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Molecular and biochemical analysis of phthalate and terephthalate degradation by *Rhodococcus* sp. strain DK17

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Abstract

Alkylbenzene-degrading *Rhodococcus* sp. strain DK17 is able to utilize phthalate and terephthalate as growth substrates. The genes encoding the transformation of phthalate and terephthalate to protocatechuate are organized as two separate operons, located 6.7 kb away from each other. Interestingly, both the phthalate and terephthalate operons are induced in response to terephthalate while expression of the terephthalate genes is undetectable in phthalate-grown cells. In addition to two known plasmids (380-kb pDK1 and 330-kb pDK2), a third megaplasmid (750-kb pDK3) was newly identified in DK17. The phthalate and terephthalate operons are duplicated and are present on both pDK2 and pDK3. RT-PCR experiments, coupled with sequence analysis, suggest that phthalate and terephthalate degradation in DK17 proceeds through oxygenation at carbons 3 and 4 and at carbons 1 and 2 to form 3,4-dihydro-3,4-dihydroxyphthalate and 1,2-dihydro-1,2-dihydroxyterephthalate, respectively. The 3,4-dihydroxyphthalate pathway was further corroborated through colorometric tests. Apparently, the two dihydrodiol metabolites are subsequently dehydrogenated and decarboxylated to form protocatechuate, which is further degraded by a protocatechuate 3,4-dioxygenase as confirmed by a ring-cleavage enzyme assay.

Keywords: *Rhodococcus*; Phthalate/terephthalate operon; Gene duplication; Protocatechuate 3,4-dioxygenase

1. Introduction

Many bacterial strains have been isolated for their ability to degrade the three benzene dicarboxylate isomers (phthalate, isophthalate, and terephthalate). In general, Gram-positive bacteria initially oxidize phthalate to 3,4-dihydro-3,4-dihydroxyphthalate, which is subsequently dehydrogenated and decarboxylated to form protocatechuate [1–3]. On the other hand, phthalate degradation in Gram-negative bacteria proceeds through oxygenation and dehydrogenation at carbons 4 and 5 to form 4,5-dihydroxyphthalate, followed by decarboxylation to yield protocatechuate [4,5]. The current known pathway for terephthalate degradation involves oxygenation at carbons 1 and 2 to form 1,2-dihydro-1,2-dihydroxyterephthalate, which is subsequently dehydrogenated with concomitant decarboxylation [6,7].

Rhodococcus sp. strain DK17 metabolizes various alkylbenzenes and possesses two previously identified

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megaplasmids (380-kb pDK1 and 330-kb pDK2) [8]. The alkylbenzene operons located on pDK2 have been well studied at both the molecular and biochemical levels [9–12]. Further characterization of the ability of DK17 to grow on aromatic compounds revealed that the strain is capable of growth on phthalate and terephthalate. Recently, we isolated a cosmid clone (pKEB2003) containing the genes encoding conversion of phthalate and terephthalate to protocatechuate from the previously constructed cosmid library [10]. The entire pKEB2003 cosmid clone was sequenced [13] and yielded an insert size of 40,536 base pairs (GenBank Accession No. AY502076). The present work was thus initiated to understand the molecular basis underlying metabolism of phthalate and terephthalate by DK17. (A preliminary report of this work was presented at the Annual Meeting of the American Society for Microbiology, May 2004 [13].)

2. Materials and methods

2.1. Growth conditions and protocatechuate dioxygenase enzyme assays

Rhodococcus sp. strain DK17 is the wild-type strain [8] and was grown on mineral salts basal (MSB) medium [14] containing 5 mM phthalate or terephthalate at 30 °C. Cell extracts for enzyme assays were prepared from bacterial cells grown in 1-l Erlenmeyer flasks containing 200 ml of medium and harvested at the mid-exponential growth phase. The enzyme solution was prepared as described previously [8]. Protocatechuate 3,4-dioxygenase (P34O) activity was assayed spectrophotometrically by measuring the disappearance of protocatechuate ($\lambda_{max} = 290$ nm and $\varepsilon = 4270\ M^{-1}\ cm^{-1}$) at 25 °C [15]. The colorimetric Rothera reaction [16] was also performed to confirm the formation of the *ortho* pathway intermediate β-ketoadipate according the method of Ottow and Zolg [17]. Activities of the two extradiol dioxygenases (protocatechuate 2,3- and 4,5-dioxygenases) were checked by monitoring the appearance of the corresponding ring-fission product according to the published methods [18,19].

2.2. DNA manipulation and pulsed field gel electrophoresis (PFGE)

Total DNA from *Rhodococcus* sp. strain DK17 was prepared according to the method of Asturias and Timmis [20]. Plasmid DNA was purified by a plasmid spin kit (Genenmed, Korea). Agarose gel electrophoresis was performed in TAE buffer. Transfer of DNA from agarose gels to Hybond-N+ membranes (Amersham Biosciences, UK) was carried out using the TurboBlotter transfer system as recommended by the supplier (Schleicher & Schuell, Germany). PCR DNA products to be used as probes in Southern blotting experiments were separated by gel electrophoresis and eluted from agarose gels using a gel extraction kit (Genenmed, Korea). Southern hybridizations were performed as recommended by the supplier of the DIG nonradioactive nucleic acid labeling and detection system (Boehringer Mannheim, Germany).

Agarose plugs containing genomic DNA were prepared as described previously [8]. Gels [1.0% agarose in 0.5× TBE buffer (1× TBE is 89 mM Tris borate, 2.5 mM EDTA, pH 8.0)] were run at 6 V/cm at 14 °C. The pulse duration increased from 15 to 60 s and from 50 to 100 s during a 16-h run and a 18-h run, respectively.

2.3. Polymerase chain reaction and reverse transcription (RT)-polymerase chain reaction

PCR amplification was carried out in a PTC-200 Peltier Thermal Cycler (MJ Research, USA). The PCR reaction was performed in 20 μl of reaction mixture containing approximately 100 ng of template DNA and 10 pmol of each primer with ReadyMix *Taq* PCR Reaction Mix (Sigma, USA) according to instructions of the manufacturer. The thermal cycling program was a 10 min hot start (95 °C), 30 cycles of 30 s of denaturation (95 °C), 30 s of annealing (55 °C), and 1 min of extension (72 °C), and a final 10 min of extension (72 °C). The following primers were designed to amplify probes for the Southern blot: *ophA1A2*, 5′-ATGGAAGACATTCGCCGC-3′ (forward), 5′-TCATAGGAACACCGCGAG-3′ (reverse); *tphA1A2*, 5′-ATGGAAAGCAGCGTTGTC-3′ (forward), 5′-TCATAGCGGGAGCGCCAA-3′ (reverse); *ophBA3A4C*, 5′-ATGACTGGCTGGCTCGAC-3′ (forward), 5′-CTATACCGTCGCTGCGCG-3′ (reverse); *ptrA*, 5′-ATGACCACTACACAAGTC-3′ (forward), 5′-TCATGCGGCATCCGTCCC-3′ (reverse). The RT-PCR reactions were performed in 20 μl with 100 ng of total RNA and 25 pmol of each primer with ONE-STEP RT-PCR PreMix Kit (iNtRON, Korea). Total RNA extraction was performed as described by Mahenthiralingam [21]. The extracted total RNA was further purified by spin column and DNase I treatment according to the manufacturer's instructions (QIAGEN, Germany). The thermocycler program used for the RT-PCR reactions were as follows: 45 °C for 30 min, 94 °C for 5 min, 30 cycles (94 °C for 45 s, 55 °C for 45 s, 72 °C for 3 min), and 72 °C for 5 min. The following primers were designed to amplify neighboring *oph* and *tph* genes: *ophA1A2orf0ophBA3*, 5′-GAACGACGTACAAGTTGC-3′ (forward), 5′-TCAGTCTTCGATCCAGAG-3′ (reverse); *ophA3A4C*, 5′-ATGGCTGTCGTCAAAGCG-3′ (forward), 5′-CTATACCGTCGCTGCGCG-3′ (reverse); *tphA1A2BA4*, 5′-ATGGAAAGCAGCGTTGTCGAC-3′ (forward), 5′-TCACGCGGTGACAACGGGCTC-3′ (reverse). Transcriptional induction

of the gene encoding P34O was also analyzed by RT PCR using the following primers: 5′-ATGACCCACGCCA-ACGGC-3′ (forward), 5′-TTACAGGGCGAAGAAC-GG-3′ (reverse).

3. Results and discussion

3.1. Gene organization and expression of the phthalate and terephthalate operons

Analysis of the sequenced region of pKEB2003 revealed that the genes encoding the transformation of phthalate and terephthalate to protocatechuate are organized as two separate gene clusters (Fig. 1). The putative phthalate operon contains the genes *ophA1–ophA2–orf0–ophB–ophA3–ophA4–ophC* encoding large and small subunits of a terminal oxygenase component of phthalate 3,4-dioxygenase, a protein of unknown function, a phthalate dihydrodiol dehydrogenase, a ferredoxin component of phthalate 3,4-dioxygenase, a ferredoxin reductase component of phthalate 3,4-dioxygenase, and a decarboxylase. Identification of the *ophA1A2* and *ophA3A4* genes expands the current observation that all the known phthalate dioxygenase enzyme from Gram-positive bacteria are three-component systems consisting of a terminal oxygenase, a ferredoxin, and a ferredoxin reductase while those from Gram-negative bacteria are two-component enzymes consisting of a bifunctional reductase and an oxygenase [2]. The genes (*tphA1–tphA2*) for the large and small subunits of terephthalate 1,2-dioxygenase are located approximately 6.7 kb away from the *ophA1* gene encoding phthalate oxygenase and are transcribed in opposite directions. A gene encoding a terephthalate dihydrodiol dehydrogenase (*tphB*) is located immediately after *tphA1A2*, and the ferredoxin reductase gene (*tphA4*) follows. Similarities between the *oph* and *tph* genes identified on pKEB2003 and representative homologs are summarized in Table 1.

In order to confirm that the identified *oph* and *tph* genes are specifically induced by phthalate and terephthalate, respectively, and to confirm the operonic nature of the *oph* and *tph* genes, RT-PCR experiments were performed with total RNA extracted from the phthalate or terephthalate-grown cells of DK17. Oligonucleotide primers were designed to generate PCR products as follows: *ophA1* to *ophA3*, 2700 bp; *ophA3* to *ophC*, 2164 bp; *tphA1* to *tphA4*, 3753 bp. Whereas PCR without RT did not show any PCR product, the RT-PCR reaction showed PCR products of the expected size (Fig. 1). These data clearly show that the *ophA1–ophA2–orf0–ophB–ophA3–ophA4–ophC* and *tphA1-tphA2-tphB-tphA4* genes are transcribed as operonic units.

Since no RT-PCR product was detectable for the *ophA1* to *ophA2* and *tphA1 to tphA2* region following growth of DK17 on glucose (data not shown), the identified *oph* and *tph* genes are specifically induced by growth on phthalate and terephthalate, respectively. More importantly, it is interesting to note that both the *oph* and *tph* operons are induced in response to terephthalate while expression of the *tph* genes was undetectable in phthalate-grown cells as demonstrated by RT-PCR analysis (Fig. 1). To date, except for the isophthalate and terephthalate operons from *Comamonas testosteroni* YZW-D [7], no studies have been done at

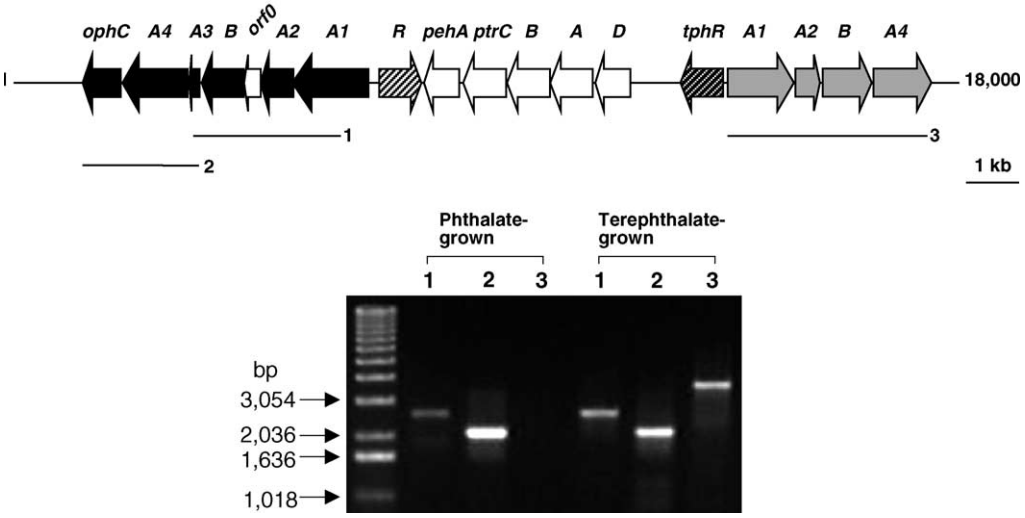

Fig. 1. Gene organization of the phthalate and terephthalate operons from *Rhodococcus* sp. strain DK17. Black and gray arrows indicate structural genes for the metabolism of phthalate and terephthalate, respectively. Putative regulatory genes are shown as hatched arrows. Genes for miscellaneous or unknown functions are shown as white arrows. The direction of transcription is indicated by arrowheads. The nucleotide number is marked on the beginning and the end of the line. The lower panel shows agarose gel electrophoresis of RT-PCR products. The expected PCR products for each well are indicated in the gene map. The first lane was loaded with a molecular weight marker.

Table 1
Identification of the phthalate and terephthalate operon genes from DK17 based on the comparison with functionally characterized homologs

Gene	Start–stop codons	Representative homolog (gene)	Identity (%)[a]	Organism	GenBank Accession No.
ophC	2205–1474	3,4-Dihydroxyphthalate decarboxylase (*phtC*)	55	*A. keyseri* 12B	AAK16538
ophA4	3443–2202	Phthalate 3,4-dioxygenase reductase component (*phtAd*)	53	*A. keyseri* 12B	AAK16537
ophA3	3637–3443	Phthalate 3,4-dioxygenase ferredoxin component (*phtAc*)	45	*A. keyseri* 12B	AAK16536
ophB	4481–3675	Phthalate dihydrodiol dehydrogenase (*phtB*)	71	*M. vanbaalenii* PYR1	AAQ91917
orf0	4478–4489	Unknown protein (*orf11*)	69	*Terrabacter* sp. DBF63	BAC54158
ophA2	5412–4786	Phthalate 3,4-dioxygenase oxygenase component small subunit (*phtAb*)	71	*A. keyseri* 12B	AAK16535
ophA1	6854–5409	Phthalate 3,4-dioxygenase oxygenase component large subunit (*phtAa*)	77	*A. keyseri* 12B	AAK16534
ophR	7045–7851	Phthalate operon regulator (*phtR*)	61	*M. vanbaalenii* PYR1	AAQ91913
pehA	8564–7899	Putative phthalate ester hydrolase (*pehA*)	76	*A. keyseri* 12B	AAK16532
ptrC	9455–8640	Transporter permease 2 (*ptrC*)	68	*A. keyseri* 12B	AAK16531
ptrB	10,261–9452	Transporter permease 1 (*ptrB*)	65	*A. keyseri* 12B	AAK16530
ptrA	11,070–10,258	Transporter ATPase (*ptrA*)	75	*A. keyseri* 12B	AAK16529
ptrD	11,762–11,085	Substrate-binding transporter (*ptrD*)	64	*A. keyseri* 12B	AAK16540
tphR	13,524–12,718	Terephthalate operon regulator (*tphR*)	34	*C. testosteroni* YZW-D	AAX18939
tphA1	13,617–14,882	Terephthalate 1,2-dioxygenase oxygenase component large subunit (*tphA2*)	68	*C. testosteroni* YZW-D	AAX18941
tphA2	14,879–15,349	Terephthalate 1,2-dioxygenase oxygenase component small subunit (*tphA3*)	48	*C. testosteroni* YZW-D	AAX18942
tphB	15,427–16,362	Terephthalate dihydrodiol dehydrogenase (*tphB*)	56	*C. testosteroni* YZW-D	AAX18943
tphA4	16,359–17,369	Terephthalate 1,2-dioxygenase reductase component (*tphA1*)	42	*C. testosteroni* YZW-D	AAX18944

[a] Percentage of identity was obtained by aligning the deduced amino acid sequences using Blastp.

the molecular level on the metabolism of two phthalate isomers in the same organism. Wang et al. [7] identified two separate operons, encoding degradation of isophthalate and terephthalate to protocatechuate in YZW-D, and showed that these two operons are separately regulated and the enzymes involved are specific for only the one isomer. However, this is unlike DK17 where both the *oph* and *tph* operons are simultaneously induced by terephthalate.

3.2. Duplication of the genes for phthalate and terephthalate degradation

We previously reported that *Rhodococcus* sp. strain DK17 has at least two megaplasmids (380-kb pDK1 and 330-kb pDK2) [8]. However, during the multiple PFGE analysis, we noticed that the intensity of the DK17 chromosome band is unusually strong on our standard gels (1.0% agarose in 0.5× TBE) run at 6 V/cm at 14 °C with the pulse duration increasing from 15 to 60 s during a 16-h run (Fig. 2A). This observation suggests coexistence of other genetic elements at the same location in the gel. Indeed, PFGE under different conditions as described in Section 2 resolved an approximately 750-kb megaplasmid now designated pDK3 from the chromosome band (Fig. 2B).

A Southern hybridization experiment was performed with the cosmid clone pKEB2003 as a probe against PFGE-separated total genomic DNA from DK17. As

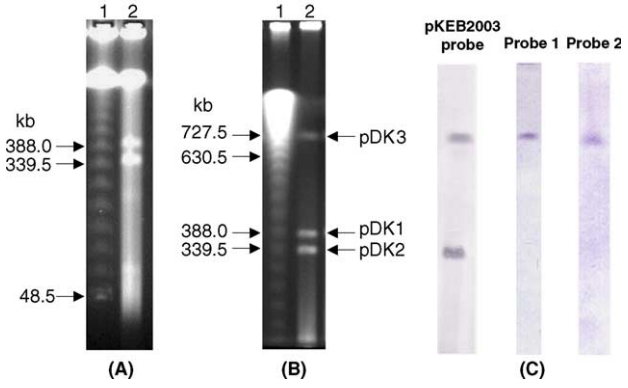

Fig. 2. Duplication of the *oph* and *tph* genes in *Rhodococcus* sp. strain DK17. (A) PFGE separation of genomic DNA prepared from DK17 with the pulse duration increased from 15 to 60 s during a 16-h run. (B) PFGE separation of genomic DNA prepared from DK17 with the pulse duration increased from 50 to 100 s during a 18-h run. Lane 1, λ ladder standard; lane 2, *Rhodococcus* sp. DK17. (C) Southern blot of the PFGE gels run under conditions shown in panel B with different probes: pKEB2003 probe, the entire pKEB2003 cosmid clone; probe 1, a 400-bp DNA fragment from bases 180 to 580 of the cloned region in pKEB2003; probe 2, a 742-bp DNA fragment from bases 39,473 to 40,220 of the cloned region in pKEB2003.

shown in Fig. 2C, DK17 has two hybridizing bands, namely pDK2 and pDK3. This Southern hybridization experiment demonstrated that there are two separate genomic regions homologous to at least part of pKEB2003. In order to determine whether the entire cloned region in pKEB2003 or only particular genes in the cloned region are homologous, smaller DNA fragments were utilized as probes in additional Southern hybridization experiments. The DNA probes were amplified by PCR using primers designed to generate PCR products as follows: *ophA1A2*, *tphA1A2*, *tphBA4*, *ophBA3A4C*, and *ptrA*. Since all the probes were found to hybridize to both pDK2 and pDK3 plasmid DNAs two more probes were prepared by amplifying each end of the cosmid clone pKEB2003, probe 1 from bases 180 to 580 (400 bp) and probe 2 from bases 39,473 to 40,220 (747 bp). Both probes 1 and 2 hybridize only to pDK3 (Fig. 2C). This means that the cloned region in pKEB2003 originated from pDK3 and that DK17 has two copies of the *oph* and *tph* genes found in the middle of the cloned region of pKEB2003. As mentioned in Section 1, the 330-kb pDK2 carries the genes for alkylbenzene degradation, and thus the pDK2-cured mutant strain DK176 loses the ability to grow not only on *o*-xylene but also on benzene, toluene, and ethylbenzene [8]. However, DK176 was found to retain the ability to grow on both phthalate and terephthalate, indicating that the *oph* and *tph* genes on pDK3 are functional in vivo.

It is interesting to note that the completed genome sequence of *Rhodococcus* sp. strain RHA1 also has two copies of the genes encoding phthalate and terephthalate degradation. A Blast search against the RHA1 genome database (http://www.rhodococcus.ca/index.html) identified two DNA regions showing a high degree of identity with the phthalate/terephthalate-degrading genes from DK17. RHA1 has three megaplasmids (1.1-Mb pRHL1, 443-kb pRHL2, and 332-kb pRHL3). Bases 1–37,071 of the cloned region in pKEB2003 are 99% identical to the pRHL1 plasmid region from 157,631 to 194,701 and pKEB2003 bases 642–20,101 are 99% identical to the pRHL2 from 157,631 to 194,701.

3.3. Key physiological aspects of phthalate and terephthalate degradation by DK17

As explained in Section 1, all the currently known Gram-positive species metabolizes phthalate through the 3,4-dihydroxyphthalate pathway. The *oph* gene sequence analysis suggests that DK17 also employs the 3,4-pathway for phthalate degradation. Eaton [1] reported that several 2-substituted benzoates were transformed to 2-substituted protocatechuates by the combined actions of enzymes including phthalate 3,4-dioxygenase and phthalate dihydrodiol dehydrogenase from *Arthrobacter keyseri* 12B. Due to the absence of a carboxyl group at the 2 position, such substituted protocatechuates fail to serve as substrates for the next enzyme of the pathway, 3,4-dihydroxyphthalate 2-decarboxylase, and form colored chelates with iron. Thus, DK17 was grown overnight on MSB medium containing 20 mM glucose and 2.5 mM 2-chlorobenzoate in the presence and absence of 5 mM phthalate, respectively. When added with $FeCl_3$ at a final concentration of 1.5 mM, only the culture supernatant obtained by harvesting cells grown in the presence of phthalate turned dark blue. In contrast, no color development was detected when the 4,5-pathway-specific color reaction test [23] was performed.

An examination of the currently known catabolic pathways for phthalate and terephthalate degradation indicates that metabolism proceeds by separate upstream degradation pathways that intersect at the common downstream pathway for protocatechuate degradation. Protocatechuate is known to be further metabolized through either an *ortho* or *meta* cleavage pathway. In *A. keyseri* 12B [1,24] protocatechuate is metabolized both by the 4,5-extradiol (*meta*) and 3,4-intradiol (*ortho*) ring cleavage pathways while *B. cepacia* DBO1 [22] employs the *ortho*-cleavage pathway. These strains are the two best-studied phthalate-degrading strains representing Gram-positive and Gram-negative bacteria. In terephthalate-degrading *Comamonas testosteroni* T2, protocatechuate undergoes 4,5-dioxygenation reactions [6]. *Rhodococcus* sp. strain DK17 was analyzed for the type of ring-cleavage enzyme that is physiologically produced during growth on phthalate or terephthalate. Extradiol 2,3- and 4,5-ring-cleavage dioxygenase activity was not detected. However, intradiol-cleaving P34O activity was significantly detected in cells of the wild type DK17 grown on phthalate (1121.1 ± 112.9 U/mg of protein/min) and terephthalate (956.3 ± 45.3 U/mg of protein/min). Also, by subsequent Rothera reaction test, positive reactions were observed only with enzyme solutions obtained from phthalate- or terephthalate-grown cells. It should be noted that P34O activity was not detected in cells grown in the presence of *o*-xylene, toluene, or benzene. These results clearly indicate that DK17 degrades phthalate and terephthalate through an *ortho*-cleavage pathway for protocatechuate degradation.

The current work utilized a combination of molecular and biochemical approaches to elucidate the catabolic pathways for phthalate and terephthalate degradation by *Rhodococcus* sp. DK17. Sequence analysis combined with RT-PCR data indicates that the initial oxidation of phthalate and terephthalate is catalyzed by phthalate 3,4-dioxygenase and terephthalate 1,2-dioxygenase to form 3,4-dihydro-3,4-dihydroxyphthalate and 1,2-dihydro-1,2-dihydroxyterephthalate, respectively. Apparently, the two dihydrodiol metabolites are subsequently dehydrogenated and decarboxylated to form protoca-

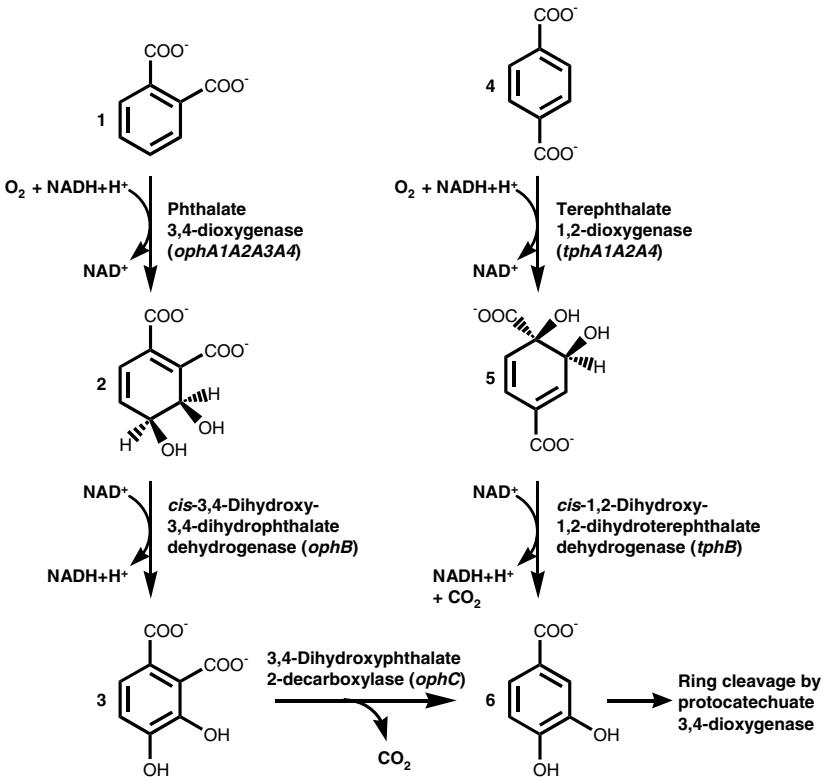

Fig. 3. Proposed pathways for the degradation of phthalate and terephthalate by *Rhodococcus* sp. DK17. Chemical designations: (1) phthalate; (2) *cis*-3,4-dihydroxy-3,4-dihydrophthalate; (3) 3,4-dihydroxyphthalate; (4) terephthalate; (5) *cis*-1,2-dihydroxy-1,2-dihydroterephthalate; (6) protocatechuate. Parentheses indicate gene designations.

techuate, which is further degraded by a protocatechuate 3,4-dioxygenase (Fig. 3). It should be noted that a gene encoding an enzyme for the decarboxylation of dihydroxyterephthalate is not found within the terephthalate gene cluster. Considering that terephthalate dihydrodiol can be dehydrogenated with concomitant decarboxylation in *C. testosteroni* strains T-2 [6] and YZW-D [7], a specific decarboxylase might also not be required for terephthalate degradation by DK17. To our best knowledge, this paper is the first report to identify and characterize two functional operons involved in phthalate and terephthalate metabolism in the same bacterial strain.

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