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Isolation and Characterization of the Genes Encoding a Novel Oxygenase Component of Angular Dioxygenase from the Gram-Positive Dibenzofuran-Degrader *Terrabacter* sp. Strain DBF63

Kano Kasuga,* Hiroshi Habe,† Jin-Sung Chung,† Takako Yoshida,† Hideaki Nojiri,† Hisakazu Yamane,† and Toshio Omori†,[1]

†*Biotechnology Research Center, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan; and*
**Department of Biotechnology, Faculty of Bioresource Sciences, Akita Prefectural University, 247-7 Kaidobata-nishi, Shimoshinjo-nakano, Akita 010-0195, Japan*

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A gram-positive bacterium *Terrabacter* sp. strain DBF63 is able to degrade dibenzofuran (DF) via initial dioxygenation by a novel angular dioxygenase. The *dbfA1* and *dbfA2* genes, which encode the large and small subunits of the dibenzofuran 4,4a-dioxygenase (DFDO), respectively, were isolated by a polymerase chain reaction-based method. DbfA1 and DbfA2 showed moderate homology to the large and small subunits of other ring-hydroxylating dioxygenases (less than 40%), respectively, and some motifs such as the Fe(II) binding site and the [2Fe-2S] cluster ligands were conserved in DbfA1. DFDO activity was confirmed in *Escherichia coli* cells containing the cloned *dbfA1* and *dbfA2* genes with the complementation of nonspecific ferredoxin and ferredoxin reductase component of *E. coli*. Under this condition, these cells exhibited angular dioxygenation of DF and dibenzo-*p*-dioxin, and monooxygenation of fluorene, but not angular dioxygenation of carbazole, xanthene, and phenoxathiin. Phylogenetic analysis revealed that DbfA1 formed a branch with recently reported large subunits of polycyclic aromatic hydrocarbon (PAH) dioxygenase from gram-positive bacteria but did not cluster with that of other angular dioxygenases, i.e., DxnA1 from *Sphingomonas* sp. strain RW1 [Armengaud, J., Happe, B., and Timmis, K. N. *J. Bacteriol.* 180, 3954–3966, 1998] and CarAa from *Pseudomonas* sp. strain CA10 [Sato, S., Nam, J.-W., Kasuga, K., Nojiri, H., Yamane, H., and Omori, T. *J. Bacteriol.* 179, 4850–4858, 1997].

Key Words: **angular dioxygenase; oxygenase component; *Terrabacter* sp.; dibenzofuran degradation; dibenzo-*p*-dioxin degradation; fluorene degradation.**

Polychlorinated dibenzo-*p*-dioxins and dibenzofurans are highly toxic compounds (1, 2), and are known to be carcinogenic. Large quantities of dioxins are released into the environment as contaminants in pesticides and herbicides, and as a result of the combustion of waste. Dibenzofuran (DF) has been used as a model compound in the biodegradation of dibenzo-*p*-dioxin (DD) (3–6), and the metabolism of DF has been studied intensively in recent years. Thus, the initial step of this metabolic pathway has been revealed to be an angular dioxygenation catalyzed by dibenzofuran 4,4a-dioxygenase (DFDO), which leads to a chemically unstable intermediate that is spontaneously rearomatized to 2,2′,3-trihydroxybiphenyl (THB). Subsequent ring cleavage of THB is catalyzed by extradiol dioxygenases yielding 2-hydroxy-6-(2-hydroxyphenyl)-6-oxo-2,4-hexadienoic acid, which is then hydrolyzed to 2-hydroxypenta-2,4-dienoic acid and salicylic acid. Salicylic acid is then metabolized via catechol or gentisic acid (Fig. 1). Among these metabolic enzymes, initial angular dioxygenase, which can act at the angular position adjacent to the oxygen atom of DF, is a key determinant of the substrate range in the degradation of heterocyclic aromatic compounds including chlorinated DDs and DFs (7, 8, Habe, H., Chung, J.-S., Lee, J.-H, Kasuga, K., Yoshida, T., Nojiri, H., and Omori, T., submitted). However, as compared with other initial dioxygenases such as naphthalene dioxygenases, biphenyl dioxygenases, and toluene dioxygenases, there is still a lack of knowledge concerning angular dioxygenases.

Until recently, genetic analyses of angular dioxygenases involved in the degradation of dioxin-related compounds have been performed with only gram-negative bacteria of the genera *Sphingomonas* and *Pseudomonas*. Bünz and Cook (7) who purified DFDO

[1] To whom correspondence should be addressed. Fax: +81-3-5841-8030. E-mail: aseigyo@mail.ecc.u-tokyo.ac.jp.

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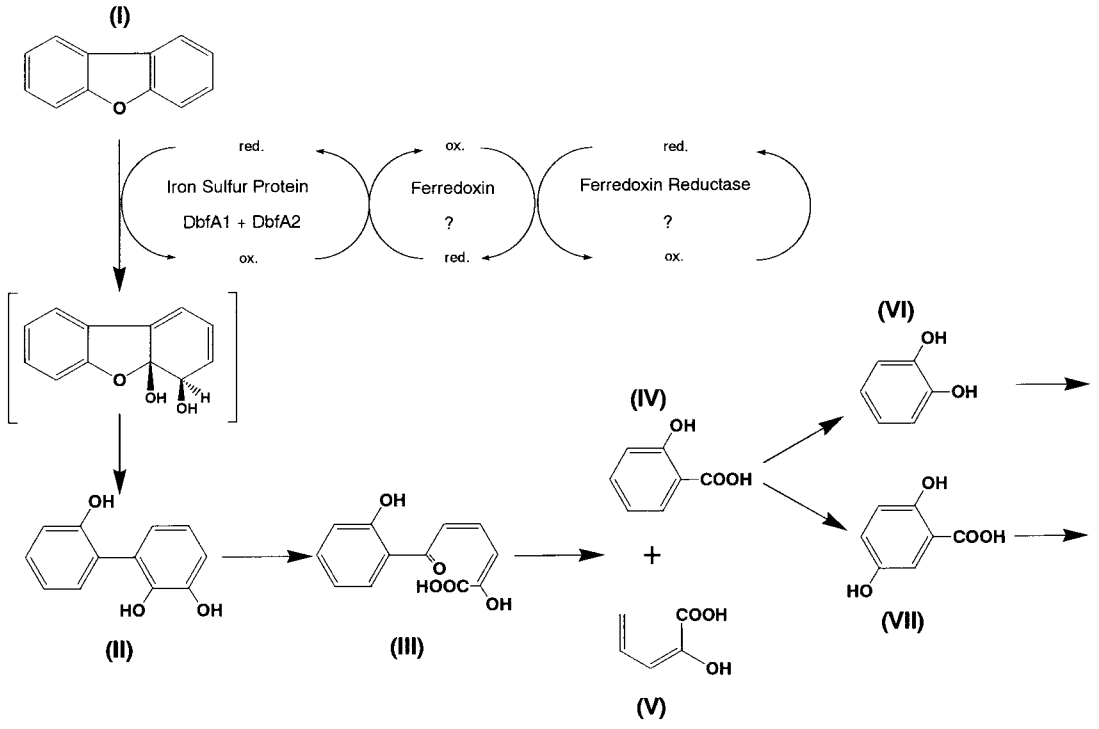

FIG. 1. Proposed degradation pathway for dibenzofuran in *Terrabacter* sp. strain DBF63. Compound designations: I, dibenzofuran; II, 2,2′,3-trihydroxybiphenyl; III, 2-hydroxy-6-(2-hydroxyphenyl)-6-oxo-2,4-hexadienoic acid; IV, salicylic acid; V, 2-hydroxypenta-2,4-dienoic acid; VI, catechol; VII, gentisic acid.

(dioxin dioxygenase) from *Sphingomonas* sp. strain RW1, revealed that this enzyme consists of three components and belongs to the class IIA dioxygenases in Batie classification (9). The genes encoding the components of this enzyme, *dxnA1* and *dxnA2,* were cloned and identified; a number of identified catabolic genes were found to be dispersed over different genome segments (10). In an other study, the genes encoding another type of angular dioxygenase, carbazole 1,9a-dioxygenase (*carAaAcAd*), were isolated from *Pseudomonas* sp. strain CA10 which degrades carbazole (CAR), a structural analogue of DF, via anthranilic acid and catechol (11). CarAaAcAd was revealed to be a three-component dioxygenase system belonging to the class III dioxygenases, and was capable of catalyzing the conversion of DF and DD into THB and 2,2′,3-trihydroxydiphenyl ether (THDE), respectively. Between these two angular dioxygenases, the substrate specificities and the organization of terminal oxygenase component were quite different from each other (7, 8, 10, 11).

On the other hand, in gram-positive bacteria, three distinct extradiol dioxygenases (*meta*-cleavage enzymes) from DF-degrader *Terrabacter* sp. strain DPO360 were purified and shown to be involved in total mineralization of DF (12). However, there have been no reports on either the purification of DFDO or the cloning of its genes, and angular dioxygenases from gram-positive bacteria remain poorly understood.

Previously, we isolated *Terrabacter* sp. strain DBF63 (strain DBF63 was formerly identified as *Staphylococcus auriculans*) on the basis of the ability to utilize DF or fluorene (FN) as the sole source of carbon and energy, and proposed degradation pathways of DF and FN (6). We also have cloned and analyzed the genes for extradiol dioxygenase (*dbfB*) and *meta*-cleavage compound hydrolase (*dbfC*) that were thought to be involved in the degradation of DF (13). However, no candidates for angular dioxygenase gene were found in the neighboring region of the genes previously reported.

In this paper, we report the isolation of the *dbfA1* and *dbfA2* genes, which are terminal oxygenase components of the angular dioxygenase system in strain DBF63, and the determination of their nucleotide sequences. This is the first report on the cloning and characterization of the terminal oxygenase component of angular dioxygenase from gram-positive bacteria.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Strain DBF63 is a DF-degrading bacterium which was isolated from a soil sample in Japan on the basis of its ability to grow on DF as the sole source of carbon and energy (6). *E. coli* strain JM109 [*recA1,* Δ(*lac-proAB*), *endA1, gyrA96, thi-1, hsdR17, relA1, supE44,* F' (*traD36, proAB, lacI*^{q}*ZΔM15*)] was used as host strains in DNA manipulation.

Strain DBF63 was cultivated as reported previously (13). Nutrient broth, commercially available from Eiken Chemical (Tokyo, Japan),

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.* (14). Ampicillin sodium salt and isopropyl-β-D-thiogalactopyranocide (IPTG) were added to the media at final concentrations of 100 μg/ml and 100 μM, respectively, if necessary. The cloning vector pUC119 was obtained from Takara Shuzo Co. (Shiga, Japan).

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).

DNA manipulations. Total DNA from strain DBF63 was prepared as reported previously (13). Plasmid DNAs were prepared by the method of Birnboim (15) and purified as described by Sambrook *et al.* (14). *E. coli* cells were transformed with plasmids according to the method of Hanahan with some modifications (16). DNA fragments were purified using a Geneclean II kit (Bio 101, Vista, CA) as indicated by the manufacturer. Other DNA manipulations were carried out as described by Sambrook *et al.* (14), and commercially available enzymes and kits were used as recommended by the respective manufacturers.

Polymerase chain reaction (PCR). Degenerated primers for amplifying the DNA fragments containing the region homologous to angular dioxygenase genes were designed on the basis of the conserved amino acid sequence for Rieske [2Fe-2S] cluster in the large subunits of oxygenase components. Two degenerated primers, RieskeF (5′-TGYCGBCAYCGBGGSAWG-3′) and RieskeR (5′-CCAGCCGTGRTARSTGCA-3′) were synthesized by commercial service (Cruachem, Kyoto, Japan). PCR was carried out with Ampli*Taq* Gold (PE Biosystems, Urayasu, Japan) and GeneAmp PCR System 9700 (PE Biosystems). Appropriate negative controls were included in each experiment. The cycling conditions for PCR amplification were as follows: 96°C for 10 min; followed by 40 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min; then by 72°C for 10 min, followed by cooling down to 4°C. After the ligation of resultant PCR product to the pT7Blue T-vector (Clontech Lab. Inc., Palo Alto, CA), the nucleotide sequence of PCR products were determined, and the deduced amino acid sequences were verified for the conserved amino acid residues and frames of codons.

Cloning of the DNA fragments containing genes encoding iron-sulfur protein. Total DNA of strain DBF63 was partially digested with *Bam*HI to obtain DNA fragments from 10 to 40 kb. The DNA fragments ranging from 20 to 30 kb were extracted from an agarose gel following electrophoresis of the digested DNA, purified and ligated into a cosmid vector SuperCos1 (17) which had been previously digested with *Xba*I, treated with calf intestine alkaline phosphatase, and then digested with *Bam*HI. The resultant constructs were packaged into λ phage with Gigapack III Packaging Extracts XL (Stratagene) to infect the *E. coli* host strain. To screen for positive clones carrying DNA fragments containing the region homologous to PCR product, colony hybridization experiments were carried out as described previously (13). The hybridizing probe was prepared from the PCR product of 78-bp products and labeled using Megaprime DNA labeling system (Amersham Pharmacia Biotech, Chalfont, Buckinghamshire, England) with [α-^{32}P]dCTP (3000 Ci/mol, ICN Biomedicals, Costea, CA). Obtained positive clones were checked for the DNA fragment that was hybridized with the probe. A restriction map of a 40-kb DNA region including angular dioxygenase genes was constructed by using several restriction enzymes.

Resting cell reactions and analysis of the products. *E. coli* cells harboring appropriate plasmids used for resting cell reaction were grown on 100 ml of LB broth supplemented with ampicillin in a 500-ml Sakaguchi flask at 30°C on a reciprocal shaker at 120 strokes per min. IPTG was added to induce the gene located down stream of the *lac* promoter, if necessary. The culture broth was centrifuged (4000*g*), and the pelleted cells were washed twice with 100 ml of CFMM buffer (containing 2.2 g of Na_2HPO_4, 0.8 g of KH_2PO_4, and 3.0 g of NH_4NO_3 per liter of distilled water) and resuspended in the same buffer to an optimum density (O.D.) 600 of approximately 10. To 5 ml of the resting cell suspension in test tubes (18 mm in diameter), each substrate from stock solutions dissolved in dimethyl sulfoxide (1 mg/ml) was added to a final concentration of 1 mM, and the tubes were incubated at 30°C for 18 h. The reaction mixtures were directly extracted with 5 ml of ethyl acetate. The extracts were analyzed by gas chromatography-mass spectrometry (GC-MS) after derivatization with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA). GC-MS analyses were performed as described previously (8, 13).

SDS–PAGE. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed by the method of Laemmli (18), using 13% polyacrylamide gels. After the electrophoresis, protein staining of the gels was stained with Coomassie brilliant blue R-250.

Nucleotide sequencing and phylogenetic analyses. Nucleotide sequencing was carried out using Dye-Terminator Cycle Sequencing Kit (PE Biosystems) and 373A DNA Sequencing System (PE Biosystems). A series of unidirectionally deleted DNA fragments for the sequencing reaction were constructed using Kilo-sequence deletion kit (Takara Shuzo). The nucleotide sequence of PCR products was determined using a pT7Blue T-vector clone (Clontech) of PCR products as a template. The results of sequencing were analyzed using DNASIS ver. 3.7 (Hitachi Software Engineering, Yokohama) and FramePlot 2.3 (19). Homology was searched using Swiss-Prot and DDBJ, EMBL, and GenBank databases. Multiple sequence alignment was carried out using Clustal W ver. 1.8 (20), and a phylogenetic tree was constructed using PHYLIP ver. 3.5c (21), by the neighbor-joining method (22). The nucleotide sequence data reported in this article have been submitted to the DDBJ, EMBL, and GenBank nucleotide sequence databases under the accession number AB054975.

RESULTS

PCR Amplification and Detection of the Genes Encoding Terminal Oxygenase Components for Ring Hydroxylating Dioxygenases

The gene encoding a large subunit of oxygenase component in strain DBF63 was presumed to be homologous to either gene for large subunits of oxygenases involved in PAH degradation, because strain DBF63 could metabolize FN, phenanthrene and anthracene, and cometabolize biphenyl and naphthalene. Therefore, we tried to clone the genes directly by PCR-based method. Major members of the genes for PAH-degradation were included in multiple alignment of nucleotide sequence for oxygenase to design a set of degenerated PCR primers. Resultant multiple alignment for the amino acid sequences of large subunits of terminal oxygenase involved in the degradation of the compounds containing more than two aromatic rings revealed the existence of two conserved regions in Rieske-type [2Fe-2S] cluster-binding sites [CRHRG(K/M)X_{14}C(S/T)YHGW; X, any amino acid]. Therefore, a set of degenerated primers was designed to amplify nucleotide sequence for Rieske [2Fe-2S] binding sites in oxygenase components.

The DNA fragment smaller than 100 bp was obtained by PCR amplification and nucleotide sequence

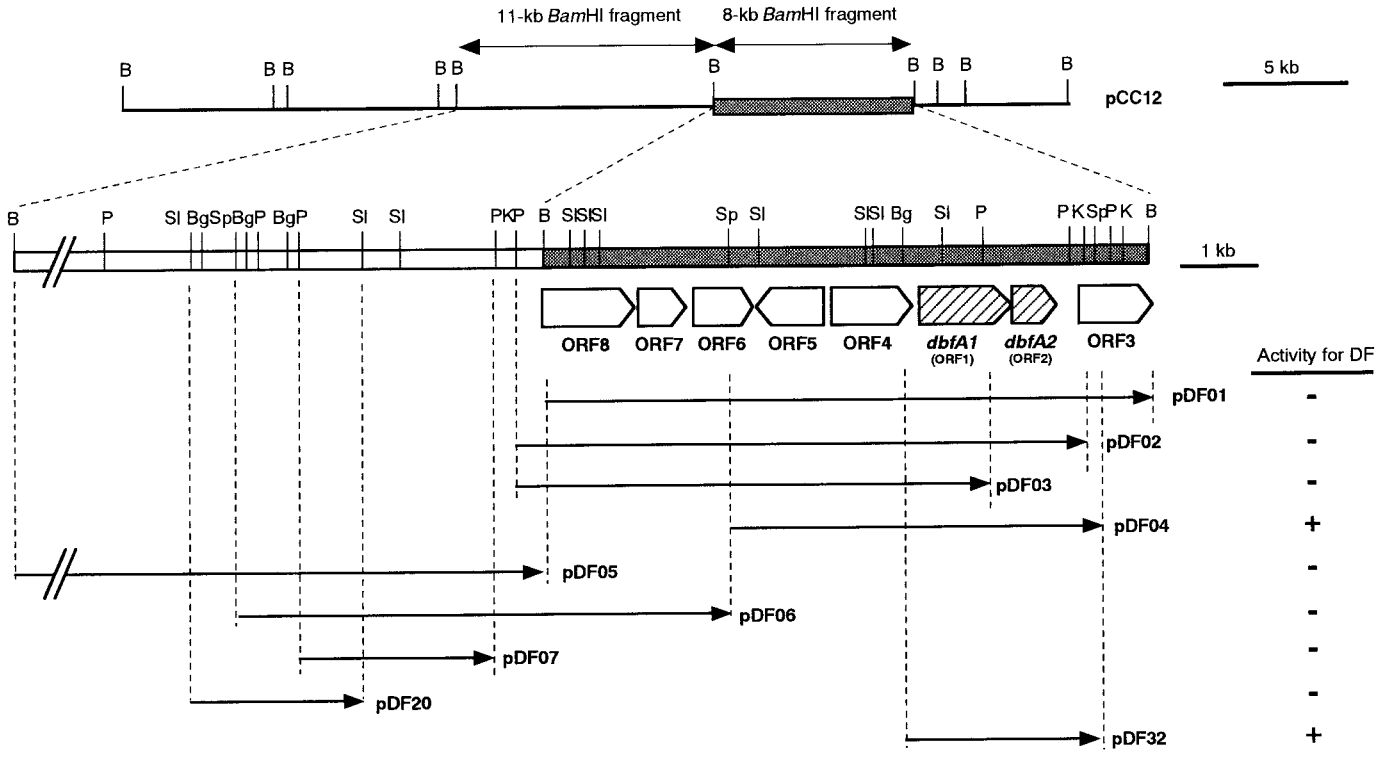

FIG. 2. Physical map of the 19-kb DNA region containing two ring-hydroxylating dioxygenases from *Terrabacter* sp. strain DBF63. The pentagons in the physical map indicate the size, location, and the direction of transcription of the ORFs. The solid arrows indicate the direction of transcription from the *lac* promoter of the cloning vector pUC119. pCC12 is one of cosmid clones shown. The presence (+) or absence (−) of angular dioxygenase activity for dibenzofuran is shown at the right. Restriction site designations: B, *Bam*HI; Bg, *Bgl*II; K, *Kpn*I; Sl, *Sal*I; Sp, *Sph*I; P, *Pst*I.

of the PCR product was determined. The size of the PCR product was 78 bp, indicating that the two primer regions were separated in frame. The nucleotide sequences of four PCR products determined using the pT7Blue T-vector, were completely identical. The deduced amino acid sequence of this frame contained no termination codon and showed the highest identity (69.2%) with BphA1 from *Pseudomonas* sp. strain KKS102 (23).

When the PCR product was used for the probe in a Southern blotting experiment of *Bam*HI-digested total DNA of strain DBF63 at a high stringency, a strong signal at a size of approximately 8-kb fragment was detected together with a weaker signal around 11 kb and a slight signal at 2.5 kb (data not shown). This result indicated that strain DBF63 contains at least three homologous genes encoding the components of ring-hydroxylating dioxygenase.

Cloning of the Gene Involved in the Angular Dioxgenation of DF

A cosmid library of the genomic DNA from strain DBF63 was constructed, and out of approximately 3000 of the resultant colonies, more than 30 positive clones having the sequence homologous to the PCR product were obtained. Twenty cosmid clones were selected for subjected to restriction mapping and hybridization analysis. As a result, nineteen of 20 cosmid clones were found to contain both of the above mentioned 8- and 11-kb *Bam*HI fragments containing proposed terminal oxygenase genes. Mapping of these cosmid inserts revealed that the two *Bam*HI fragments were linked to each other as shown in Fig. 2. The DNA fragment corresponding to a slight 2.5-kb signal in the Southern blotting experiment could not be obtained in this study.

The angular dioxygenase activity of these cosmid clones for DF to produce THB was tested in resting cell reaction and GC-MS analysis of the metabolite, but no cosmid clone exhibited the activity. Therefore, within the region of the linked 8- and 11-kb *Bam*HI fragments, a series of subclones designated as pDF01 to pDF07, pDF20, and pDF32 were constructed using vector pUC119 with *lac* promoter (Fig. 2). As all these constructs were hybridized with the PCR products, they were thought to contain the whole or part of the region of the large subunit of the terminal oxygenase component. From hybridization analysis, the conserved regions of Rieske [2Fe-2S] cluster binding site in large subunit were revealed to exist in approxi-

TABLE 1

GC Retention Times and MS Spectral Properties of Major Compounds Formed from Dibenzofuran and Its Analogs by Resting Cell Reactions of *E. coli* Carrying DFDO

Substrate	RT[a] (min)	Principle fragment ions[b]	Relative conversion rate (%)[c]	Identified or possible product[d]
Dibenzofuran	10.1	418 (M^+, 34), 315 (67), 73 (100)	100	2,2′,3-Trihydroxybiphenyl
Dibenzo-*p*-dioxin	10.7	434 (M^+, 23), 331 (49), 166 (15), 73 (100)	98.7–99.6	2,2′,3-Trihydroxydiphenyl ether
Fluorene	8.9	254 (M^+, 88), 239 (73), 165 (100), 74 (52)	97.7–102	9-Fluorenol
9-Fluorenone	10.3	358 (M^+, 34), 343 (6), 270 (17), 253 (47), 223 (5), 180 (9), 147 (51), 73 (100)	79.4–84.2	1-Hydro-1,1a-dihydroxy-9-fluorenone or 1,2-Dihydro-1,2-dihydroxy-9-fluorenone

[a] All products were analyzed by GC-MS after derivatization of MSTFA.
[b] Fragment ions are expressed as *m/z* values; relative intensities expressed as percentages are given in parentheses.
[c] Relative conversion rate was calculated using the conversion rate of dibenzofuran as 100%.
[d] Possible products of 9-fluorenone were suggested based on the results reported by Monna *et al.* (1993).

mately 2.5-kb *Bgl*II-*Sph*I region within the 8-kb *Bam*HI fragment (Fig. 2, pDF32) and 850-bp *Pst*I-*Sal*I region within 11-kb *Bam*HI fragment (Fig. 2, the region common to pDF07 and pDF20). Then, to investigate whether *E. coli* harboring these constructs catalyze the angular dioxygenation of DF, we performed expression experiments in the presence of IPTG. As a result, *E. coli* harboring pDF04 exhibited activity to produce a small amount of THB, and *E. coli* harboring pDF32 showed strong activity for DF (Fig. 2). In both cases, the mass spectrum having the principle fragment ions at *m/z* 418 (M^+, 34), 315 (67), and 73 (100) was detected by GC-MS analysis of the product from DF (relative intensity expressed as percentage is given in parentheses) (Table 1). This fragmentation pattern and the retention time (RT) of the trimethylsilyl (TMS) derivative of the metabolite were identical to those of THB (13). This confirmed that an approximately 2.5-kb *Bgl*II-*Sph*I fragment contained the components of DFDO, which was capable of converting DF to THB by incorporating two oxygen atoms into an angular position and the adjacent position of the DF molecule. However, *E. coli* JM109 harboring pDF01 and pDF02 containing a larger fragment including the whole region of the 4-kb *Sph*I fragment did not exhibit any activities of angular dioxygenation. In addition, *E. coli* JM109 harboring pDF05 and pDF06, which were thought to contain the whole region of another large subunit, did not show angular dioxygenase activity for DF (Fig. 2).

Nucleotide Sequence of the Gene Encoding the Angular Oxygenase Component

Sequence analysis revealed that the 2.5-kb *Bgl*II-*Sph*I fragment contained two possible ORFs (ORF1 and ORF2), that had the same orientation (Fig. 2). The deduced amino acid sequence of ORF1 showed the highest identity (38%) with NidA from *Rhodococcus* sp. strain I24 [Accession No. AF121905] which is a large subunit of terminal oxygenase component of indene dioxygenase, and shared homology with amino acid sequences of the large subunit of oxygenases involved in the degradation of polycyclic aromatic hydrocarbons (PAHs) [36–37% identity with PhdA from *Nocardioides* sp. strain KP7 (No. AB017794), and NarAa from *Rhodococcus* sp. strain NCIMB12038 (No. AF082663)]. The consensus sequence of Rieske-type iron sulfur proteins for the binding of a [2Fe-2S] cluster ($CXHX_{16-17}CXXH$) (24) was found in the deduced amino acid sequence of ORF1. The critical motif $DX_2HX_{4-5}H$, which was considered to act as a mononuclear iron ligand at the site of oxygen activation (25), was also conserved in the deduced amino acid sequence of ORF1 (data not shown).

The deduced amino acid sequence of ORF2, which was found just downstream of ORF1, showed homology with the amino acid sequence of the small subunit of the oxygenase involved in the hydroxylation of aromatic ring compounds. The deduced amino acid sequence of ORF2 showed the highest identity of 40% with BphA2 from *R. erythropolis* strain TA421 (No. D88020) which is a small subunit of terminal oxygenase component of biphenyl dioxygenase, and shared homology with amino acid sequences of the small subunit of oxygenases involved in the degradation of monoaromatics [34–36% identity with BnzB from *P. putida* (No. P08085), TodC2 from *P. putida* strain F1 (No. Y18245), McbAb from *Ralstonia* sp. strain JS705 (No. AJ006307), BedC2 from *P. putida* strain ML2 (No. AF148496), and IpbA2 from *R. erythropolis* strain BD2 (No. U24277)].

From these findings, the products of two ORFs were suggested to be involved in the initial dioxygenation of DF molecule, thus ORF1 and ORF2 were designated as *dbfA1* and *dbfA2*, respectively.

Detection of the Products of DbfA1 (ORF1) and DbfA2 (ORF2)

To confirm whether these genes could be translated to polypeptides with the predicted sizes in *E. coli* cell,

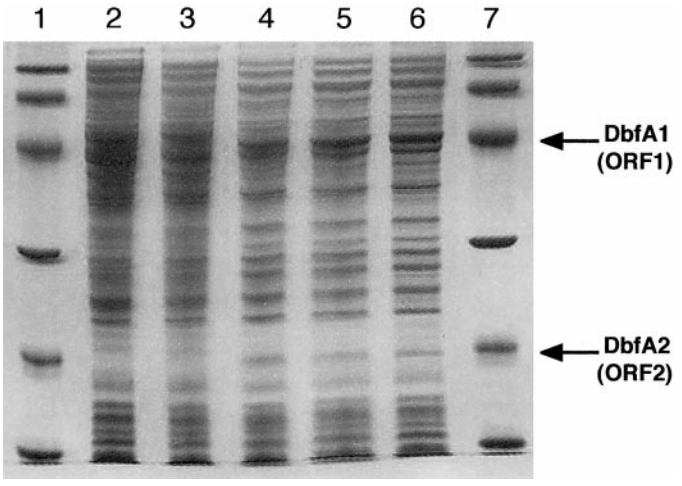

FIG. 3. Detection of products of DbfA1 (ORF1) and DbfA2 (ORF2) expressed in *E. coli* JM109. Total cellular proteins of *E. coli* strains were analyzed by SDS–PAGE. Lanes: 1 and 7, molecular mass standards of 96,000, 67,000, 43,000, 30,000, 20,100, and 14,400 (top to bottom); 2, JM109 (pUC119); 3, JM109 (pDF32) IPTG 0 mM; 4, JM109 (pDF32) IPTG 0.1 mM; 5, JM109 (pDF32) IPTG 0.5 mM; 6, JM109 (pDF32) IPTG 1 mM.

the cellular extract from the recombinant *E. coli* cells harboring a plasmid containing ORF1 and ORF2 (pDF32) (Fig. 2) were loaded on SDS-PAGE. As shown in Fig. 3, the results indicate that the products of ORF1 (49-k) and ORF2 (20-k) which corresponds to the predicted molecular mass of ORF1 (49,518) and ORF2 (19,761), respectively were expressed in the presence of IPTG at final concentrations of 0.1, 0.5, and 1 mM.

Substrate Specificity of DbfA1A2

By means of the resting cell reaction using *E. coli* harboring pDF32, the conversion of other aromatic compounds by DFDO was investigated (Table 1). *E. coli* harboring pUC119 was used as a control. When DD was added as a substrate in transformation experiments, we detected a product exhibiting fragment ions at *m/z* 434 (M^+, 23), 331 (49), 166 (15), and 74 (100). This fragmentation pattern and the RT of the TMS derivative of the metabolite were the same as those observed for the TMS derivatives of THDE (13). In addition, FN was converted to a compound having the fragment ions at *m/z* 254 (M^+, 88), 239 (73), 165 (100), and 74 (52) and the RT of 8.9 min. These data were identical to those of the TMS derivative of the authentic 9-fluorenol, which is metabolic intermediate in FN degradation of strain DBF63 (6). Also, 9-fluorenone was converted to a compound exhibiting major fragment ions at *m/z* 358 (M^+, 34), 253 (47), 147 (51), and 73 (100). This fragmentation pattern and relative intensity were identical with those of the metabolic intermediate of FN (designated FN-P5) reported by Monna *et al.* (1993), which is determined to be 1-hydro-1,1a-dihydroxy-9-fluorenone or 1,2-dihydro-1,2-dihydroxy-9-fluorenone. On the other hand, it was revealed by GC-MS analysis that CAR, xanthene, and phenoxathiin were not converted to 2′-aminobiphenyl-2,3-diol, 2,2′,3-trihydroxydiphenylmethane, and 2,2′,3-trihydroxydiphenyl sulfide, respectively, by this angular dioxygenase. Moreover, no oxygenation products were detected when PAHs, such as naphthalene, phenanthrene, and anthracene, were used as substrates.

Homology Analysis of the Neighboring Region of dbfA1A2 Genes

The genes for the electron transport proteins, ferredoxin and ferredoxin reductase, that transport electron from NAD(P)H to the oxygenase component, were not found within the 2.5-kb *Bgl*II-*Sph*I region. In many cases, these components of dioxygenase are clustered with the terminal oxygenase gene. Therefore, the nucleotide sequence of an approximately 8-kb *Bam*HI fragment containing *dbfA1A2* was determined. Open reading frame analysis of the region revealed the existence of 8 additional ORFs (Fig. 2).

(i) Putative hydrolase and dehydrogenase genes (ORF3 and ORF4). Although the nucleotide sequence of the 3′-terminal region has not been determined completely (about 30-bp lack), the deduced amino acid sequence of ORF3, which was located just downstream region of *dbfA1A2* genes, revealed to be closely related to *meta*-cleavage compound hydrolase involved in the degradation of monoaromatic, such as OhpC from *Rhodococcus* sp. strain V49 [3-(2-hydroxybiphenyl)propionic acid degradation] (No. AF274045), CmtE from *P. putida* strain F1 (*p*-cumate degradation) (No. U24215), EtbD1 from *Rhodococcus* sp. strain RHA1 (ethylbenzene degradation) (No. AB004320), and NahN from *P. stutzeri* strain AN10 (catechol degradation) (No. AF039534), showing 38, 35, 33, and 32% identity, respectively. These homologies strongly indicated that ORF3 encodes the *meta*-cleavage compound hydrolase, and a conserved pentapeptide motif of serine hydrolase is also conserved (GXSXG) (data not shown). On the other hand, the deduced amino acid sequence of ORF4, which was located just upstream of the *dbfA1* and *dbfA2* genes, exhibited homology to acetoacetyl-CoA reductase (PhbB) from *Pseudomonas* sp. strain 61-3 (No. AB014757) (33% identity), and alcohol dehydrogenase (CymB) from *P. putida* strain F1 (No. U24215) (32% identity). However, ORF4 did not show homology to dihydrodiol dehydrogenase involved in the aromatic compound degradation.

(ii) Two putative regulatory genes (ORF5 and ORF6). The deduced amino acid sequences of ORF5 and ORF6, that had the opposite direction of transcription (Fig. 2), showed homology to transcriptional regulators. Com-

parison of the deduced amino acid sequence of ORF5 with those of the members of LysR-type transcriptional regulators, such as TfdT of *R. eutropha* strain JMP134 (No. U16782), ClcR of *Ralstonia* sp. strain JS705 (No. AJ006307), and AphT of *Comamonas testosteroni* strain TA441 (No. AB029044) revealed 32, 27, and 26% identity, respectively. The homology was the highest in the N-terminal helix-turn-helix motif which is presumed to be the DNA-binding region of these proteins (26) (data not shown). On the other hand, the predicted gene products of ORF6 shared 27-28% identity with those of PcaR of *Rhodococcus opacus* strain 1CP (No. AF003947) and *P. putida* (No. AJ252090), 28% with those of KdgR of *Erwinia chrysanthemi* (No. X62072), and 26% with those of IclR of *E. coli* (No. M31761). All these proteins belong to the IclR family of regulators, and these are activators of protocatechuate catabolic gene cluster (*pca*) from the above two strains (27, 28), repressor of the pectin catabolism pathway genes (29), and repressor of the acetate operon (30), respectively. The ORF6 product possessed possible helix-turn-helix, DNA-binding motif in the N-terminus, and a conserved region in the C-terminus that corresponds to the signature pattern for the IclR family of proteins: $[G/A]X_3[D/S]X_2EX_6[C/S/A][L/I/V/M][G/S/A]X_2[L/I/V/M]$-$[F/Y/H][D/N]$ (31), except for Ala222 and Ser223 in ORF6 product (data not shown). The putative helix-turn helix and IclR family signature motifs are located in ORF6 product between residues 42-61 (LTDVSKYLGVASSTAHRLLA) and 202-223 (GFATSKEESEEGVASLAVALAS), respectively (data not shown).

(iii) Ferredoxin reductase genes (ORF8). The deduced amino acid sequence of ORF8 exhibited homology (37–39% identity) to the ferredoxin reductase of the ring-hydroxylating dioxygenases such as BphA4 from *R. erythropolis* strain TA421 (biphenyl degradation) (No. D88021) and PhdD from *Nocardioides* sp. strain KP7 (phenanthrene degradation) (No. AB017795), although the nucleotide sequence of the 5′-terminal region has not been determined completely. The putative ADP-binding sites of FAD (FAD-binding sites) and NAD (NAD-binding sites) in the ferredoxin reductase were predicted from amino acid sequences in comparison with the proteins of the reductase family. The consensus sequences of the putative FAD- and NAD-binding site (32) are conserved in the deduced amino acid sequence of ORF8 with the completely conserved motif $GXGX_2GX_3A$ (33) (data not shown).

(iv) Other gene (ORF7). 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).

DISCUSSION

In this study, by PCR method with Rieske primers, we successfully cloned the *dbfA1* and *dbfA2* genes, which is the first example of the genes encoding terminal oxygenase components of angular dioxygenase from gram-positive bacteria. Although sequence analysis revealed that the insert of pDF32 (2.5-kb *Bgl*II-*Sph*I fragment) contained neither the gene encoding ferredoxin nor that encoding ferredoxin reductase of DFDO, *E. coli* cells harboring pDF32 exhibited angular dioxygenase activity for DF (Fig. 2). Until now, there were some examples showing that the absence of the electron transporting proteins was complemented with the presence of some electron transporters derived from *E. coli* host cells, e.g., incomplete naphthalene dioxygenases from *P. putida* strain NCIB9816 (34), isopropylbenzene dioxygenases from *P. putida* strain RE204 (35), phenanthrene dioxygenase from *Burkholderia* sp. strain RP007 (36), and toluene-4-monooxygenase from *P. mendocina* strain KR1 (37) (these were encoded by recombinant plasmids lacking the ferredoxin and/or ferredoxin reductase genes). In DFDO from strainDBF63, also angular dioxygenase activity was achieved with the complementation of nonspecific ferredoxin and ferredoxin reductase component of *E. coli*.

Phylogenetic analysis revealed that DbfA1 did not cluster with most of the known large subunits of dioxygenases but formed a branch with recently reported large subunits of PAH dioxygenase from gram-positive bacteria, i.e., NidA from *Rhodococcus* sp. strain I24 (38), PhdA from *Nocardioides* sp. strain KP7 (39), and NarAa from *Rhodococcus* sp. strain NCIMB12038 (No. AF082663) (Fig. 4). On the other hand, phylogenetic analysis of the small subunit, DbfA2, did not give a cluster similar to that for large subunits, i.e., DbfA2 did not form a cluster with NidB from strain I24 (38) and PhdB from strain KP7 (39), but with BphA2 from *Rhodococcus* sp. strains TA421 (No. 88020) and RHA1 (40), and TodC2 from *P. putida* strain F1 (41), that are small subunits of biphenyl or monoaromatics dioxygenases (data not shown). Whether this phylogenetic difference between DbfA1 and DbfA2 has a physiological or evolutionary significance remains unclear.

The substrate specificity of DbfA1A2 was investigated and compared with those of the well-investigated angular dioxygenases. DFDO (dioxin dioxygenase), which was purified from *Sphingomonas* sp. strain RW1, was reported to catalyze the angular dioxygenation of DF and DD but not of CAR, and this enzyme could not oxidize PAHs such as naphthalene (7). Similar to DFDO from strain RW1, DbfA1A2 also catalyzed the angular dioxygenation of DF and DD but not of CAR, and produced no oxygenation products of PAHs, such as naphthalene, phenanthrene and anthracene. It converted 9-fluorenone to a compound having major fragment ions at *m/z* 358 (M^+, 34), 253 (47),

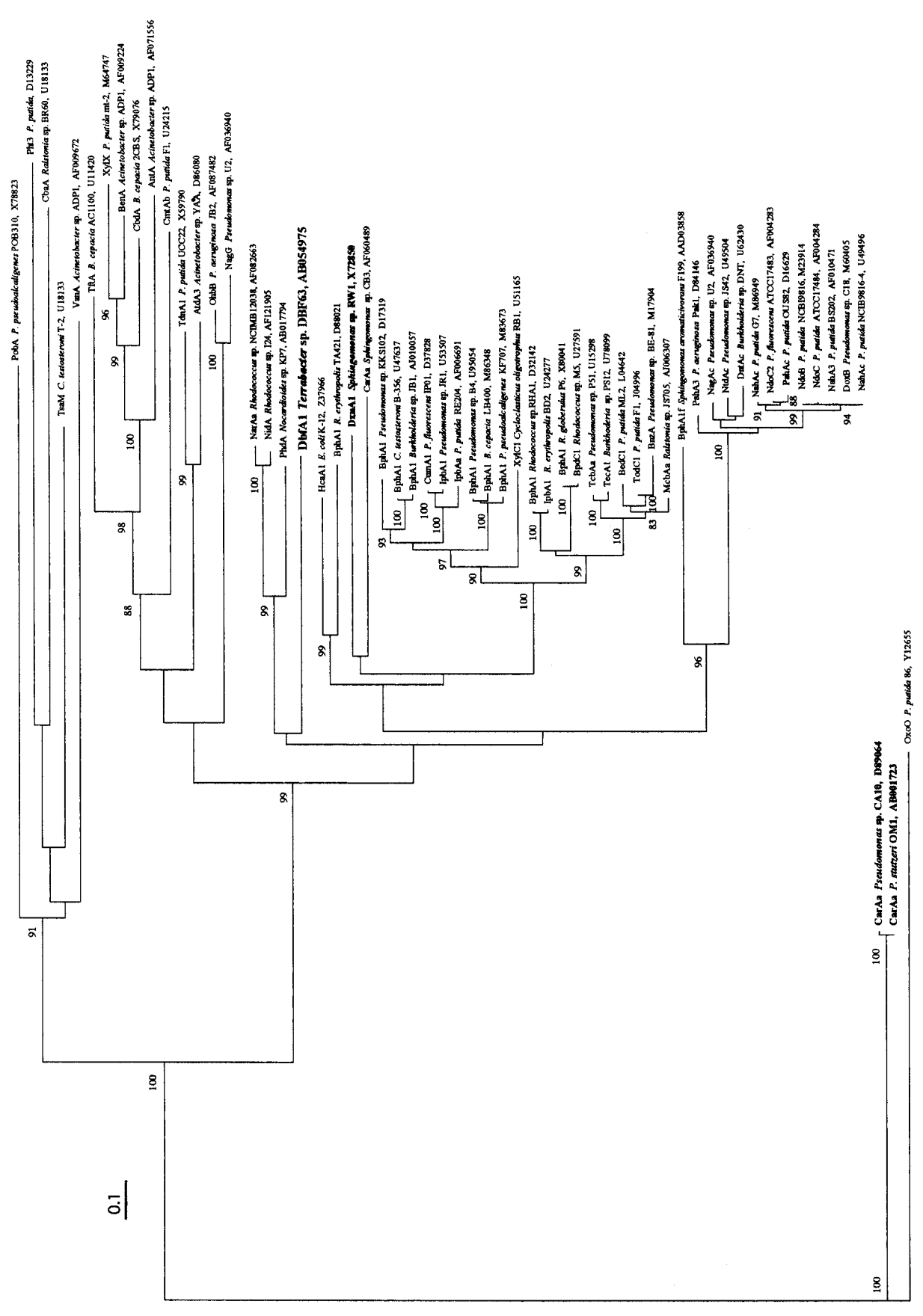

FIG. 4. Phylogenetic tree of large subunits from ring-hydroxylating dioxygenases based on the multiple alignment of related proteins. The phylogenetic affiliation was calculated using PHYLIP ver. 3.5c (21), by the neighbor-joining method (22). The numbers on the branches represent percent confidence of 100 replicate analysis. The scale bar indicates percent divergence. Bold characters indicate angular dioxygenases, which have already been characterized for their enzyme activity.

147 (51), and 73 (100) by DbfA1A2 (Table 1). This fragmentation pattern was identical with that of a metabolite (designated FN-P5), which was determined to be 1-hydroxy-1,1a-dihydroxy-9-fluorenone or 1,2-dihydro-1,2-dihydroxy-9-fluorenone, by Monna *et al.* (1993). Considering the facts that DbfA1A2 apparently showed angular dioxygenase activity and did not exhibit *cis*-dihydroxylase activity toward FN, naphthalene, phenanthrene and anthracene, this metabolite converted by DbfA1A2 was speculated to be 1-hydroxy-1,1a-dihydroxy-9-fluorenone. Also, by DFDO from strain RW1, 9-fluorenone was converted to the compound, which was presumed to be *cis*-diol derivative of 9-fluorenone (7). The above results indicated that the substrate specificity of DFDO from strain DBF63 is very similar to DFDO from strain RW1. However, while FN was not oxygenated by DFDO from strain RW1 (7), DbfA1A2 could convert FN to 9-fluorenol. This finding showed that DFDO from strain DBF63 could catalyze the monooxygenation of the methylenic group of FN. On the other hand, the substrate specificity of DbfA1A2 was quite different from that of another type of angular dioxygenase, CarAaAcAd from *Pseudomonas* sp. strain CA10 (11). Both DbfA1A2 and CarAaAcAd could catalyze the angular dioxygenation of DF and DD, and the monooxygenation of FN. However, different from DbfA1A2, CarAaAcAd could catalyze the angular dioxygenation of CAR, xanthene, and phenoxathiin, and the *cis*-dihydroxylation of PAHs including naphthalene, anthracene, and fluoranthene (8). These results suggested that the amino acid residues involved in the substrate recognition were probably different among these angular dioxygenases.

In our previous study, strain DBF63W, which is a derivative of strain DBF63, was obtained by the continuous culture of wild-type strain on nutrient broth. This mutant utilized salicylic acid, but not DF as sole sources of carbon and energy (13). We reported that in Southern blotting analysis using a DNA fragment containing *dbfBC* genes as a probe, no signals were detected in the total DNA from strain DBF63W, whereas reasonably strong signals were detected in the total DNA from wild-type strain DBF63 (13). Similarly, in Southern blotting analysis using the PCR product as a probe, by which *dbfA1* gene could be cloned, no signals were detected in the *Bam*HI-digested total DNA from strain DBF63W, whereas a strong signal at a size of approximately 8-kb (DNA fragment containing both *dbfA1* and *dbfA2* genes), a weaker signal around 11-kb (DNA fragment containing another multicomponent dioxygenase genes) and a slight signal at 2.5-kb were detected in the *Bam*HI-digested total DNA from wild-type strain (data not shown). These results indicate a possibility that DbfA1A2 as well as DbfBC is involved in the degradation of DF by strain DBF63.

We also performed homology analysis of the neighboring region of the *dbfA1A2* gene. In the 3.8-kb upstream region of the *dbfA1A2* gene, we found the ORF8 which was homologous to the genes encoding ferredoxin reductase component of multicomponent dioxygenase. Although there is a possibility that DbfA1A2 is complemented with the product of ORF8, two putative regulatory genes that had the opposite direction of transcription exist between *dbfA1A2* gene and ORF8. This fact indicates that *dbfA1A2* gene and ORF8 are located on different transcriptional units. In addition, based on hybridization analysis, another large subunit of multicomponent dioxygenase system was thought to exist around 850-bp *Pst*I-*Sal*I fragment (Fig. 2, region common to pDF07 and pDF20). Therefore, the product of ORF8 is considered to function with another terminal oxygenase component. We are currently carrying out sequencing analyses of both upstream and downstream regions of 8-kb *Bam*HI fragment to search for ferredoxin and ferredoxin reductase component of DFDO, and to reveal the genetic structure of another dioxygenase system. These studies will reveal the function of these two dioxygenase systems and the relationship between them.

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