cys-X_2-His, in agreement with the consensus sequence for the Rieske-type [2Fe-2S] cluster. The amino acid sequence alignment between $TphA2_{I/II}$ and the oxygenase large subunit of naphthalene dioxygenase (NahAc) of *Pseudomonas putida* NCIB 9816-4 shows the conserved residues His210, His215, and Asp356, which may be involved in the binding of mononuclear iron (18).

The NCBI conserved-domain search revealed that $TphA1_I$ and $TphA1_{II}$ contain the motifs for a plant-type [2Fe-2S] iron-sulfur cluster (cd00207), a FAD binding domain (pfam00970), and a NAD binding domain (pfam00175). The presence of a motif for a [2Fe-2S] cluster in $TphA1_I$ and $TphA1_{II}$ and the fact that the ferredoxin gene was not found in the cloned regions may suggest that TPADO is a two-component dioxygenase classified as a class I dioxygenase (4). However, with naphthalene dioxygenase, ferredoxin is required for activity, although the ferredoxin reductase contains the plant-type [2Fe-2S] cluster (11, 12).

$TphB_I$ and $TphB_{II}$ showed 35 to 36% similarity with the putative 4-hydroxythreonine-4-phosphate dehydrogenase (PdxA) from *Rhizobium* sp. strain NGR234.

$TphC_I$ and $TphC_{II}$ showed 35% similarity to BugT from *Bordetella pertussis*, which is a periplasmic solute binding receptor of the tripartite tricarboxylate transporter family (2). The tripartite tricarboxylate transporter system consists of three proteins: a periplasmic solute binding receptor, a membrane protein with 12 putative transmembrane α-helical spanners, and a small poorly conserved membrane protein with four putative transmembrane α-helical spanners (37). The similarity between $TphC_{I/II}$ and a periplasmic solute binding receptor suggests that $tphC_I$ and $tphC_{II}$ each code for a periplasmic TPA binding receptor.

The $tphR_I$ and $tphR_{II}$ genes are carried on opposite strands in other *tph* genes and were located upstream of $tphC_I$ and $tphC_{II}$, respectively. $TphR_I$ and $TphR_{II}$ showed 39% similarity to the PcaR from *Rhodococcus opacus* 1CP (10), which is a member of the IclR-type transcriptional regulators.

Downstream of $tphR_I$, an ORF (*orf1*) showed 31% identity with a serine protease, DegP, from *Arabidopsis thaliana* (17); however, this ORF may not be involved in TPA degradation. *orf2* and *orf3*, which are located downstream of $tphR_{II}$, showed 55% and 40% identity, respectively, to a putative transposase from *Ralstonia eutropha* H16 (Q7WWT0) and an IS*1353* putative transposase from *Klebsiella pneumoniae* (Q8GEE9).

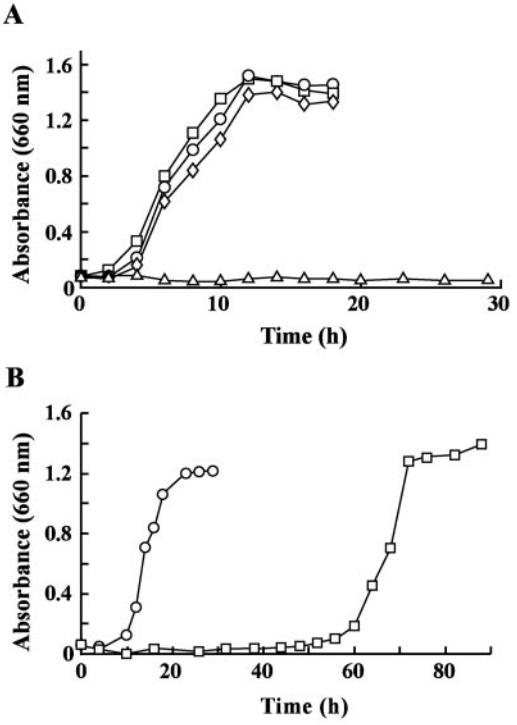

FIG. 3. Disruption of $tphA2_I$ and $tphA2_{II}$ in *Comamonas* sp. strain E6. (A) Growth on TPA of E6 (circles), the $tphA2_I$ mutant (DE61) (squares), the $tphA2_{II}$ mutant (DE62) (diamonds), and the $tphA2_I$ $tphA2_{II}$ mutant (DE612) (triangles). These mutants were incubated in 10 ml W medium containing 10 mM TPA. (B) Complementation of $tphA2_FA3_I$ in the DE612 mutant. Growth of E6 cells harboring pJB866 (circles) and of DE612 cells harboring pJSK432 carrying $tphA2_FA3_I$ (squares) is shown. The cells were incubated in W medium containing 10 mM TPA, tetracycline, and *m*-toluate.

These putative transposase genes may be involved in the duplication of the *tph* genes in *Comamonas* sp. strain E6.

Disruption of $tphA2_I$ and $tphA2_{II}$ in *Comamonas* sp. strain E6. To examine the function of $tphA2_I$ and $tphA2_{II}$ in TPA degradation by *Comamonas* sp. strain E6, $tphA2_I$ and $tphA2_{II}$ were disrupted using a gene replacement technique with pDSR43 and pDSR63, where $tphA2_I$ and $tphA2_{II}$ were inactivated using the insertion of *kan* and *cat*, respectively (Fig. 2). The disruption of $tphA2_I$ and $tphA2_{II}$ in the mutant candidates was confirmed using Southern hybridization analysis with the 0.7-kb SalI fragment carrying $tphA2_I$, the 1.2-kb PstI fragment carrying *kan*, and the 1.0-kb PstI fragment carrying *cat*. The resulting $tphA2_I$ mutant (strain DE61) and $tphA2_{II}$ mutant (strain DE62) were cultured in W medium containing 10 mM TPA; however, the growth rates of both mutants were not affected (Fig. 3A). Therefore, the $tphA2_{II}$ gene in DE61 was disrupted by introducing pDSR63 into DE61 cells to obtain the $tphA2_I$ $tphA2_{II}$ double mutant (strain DE612). As a result, DE612 no longer grew on TPA (Fig. 3A). To determine if this growth deficiency was caused by the disruption of $tphA2_I$ and $tphA2_{II}$, pJSK432 containing the 2.0-kb HindIII-Eco47III fragment carrying $tphA2_FA3_I$ in pJB866 was introduced into DE612. The $tphA2_FA3_I$ genes were expected to be expressed by using the P_m promoter regulated by XylS in the presence of *m*-toluate. The DE612 cells harboring pJSK432 grew on TPA in the presence of *m*-toluate, which is not a growth substrate of E6 (Fig. 3B). Although it took approximately 3.5 times longer for DE612 cells harboring pJSK432 than for the E6 cells harboring pJB866 to enter stationary phase, this indicates that both $tphA2_I$ and $tphA2_{II}$ are functional in E6 and that one of these genes is essential for the growth of E6 on TPA.

Expression of tph_{II} genes in *C. testosteroni* IAM 1152. To examine the role of the *tph* genes, pJSK63, containing the 6.3-kb HindIII fragment carrying $tphA2_{II}A3_{II}B_{II}A1_{II}$ on pJB866 (Fig. 2), was introduced into *C. testosteroni* IAM 1152, which is able to grow on PCA but not on TPA. If the IAM 1152 cells harboring pJSK63 grow on TPA as a sole source of carbon and energy, the $tphA2_{II}A3_{II}A1_{II}$, and $tphB_{II}$ genes may code for TPADO and DCD dehydrogenase, respectively. However, this transformant was not able to grow on TPA even in the presence of 3 mM *m*-toluate. We found that IAM 1152 was able to grow on TPA in the absence of *m*-toluate when the IAM 1152 cells possessed pEJ89 containing the 8.9-kb EcoRI fragment carrying $tphR_{II}$ and $tphC_{II}$ in addition to $tphA2_{II}A3_{II}B_{II}A1_{II}$ (Fig. 4A). This indicates that either $tphC_{II}$ or $tphR_{II}$ or both genes are required for the growth of IAM 1152 on TPA.

To confirm the requirement of $tphC_{II}$ or $tphR_{II}$, we constructed pEJ80 and pEJ84, where $tphC_{II}$ and $tphR_{II}$, respectively, were disrupted by a deletion in each structural gene (Fig. 2). *C. testosteroni* IAM 1152 cells harboring each of these plasmids could not grow on TPA (Fig. 4B and C). The introductions of pKTC carrying $tphC_{II}$ and pKTR carrying $tphR_{II}$ into IAM 1152 cells harboring pEJ80 and pEJ84, respectively, conferred the TPA utilization phenotype on both transformants (Fig. 4B and C). This indicates that $tphC_{II}$ and $tphR_{II}$ are essential for IAM 1152 to grow on TPA. The fact that IAM 1152 harboring pEJ84 could not grow on TPA suggests that the transcription of the $tphC_{II}A2_{II}A3_{II}B_{II}A1_{II}$ genes from the P_m promoter was very weak or that *m*-toluate inhibited the growth of the IAM 1152 transformant on TPA. When IAM 1152 harbors pEJ89, the transcriptions of the $tphR_{II}$ and $tphC_{II}A2_{II}A3_{II}B_{II}A1_{II}$ genes appear to be initiated from their own promoters, and the transcription of the latter genes seem to be activated by the $tphR_{II}$ product.

The effect of pH on growth of IAM 1152 harboring pEJ80 was tested, since the lower the pH, the more TPA is in its undissociated form, which was expected to diffuse to the cells (8). This strain grew on TPA when the pH of the W medium containing TPA (pH 7.3) was adjusted to 6.0 (Fig. 4B). This result also suggests that $tphC_{II}$ codes for a periplasmic TPA binding receptor involved in TPA uptake.

Expression of $tphA2_{II}A3_{II}$ and $tphA1_{II}$ in *E. coli*. The $tphA2_{II}A3_{II}$ and $tphA1_{II}$ genes were cloned into pETDuet-1 and pRSFDuet-1 as described in Materials and Methods to obtain pETtpa23 and pRSFtpa1, respectively. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis showed the production of 46-kDa, 17-kDa, and 36-kDa proteins in the cell extract of *E. coli* BL21(DE3) harboring both pETtpa23 and pRSFtpa1 (data not shown). These values were close to the molecular masses of the deduced amino acid sequences of gene products from $tphA2_{II}$ (46,191 Da), $tphA3_{II}$ (17,217 Da), and $tphA1_{II}$ (36,299 Da), respectively. However, a substantial amount of $TphA1_{II}$ was produced in an insoluble fraction.

To examine whether a cell extract containing $TphA2_{II}$, $TphA3_{II}$, and $TphA1_{II}$ shows TPA transformation activity, 100

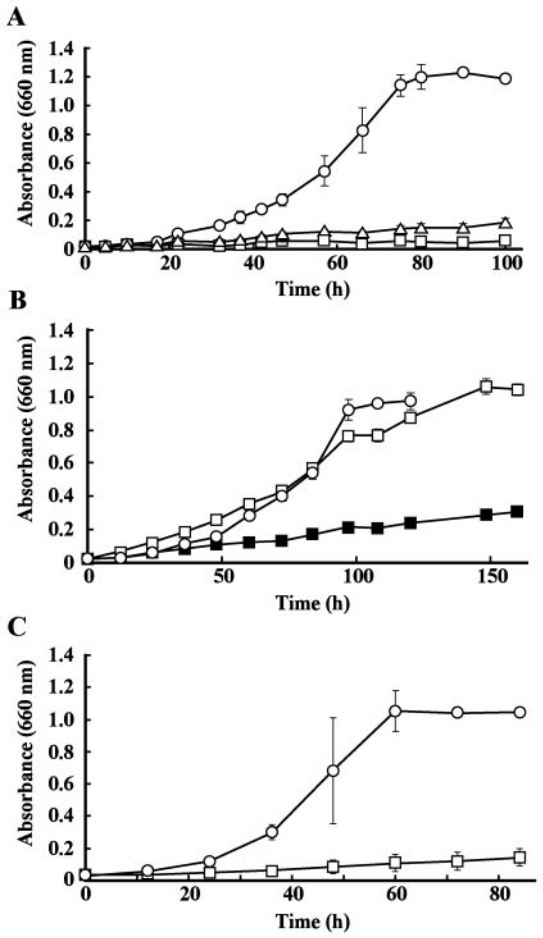

FIG. 4. Expression of $tphR_{II}C_{II}A2_{II}A3_{II}B_{II}A1_{II}$ in *C. testosteroni* IAM 1152. (A) Growth on TPA of IAM 1152 cells harboring pEJ89 carrying $tphR_{II}C_{II}A2_{II}A3_{II}B_{II}A1_{II}$ (circles), pJSK63 carrying $A2_{II}A3_{II}B_{II}A1_{II}$ (squares), and pJB866 (vector) (triangles). (B) Disruption of $tphC_{II}$ in pEJ89. Growth on TPA of IAM 1152 cells harboring pEJ80 ($\Delta tphC_{II}$) and pKT230 (vector) at pH 7.3 (closed squares), harboring pEJ80 and pKT230 at pH 6.0 (open squares), and harboring pEJ80 and pKTC ($tphC_{II}$) at pH 7.3 (circles) is shown. (C) Disruption of $tphR_{II}$ in pEJ89. Growth on TPA of IAM 1152 cells harboring pEJ84 ($\Delta tphR_{II}$) and pKT230MC (vector) (squares) and harboring pEJ84 and pKTR ($tphR_{II}$) (circles) is shown.

μM TPA was incubated with the cell extract of *E. coli* harboring both pETtpa23 and pRSFtpa1 (100 μg of protein/ml) in the presence of 1 mM NADPH. TPA was completely degraded by the cell extract within 30 min, whereas the cell extract from *E. coli* harboring pETtpa23 and pRSFDuet-1 did not transform TPA. This suggests that TPADO consists of $TphA2_{II}$, $TphA3_{II}$, and $TphA1_{II}$.

To determine the reaction product of TPADO, 1 mM of TPA was incubated with the cell extract of *E. coli* harboring pETtpa23 and pRSFtpa1 (1 mg of protein/ml) with 5 mM NADPH, and the reaction mixture was analyzed using HPLC. The generation of a peak with a retention time of 3.0 min (compound I) was observed (data not shown). Negative-mode electrospray ionization-mass spectrometry analysis of compound I showed a fragment at *m/z* 199 that corresponds to the deprotonated molecular ion of DCD. This suggests that the production of DCD from TPA is catalyzed by TPADO.

The TPA transformation rates of TPADO using NADPH and NADH were compared. When 100 μM TPA was incubated with the mixture of crude extracts of *E. coli* harboring pETtpa23 and *E. coli* harboring pRSFtpa1 (each 100 μg of protein/ml) in the presence of 1 mM NADPH, the TPA transformation activity of the crude extract measured in a 1-min reaction (560 ± 70 mU/mg protein) was 1.3 times higher than that with 1 mM NADH. Further, it required more than 5 min to degrade 100 μM TPA by TPADO with NADH, while TPA was degraded within 2 min in the presence of 1 mM NADPH. This indicates that NADPH is the preferred electron donor compared to NADH.

The TPA transformation activity of TPADO was lost when the crude enzymes were treated with 500 μM EDTA. The metal ions Fe^{2+}, Fe^{3+}, Mn^{2+}, Mg^{2+}, Cu^{2+}, Co^{2+}, and Zn^{2+} were added to the reaction mixture to a final concentration of 1 mM, and the resulting solutions were kept on ice for 1 h. The TPADO activity was partially recovered only when Fe^{2+} was added to the reaction mixture (39 ± 7 mU/mg). This suggests that TPADO requires Fe^{2+} for activity.

The substrate preference of TPADO was examined by using phthalate, isophthalate, benzoate, *p*-hydroxybenzoate, biphenyl, benzene, and toluene. However, the conversion of these substrates and product formation were not observed using HPLC analysis, suggesting that TPADO is specific for only TPA.

Expression of $tphB_{II}$ in *E. coli*. The $tphB_{II}$ gene was cloned in pETDuet-1 to obtain pETtpb. Expression of a 33-kDa protein was observed in *E. coli* BL21(DE3) harboring pETtpb (data not shown); the size is close to the predicted molecular mass of $TphB_{II}$ (33,050 Da).

To examine whether $tphB_{II}$ codes for the DCD dehydrogenase, the cell extract of *E. coli* harboring pETtpb was incubated with the reaction product from TPA (100 μM). This reaction product (compound I) from TPA was prepared by incubating 1 mM TPA with the crude extract of *E. coli* harboring pETtpa23 and pRSFtpa1 (1 mg of protein/ml) and 5 mM NADPH. After a 60-min incubation of the reaction mixture containing compound I with the crude $TphB_{II}$ (100 μg of protein/ml) in the presence of 1 mM $NADP^+$, compound I was completely degraded, and a new peak with a retention time of 4.4 min was generated (data not shown). By comparison of the retention times and the UV-visible spectra of the product and standard compounds, this product was identified as PCA. These results indicated that TPA was converted to PCA by two successive reactions catalyzed by TPADO and $TphB_{II}$. This suggests that $tphB_{II}$ codes for DCD dehydrogenase.

Conclusions. This study reports the isolation of two sets of genes involved in the transformation of TPA to PCA from *Comamonas* sp. strain E6, and one of the gene sets, the tph_{II} genes, was characterized. Recently, the presence of two sets of *tph* genes on different plasmids in *Rhodococcus* was reported (7). Our preliminary experiment suggested that the E6 *tph* gene clusters were located on chromosome.

TPADO activity was obtained using a mixture of the crude $TphA1_{II}$, $TphA2_{II}$, and $TphA3_{II}$ enzymes. From this, it appears that E6 TPADO may be a two-component dioxygenase. To confirm this, we are now purifying $TphA1_{II}$ and $TphA2_{II}A3_{II}$

to reconstitute TPADO activity using the purified gene products. $TphB_{II}$ apparently converts the reaction product from TPA catalyzed by TPADO to PCA, and thus TphB was determined to be a DCD dehydrogenase. However, more convincing evidence that the reaction product from TPA catalyzed by TPADO is DCD is necessary. From studies of the expression of tph_{II} genes in *C. testosteroni* IAM 1152, $tphR_{II}$ and $tphC_{II}$ are suggested to code for a positive regulator of the tph_{II} genes and the TPA binding receptor, respectively. Further study is required to gain a better understanding of the regulation of tph_{II} genes by $TphR_{II}$, the TPA uptake system, and the reason for the presence of redundant *tph* gene sets in this strain.

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