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Plasmid pCAR3 Contains Multiple Gene Sets Involved in the Conversion of Carbazole to Anthranilate†

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The carbazole degradative *car-I* gene cluster (*carAaIBaIBbICIAcI*) of *Sphingomonas* sp. strain KA1 is located on the 254-kb circular plasmid pCAR3. Carbazole conversion to anthranilate is catalyzed by carbazole 1,9a-dioxygenase (CARDO; CarAaIAcI), *meta*-cleavage enzyme (CarBaIBbI), and hydrolase (CarCI). CARDO is a three-component dioxygenase, and CarAaI and CarAcI are its terminal oxygenase and ferredoxin components. The *car-I* gene cluster lacks the gene encoding the ferredoxin reductase component of CARDO. In the present study, based on the draft sequence of pCAR3, we found multiple carbazole degradation genes dispersed in four loci on pCAR3, including a second copy of the *car* gene cluster (*carAaIIBaIIBbIICIIAcII*) and the ferredoxin/reductase genes *fdxI-fdrI* and *fdrII*. Biotransformation experiments showed that FdrI (or FdrII) could drive the electron transfer chain from NAD(P)H to CarAaI (or CarAaII) with the aid of ferredoxin (CarAcI, CarAcII, or FdxI). Because this electron transfer chain showed phylogenetic relatedness to that consisting of putidaredoxin and putidaredoxin reductase of the P450cam monooxygenase system of *Pseudomonas putida*, CARDO systems of KA1 can be classified in the class IIA Rieske non-heme iron oxygenase system. Reverse transcription-PCR (RT-PCR) and quantitative RT-PCR analyses revealed that two *car* gene clusters constituted operons, and their expression was induced when KA1 was exposed to carbazole, although the *fdxI-fdrI* and *fdrII* genes were expressed constitutively. Both terminal oxygenases of KA1 showed roughly the same substrate specificity as that from the well-characterized carbazole degrader *Pseudomonas resinovorans* CA10, although slight differences were observed.

Carbazole is an N-heterocyclic aromatic compound derived from coal tar and shale oil (26) and is known to possess mutagenic and toxic activities (2, 16). To remediate carbazole-contaminated environments using biotechnological approaches, a wide variety of carbazole-degrading bacteria have been isolated and characterized (13, 14, 18, 28). Among them, the carbazole-catabolic *car* genes of *Pseudomonas resinovorans* CA10 (car_{CA10} genes) have been studied most extensively. Carbazole is first dioxygenated at the angular (C-9a) and adjacent (C-1) positions to yield an unstable *cis*-hydrodiol (Fig. 1) (28). Such initial dioxygenation is called an angular dioxygenation. Unstable *cis*-hydrodiol is spontaneously converted to 2′-aminobiphenyl-2,3-diol, which is further converted to anthranilate via *meta*-cleavage and hydrolysis (Fig. 1). Genes encoding carbazole 1,9a-dioxygenase (CARDO) were first cloned from CA10, and $CARDO_{CA10}$ was found to be a three-component system, in which NAD(P)H-dependent ferredoxin reductase (CARDO-R_{CA10}; $CarAd_{CA10}$ monomer) and ferredoxin (CARDO-F_{CA10}; $CarAc_{CA10}$ monomer) transfer electrons from NAD(P)H to oxygenase (CARDO-O_{CA10}; $CarAa_{CA10}$ trimer) (Fig. 1) (25, 36). This multicomponent enzyme is a member of the Rieske non-heme iron oxygenase system (ROS). ROS members are classified into three classes and several subclasses based on the features of the electron transport chain (7). CARDO-R_{CA10} contains both a flavin adenine dinucleotide (FAD) and a plant-type [2Fe-2S] cluster, and CARDO-F_{CA10} is a ferredoxin having a Rieske-type [2Fe-2S] cluster (Rieske ferredoxin) (25). Thus, the $CARDO_{CA10}$ is classified in class III.

Sphingomonas sp. strain KA1 was isolated as a versatile carbazole-degrading bacterium (9) whose degradation pathway of carbazole is similar to that by CA10 (14). Previous study of the carbazole degradation (car_{KA1}) gene cluster of KA1 (14) revealed that, unlike the car_{CA10} gene cluster, the car_{KA1} gene cluster does not contain the CARDO-R gene but does contain the genes for CARDO-O (*carAa*) and CARDO-F (*carAc*), *meta*-cleavage enzyme (*carBaBb*), and *meta*-cleavage compound hydrolase (*carC*). Interestingly, although $CarAa_{KA1}$ shows high homology with $CarAa_{CA10}$ (60% identity), $CarAc_{KA1}$ had no relatedness with the Rieske ferredoxin, including $CarAc_{CA10}$, and showed similarity to the putidaredoxin-type ferredoxins. Because CARDO-O_{KA1} can receive electrons from CARDO-F_{KA1} and catalyze angular dioxygenation of carbazole (14), $CARDO_{KA1}$ consisting of $CarAa_{KA1}$ and $CarAc_{KA1}$ is likely to be assigned to class IIA. To confirm this consideration, we should definitely clarify all CARDO components, especially ferredoxin reductase CARDO-R, that are involved in carbazole metabolism by KA1.

In the genus *Sphingomonas*, it has been reported that cata-

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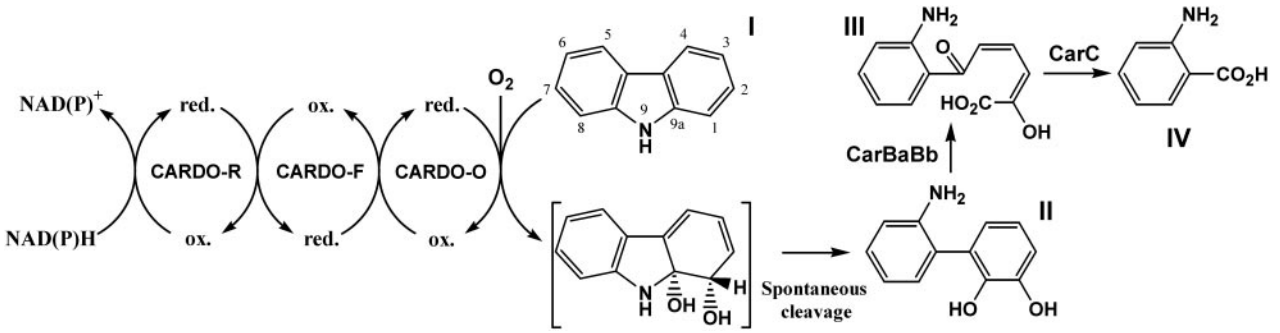

FIG. 1. Carbazole degradation by Car enzymes harbored by various carbazole degraders. The product of angular dioxygenation of carbazole shown in brackets is unstable and has not been detected directly. Enzyme (or protein) names: terminal oxygenase (CARDO-O), ferredoxin (CARDO-F), and reductase (CARDO-R) components of CARDO; *meta*-cleavage enzyme (CarBaBb); *meta*-cleavage compound hydrolase (CarC). ox. and red., oxidized and reduced states of the CARDO components, respectively. Compounds: I, carbazole; II, 2′-aminobiphenyl-2,3-diol; III, 2-hydroxy-6-oxo-6-(2′-aminobiphenyl)-hexa-2,4-dienoic acid; IV, anthranilic acid.

bolic genes are sometimes dispersed on the genome (4, 23). Considering that the car_{KA1} gene cluster is located on the 254-kb circular plasmid pCAR3 (9), it is possible that the ferredoxin reductase gene is also located on this plasmid. In the present study, using the draft sequence covering >99.9% of pCAR3, we found the multiple gene sets involved in carbazole degradation. In addition, based on the reverse transcription-PCR (RT-PCR) analysis of each $CARDO_{KA1}$ component gene and reconstitution assays of $CARDO_{KA1}$ components in *Escherichia coli* cells, we discuss the multiplicity of the carbazole degradation function of pCAR3.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 1. LB or 2×YT medium (35) was used for bacterial growth. To prepare the KA1 RNA, nitrogen-containing mineral medium NMM1 supplemented with carbazole or succinate was used. NMM1 has the same composition as CNFMM (29) except for the addition of 3.0 g of NH_4NO_3 per liter. Carbazole was added to NMM1 as described previously (29). *E. coli* JM109 (35) and DH5α (35) were used as hosts for pUC119 and its derivatives. Ampicillin (Ap) was added to selective media at a final concentration of 50 μg/ml. For plate cultures, the media mentioned above, solidified with 1.6% (wt/vol) agar, were used.

DNA manipulations. Total DNA of KA1 was prepared as described previously (29). Plasmids were prepared from *E. coli* by the alkaline lysis method (35) or with a Quantum Prep plasmid miniprep kit (Bio-Rad Laboratories, Hercules, CA). DNA fragments were extracted from agarose gels with an EZNA gel extraction kit (Omega Bio-tek, Inc., Doraville, GA). Other DNA manipulations were performed according to standard protocols (35).

Sequencing of pCAR3 and annotation. Shotgun sequencing of pCAR3 was performed by Dragon Genomics Co. Ltd. (Shiga, Japan). Open reading frames (ORFs) were found by DNASIS-Mac, version 3.7 (Hitachi Software Engineering Co. Ltd., Yokohama, Japan). Homologous sequences were searched from the DDBJ/EMBL/GenBank DNA databases using the BLAST program (version 2.2.10) (1). The deduced amino acid sequences of ORFs were aligned using CLUSTAL W (version 1.83) (15).

RNA preparation and RT-PCR. After the precultivation of KA1 in 5 ml of NMM1 supplemented with 10 mM succinate at 30°C, cells were gathered by centrifugation at 5,000 × *g* and then washed twice using CFMM (17). The washed cells were suspended in 500 μl of CFMM. Fifty microliters of the resultant cell suspension was added to 5 ml of NMM1 supplemented with 10 mM succinate, 10 mM each succinate and carbazole, or 10 mM carbazole (all media contained 1% [vol/vol] dimethyl sulfoxide [DMSO]). After a 2-h incubation with reciprocal shaking (300 strokes/min) at 30°C, the cells were harvested and used for extraction of total RNA by a NucleoSpin RNA II (Macherey-Nagel & Co., Düren, Germany) combined with RQ1 RNase-free DNase (Promega, Madison, WI). For RT-PCR, a One Step RNA PCR kit (AMV) (Takara Bio Inc.) was used. In RT-PCR, 100 ng of total RNA was used as a template. Detailed information on the RT-PCR primer sets and the conditions employed for respective gene amplifications are provided in Table S1 in the supplemental material. Control experiments without the addition of reverse transcriptase were also performed.

qRT-PCR. The primer sets used in quantitative RT-PCR (qRT-PCR) and RT conditions are provided in Table S1 in the supplemental material. To synthesize each cDNA, ThermoScript reverse transcriptase (Invitrogen Corp., Carlsbad, CA) and 10 ng of total RNA (as a template) were used. After the RT reaction, quantitative real-time PCR with SYBR GREEN PCR Master Mix (Applied Biosystems, Foster City, CA) was performed using the synthesized cDNA as a template on the ABI7700 sequence detection system (Applied Biosystems). The copy number of each mRNA was determined by a standard curve using a series of known concentrations of the target sequence according to the method of Habe et al. (10). For normalization, 16S rRNA of KA1 was used as an internal standard. For each sample, the mean value from triplicate real-time PCRs was used to calculate the transcript abundance. The mRNA levels of each gene in the control sample (NMM1 supplemented with succinate) were set at 1.0.

Construction of expression plasmids for *car* genes. Each of the *carAaI*, *carAaII*, *carAcI*, *carAcII*, *fdxI*, *fdrI*, and *fdrII* genes was separately amplified by PCR using the respective primer sets shown in Table S2 in the supplemental material, which were designed to introduce appropriate restriction sites and the effective Shine-Dalgarno sequence for the *E. coli* transcription system. In PCR amplification, total DNA of KA1 was used as a template. The amplified products were digested at the introduced restriction sites and were ligated into the corresponding sites of pUC119 to produce plasmids for the expression of single $CARDO_{KA1}$ components. After their nucleotide sequences were confirmed to be identical to those designed, their insert fragments were used for the construction of plasmids to direct the expression of each of the $CARDO_{KA1}$ components. For example, pUKA248 was constructed by cloning the 0.3-kb XbaI-KpnI fragment from pUKAcarAcI into the corresponding site of pUKAcarAaI. For the construction of pUKA249 and pUKA253, the 1.2-kb KpnI-EcoRI fragment from pUKAfdrII was ligated into the corresponding sites of pUKAcarAaI and pUKA248, respectively. Other plasmids were constructed similarly.

Biotransformation of carbazole by *E. coli* cells expressing putative CARDO components. *E. coli* JM109 harboring appropriate plasmids was cultivated in 5 ml of LB supplemented with Ap at 37°C with reciprocal shaking (300 strokes min^{-1}), and then 100 μl of the culture was transferred to 100 ml of the same medium. After cultivation at 30°C with shaking at 120 rpm until the optical density at 600 nm (OD_{600}) reached 0.4 to 0.5, isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to 0.5 mM and the cells further cultivated at 30°C for 15 h. Then the cells were harvested by centrifugation (5,000 × *g*, 10 min, 4°C), washed twice with CNF buffer (2.2 g Na_2HPO_4, 0.8 g KH_2PO_4 per liter), and resuspended in the same buffer to an OD_{600} of 12 to 13. Carbazole was dissolved at 100 mM in DMSO and 50 μl added to 5-ml cell suspensions in the reaction tube. After incubation on a reciprocal shaker (300 strokes min^{-1}) at 30°C for 20 h, the mixtures were extracted with an equal volume of ethyl acetate. The extracts were analyzed by gas chromatography-mass spectrometry (GC-MS) after derivatization with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) as described previously (27, 39). To define the conversion ratio, we used the following formula: conversion ratio (%) = 100 × (peak area for the TIC of the product)/(peak area for the TIC of the carbazole + peak area for the TIC of the product),

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristic(s)[a]	Reference or source
Bacterial strains		
E. coli JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*)/F$^-$[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*]	35
E. coli DH5α	*supE44* Δ*lacU169* (ϕ80 *lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	35
Sphingomonas sp. strain KA1	Car$^+$	9
Plasmids		
pUC119	Ap^r *lacZ*, pMB9 replicon, M13IG	35
pT7Blue(R)	Ap^r *lacZ*	Novagen
pUKAcarAaI	Ap^r; pUC119 with 1.2-kb SphI-XbaI DNA fragment containing the *carAaI* gene of strain KA1	This study
pUKAcarAaII	Ap^r; pUC119 with 1.2-kb SphI-XbaI DNA fragment containing the *carAaII* gene of strain KA1	This study
pUKAcarAcI	Ap^r; pUC119 with 0.3-kb XbaI-KpnI DNA fragment containing the *carAcI* gene of strain KA1	This study
pUKAcarAcII	Ap^r; pUC119 with 0.3-kb XbaI-KpnI DNA fragment containing the *carAcII* gene of strain KA1	This study
pUKAfdxI	Ap^r; pUC119 with 0.3-kb XbaI-KpnI DNA fragment containing the *fdxI* gene of strain KA1	This study
pUKAfdrI	Ap^r; pUC119 with 1.2-kb KpnI-EcoRI DNA fragment containing the *fdrI* gene of strain KA1	This study
pUKAfdrII	Ap^r; pUC119 with 1.2-kb KpnI-EcoRI DNA fragment containing the *fdrII* gene of strain KA1	This study
pUKA248	Ap^r; pUC119 with 1.2-kb SphI-XbaI fragment (*carAaI*) from pUKAcarAaI and 0.3-kb XbaI-KpnI fragment (*carAcI*) from pUKAcarAcI	This study
pUKA249	Ap^r; pUC119 with 1.2-kb SphI-XbaI fragment (*carAaI*) from pUKAcarAaI and 1.2-kb KpnI-EcoRI fragment (*fdrII*) from pUKAfdrII	This study
pUKA250	Ap^r; pUC119 with 1.2-kb SphI-XbaI fragment (*carAaI*) from pUKAcarAaI and 1.2-kb KpnI-EcoRI fragment (*fdrI*) from pUKAfdrI	This study
pUKA253	Ap^r; pUC119 with 1.5-kb SphI-KpnI fragment (*carAaI* and *carAcI*) from pUKA248 and 1.2-kb KpnI-EcoRI fragment (*fdrII*) from pUKAfdrII	This study
pUKA254	Ap^r; pUC119 with 1.5-kb SphI-KpnI fragment (*carAaI* and *carAcI*) from pUKA248 and 1.2-kb KpnI-EcoRI fragment (*fdrI*) from pUKAfdrI	This study
pUKA255	Ap^r; pUC119 with 1.2-kb SphI-XbaI fragment (*carAaII*) from pUKAcarAaII and 0.3-kb XbaI-KpnI fragment (*carAcII*) from pUKAcarAcII	This study
pUKA256	Ap^r; pUC119 with 1.2-kb SphI-XbaI fragment (*carAaII*) from pUKAcarAaII and 1.2-kb KpnI-EcoRI fragment (*fdrII*) from pUKAfdrII	This study
pUKA257	Ap^r; pUC119 with 1.2-kb SphI-XbaI fragment (*carAaII*) from pUKAcarAaII and 1.2-kb KpnI-EcoRI fragment (*fdrI*) from pUKAfdrI	This study
pUKA260	Ap^r; pUC119 with 1.5-kb SphI-KpnI fragment (*carAaII* and *carAcII*) from pUKA255 and 1.2-kb KpnI-EcoRI fragment (*fdrII*) from pUKAfdrII	This study
pUKA261	Ap^r; pUC119 with 1.5-kb SphI-KpnI fragment (*carAaII* and *carAcII*) from pUKA255 and 1.2-kb KpnI-EcoRI fragment (*fdrI*) from pUKAfdrI	This study
pUKA263	Ap^r; pUC119 with 1.2-kb SphI-XbaI fragment (*carAaI*) from pUKAcarAaI, 0.3-kb XbaI-KpnI fragment (*fdxI*) from pUKAfdxI, and 1.2-kb KpnI-EcoRI fragment (*fdrI*) from pUKAfdrI	This study
pUKA265	Ap^r; pUC119 with 1.2-kb SphI-XbaI fragment (*carAaII*) from pUKAcarAaII, 0.3-kb XbaI-KpnI fragment (*fdxI*) from pUKAfdxI, and 1.2-kb KpnI-EcoRI fragment (*fdrI*) from pUKAfdrI	This study
pUCARA	Ap^r; pUC119 with 5.3-kb EcoRI DNA fragment containing *carAaAaAc* (ORF7) *carAd* genes of *Pseudomonas resinovorans* strain CA10	36

[a] Car$^+$ represents an ability to grow on CAR as the sole source of carbon, nitrogen, and energy. Ap^r represents resistance to ampicillin.

where TIC is the total ion current. All experiments were conducted independently at least three times.

Biotransformation analysis to determine substrate specificity. *E. coli* JM109 harboring pUKA253 or pUKA260 was cultivated as described above, and then 5 ml of the culture was transferred to 1 liter of the same medium. A resting cell suspension (OD_{600}, 17 to 18) was prepared as described above except for the incubation temperature (25°C) and induction time after the addition of IPTG (12 h). Anthracene was dissolved in *N*,*N*-dimethylformamide at 10 mg/ml (wt/vol), and the other putative substrates shown in Table S3 in the supplemental material were dissolved in DMSO at the same concentration. Addition of substrate, subsequent incubation, and GC-MS analysis were performed as described above.

Nucleotide sequence accession numbers. The nucleotide sequences of the *car-I*, *car-II*, *fdxIfdrI*, and *fdrII* loci were deposited in the DDBJ DNA database under accession numbers AB095953, AB220949, AB220950, and AB220951, respectively.

RESULTS

pCAR3 loci encompassing the carbazole degradation genes. The draft sequence of pCAR3 having a single gap (estimated to be <80 bp) revealed the loci encompassing the genes that could be involved in the transformation of carbazole to anthranilate. In addition to the previously characterized *car* gene cluster (14), probable carbazole degradation genes were found in three other distinct loci of pCAR3 (Fig. 2 and Table 2).

The deduced amino acid sequences of six ORFs (ORFB1 to ORFB6) in locus B exhibited 51 to 75% identity with those of the previous *car* genes on pCAR3, and we designated this cluster the *car-II* gene cluster, *carAaIIBaIIBbIICIIAcII-carRII*. The previous *car* gene cluster was renamed the *car-I* gene cluster (Fig. 2, locus A). The amino acid sequence of CarAaII displayed 75 and 59% identity with those of CarAaI and $CarAa_{CA10}$, respectively, and contained both the Rieske-type [2Fe-2S] cluster consensus and Fe(II)-binding motifs (31). The amino acid sequence of CarAcII showed similarity to that of several putidaredoxin-type ferredoxins and 55% identity with that of CarAcI. CarAcII contained four Cys residues ($CX_5CX_2CX_nC$), typical of putidaredoxin-type [2Fe-2S] cluster ligands (3, 5, 38). ORFC2 (designated *fdxI*) at locus C (Fig.

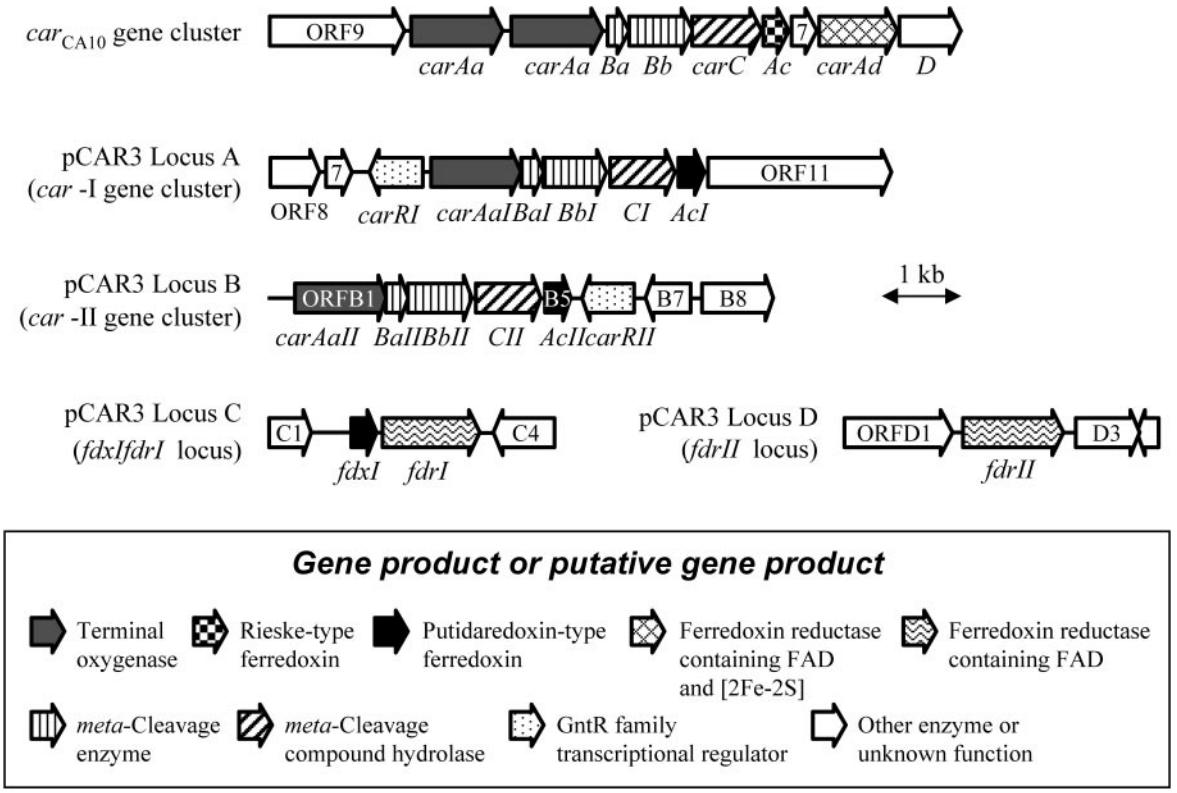

FIG. 2. Genetic organization of the *car-I* gene cluster (locus A), *car-II* gene cluster (locus B), *fdxIfdrI* locus (locus C), and *fdrII* locus (locus D) encompassing the pCAR3 genes involved in the degradation of carbazole by *Sphingomonas* sp. strain KA1. The genetic organization of the *car* gene cluster responsible for carbazole degradation in *P. resinovorans* CA10 is also shown. The *carAa* gene of CA10 is tandemly duplicated (36). The arrows in the physical maps indicate the size, location, and direction of transcription of the ORFs derived from the nucleotide sequence data.

2) showed similarity to CarAcI and CarAcII. The deduced amino acid sequences of ORFC3 (designated *fdrI*), which may constitute an operon with the *fdxI* gene in locus C, and ORFD2 (designated *fdrII*) at locus D (Fig. 2) displayed 59 and 76% identities with RedA2, the ferredoxin reductase component of the dioxin dioxygenase of *Sphingomonas wittichii* RW1 (6). FdrI and FdrII shared 38 to 41% identity with putidaredoxin reductase (20, 32) and rhodocoxin reductase (24), and also had a FAD-binding motif and two ADP-binding motifs (for FAD and NADH) (40).

As well as CarBaI, CarBbI, and CarCI in the *car-I* gene cluster, CarBaII, CarBbII, and CarCII shared moderate homology with the CarBa, CarBb, and CarC from CA10 as summarized in Table 2. Their functions were predicted as follows: CarBaII and CarBbII, structural and catalytic subunits, respectively, of the *meta*-cleavage enzyme; CarCII, *meta*-cleavage compound hydrolase.

Transcriptional analyses of the putative CARDO component genes. The primer set for the *carAaIBaIBbICIAcI*, *carAaIIBaIIBbII*, *carBbIICII*, *carCIIAcII*, *fdxIfdrI*, or *fdrII* genes could amplify DNA fragments with the expected sizes from the RNA of carbazole-grown KA1 cells (data not shown). Therefore, it was revealed that all seven genes (*carAaI*, *carAaII*, *carAcI*, *carAcII*, *fdxI*, *fdrI*, and *fdrII*) are expressed in carbazole-grown KA1 cells, suggesting that the gene products could be involved in CARDO system formation. Also, these results indicated that the *carAaIBaIBbICIAcI* and *fdxIfdrI* genes are transcribed as single transcriptional units. Furthermore, although we could not detect the RT-PCR product spanning the region from *carAaII* to *carAcII* (data not shown), the amplifications of three segmental fragments covering the entire *car-II* gene cluster (*carAaIIBaIIBbII*, *carBbIICII*, and *carCIIAcII* regions) were detected, and we concluded that the *car-II* gene cluster was also cotranscribed.

To analyze the inducibilities of the putative CARDO component genes, the mRNA levels of each gene after 2 h in carbazole-exposed or nonexposed KA1 were investigated by qRT-PCR. The mRNAs of *carAaI*, *carAcI*, *carAaII*, and *carAcII* in carbazole-exposed cells were about 13-, 10-, 15-, and 11-fold more abundant than those in nonexposed cells. Together with the results of RT-PCR analyses, these findings clearly indicated that transcription of the *car-I* and *car-II* gene clusters was induced when KA1 cells were exposed to carbazole. In contrast, the expression levels of ferredoxin and ferredoxin reductase genes were not elevated in response to carbazole exposure (1.0- to 1.2-fold induction).

Functional analyses of putative CARDO. Biotransformation experiments were performed with carbazole using *E. coli* cells expressing putative CARDO components in various combinations. Although we could not detect their expression by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown), considering that the same promoter and Shine-Dalgarno sequence were used, the expression level of each gene was assumed to be similar. The production ratios of 2′-ami-

TABLE 2. ORFs found in four pCAR3 loci, which contain the genes probably involved in carbazole conversion to anthranilate

Locus and accession no.	Position (bp) in sequence (direction[a])	Name of gene	GC content (%)	No. of amino acids	Probable function	Homologous protein			
						Identity (%)[b]	Protein or ORF name	Source	Accession no.
Locus A									
AB095953	3162–3785 (n)	ORF8	57.2	207	Conserved hypothetical protein similar to SMa10599 (truncated)	27	SalaDRAFT_3075	*Sphingopyxis alaskensis* RB2256	ZP_00577426
AB095953	3862–4188 (n)	ORF7	52.6	108	Putative RNA-binding protein (truncated)	68	Atlg32790	*Arabidopsis thaliana*	NP_174556
AB095953	4409–5089 (c)	*carRI* (ORF6)	60.8	226	Transcriptional regulator of *car* operon	51	CarRII	*Sphingomonas* sp. strain KA1	
						36	CarR	*Janthinobacterium* sp. strain J3	BAC56739
AB095953	5192–6328 (n)	*carAaI* (ORF1)	57.1	378	Terminal oxygenase component of CARDO	75	CarAaII	*Sphingomonas* sp. strain KA1	
						59	CarAa	*P. resinovorans* CA10	BAC41545
AB095953	6328–6609 (n)	*carBaI* (ORF2)	55.3	93	Subunit of *meta*-cleavage enzyme	66	CarBaII	*Sphingomonas* sp. strain KA1	
						36	CarBa	*P. resinovorans* CA10	BAC41546
AB095953	6602–7405 (n)	*carBbI* (ORF3)	59.5	267	Subunit of *meta*-cleavage enzyme	72	CarBbII	*Sphingomonas* sp. strain KA1	
AB095953	7448–8272 (n)	*carCI* (ORF4)	59.6	274	*meta*-Cleavage compound hydrolase	67	CarCII	*Sphingomonas* sp. strain KA1	
						57	CarC	*P. resinovorans* CA10	BAC41548
AB095953	8313–8642 (n)	*carAcI* (ORF5)	62.7	109	Ferredoxin component of CARDO	56	CarAcII	*Sphingomonas* sp. strain KA1	
						42	CamB	*Pseudomonas putida*	P00259
AB095953	8684–10999 (n)	ORF11	58.1	771	Outer membrane receptor	44	Saro02002648	*Novosphingobium aromaticivorans* DSM12444	ZP_00302784
Locus B									
AB220949	344–1495 (n)	*carAaII* (ORFB1)	67.4	383	Terminal oxygenase component of CARDO	75	CarAaI	*Sphingomonas* sp. strain KA1	BAC56759
						55	CarAa	*P. resinovorans* CA10	BAC41545
AB220949	1492–1773 (n)	*carBaII* (ORFB2)	68.5	93	Subunit of *meta*-cleavage enzyme	66	CarBaI	*Sphingomonas* sp. strain KA1	BAC56760
						32	CarBa	*P. resinovorans* CA10	BAC41546
AB220949	1766–2575 (n)	*carBbII* (ORFB3)	69.8	269	Subunit of *meta*-cleavage enzyme	72	CarBbI	*Sphingomonas* sp. strain KA1	BAC56761
						44	CarBb	*P. resinovorans* CA10	BAC41547
AB220949	2626–3444 (n)	*carCII* (ORFB4)	67.5	272	*meta*-Cleavage compound hydrolase	67	CarCI	*Sphingomonas* sp. strain KA1	BAC56762
						59	CarC	*P. resinovorans* CA10	BAC41548
AB220949	3499–3828 (n)	*carAcII* (ORFB5)	70.6	109	Ferredoxin component of CARDO	56	CarAcI	*Sphingomonas* sp. strain KA1	BAC56763
						45	CamB	*P. putida*	P00259
AB220949	3968–4630 (c)	*carRII* (ORFB6)	71.3	220	Transcriptional regulator of *car* operon	51	CarRI	*Sphingomonas* sp. strain KA1	BAC56758
						37	CarR	*Janthiobacterium* sp. strain J3	BAC56739
AB220949	4771–5340 (c)	*tnpR* (ORFB7)	59.4	189	Resolvase	95	TnpR	*Flavobacterium* sp. strain ATCC 27551(pPDL2)	CAD13184
AB220949	5481–6383 (n)	*tnpA* (ORFB8)	61.8	300	Transposase (truncated)	58	TnpA	*Flavobacterium* sp. strain ATCC 27551(pPDL2)	CAD13183
Locus C									
AB220950	1–534 (n)	ORFC1	58.0	177	Resolvase/integrase (truncated)	76	mlr9024	*Mesorhizobium loti* MAFF303099(pMLa)	NP_085611
AB220950	1038–1355 (n)	*fdxI* (ORFC2)	56.8	105	Putidaredoxin-type [2Fe-2S] ferredoxin	66	ELI2682	*Erythrobacter litoralis* HTCC2594	ZP_00377441
						40	CarAcI	*Sphingomonas* sp. strain KA1	BAC56763
						38	CarAcII	*Sphingomonas* sp. strain KA1	
						32	CamB	*P. putida*	P00259
AB220950	1432–2655 (n)	*fdrI* (ORFC3)	60.8	407	Ferredoxin reductase component of CARDO	64	Saro02000237	*N. aromaticivorans* DSM12444	ZP_00304456
						59	FdrII	*Sphingomonas* sp. strain KA1	

					59	RedA2	*S. wittichii* RW1	CAA05635
					38	CamA	*P. putida*	P16640
AB220950	2831–3607 (c)	ORFC4	62.1	258	Transcriptional regulator (IcIR family)	28	Chlo02004436	*Chloroflexus aurantiacus*	ZP_00356137
Locus D									
AB220951	1–1368 (n)	ORFD1	63.9	455	Pyruvate/2-oxoglutarate dehydrogenase complex, dehydrogenase (E1) component, β subunit	80	Saro02001437	*N. aromaticivorans* DSM12444	ZP_00303572
AB220951	1504–2766 (n)	*fdrII* (ORFD2)	62.4	420	Ferredoxin reductase component of CARDO	76	RedA2	*S. wittichii* RW1	CAA05635
						59	FdrI	*Sphingomonas* sp. strain KA1	
						41	CamA	*P. putida*	P16640
AB220951	2923–3729 (n)	ORFD3	58.0	268	Transglycosylase	49	ORF925	*N. aromaticivorans* F199(pNL1)	AAD03969
AB220951	3696–3971 (c)	ORFD4	60.5	91	Hypothetical protein	62	ORF921	*N. aromaticivorans* F199(pNL1)	AAD03968

[a] n, normal; c, complementary.
[b] Percentage of amino acids that are identical when sequences are aligned with sequences listed in the DDBJ/EMBL/GenBank databases by using the CLUSTAL W multiple sequence alignment program (version 1.83) (15).

TABLE 3. Carbazole conversion by *E. coli* expressing a putative CARDO component(s)

Plasmid used	Protein(s) expressed in *E. coli* JM109			Conversion ratio (%)[a]
	Oxygenase	Ferredoxin	Reductase	
pUKAcarAaI	CarAaI			0.090 (±0.030)
pUKA248	CarAaI	CarAcI		0.34 (±0.21)
pUKA250	CarAaI		FdrI	0.19 (±0.089)
pUKA254	CarAaI	CarAcI	FdrI	96 (±1.4)
pUKA263	CarAaI	FdxI	FdrI	11.7 (±6.9)
pUKA249	CarAaI		FdrII	0.19 (±0.067)
pUKA253	CarAaI	CarAcI	FdrII	95 (±4.4)
pUKAcarAaII	CarAaII			ND[b]
pUKA255	CarAaII	CarAcII		0.24 (±0.026)
pUKA257	CarAaII		FdrI	ND
pUKA261	CarAaII	CarAcII	FdrI	40 (±13)
pUKA265	CarAaII	FdxI	FdrI	0.18 (±0.052)
pUKA256	CarAaII		FdrII	ND
pUKA260	CarAaII	CarAcII	FdrII	61 (±1.7)

[a] Conversion ratio (%) = 100 × (peak area for TIC of 2′-aminobiphenyl-2,3-diol)/(peak area for TIC of carbazole + peak area for TIC of 2′-aminobiphenyl-2,3-diol). Values are means ± standard deviations calculated from at least triplicate assays.
[b] ND, no product was produced from carbazole.

nobiphenyl-2,3-diol from carbazole detected for cells harboring pUKA254 (*carAaI*, *carAcI*, and *fdrI*) and pUKA253 (*carAaI*, *carAcI*, and *fdrII*) were much higher than those seen with cells harboring any other recombinant plasmids containing *carAaI* (Table 3). These results clearly indicated that CarAaI can catalyze angular dioxygenation of carbazole efficiently in conjunction with both CarAcI and FdrI (or FdrII). Similarly, as shown in Table 3, *E. coli* cells expressing CarAaII exhibited a much higher conversion ratio when both CarAcII and FdrI (or FdrII) were coexpressed, indicating that CarAaII could function as a terminal oxygenase of CARDO with the aid of both CarAcII and FdrI (or FdrII). In addition, the expression of FdxI with CarAaI/FdrI or CarAaII/FdrI also increased the conversion ratio (see pUKA263 versus pUKA250 and pUKA265 versus pUKA257 in Table 3), although it was markedly lower than the expression observed when CarAcI or CarAcII was supplied as ferredoxin. These results suggested that FdxI could also transfer electrons from FdrI to CarAaI/CarAaII, but the efficiency of electron transfer was markedly lower than those of CarAcI and CarAcII.

We further examined whether CarAaI and CarAaII could receive electrons from ferredoxins not encoded on the same *car* gene clusters. When CarAcII was expressed with CarAaI/FdrI (pUKA268) or CarAaI/FdrII (pUKA267), 77% ± 3.3% and 87% ± 5.2% conversion occurred, respectively. Both of these figures were only a little lower than those seen with CarAcI (pUKA254 and pUKA253) (Table 3). These results suggested that CarAaI could receive electrons from CarAcII and CarAcI with similar efficiencies. However, the conversion ratio of carbazole by *E. coli* expressing CarAaII/CarAcI/FdrI (or FdrII) was markedly lower than that detected when Car AcII was replaced with CarAcI (8.3% ± 2.0% and 6.2% ± 1.2% conversion were observed for *E. coli* harboring pUKA270 [CarAaII/CarAcI/FdrI] and pUKA269 [CarAaII/CarAcI/FdrII], respectively). Therefore, we concluded that CarAcI could transfer electrons to CarAaII but that the electron transferability to CarAaII is substantially lower than that to CarAcII.

Based on these results, although all CARDO components found on pCAR3 can theoretically function in various combinations, we concluded that two CARDO systems, composed of CarAaI/CarAcI/(FdrI or FdrII) (designated $CARDO_{KA1-1}$) and CarAaII/CarAcII/(FdrI or FdrII) (designated $CARDO_{KA1-2}$) primarily function as initial oxygenases for carbazole in KA1.

Substrate ranges of two CARDOs of KA1. The substrate ranges of $CARDO_{KA1-1}$ and $CARDO_{KA1-2}$ were determined by biotransformation analyses using recombinant *E. coli*. (Detailed data are provided in Table S3 in the supplemental material). Both CARDOs catalyzed angular dioxygenation (28) of carbazole, dibenzofuran, dibenzo-*p*-dioxin (DD), and phenoxathiin. The ratio of angular dioxygenation of 9-fluorenone was markedly poorer in comparison with carbazole, dibenzofuran, DD, or phenoxathiin. Neither $CARDO_{KA1-1}$ nor $CARDO_{KA1-2}$ could catalyze any oxygenation reactions for dibenzothiophene sulfone. With fluorene, both CARDOs catalyzed the monooxygenation of the methylene carbon and probable lateral dioxygenation (28) at unidentified positions. For biphenyl, naphthalene, and anthracene, both CARDOs catalyzed lateral dioxygenations as was the case with $CARDO_{CA10}$ (28).

In conclusion, $CARDO_{KA1-1}$ and $CARDO_{KA1-2}$ have a wide substrate range, which is similar to that of $CARDO_{CA10}$. However, there are two noteworthy differences (see Table S3 in the supplemental material). (i) $CARDO_{KA1-1}$ preferably oxygenates anthracene, probably at C-2 and C-3, whereas $CARDO_{KA1-2}$ and $CARDO_{CA10}$ preferably oxygenate at C-1 and C-2 to yield *cis*-1,2-dihydroxy-1,2-dihydroanthracene. (ii) It is likely that the angular dioxygenation activities of $CARDO_{KA1-2}$ for DD or phenoxathiin are relatively lower than those in the other two CARDO systems.

DISCUSSION

Two *car* gene clusters were present on plasmid pCAR3, and two CARDOs, composed of CarAaI/CarAcI/(FdrI or FdrII) ($CARDO_{KA1-1}$) and CarAaII/CarAcII/(FdrI or FdrII) ($CARDO_{KA1-2}$), function in KA1. Plural isofunctional degradation genes on a single bacterial genome have been reported. For example, multiple gene sets for biphenyl/polychlorinated biphenyl degradation are known to be dispersed on the chromosome and large linear plasmids in *Rhodococcus* sp. strain RHA1 (11, 19, 22, 42). Duplication of degradation genes has also been reported in several degradation plasmids, such as the 2,4-dichlorophenoxyacetic acid-degrading plasmids pJP4 (21) and pEST4011 (41) and the toluene/xylene-degrading plasmid pWW53 (30). It would be of great interest to determine the physiological roles of the two copies of the *car* gene cluster in carbazole metabolism by KA1. As described above, the substrate specificities of the two CARDOs are nearly identical, although slight differences were observed. These facts suggest that duplication does not broaden the degradation (or growth) substrate range of KA1 but raises its degradation rate of carbazole. Estimation of the in vivo concentration of each CARDO component and generation of knockouts of either *car-I* or *car-II* (or either *fdrI* or *fdrII*) followed by quantitative determination of the carbazole catabolic capacities will yield information to reveal the importance of multiple degradation genes.

The draft sequence of pCAR3 showed that the *fdxIfdrI* cistron is located 50 and 85 kb downstream of the *car-I* and *car-II* gene clusters, respectively (data not shown). The *fdrII* gene is located about 80 and 115 kb downstream of the *car-I* and *car-II* gene clusters, respectively (data not shown). The dispersion of the ROS component genes in pCAR3 contrasts with the well-organized degradation operon in pseudomonads, where the four or three genes are assembled, or at least cotranscribed, as is observed with the car_{CA10} gene cluster (Fig. 2) (36). Because similar dispersions of ROS component genes have been reported many times in sphingomonads (4, 23, 33, 34), it is possible that gene dispersion is one of the characteristics of sphingomonads. It is possible that constitutive expression of the *fdrI* and *fdrII* genes provides enough reductase to transfer electrons from NAD(P)H to ferredoxin. If this is the case, a well-organized operon is not necessary to achieve the appropriate carbazole degradation capacity. Another possibility is that the organization of *car* gene clusters of pCAR3 has not yet fully evolved and been optimized and that there is room for further evolution of the carbazole catabolic operon when KA1 is exposed to some selective pressure. On the other hand, the ferredoxin reductases, FdrI and FdrII, may be shared with other redox systems, possibly to maximize the catabolic potential while limiting its genetic burden. In this case, carbazole-dependent control of their expression would be disadvantageous for KA1.

According to the classification of Batie et al. (7), both CARDOs of KA1 are classified in class IIA. Class IIA ROS is a three-component oxygenase, in which the electron transfer components comprise a simple flavoprotein and a putidaredoxin-type ferredoxin. Until now, almost all ROSs have been assigned to class IIB or III. As for class IIA ROSs, only a few examples have been reported, such as the pyrazon dioxygenase of an unidentified bacterium (37), the dioxin dioxygenase of *S. wittichii* RW1 (6), and the dicamba *O*-demethylase of *Pseudomonas maltophilia* DI-6 (8, 12). Whereas $CARDO_{KA1-1/2}$ belongs to class IIA, according to the properties of ferredoxin and ferredoxin reductase, $CARDO_{CA10}$ is a class III ROS. Considering that the ferredoxins contained in $CARDO_{KA1-1/2}$ and $CARDO_{CA10}$ are different from one another, it is likely that the respective oxygenases have different ferredoxin selectivity, although markedly high amino acid sequence homology was observed between the two oxygenases (CarAaI and Car AaII) and $CarAa_{CA10}$ (Table 2). To confirm this, ferredoxin interchangeability between CARDO-$O_{KA1-1/2}$ and CARDO-O_{CA10} should be determined.

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