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ORIGINAL ARTICLE

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Phthalate catabolic gene cluster is linked to the angular dioxygenase gene in *Terrabacter* sp. strain DBF63

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Abstract Phthalate is a metabolic intermediate of the pathway of fluorene (FN) degradation via angular dioxygenation. A gene cluster responsible for the conversion of phthalate to protocatechuate was cloned from the dibenzofuran (DF)- and FN-degrading bacterium *Terrabacter* sp. strain DBF63 and sequenced. The genes encoding seven catabolic enzymes, oxygenase large subunit of phthalate 3,4-dioxygenase (*phtA1*), oxygenase small subunit of phthalate 3,4-dioxygenase (*phtA2*), *cis*-3,4-dihydroxy-3,4-dihydrophthalate dehydrogenase (*phtB*), [3Fe-4S] or [4Fe-4S] type of ferredoxin (*phtA3*), ferredoxin reductase (*phtA4*), 3,4-dihydroxyphthalate decarboxylase (*phtC*) and putative regulatory protein (*phtR*), were found in the upstream region of the angular dioxygenase gene (*dbfA1A2*), encoded in this order. *Escherichia coli* carrying *phtA1A2BA3A4* genes converted phthalate to 3,4-dihydroxyphthalate, and the 3,4-dihydroxyphthalate decarboxylase activity by *E. coli* cells carrying *phtC* was finally detected with the introduction of a Shine-Dalgarno sequence in the upstream region of its initiation codon. Homology analysis on the upstream region of the *pht* gene cluster revealed that there was an insertion sequence (IS) (ISTesp2; ORF14 and its flanking region), part of which was almost 100% identical to the *orf1* and its flanking region adjacent to the extradiol dioxygenase gene (*bphC1*) involved in the DF degradation of *Terrabacter* sp. strain DPO360 [Schmid et al. (1997) J Bacteriol 179:53–62]. This suggests that ISTesp2 plays a role in the metabolism of aromatic compounds in *Terrabacter* sp. strains DBF63 and DPO360.

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Introduction

Phthalate has been known for many years as a growth substrate for bacterial strains, and also as an intermediary metabolite in the degradation of certain polycyclic aromatic hydrocarbons (PAHs), e.g. phenanthrene (Kiyohara et al. 1976; Barnsley 1983), fluoranthene (Šepič et al. 1998), and fluorene (FN) (Grifoll et al. 1994). The metabolism of phthalate by aerobic bacteria has been studied in detail, and two catabolic pathways have been identified (Ribbons and Evans 1960; Nakazawa and Hayashi 1977; Eaton and Ribbons 1982; Nomura et al. 1989) (Fig. 1). While phthalate is metabolized to protocatechuate via 4,5-dihydroxyphthalate in gram-negative bacteria (Fig. 1a), gram-positive bacteria degrade phthalate via 3,4-dihydroxyphthalate and protocatechuate (Fig. 1b). Protocatechuate is further metabolized through either an *ortho* or *meta* cleavage pathway.

Specific enzymes involved in the catabolism of phthalate and the genes encoding for these enzymes have been characterized in some bacteria. Among them, phthalate-utilizing bacterium *Burkholderia cepacia* strain DBO1 has been well studied. Phthalate dioxygenase in strain DBO1 is a two-component enzyme consisting of a reductase component and an oxygenase component (Batie et al. 1987), and the crystal structure of the reductase has been already determined at 2.0-Å resolution (Correll et al. 1992). Also, the biophysical characteristics of the oxygenase have been well investigated (Cline et al. 1985; Kuila et al. 1987; Tsang et al. 1989). The genes for phthalate degradation in strain DBO1 (*oph* genes) were revealed to be organized separately and arranged in at least three transcriptional units [*ophA1* (phthalate oxygenase reductase), *ophDC* (transporter and 4,5-dihydroxyphthalate decarboxylase), and *ophA2B* (phthalate oxygenase and *cis*-phthalate dihydrodiol dehydrogenase)] (Chang and Zylstra 1998). By contrast, the phthalate-degradation genes from *Pseudomonas putida* strain NMH102–2 (*pht* genes) were reported to be clustered and transcribed in the same direction [*pht12345* (permease, reductase, oxygenase, dehydrogenase, and decarboxylase, respectively)]

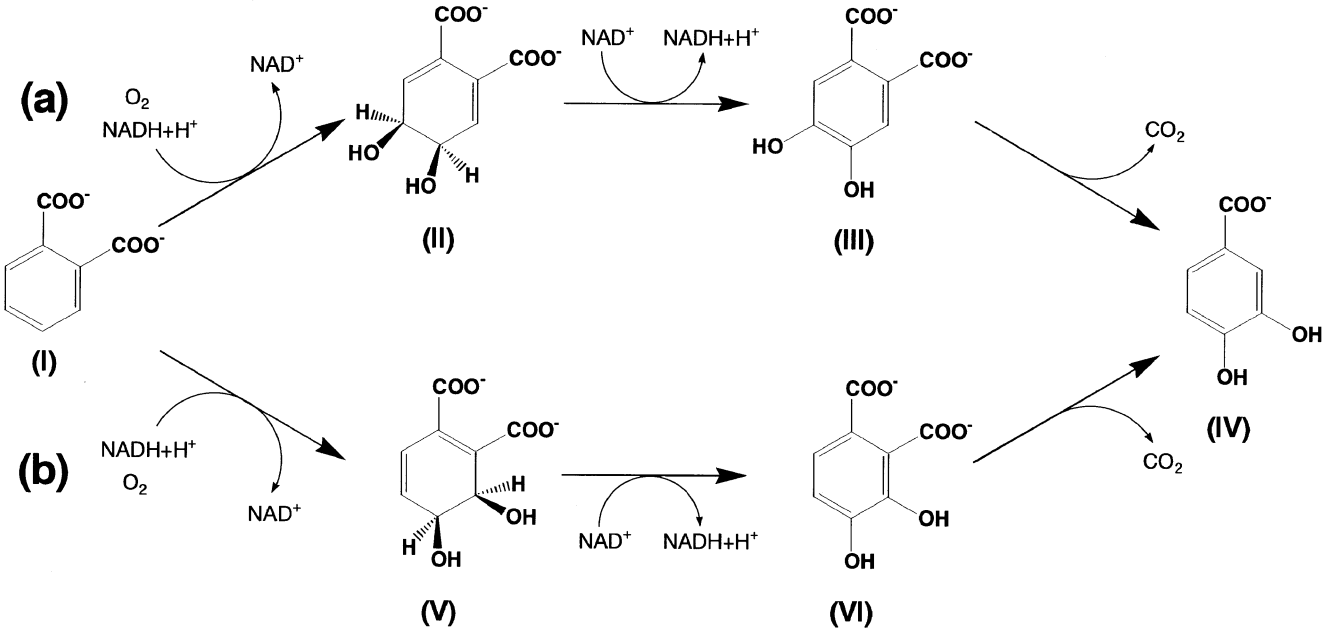

Fig. 1 Catabolic pathways of phthalate to protocatechuate by gram-negative bacteria (**a**) and by gram-positive bacteria (**b**). *I* Phthalate, *II cis*-4,5-dihydro-4,5-dihydroxyphthalate, *III* 4,5-dihydroxyphthalate, *IV* protocatechuate, *V cis*-3,4-dihydro-3,4-dihydroxyphthalate, *VI* 3,4-dihydroxyphthalate

(Nomura et al. 1992), although phthalate catabolic genes in both strains are closely related, based on their nucleotide sequences. The genes for 4,5-dihydroxyphthalate decarboxylase from *Comamonas teststeroni* M4–1 have also been cloned and sequenced (Lee et al. 1994).

The enzymes responsible for initial phthalate dioxygenation in gram-positive bacteria had features different from those in gram-negative bacteria. Phthalate oxygenase was purified and characterized from *Rhodococcus erythropolis* strain S-1; this enzyme catalyses dihydroxylation of phthalate to form 3,4-dihydro-3,4-dihydroxyphthalate (Suemori et al. 1993, 1995). Interestingly, the enzyme consists of a tetramer of identical 56-kDa monomers and does not require an oxidoreductase for electron transfer from NADH. Therefore, Suemori et al. (1993) suggested that the phthalate oxygenase from strain S-1 might be a dual-function enzyme, which has both the oxygenase and the reductase functions. By contrast, plasmid-encoded phthalate catabolic genes (*pht* genes) were recently cloned and characterized from *Arthrobacter keyseri* strain 12B (Eaton 2001). The phthalate catabolism in strain 12B is initiated by phthalate 3,4-dioxygenase, which is a three-component dioxygenase encoded by *phtAaAbAcAd* (oxygenase large subunit, oxygenase small subunit, ferredoxin, and ferredoxin reductase). The genes encoding *cis*-3,4-dihydro-3,4-dihydroxyphthalate dehydrogenase (*phtB*) and 3,4-dihydroxyphthalate decarboxylase (*phtC*) were located just upstream and downstream of the region of the *phtAaAbAcAd* gene cluster, respectively. However, the *pht* gene cluster from strain 12B is the only reported phthalate catabolic gene in gram-positive bacteria, and therefore, little is known about the genetic basis of the phthalate degradation in gram-positive bacteria.

A gram-positive bacterium *Terrabacter* sp. strain DBF63 was originally isolated on the basis of its ability to utilize dibenzofuran (DF) and FN as the sole sources of carbon and energy, and the degradation pathways of DF and FN have been proposed (Monna et al. 1993). DF and FN are thought to be degraded to salicylate and phthalate, metabolic intermediates of respective substrates, via angular dioxygenase attack. Recently, new terminal oxygenase genes of angular dioxygenase (*dbfA1* and *dbfA2*), whose products can catalyse the angular dioxygenation of DF with the complementation of non-specific ferredoxin and a reductase component of *E. coli*, were isolated from strain DBF63 by a polymerase chain reaction (PCR)-based strategy, and the possibility that DbfA1A2 can act on the angular position of 9-fluorenone has also been suggested (Kasuga et al. 2001). In addition, hybridization analysis using the PCR product as a probe revealed that the gene encoding the large subunit of multicomponent dioxygenase other than *dbfA1* was located about 6 kb upstream from the *dbfA1* gene (Kasuga et al. 2001).

In this study, we sequenced the upstream region of the *dbfA1A2* gene in strain DBF63 by gene walking and characterized the phthalate catabolic genes in the region.

Table 1 Bacterial strains, plasmids, and cosmids used in this study

Strains and plasmids	Relevant characteristics	References/sources
Bacterial strains		
Terrabacter sp. strain DBF63	DF+[a]	Monna et al. (1993)
Escherichia coli strain JM 109	*recA1*, (*lac-proAB*), *endA1*, *gyrA96, thi-1, hsdR17, relA1, supE44*, F (*traD36, proAB, laqIqZM15*)	Yanisch-Perron et al. (1985)
Plasmids		
pUC118/119	Ap^r, *lacZ*, pMB9 replicon	Yanisch-Perron et al. (1985)
pSTV28/29	Cm^r, *lacZ*, pMB9 replicon	Takara Shuzo
pBluescript II KS(+/–)	Ap^r, *lacZ*, pMB9 replicon	Stratagene
SuperCos1	Ap^r, Km^r, cos	Stratagene
pT7Blue(R)	Ap^r, *lacZ*	Novagene
pDF06	Ap^r, 6.6-kb *Sph*I fragment from DBF63 DNA inserted into *Sph*I-digested pUC119; carries ORF13-ORF7, *lac* orientation	Kasuga et al. (2001)
pDF201	Ap^r, 6.5-kb *ClaI* fragment from DBF63 DNA inserted into *Cla*I-digested pBluescript II KS; carries ORF13-ORF7, *lac* orientation	This study
pDF202	Ap^r, 0.5-kb *Apa*I-*Afl*II fragment was deleted from pDF201; carries ORF13-ORF7, *lac* orientation	This study
pDF203	Ap^r, 0.9-kb *Pma*CI-*Eco*RV fragment was deleted from pDF202; carries ORF13-ORF8, *lac* orientation	This study
pDFS204	Cm^r, 1.3-kb *Hpa*I-*Sph*I fragment from pDF06 inserted into *Hin*cII-*Sph*I double-digested pSTV28; carries ORF7, *lac* orientation	This study
pT7-ORF7	Ap^r, 0.7-kb PCR product of ORF7 inserted into pT7Blue(R)	This study
pDFS205	Cm^r, 0.7-kb *Hin*dIII-*Eco*RI fragment from pT7-ORF7 inserted into *Hin*dIII-*Eco*RI double-digested pSTV29; carries ORF7, *lac* orientation	This study
Cosmids		
pCC12	Ap^r, Km^r, SuperCos1 with *Bam*HI insert of strain DBF63 DNA	Kasuga et al. (2001)

[a] DF+ indicates the ability to use DF as sole carbon and energy sources

Materials and methods

Bacterial strains, plasmids, and culture conditions

Bacterial strains, plasmids, and cosmids used in this study are listed in Table 1. A physical map of the plasmids constructed in this study is also shown in Fig. 2. Strain DBF63 was cultivated as reported previously (Kasuga et al. 1997). For extraction of total DNA from strain DBF63, nutrient broth (Eiken Chemical, Tokyo) was used as indicated by the manufacturer. *E. coli* strains were cultivated using LB broth, 2xYT medium or Terrific broth, as described by Sambrook et al. (Sambrook et al. 1989). Ampicillin sodium salt, chloramphenicol, and isopropyl-β-D-thiogalactopyranoside (IPTG) were added to media at final concentrations of 100 μg/ml, 30 μg/ml, and 100 μM, respectively, if necessary.

Chemicals

Chemicals used in this study were of the highest purity commercially available (Merck, Darmstadt, Germany; Sigma-Aldrich, Steinheim, Germany; Kanto Chemical, Tokyo; Wako Pure Chemical, Osaka; Nacalai Tesque, Kyoto). Diazomethane was generated by alkaline decomposition of *p*-toluenesulfonyl-*N*-methyl-*N*-nitrosoamide.

DNA manipulations

Preparation of total DNA of strain DBF63 and recombinant DNA manipulation were performed as reported previously (Kasuga et al. 1997). Plasmid DNAs were prepared according to the method by Birnboim (1983) and purified as described by Sambrook et al. (1989). *E. coli* cells were transformed with plasmids according to the method of Hanahan with some modifications (Hanahan et al. 1983). DNA fragments were purified by using Concert Rapid Gel Extraction Systems (Invitrogen, Groningen, The Netherlands) as indicated by the manufacturer. Other DNA manipulations were carried out as described by Sambrook et al. (1989), and commercially available enzymes and kits were used as indicated by the respective manufacturers.

Gene walking, nucleotide sequencing and sequence comparisons

From cosmid clone pCC12, we have already subcloned the 11-kb *Bam*HI fragment (pDF05), located next to the 8-kb *Bam*HI fragment containing *dbfA1A2* genes (pDF01 insert), and we have also determined the physical map of this 11-kb *Bam*HI fragment (Kasuga et al. 2001). A series of unidirectionally deleted DNA fragments of this pDF05 insert for the sequencing reaction were constructed using a kilo-sequence deletion kit (Takara Shuzo, Kyoto). Then the nucleotide sequence of PCR products was determined using a pT7Blue(R) vector derivative clone (Novagene, Madison, Wis.) containing a PCR product as template. Nucleotide sequencing was carried out using a Dye-Terminator Cycle Sequencing Kit (PE Biosystems, Chiba, Japan) and 373A DNA Sequencing System (PE Biosystems). The results of sequencing were analysed using DNASIS ver. 3.7 (Hitachi Software Engineering, Yokohama) and FramePlot 2.3 (Ishikawa and Hotta 1999). We searched for homology in the SWISS-PROT amino acid sequence data bank or the DDBJ, EMBL and GenBank nucleotide sequence databases with the BLAST program (version 2.0.10) (Altschul et al. 1997). Multiple sequence alignment was carried out using Clustal W version 1.6 (Thompson et al. 1994). The nucleotide sequence data reported in this article have been submitted to the DDBJ, EMBL and GenBank nucleotide sequence databases under the accession no. AB084235.

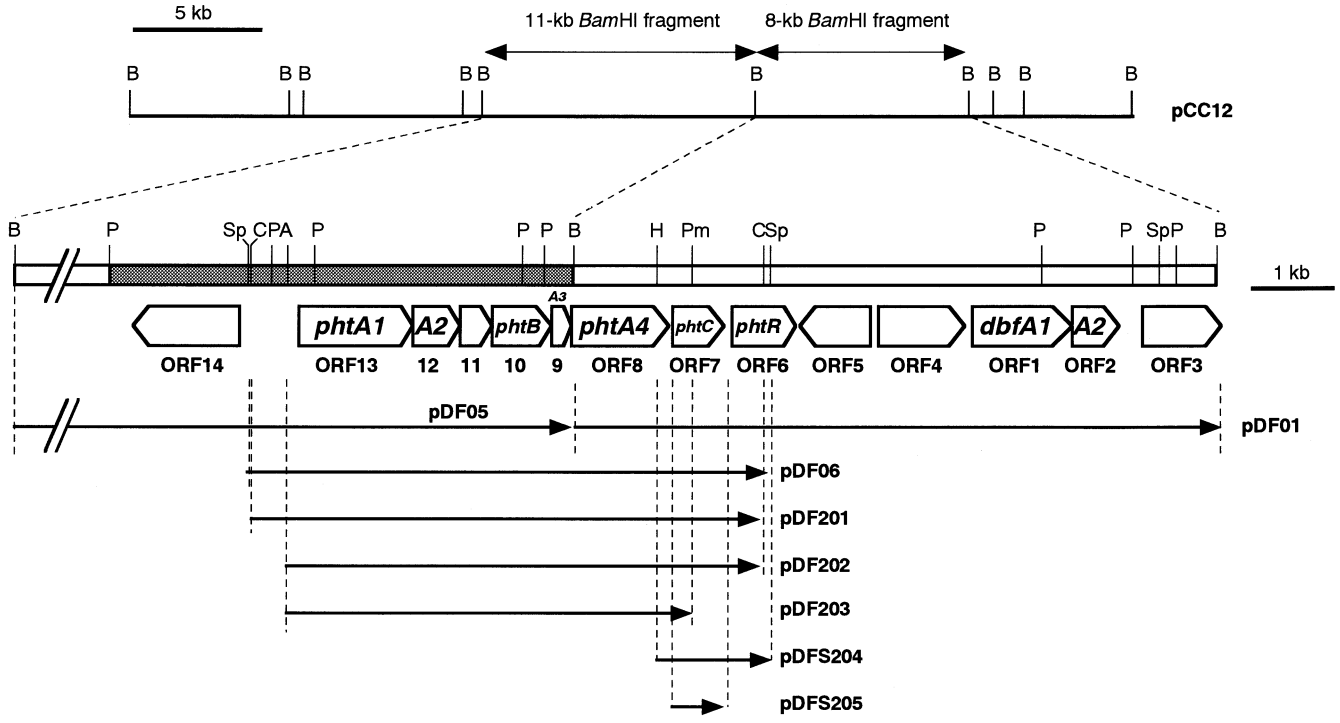

Fig. 2 Physical map of the 14-kb DNA region containing two ring-hydroxylating dioxygenase genes, *dbfA1A2* and *phtA1A2A3A4*, in *Terrabacter* sp. strain DBF63. The *pentagons* in the physical map indicate the size, location, and the direction of transcription of the ORFs. The *solid arrows* indicate the direction of transcription from the *lac* promoter of the cloning vector [pUC119 for pDF01, pDF05 and pDF06; pBluescript II KS(–) for pDF201, pDF202 and pDF203; pSTV28 for pDFS204; pSTV29 for pDFS205]. *A Afl*II, *B Bam*HI, *C Cla*I, *H HpaI*, *Sp Sph*I, *Pm Pma*CI *P Pst*I

Construction of plasmid for *phtC* expression

The 720-bp DNA fragment containing *phtC* was prepared by PCR using pDF06 as a template. In PCR amplification, the primer set having the nucleotide sequence, 5-TTAAGCTT*AAGGAGG*CGC-CGGCATGAGCGGCGGGTACGCCGC-3 [the underlined and italic sequences are the *Hin*dIII site and an efficient Shine-Dalgarno (SD) sequence (Shine and Dalgarno 1975), respectively] and 5-AAGAATTCTCACCCCATCCTGGCGACTTCATGCCGC-CACGCCAC-3 (the underlined sequences show the *Eco*RI site), was used. PCRs were carried out with LA Taq (Takara Shuzo) and GeneAmp PCR system 9700 (PE Biosystems). The cycling conditions for PCR amplification were as follows: 96°C for 1 min; followed by 30 cycles of 96°C for 30 s, 64°C for 30 s and 72°C for 80 s; followed by 72°C for 7 min, and followed by cooling down to 4°C. The PCR products were cloned using the pT7Blue (R) vector (Novagene) and the nucleotide sequence of PCR products were confirmed by sequencing. The clone was cut with both the *Hin*dIII and the *Eco*RI sites (derived from the primers), and then the fragment was cloned to the corresponding sites of pSTV29 to give pDFS205 (Fig. 2).

Resting cell reactions and analysis of the products

E. coli strains used for the resting cell reaction were grown on 100 ml of LB broth in a 500-ml Sakaguchi flask supplemented with ampicillin, chloramphenicol, and IPTG if necessary at 30°C and 120 strokes/min on a reciprocal shaker. The resultant culture was subjected to centrifugation (4,000 *g*), and the pelleted cells were washed twice with 100 ml of 50 mM phosphate buffer (pH 6.8) and resuspended in the same buffer to an OD_{600} of approximately 10. To 5 ml of the resting cell suspension in test tubes (18 mm in diameter), each substrate was added to a final concentration of 0.1% (wt/vol) and the tubes were incubated at 30°C for 18 h. The reaction mixtures were centrifuged (4,000 *g*), and the resultant supernatants were extracted with 5 ml of ethyl acetate. The extracts were analysed by gas chromatography-mass spectrometry (GC-MS) after derivatization with methylsilyltrifluoroacetamide (MSTFA) or diazomethane. GC-MS analyses were performed with a model JMS-Automass 150 GC-MS system (JOEL, Tokyo) fitted with a fused-silica chemically bonded capillary column [DB-5; 0.25 mm (inside diameter) by 15 m, 0.25-μm film thickness; J&W Scientific, Folsom, Calif.). Each sample was injected into the column at 80°C in the splitless mode. After 2 min at 80°C, the column temperature was increased at 16°C/min to 280°C. The head pressure of the helium carrier gas was 65 kPa.

Results

Nucleotide sequencing of the upstream region of angular dioxygenase genes, *dbfA1A2*

To determine the nucleotide sequence of the upstream region of the *dbfA1A2* genes, we constructed several deletion plasmids from the 11-kb *Bam*HI fragment (Fig. 2, pDF05 insert). Consequently, the nucleotide sequences of the 5,880-bp long *Pst*I-*Bam*HI region within pDF05 were determined. By adding the nucleotide sequence of the 8-kb *Bam*HI fragment (pDF01 insert) we previously determined (Kasuga et al. 2001), we revealed the gene organization of the total 14,055-bp *Pst*I-*Bam*HI region (Fig. 2). There were six putative ORFs [ORF9, ORF10,

Table 2 Deduced amino acid sequences of respective ORFs compared with the genes for other aromatic-degrading enzymes

Gene[a]	Probable function or product	Molecular mass	Homologous protein	Source	Identity (%)[b]	Accession number[c]
ORF14[d]	Transposase	50,636	ORF1[e]	*Terrabacter* sp. strain DPO360	100	U87649
			Transposase	*Mycobacterium tuberculosis* strain H37Rv	67	NC_006962
			Transposase	*Methanosarcina acetivorans* strain C2A	34	AE010931
phtA1 (ORF13)	Large subunit of phthalate dioxygenase	52,534	PhtAa	*Arthrobacter keyseri* strain 12B	74	AF331043
			NidA	*Rhodococcus* sp. strain I24	43	AF121905
			NarAa	*Rhodococcus* sp. strain NCIMB12038	43	AF082663
phtA2 (ORF12)	Small subunit of phthalate dioxygenase	22,404	PhtAb	*A. keyseri* strain 12B	66	AF331043
			NarB	*Rhodococcus* sp. strain 1BN	44	AJ401612
			NidB	*Rhodococcus* sp. strain I24	44	AF121905
ORF11	Unknown	13,363	ORF72	*Nocardioides* sp. strain KP7	31	AB031319
phtB (ORF10)	*cis*-Dihydrodiol dehydrogenase	27,594	NarB	*Rhodococcus* sp. strain NCIMB12038	50	AF082663
			NidC	*Rhodococcus* sp. strain I24	47	AF121905
			PhdE	*Nocardioides* sp. strain KP7	47	AB031319
phtA3 (ORF9)	Ferredoxin	6,838	PhtAc	*A. keyseri* strain 12B	63	AF331043
			NysM	*Streptomyces noursei*	42	AF263912
			SuaB	*Streptomyces griseolus*	41	M32238
phtA4 (ORF8)	Ferredoxin reductase	42,882	PhtAd	*A. keyseri* strain 12B	55	AF331043
			MocF	*Sinorhizobium melilot*	39	AF076471
			BphA4	*Rhodococcus erythropolis* strain TA421	38	D88021
phtC (ORF7)	Decarboxylase	24,665	PhtC	*A. keyseri* strain 12B	68	AF331043
			Aldolase	*Methanobacterium thermoautotrophicum*	31	AE000903
			CmtD	*Pseudomonas putida* strain F1	25	U24215
phtR (ORF6)	Regulatory protein	28,947	PhtR	*A. keyseri* strain 12B	54	AF331043
			KdgR	*Bacillus halodurans*	31	AP001519
			PcaR	*Rhodococcus opacus* strain 1CP	28	AF003947

[a] Accession no. AB084235
[b] Multiple sequence alignment was carried out using Clustal W version 1.6 (Thompson et al. 1994)
[c] Accession number in the SwissProt and DDBJ/EMBL/GenBank databases
[d] The ORF is encoded in the complementary strand
[e] Only the 163 amino acid-long N-terminal region of ORF1 from strain DPO360 was compared (see Fig. 4)

ORF11, ORF12, ORF13 and ORF14 (encoded in the opposite direction)] in this region as shown in Fig. 2. In addition, although the nucleotide sequence of the 5-terminal region of ORF8 had not been determined completely in our previous study (Kasuga et al. 2001), it was revealed in this study that the ORF8 encodes a protein with a size of 412 amino acids and calculated molecular mass of 42,882. The putative SD sequences (Shine and Dalgarno 1975) were found in the upstream regions of the putative initiation codons of ORF9, ORF13 and ORF14 (data not shown).

Sequence comparisons with other related proteins

The deduced amino acid sequences of respective ORFs were compared with the genes for other aromatic-degrading enzymes (Table 2).

Phthalate dioxygenase genes (ORF13, ORF12, ORF9 and ORF8)

The ORF13 encodes a protein consisting of 492 amino acid residues showing the highest identity of 74% with the oxygenase large subunit of phthalate dioxygenase from *A. keyseri* strain 12B (PhtAa) (accession number AF331043) (Eaton 2001). The deduced amino acid sequence of ORF13 also shows moderate similarity to the large subunit of oxygenases involved in the degaradation of aromatics, including indene dioxygenase from *Rhodococcus* sp. strain I24 (NidA) (no. AF121905) and naphthalene dioxygenase from *Rhodococcus* sp. strain NCIMB12038 (NarAa) (no. AF082663) (Table 2). The consensus sequence of Rieske-type iron sulphur proteins for the binding of a [2Fe-2S] cluster ($CXHX_{16–17}CXXH$) (Kauppi et al. 1998) was found in the deduced amino acid sequence of ORF13 (X represents any amino acid). The critical motif, which was considered to act as a mononuclear iron ligand at the site of oxygen activation (Kauppi et al. 1998), was also conserved in the deduced amino acid sequence of ORF13 (data not shown).

The deduced amino acid sequence of ORF12 is most similar to the small subunit of the phthalate dioxygenase component from strain 12B (PhtAb) (no. AF331043) (Eaton 2001), and is also homologous with the small subunit of oxygenase involved in the degradation of naphthalene degradation from *Rhodococcus* sp. strain 1BN (NarB) (no. AJ401612) and indene degradation from *Rhodococcus* sp. strain I24 (NidB) (no. AF121905) (Table 2). Thus, the ORF12 was shown to encode a small subunit of the oxygenase component consisting of 197 amino acid residues.

The 64-amino acid sequence of ORF9 showed significant homology to the [3Fe-4S] and [4Fe-4S] ferredoxins, although most ferredoxin components of ring-hydroxy-

lating dioxygenase belong to the [2Fe-2S] type of ferredoxin. The deduced amino acid sequence of ORF9 showed the highest identity with PhtAc from strain 12B (no. AF331043) (Eaton 2001). A homology search also showed that the product of ORF9 was related to the [3Fe-4S] type of electron transport proteins in the multicomponent P-450 system in *Streptomyces griseolus* (SuaB) (no. M32238), *S. noursei* (NysM) (no. AF263912), and *S. tendae* strain Tue901 (NikG) (no. Y18574) (41–42% identities). These ferredoxins are involved in the herbicide monooxygenation (O'Keefe et al. 1991) and biosynthesis of antibiotics such as nystatin and nikkomycin (Brautaset et al. 2000; Bruntner et al. 1999). Also, the deduced amino acid sequence of ORF9 exhibited 36% and 35% identities to DitA3 from *P. abietaniphila* strain BKME-9 (no. AF119621) and PhdC from *Nocardioides* sp. strain KP7 (no. AB017795), which have been reported as a ferredoxin component of the diterpenoid dioxygenase (Martin and Mohn 1999) and phenanthrene (Saito et al. 2000), respectively. All these proteins contained three conserved cystein residues that probably provide ligands to the [3Fe-4S] iron-sulphur cluster (O'Keefe et al. 1991) (data not shown).

Previously, we reported the deduced amino acid sequence of ORF8 exhibited homology (37–39% identity) to the ferredoxin reductase of the ring hydroxylating dioxygenases involved in the biphenyl degradation from *R. erythropolis* strain TA421 (BphA4) (no. D88021) and phenanthrene degradation from *Nocardioides* sp. strain KP7 (PhdD) (no. AB017795) (Kasuga et al. 2001). However, after the sequences of the phthalate catabolic pathway gene cluster on plasmid pRE1 from *A. keyseri* strain 12B (Eaton 2001) became available on databases, the ORF8 product showed the highest identity of 55% with PhtAd from strain 12B (no. AF331043) (Table 2).

cis-Dihydrodiol dehydrogenase genes (ORF10)

The deduced amino acid sequence of ORF10, located within the multicomponent dioxygenase genes, was homologous to *cis*-dihydrodiol dehydrogenase involved in the PAHs degradation, e.g. NarB from *Rhodococcus* sp. strain NCIMB12038 (no. AF082663) (50% identity), NidC from *Rhodococcus* sp. strain I24 (no. AF121905) (47% identity), and PhdE from *Nocardioides* sp. strain KP7 (no. AB031319) (47% identity), and the monoaromatics degradation, e.g. TodD from *P. putida* strain F1 (no. P13859), BnzE from *P. putida* (no. P08088), and TcbB from *Pseudomonas* sp. strain P51 (no. U15298) (42–44% identity). By contrast, the ORF10 product showed only low homology (30%) to PhtB from strain 12B (no. AF331043). These results indicate that the ORF10 encodes a *cis*-dihydrodiol dehydrogenese consisting of 269 amino acid residues.

3,4-Dihydroxyphthalate decarboxylase genes (ORF7)

As described previously (Kasuga et al. 2001), the deduced amino acid sequence of ORF7 showed homology to fuculose-1-phosphate aldolase from *Methanobacterium thermoautotrophicum* strain Delta H (no. AE000903) (31% identity) and 2-hydroxy-3-carboxy-6-oxo-7-methylocta-2,4-dienoate decarboxylase (CmtD) from *P. putida* strain F1 (no. U24215) (25% identity). In addition, the gene product exhibited the highest identity of 68% to 3,4-dihydroxyphthalate decarboxylase (PhtC) from *A. keyseri* strain 12B (no. AF331043), whose sequence data was more recently published (Eaton 2001).

Putative regulatory genes (ORF6)

In our previous paper (Kasuga et al. 2001), we reported that the deduced amino acid sequences of ORF6 showed homology to transcriptional regulators belonging to the IclR family. The gene product of ORF6 shared 27–28% identities with PcaRs of *R. opacus* strain 1CP (no. AF003947) and *P. putida* (no. AJ252090), 28% with KdgR of *Erwinia chrysanthemi* (no. X62072), and 26% with IclR of *E. coli* (no. M31761). In addition, the ORF6 product now shows the highest identity of 54% with *PhtR* from strain 12B (no. AF331043) (Table 2), although its activity in regulating the expression of the *pht* operon has not been investigated (Eaton 2001).

Insertion sequence (ORF14 and its flanking region)

In the upstream region of a set of gene clusters encoding the multicomponent dioxygenase system, there is an ORF14 with the highest identity of 67% with putative transposases from *Mycobacterium tuberculosis* strain H37Rv (no. NC_006962) and *M. tuberculosis* strain CDC1551 (no. NC_002755). A homology search also showed that the deduced amino acid sequence of ORF14 was related to transposases MA2406 (no. AE010931) and MA3988 (no. AE011111) from *Methanosarcina acetivorans* strain C2A (34% identity), the transposase in ISTfe1 from *Acidithiobacillus ferroxidans* strain ATCC19859 (34% identity; no. U66426), the transposase from *E. coli* Nissle 1917 (34% identity; no. AF188737), and the transposase in ISAE1 from *Ralstonia eutrophus* (32% identity; no. M86608). The critical DDE consensus motif predicted in ISTfe1 (Holmes et al. 2001; no. U66426) was conserved in the deduced amino acid sequences of all these transposases, as shown in Fig. 3.

The ORF14 and its flanking region exhibit the features of a typical bacterial IS. It has 24-bp, imperfectly matched, terminal inverted repeats (IRs) and ORF encoding transposase of 421 amino acids with significant similarity to the ISL3 family of ISs, as described above. The ISs belonging to the ISL3 family carry IRs between 15 and 39 bp long, and generate a direct target duplication of 8 bp, and also, one long ORF is present which encodes

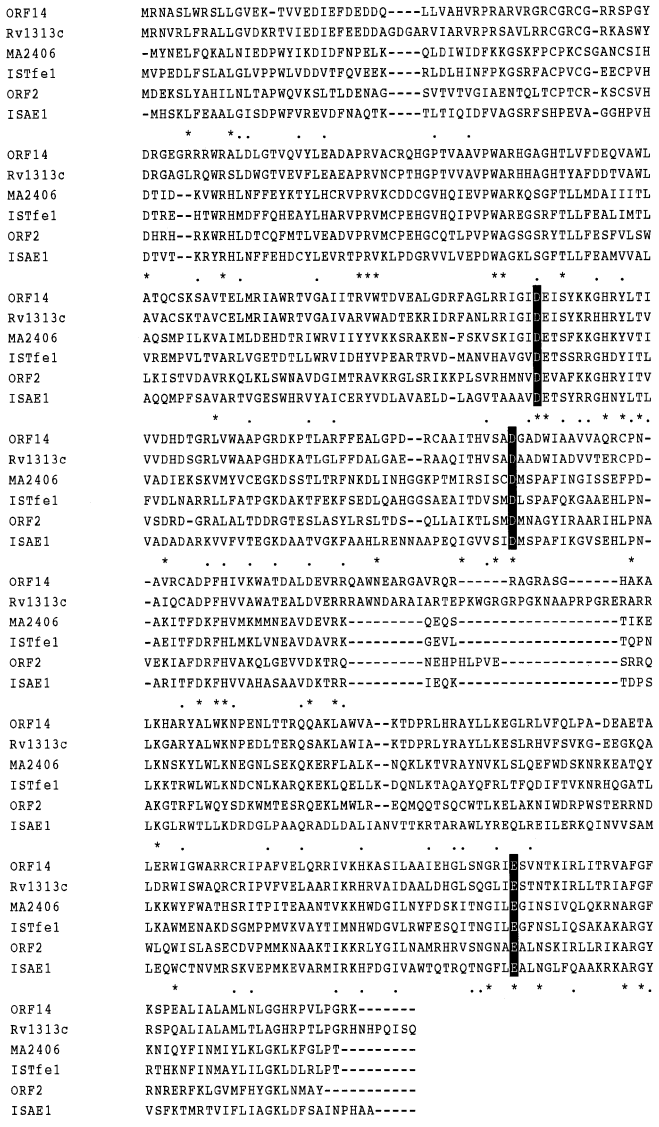

Fig. 3 Alignments of the ORF14 product from *Terrabacter* sp. strain DBF63 with their homologous transposases. The aligned transposases are in ISs belonging to the ISL3 family (Mahillon and Chandler 1998). The identical and similar amino acid residues in all proteins are indicated by *asterisks* and *dots*, respectively. The critical DDE consensus motifs predicted in ISTfe1 (Holmes et al. 2001; no. U66426) are indicated by the *white type*. The sequence abbreviations, species and DDBJ/GenBank/EMBL references are as follows: *ORF14 Terrabacter* sp. strain DBF63, no. AB084235; *Rv1313c M. tuberculosis* strain H37Rv, no. NC_006962; *MA2406 Methanosarcina acetivorans* strain C2A, no. AE010931; *ISTfe1 Acidithiobacillus ferroxidans*, no. U66426; *ORF2 E. coli*, no. AF188737; *ISAE1 R. eutrophus*, no. M86608

a transposase of roughly 400–440 amino acids (Mahillon and Chandler 1998). The ORF14 and its flanking region showed the general features of this family. Therefore, although the direct target duplication sites have not been found, we designated it as ISTesp2.

More interestingly, part of the nucleotide sequence of ISTesp2 had almost 100% identity to the DNA region (truncated *orf1* and its upstream region) found in the DF-degrader *Terrabacter* sp. strain DPO360 isolated from tar-contaminated soil in Germany (no. U87649) (Schmid et al. 1997) (Fig. 4). While there exists a gene cluster containing phthalate dioxygenase downstream of ISTesp2 in strain DBF63, the extradiol dioxygenase (*meta*-cleavage enzyme) gene involved in the DF mineralization was located in this region of strain DPO360 as shown in Fig. 4. In addition, ISTesp2 from strain DBF63 showed 56–57% identities with ISTfe1 from *A. ferroxidans* strain ATCC19859 (no. U66426) (Holmes et al. 2001) and ISAE1 from *R. eutrophus* (no. M86608) (Kung et al. 1992) at the DNA level.

Other genes (ORF11)

Just upstream of ORF10, we found a small ORF and named it ORF11. The 127-amino acid sequence of ORF11 did not show significant homology to any other proteins, but exhibited low homology (31%) to the functionally unknown ORF72 from *Nocardioides* sp. strain KP7 (no. AB031319), which was located within the phenanthrene degradation gene cluster (*phd* genes) in strain KP7 (Saito et al. 2000). Involvement of the ORF11 in the degradation of aromatic compounds has not been demonstrated.

Biotransformation of phthalate by the enzymes of the *pht* operon

Based on the results of sequence comparison, we investigated whether ORF13, ORF12, ORF10, ORF9 and ORF8 actually catalyse the transformation of phthalate to 3,4-dihydroxyphthalate. pDF203 containing these ORFs under the control of a *lac* promoter was constructed and introduced into *E. coli* JM109 cells. On GC-MS analysis of products from phthalate after treatment with MSTFA, the mass spectrum exhibited fragment ions at *m/z* 486 (M^+, 1.3), 471 {$[M\text{-}CH_3]^+$, 59}, 383 (12), 353 (3), 309 (76), 147 (48), and 73 (100) was detected at the retention time (RT) of 10.9 min (relative intensities expressed as percentages are given in parentheses). This fragmentation pattern of the trimethylsilyl (TMS) derivative of the metabolite was identical to that of 3,4-dihydroxyphthalate previously reported (Eaton 2001). In addition, on GC-MS analysis of products from phthalate after treatment with diazomethane, the molecular ion of the trimethyl derivative of the 3,4-dihydroxyphthalate (M^+=240, Suemori et al. 1993) was also detected at the RT of 8.6 min. From these findings, the products of ORF13, ORF12, ORF9 and ORF8 were suggested to be involved in the 3,4-dioxygenation of phthalate. Thus, they were designated as *phtA1*, *phtA2*, *phtA3* and *phtA4*, respectively. Also, these results indicated that ORF10 encodes the *cis*-3,4-dihydro-3,4-dihydroxyphthalate dehydrogenase. Hence, we named the ORF10 as *phtB*.

On the other hand, *E. coli* carrying pDF202, which contains all genes encoding dioxygenase (*phtA1A2A3A4*),

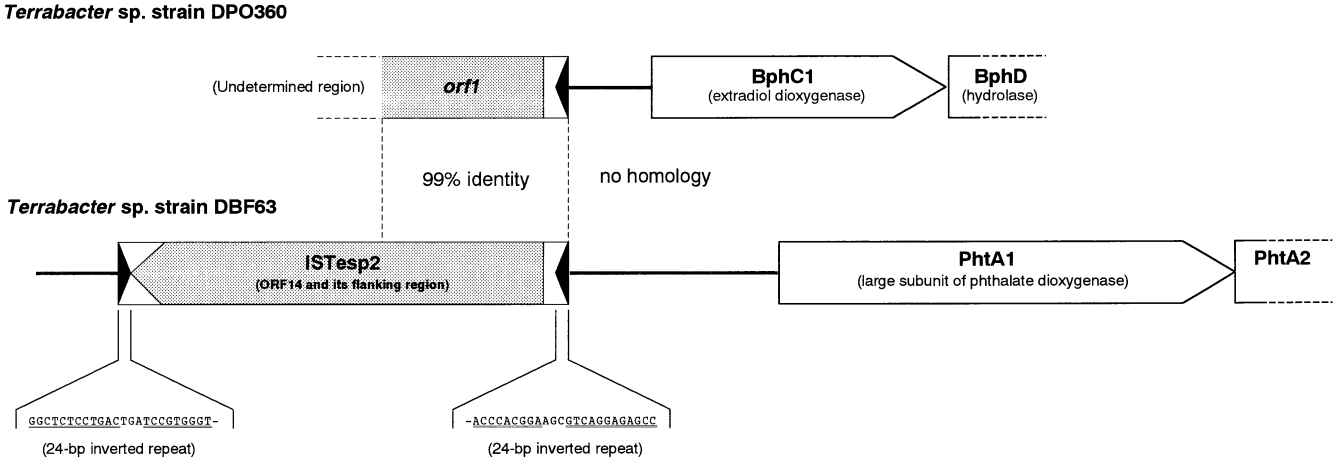

Fig. 4 Comparison of the *orf1* and its downstream region from *Terrabacter* sp. strain DPO360 (Schmid et al. 1997) and the ORF14 and its flanking region from *Terrabacter* sp. strain DBF63. *Pentagons* represent the location, length, and direction of ORFs, and especially, *shaded pentagons* represent transposase genes. *Black triangles* represent inverted repeats (IRs) of insertion sequence. The complementary nucleotides between the left and right IRs are *double underlined*

dehydrogenase (*phtB*) and putative decarboxylase (ORF7), transformed phthalate to 3,4-dihydroxyphthalate, but not to the predicted product, protocatechuate (data not shown). First, we thought that only a small amount of the ORF7 product was expressed, because ORF7 was located about 4.9 kb downstream of the *lac* promoter of the pBluescript II KS(–) vector. Therefore, we cloned the 1.3-kb *Hpa*I-*Sph*I fragment (containing ORF7) under the *lac* promoter of the pSTV28 vector (designated as pDFS204), and biotransformed phthalate using *E. coli* carrying both pDF203 and pDFS204. However, also in this experiment, we could not detect protocatechuate from phthalate. Finally, to express the ORF7 product effectively in *E. coli* cells, we constructed pDFS205, which contained a SD sequence in the upstream region of its initiation codon, using PCR. After biotransformation using *E. coli* carrying both pDF203 and pDFS205, GC-MS analysis showed the mass spectrum exhibiting fragment ions at *m/z* 370 (M^+, 58), 355 ($[M\text{-}CH_3]^+$, 34), 311 (26), 281 (11), 223 (8), 193 (100), 165 (8), and 73 (88) at the RT of 8.8 min. This fragmentation pattern of the TMS derivative of the metabolite was identical to those of authentic protocatechuate. This indicates that the ORF7 product catalyses the decarboxylation of 3,4-dihydroxyphthalate, and therefore, we designated the ORF7 as *phtC*.

Substrate specificity of PhtA1A2A3A4

We investigated the substrate specificity of PhtA for other aromatic compounds by transforming the compounds using the recombinant *E. coli* harbouring pDF203. *E. coli* harbouring pBluescript II KS(–) was used as a control. When 2-chlorobenzoate was added as a substrate in transformation experiments, we detected in the mass spectrum the fragment ions at *m/z* 406 (M^+, 29), 404 (60), 391 (19), 389 (41), 317 (5), 315 (12), 301 (6), 257 (6), 229 (100), 227 (88), 193 (7), 171 (14) and 73 (68) (these peaks represent the paired ions differing by two mass units resulting from major chlorine isotopes of mass 35 and 37). This fragmentation pattern of the TMS derivative of metabolite was the same as that observed for the TMS derivative of 2-chloro-3,4-dihydroxybenzoate previously reported (Eaton 2001). By contrast, no oxygenation products including catechol were detected when using salicylate or anthranilate as a substrate. Moreover, it was also revealed that PhtA did not catalyse oxygenation of aromatic compounds having more than two aromatic rings, e.g. biphenyl, naphthalene, phenanthlene and pyrene.

Discussion

In this study, we identified the gene cluster designated *pht*, which encode enzymes for the degradation of phthalate to protocatechuate, in the DNA region adjacent to the angular dioxygenase genes, *dbfA1* and *dbfA2,* from *Terrabacter* sp. strain DBF63. Comparison to other sequences in the databases strongly suggested that these gene products were closely related to the phthalate catabolic enzymes from the phthalate-utilizing bacterium *A. keyseri* strain 12B (Eaton 2001). The determined structure of the *pht* gene cluster (*phtA1A2BA3A4CR*) from strain DBF63 was compared with that of the *pht* gene cluster (*phtBAaAbAcAdCR*) from strain 12B (Fig. 5). These gene clusters are highly similar with respect to gene organization, size, and homology of corresponding enzymes. However, the locations of the genes encoding *cis*-3,4-dihydroxy-3,4-dihydrophthalate dehydrogenase (*phtB*) are markedly different (Fig. 5). *phtB* from strain 12B is located at the top of the cluster, and the genes encoding phthalate dioxygenase components (*phtAaAbAcAd*) were connected in tandem in strain 12B. By contrast,

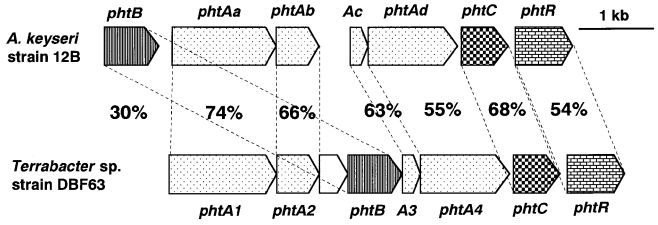

Fig. 5 Organization of the genes involved in phthalate degradation from *A. keyseri* strain 12B (Eaton 2001) and *Terrabacter* sp. strain DBF63. *phtAa and phtA1* Genes coding for large subunit of oxygenase component of phthalate 3,4-dioxygenase, *phtAb and phtA2* genes coding for small subunit of oxygenase component of phthalate 3,4-dioxygenase, *phtAc and phtA3* genes coding for [3Fe-4S] or [4Fe-4S] type ferredoxin component of phthalate 3,4-dioxygenase, *phtAd and phtA4*, genes coding for ferredoxin reductase component of phthalate 3,4-dioxygenase, *phtB* genes coding for *cis*-3,4-dihydro-3,4-dihydroxyphthalate dehydrogenase, *phtC* genes coding for 3,4-dihydroxyphthalate decarboxylase, *phtR* genes coding for putative regulatory protein of phthalate degradation

phtB from strain DBF63 exists between the genes *phtA2* and *phtA3* that encode the phthalate dioxygenase system. In addition, whereas gene products of the *pht* operon except *phtB* exhibited relatively high identity between strains 12B and DBF63 (54–74%), PhtB showed the lowest identity (30%). Although these two dehydrogenases catalyse the same reaction, PhtB from strain 12B is related to a member of a superfamily of oxidoreductases that includes morphine dehydrogenase (Eaton 2001), while PhtB from strain DBF63 shows high homology with *cis*-diol dehydrogenases involved in the degradation of aromatic compounds (Table 2). However, now we cannot postulate a mechanism for the evolutionary rearrangement of the *phtB* genes in the two strains.

Furthermore, between the *pht* genes from *Terrabacter* sp. strain DBF63 and *A. keyseri* strain 12B, their flanking regions were quite different from each other. In strain 12B, there exist genes encoding enzymes of the protocatechuate *meta*-cleavage pathway (*pcm* genes) in about the 6.5-kb upstream region of the *pht* gene cluster. Also, between *pcm* and *pht* operons, the *ptr* genes encoding polypeptides similar to those of an ABC transport system were located in strain 12B (Eaton 2001). On the other hand, the *pht* gene cluster from strain DBF63 is linked to the oxygenase component of angular dioxygenase gene (*dbfA1A2*), and a regulatory gene opposite the direction of transcription (ORF5) exists between the *pht* and *dbf* genes (Fig. 2).

In the 770-bp region upstream of *pht* genes, an ISTesp2 (ORF14 and its flanking region) carrying 24-bp imperfect IRs was found. The part of *orf1* from strain DPO360 and the corresponding region of the ORF14 from strain DBF63 exhibited an almost 100% match including their flanking regions, although these strains were isolated at geographically different sites (Japan and Germany) (Fig. 4). In strain DBF63, there exists a set of phthalate catabolic gene clusters adjacent to this IS, but the extradiol dioxygenase gene (*bphC1*) and *meta* cleavage compound hydrolase gene (*bphD*) were located next to the putative same IS in strain DPO360 (Schmid et al. 1997). This suggests that the IS elements play a role in the dispersion or the reconstruction of aromatic-degradative genes in the genus *Terrabacter*. In fact, Southern blot analysis carried out using the DNA fragment containing the inside region of ORF14 as a probe, revealed many strong signals with various sizes in the *Bam*HI-digested total DNA of strain DBF63 (data not shown).

We biotransformed phthalate with PhtA1A2BA3A4C using the recombinant *E. coli* cells. Eaton (2001) reported that phthalate has one protonated carboxyl group at a pH below 5.51 and exists as a dicarboxylate anion at a neutral pH, and that phthalate was not transformed by any *E. coli* clones at a neutral pH, probably due to the inability of the dicarboxylate anion to enter these cells. They also reported that the transformation experiment of phthalate by *E. coli* JM109 clones at pH4.7 was finally able to give its metabolites, i.e. 3,4-dihydroxyphthalate and protocatechuate (Eaton 2001). However, we detected 3,4-dihydroxyphthalate from phthalate at neutral pH 6.8 using *E. coli* JM109 cells carrying pDF203. Also, protocatechuate was detected at pH 6.8 using *E. coli* JM109 cells carrying both pDF203 and pDFS205. What causes this difference still remains unclear. At first, *E. coli* JM109 cells carrying pDF06 (Fig. 2) were used for biotransforming phthalate, but neither 3,4-dihydroxyphthalate nor protocatechuate were detected (data not shown). On the other hand, when we used pDF202 which has an approximately 0.5-kb shorter insert than pDF06, 3,4-dihydroxyphthalate was detected in the reaction mixture. This 0.5-kb DNA fragment is located in the region between the transposase gene (ORF14) and *phtA1* gene opposite the direction of transcription, and within this region, we found some possible termination signals of transcription (Platt 1986), e.g. stem and loop structure (data not shown). Considering that the 0.5-kb DNA fragment is located just downstream of the region of the *lac* promoter derived from the respective cloning vector (Fig. 2), transcription from the *lac* promoter might terminate before the *phtA1* gene is transcribed.

The substrate specificity of PhtAB from strain DBF63 was investigated and compared with those of the phthalate dioxygenase from other gram-positive bacteria. The phthalate-grown *A. keyseri* strain 12B was able to convert several 2-substituted benzoates, e.g. 2-trifluoromethyl-, 2-chloro-, 2-bromo-, 2-iodo-, 2-nitro-, 2-methoxy-, and 2-acetyl-benzoates to the corresponding 2-substituted 3,4-dihydroxybenzoates, and it was also confirmed that incubation of *E. coli* JM109 carrying *phtBAaAbAcAdCR* from strain 12B with 2-bromobenzoate yielded 2-bromo-protocatechuic acid (Eaton 2001). Similar to PhtAB from strain 12B, PhtAB from strain DBF63 also catalysed the dioxygenation and dehydrogenation of the phthalate analogue 2-chlorobenzoate to 2-chloro-3,4-dihydroxybenzoate. On the other hand, Suemori et al. (1993) reported that the purified phthalate oxygenase from *R. erythropolis* strain S-1 oxidized anthranilate and salicylate, though slightly. However, no oxygenation products including

catechol and corresponding dihydroxy derivatives from these substrates were found in the case of PhtA from strain DBF63.

Previously, we isolated the plasmid-cured derivative strain DBF63W, which is unable to grow with DF as a carbon source, following the continuous culture of a wild-type strain on nutrient broth (Kasuga et al. 1997). Southern blot analysis using the PCR product containing the Rieske [2Fe-2S] binding sites in oxygenase components, by which *dbfA1* and *phtA1* genes could be cloned, as a probe, revealed no signals in the *Bam*HI-digested total DNA from strain DBF63W. However, a strong signal was detected at a size of approximately 8 kb (DNA fragment containing *dbfA1A2* genes), a weaker signal around 11 kb (DNA fragment containing *phtA1A2* genes), and a slight signal at 2.5 kb were detected in the *Bam*HI-digested total DNA from the wild-type strain (Kasuga et al. 2001). Therefore, it was quite likely that the *pht* gene cluster in strain DBF63 was involved in the degradation of phthalate.

As described before, we have already found a regulatory gene (ORF5), which has the opposite direction of transcription, between *pht* and *dbf* genes (Fig. 2). In naphthalene degradative genes (*nah* genes) on plasmid NAH7 from *P. putida* strain PpG7, the "upper pathway" operon encodes for enzymes which transform naphthalene into salycilate and the "lower pathway" operon for enzymes which convert salicylate further to central metabolites, and a single regulatory gene *nahR* is located between the two operons and is involved in the regulation of both (Schell and Poser 1989). Considering the fact that DbfA1A2 can catalyse the angular dioxygenation for 9-fluorenone (H. Habe, H. Kato, J.-S. Chung, K. Kasuga, T. Yoshida, H. Nojiri, T. Omori, unpublished data), there is the possibility that the ORFs located downstream from ORF5 may be involved in the degradation of FN or 9-fluorenone. Sequencing and characterization of the DNA region located downstream of *dbfA1A2* genes are now underway.

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