Divergent Structures of Carbazole Degradative car Operons Isolated from Gram-negative Bacteria

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Southern hybridization analysis of the genomes from the newly-isolated 10 carbazole (CAR)-utilizing bacteria revealed that 8 of the isolates carried gene clusters homologous to the CAR-catabolic car operon of Pseudomonas resinosovars strain CA10. Sequencing analysis showed that two car operons and the neighboring regions of Pseudomonas sp. strain K23 are nearly identical to that of strain CA10. In contrast to strains CA10 and K23, carEF genes did not exist downstream of the car gene cluster of Janthinobacterium sp. strain J3. In the car gene clusters, strains CA10, K23 and J3 have Rieske-type ferredoxin as a component of carbazole dioxygenase, although Sphingomonas sp. strain KA1 possesses a putidaredoxin-type ferredoxin. We confirmed that this putidaredoxin-type ferredoxin CarAc can function as an electron mediator to CarAa of strain KA1. In the upstream regions of the carJA and carKA1 gene clusters, ORFs whose deduced amino acid sequences showed homology to GntR-family transcriptional regulators were identified.

Key words: carbazole; carbazole 1,9a-dioxygenase; car gene cluster; biodegradation

Various functional bacteria have been studied extensively for practical application in useful compound production, bioremediation, bioconversion, and biodegradation of diverse xenobiotics. The resultant information on xenobiotic degradative genes and enzymes provides us several clues to the evolution of xenobiotic-degradation systems. For example, various xenobiotic degradative gene clusters have been found to be located on mobile genetic elements, such as plasmids and transposons, indicating that such genes have undergone gene recruitment to generate xenobiotic-degrading bacteria.¹⁻³ Subsequent point mutations and various types of rearrangements, including deletion, duplication, inversion, and insertion, would have occurred, to yield the diversity of xenobiotic degradative genes.⁴ It is considered that these gene recruitment(s) and rearrangement(s) are the result of adaptation of the microorganisms to xenobiotic compounds.

Carbazole (CAR) is an N-heterocyclic aromatic compound derived from coal tar and shale oil. CAR is recalcitrant and possesses mutagenic and toxic activities.⁵ Many CAR-degrading bacteria have been isolated and characterized, and the gene clusters involved in CAR degradation have been cloned from several bacteria.⁶⁻⁷ Among them, the CAR-catabolic car genes from Pseudomonas resinosovars strain CA10 (carCA10 genes) have been studied extensively and the CAR biodegradation pathway has been elucidated (Fig. 1).⁸⁻¹⁰ Carbazole 1,9a-dioxygenase (CarAaAcAd), the meta-cleavage enzyme (CarBaBb), and the meta-cleavage compound hydrolase (CarC) catalyze the first three steps in CAR degradation (Fig. 1).⁹,¹⁰ One of the products of the third step, anthranilate, is attacked by dioxygenase (AntABC) at the 1,2 positions to yield catechol, and the other product, 2-hydroxypenta-2,4-dienoate, is converted to pyruvate and acetyl-CoA by CarDEF (Fig. 1).⁸ The above-described car genes in strain CA10 are located on a 199-kb circular plasmid, pCAR1.⁸ The complete nucleotide sequence of pCAR1 showed that the tra and trh genes potentially involved in the conjugal transfer are contained in pCAR1, suggesting that pCAR1 is a self-transmissible plasmid.¹¹ The nucleotide sequence of pCAR1 also indicated that the carCA10 gene cluster is contained in a 72.8-kb class II type transposon, Tn4676.¹¹ These genetic elements, pCAR1 and Tn4676, are thought to play an important role in the distribution (or recruitment) of the car gene cluster in nature. On the other hand, nucleotide sequence

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Abbreviations: CAR, carbazole; IS, insertion sequence; CNFMM, carbon- and nitrogen-free mineral medium; IR, inverted repeat; DR, direct repeat; GC-MS, gas chromatograph-mass spectrometry; CARDO, carbazole dioxygenase
analysis of carCA10 gene cluster-flanking regions indicated that a complicated rearrangement, such as a transposition of insertion sequence (IS), IS-dependent recombination, and gene duplication and fusion by an unknown mechanism occurred within and around this gene cluster to yield the present novel mosaic structure. 8)

Recently, Sphingomonas sp. strain KA1 was isolated as a CAR-degrading bacterium, and the 1,110-bp DNA fragment containing parts of the carAa and carBa homologs was PCR-amplified. 12) The nucleotide sequence of this DNA fragment showed 57.7% identity with the corresponding part of the car gene cluster of strain CA10. Kilbane II also reported the sphingomonad (Sphignomonas sp. strain GTIN11)-bone CAR degradative carAaBaBbCAc gene cluster (carGTIN11 operon).13)

The nucleotide sequence of the carAaBa regions of the two strains were identical. Interestingly, among car genes from the carGTIN11 operon, only the carAcGTIN11 gene showed negligible homology with that of CA10 at both the nucleotide and the amino acid sequence levels. carAcGTIN11 encodes putidaredoxin-type ferredoxin, and is believed to be functional in the carbazole 1,9a-dioxygenase system, although the electron transferability of this ferredoxin to CarAaGTIN11 has not been proven.13)

In this study, we isolated 10 carbazole-utilizing bacteria and elucidated the genetic structures of the car gene clusters and their flanking regions in Pseudomonas sp. strain K23 and Janthinobacterium sp. strain J3. Also, we genetically analyzed the whole car gene cluster and its flanking region in Sphingomonas sp. strain KA1. Comparison of the genetic structures with that of carCA10 provided new information on the divergence and evolution of the car gene cluster(s) and the CAR-degrading enzyme system(s).

Materials and Methods

Bacterial strains, plasmids, media, and culture conditions. The bacterial strains, plasmids, and cosmids used in this study are listed in Table 1. The medium used for bacterial growth was Luria-Bertani (LB) medium or 2xYT medium,14) E. coli strains JM10914) and DH5α14) were used as host strains for the plasmids, pBlueScript II SK(-), pBlueScript II KS(-), and their derivatives. Ampicillin, kanamycin, and chloramphenicol were added to selective media at final concentrations of 50, 50, and 30 μg/ml respectively. For plate cultures, the above-described media solidified with 1.6% (wt/vol) agar were used.

DNA manipulations. Total DNA of the CAR-utilizing strains grown on 2x YT or LB medium at 30°C, and plasmid and cosmid DNA from E. coli host cells was prepared as described previously.5) Restriction endonuclease and DNA Ligation Kit version 2 (Takara Shuzo Co., Ltd., Kyoto, Japan) were used according to the manufacturer’s instructions. DNA fragments were extracted from the agarose gel using Concert Rapid Gel Extraction Systems (Life Technologies, Rockville, Maryland, USA) according to the manufacturer’s instructions. Other DNA manipulations were done according to standard methods.14)

Isolation and characterization of CAR-utilizing bacteria. Enrichment cultures were set up in 100 ml of CNFMM (carbon- and nitrogen-free mineral medium)5) supplemented with CAR. When bacterial growth was observed, 100 μl of the resultant culture was transferred into similarly prepared fresh medium. After transfer 4 times to fresh medium, the appropriate dilution of the bacterial culture was spread onto solid CNFMM plate that was overlaid with CNFMM agar containing CAR. Colonies producing a clear zone, as a result of CAR degradation, were purified on nutrient broth agar medium (Eiken Chemical, Co., Ltd., Japan). The 16S rRNA gene was amplified from each strain using the forward primer 27f (5'-AGAGTTTGATC[C/A]TGG-CTCAG-3') and the reverse primer 1492r (5'-TACGG[AT/C]TACCTTGTTACGACTT-3'), which correspond to positions 8–27 and 1492–1513 of the E. coli 16S rRNA sequence respectively. Total DNA of each strain was used as a template in PCR, and the reaction conditions were those described by Hedlund et al.15)
PCR products were then digested with restriction endonucleases, *Hae*III, *Hha*I or *Alu*I, and the resulting restriction fragments were analyzed by electrophoresis on 2% agarose gels. Appropriate PCR products were cloned into pT7Blue(R) vector (Novagen, Madison, Wisconsin, USA) for sequence analysis.
Cloning of the DNA regions containing the car gene cluster. Southern hybridization analysis of the EcoRI-digested total DNAs of the isolates was performed with a probe prepared from the 6.9-kb fragment of pUCA1\(^1\)) (containing carAa through partial carD of strain CA10; carCA10 probe) according to methods described previously.\(^5\) The appropriate hybridized DNA fragments (about 5.6- to 6.9-kb for strain K23 and about 5.6-kb for strain J3) were extracted from agarose gel. The resultant DNA fragments were ligated to the corresponding site of plasmid pBluescript II KS(-), and the recombinant plasmids formed were used in the transformation of *E. coli* strain JM109. The resultant clones were screened as described previously\(^7\) and the plasmids obtained containing the above fragments hybridized with the carCA10 probe were designated pBK23001, pBK23002, and pBJ3001 respectively (Table 1 and Fig. 2).

To obtain the DNA fragments containing the *car* gene cluster and its neighboring DNA region from the genome of CAR-degrading bacteria, we constructed a genomic cosmid library of strains K23, J3, and KA1 as described previously,\(^8\) except for the use of the restriction endonuclease San3AI instead of EcoRI to prepare the vector arm of SuperCos1 and partially digested genomic DNA. Colony hybridization was performed according to methods reported previously.\(^8\)

To screen the positive cosmid clones containing the *car* gene clusters of strains K23, J3, and KA1, we used the probes prepared from the 6.9-kb EcoRI insert of pUC1 (the carCA10 probe),\(^10\) the 5.6-kb EcoRI insert of pBJ3001, and the 0.5-kb BamHI insert of pTKA001\(^12\) respectively.

Nucleotide sequence determination, homology search, and alignment analysis. The nucleotide sequences were determined using the GPS-1 Genome Priming System (New England Biolabs, Inc., Beverly, Massachusetts, USA) according to the methods reported previously.\(^8\)

The nucleotide sequences obtained were analyzed with DNASIS-Mac software (version 3.7, Hitachi Software Engineering Co., Ltd., Yokohama, Japan). We searched for homology of the sequences using the SWISS-PROT amino acid sequence data bank or the DDBJ/EMBL/GenBank DNA databases with the BLAST program (version 2.0.10). The deduced amino acid sequences of observed open reading frames (ORFs) were aligned by ClustalW through DDBJ (http://www.ddbj.nig.ac.jp).

Nucleotide sequence accession numbers. The nucleotide sequences reported in this study have been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases. The accession nos. for the 16S rRNA gene sequences are as follows: K15, no. AB089755; K22, no. AB089756; J30, no. AB088760; K23, no. AB088844; J4, no. AB097171; J3, no. AB097172; J14, no. AB097173; J11, no. AB097174; J40, no. AB097175; and M2, no. AB097176. The nucleotide sequence data for the 13,651-bp *HindIII-Xhol* fragment (the *carCA1* gene cluster and its flanking regions), the 8,096-bp *SmaI* fragment (upstream of the *carK23L* gene cluster), the 3,435-bp *SacI* fragment (downstream of the *carK23L* gene cluster), and the 20,017-bp *EcoRV* fragment (the *carJ* gene cluster and flanking region), were registered under accession nos. AB095953, AB094786, AB095951, and AB095952 respectively.

Copy number determination of the ISs. To check the copy numbers of the identified ISs in the genome of host bacteria, we carried out southern hybridization analysis using probes prepared from a PCR-amplified internal sequence. The regions of probes in deposited DNA regions were as follows: ISPsp4, 1,295–2,391 in accession no. AB094786; ISPsp7, 1,055–1,734 in accession no. AB095951; ISJsp1, 2,984–3,664 in accession no. AB095952; ISJsp2, 17,006–17,558 in accession no. AB095952; and ISHsp1, 11,706–12,454 in accession no. AB095953. Southern blotting was performed according to methods reported previously.\(^8\)

Determination of the CAR 1,9a-dioxygenase activities of *E. coli* transformants. To examine whether CarAc\(_{KA1}\) can function as an electron transporter for CarAc\(_{KA1}\), we constructed a broad-host-range vector pBBR1MCS-2\(^16\)-based plasmid for *nptII* promoter-dependent constitutive expression of CarAc\(_{KA1}\) and CarAc\(_{KA1}\). The *carAc\(_{KA1}\)* DNA fragment produced by PCR using the primer sets (5'–CTGA–GTCCAG–AGGAGGAGTGGATTGACCG–CAAAGGTCCGCGTG–3' and 5'–CTGA–ACTAGT–CA–TGACTGTGCTTCGAG–3' [SalI and SpeI restriction sites italicized]) was digested by SalI and SpeI, and ligated into the corresponding sites of pBBKA1K\(^17\) containing only carAc\(_{KA1}\) under the *nptII* promoter in the multi-cloning site of pBBR1MCS-2, to give pBBKA2K, pBBKA1K and pBBKA2K were introduced into *E. coli* strain JM109, and the resulting transformants were used in the biotransformation experiments with CAR according to the method reported previously.\(^8\)

Determination of the inducibility of the CAR degradative capacity of strain KA1. Strain KA1 cells were grown on 5-ml CNFMM medium supplemented with 0.1% (wt/vol) CAR as sole source of carbon, nitrogen, and energy. One-milliliter of culture was transferred to a similarly prepared 100-ml medium or 100-ml nutrient broth (Eiken Chemical, Tokyo, Japan), and incubated for 18 h. The resultant cells were washed twice with CNFMM, and finally suspended in CNFMM, to give an optical density of 5 at 550 nm. To 1 ml of the resulting cell suspension, 25 nmol of [UL-\(^14\)C]CAR (Sigma Chemical Co., St. Louis, Missouri, USA; 7.9 mCi/mmol) dissolved in 10 μl dimethyl sulfoxide was added, and incubated at 30 °C with reciprocal shaking at 120 strokes per min. After appropriate intervals, the reaction
was stopped by adding 100 μl of 1 N HCl on ice, and then extracted with 1 ml of ethyl acetate 3 times. The CAR remaining and the product formed were quantified by a method described previously.\(^{19}\)
Results

Isolation and phylogenetic grouping of CAR-utilizing bacteria

Ten CAR-utilizing bacteria were obtained from different environmental samples, such as soil, activated sludge, river water, and river sediment. Identification of the isolates by using 16S rRNA gene sequence analysis showed that the isolates belong to *Janthinobacterium* (strains J3 and J4), *Pantoea* (strain J14), *Pseudomonas* (strains J11, K15, K22, and K23), *Novosphingobium* (strain J30), and *Sphingomonas* (strains J40 and M2).

Diversity of the car gene clusters

Hybridization of the EcoRI-digested total DNA of the isolates with the carCA10 probe, prepared from the 6.9-kb fragment of DNA containing carAa through partial carD of strain CA10, in high stringency showed clear hybridization to the total DNA of 8 isolates, implying the presence of a car gene cluster homologous to that of strain CA10. We classified the 8 isolates into 4 types based on hybridization patterns: 2 strains (J3 and J4), in strain CA10. We classified the 8 isolates into 4 types based on hybridization patterns: 2 strains (J3 and J4), in strain CA10, 3 strains (J11, J14, and J30) that showed clear hybridization with a single 6.9-kb EcoRI fragment like strain CA10 (type 2); 2 strains (K15 and K23) that showed hybridization with two EcoRI restriction fragments (about 5.6- and 6.9-kb) and strain K22, which had three EcoRI restriction fragments (about 5.6-, 6.9-, and >20.0-kb) strongly hybridized with the carCA10 probe (type 4). Strain M2, which did not hybridize with the car genes from strain CA10, strongly hybridized with the partial carAaBa genes from strain KA1 (0.5-kb BamHI insert of pTKA001; Table 1). Strain J40 did not hybridize with the car genes from strains CA10 or KA1.

Because the results of southern hybridization showed that type 2 strains have a genetic structure very similar to that of the carCA10 gene cluster, and the type 1 and type 3 strains were expected to have different car gene clusters from strain CA10, we analyzed the car gene clusters of strains K23 (type 3) and J3 (type 1).

Genetic structures of the car gene clusters and their flanking regions in strain K23

(1) The carK23 gene clusters

To verify the fragments that hybridized with the carCA10 probe, we first cloned the 5.6-kb EcoRI fragment (pBK23002 insert) and the 6.9-kb EcoRI fragment (pBK23001 insert) from K23 (Fig. 2). The fragments were digested with several different restriction enzymes and the restriction sites were mapped (Fig. 2). The restriction maps of inserts of both pBK23001 and pBK23002 were almost the same as the 6.9-kb-long pUC1 insert from strain CA10. Except that the 5.6-kb pBK23002 insert had only one EcoRV and one Smal site at the DNA region corresponding to the carAa gene (Fig. 2). These results strongly suggest that strain K23 has two car gene clusters, one of which carries a single copy of the carAa gene (the carK23L gene cluster containing carAaBaBbCAcORF7Ad genes) and the other contains two tandemly duplicated copies of carAa gene (the carK23L gene cluster containing carAaAaBaBbCAcORF7Ad genes) like strain CA10. Partial sequencing analysis of pBK23002 confirmed that only one copy of the carAa gene is encoded in the carK23L gene cluster (data not shown).

(2) Genetic organization of the flanking regions of carK23L gene clusters

We constructed genomic cosmid libraries of strain K23 and screened for the car gene cluster using the carCA10 probe, and isolated 4 positive clones (Table 1). Southern hybridization analysis indicated that the insert DNAs of pSCosK23003 and pSCosK23072 contained carCA10, and that those of pSCosK23052 and pSCosK23121 contained carK23S. To determine the genetic structure of the upstream and downstream regions of the carK23L gene cluster, we constructed the deletion plasmids, pBK23101, pBK23102, and pBK23103, from the insert of pSCosK23052 (Fig. 2). The flanking regions of the carK23L and carCA10 gene clusters had the same restriction sites. However, the Smal-HindIII fragments and SacI fragments in the upstream and downstream regions of each car gene cluster, respectively, had different lengths (Fig. 2). We also generated the plasmid pBK23202 from pSCosK23003 to determine the genetic structure of the carK23S upstream region (Fig. 2). Restriction mapping of pBK23202 insert revealed that the 7.3-kb upstream region of the carAa gene is highly homologous with the corresponding region of carK23L (pBK23101 insert). Although the cosmid clones containing the carK23S gene cluster did not have the further downstream region in their inserts, southern hybridization analysis of the total DNA of strain K23 showed a clear single hybridization with several probes prepared from the DNA fragments located in the 16.9-kb-long downstream region from the carAd gene of strain CA10 (data not shown). These results suggest that the 16.9-kb downstream region of the carK23S gene cluster is nearly identical with that of the carK23L gene cluster.

Based on these results, we sequenced the strain K23 DNA fragments in pBK23101 (8,096-bp) and pBK23104 (3,435-bp) (Fig. 2), and the functions of the products encoded by the ORFs identified were examined by homology search analysis.

By comparing the nucleotide sequence of the upstream region of the carK23L gene cluster with that of the carCA10, we found that a 1,112-bp DNA region was inserted into the 4,898-bp upstream of the initiation codon of the carAaK23L gene, and that the nucleotide sequence of the rest of the DNA region was identical except for one base. According to the ORF numbers in strain CA10, the 4 ORFs observed in the immediately upstream of the carAaK23L gene were designated ORF9K23L to ORF13K23L (Fig. 2). ORF13K23L was a gene...
upstream of the car gene
the copy number of the regions were considered to be almost identical except for

(1) The carAa gene cluster
To investigate the fragments that hybridized with the carCA10 probe in strain J3, we cloned 5.6-kb EcoRI fragments from total DNA derived from strain J3 (pBJ3001). The restriction enzyme map of the 5.6-kb EcoRI fragment from strain J3 showed slight differences from that of the pK23002 insert (Fig. 2). To reveal the difference between the car gene structures of strains CA10 and K23, and J3, we determined the nucleotide sequence of the pBJ3001 insert. The nucleotide sequence of the 5,613-bp DNA fragment of the pBJ3001 insert (9,642 to 15,254 in accession no. AB095952) contained 7 complete ORFs, and the deduced amino acid sequences of each ORF showed 99, 92, 97, 98, 99, 99, and 95% identity with those of carAa, carBa, carBb, carC, carAc, ORF7, and carAd in strain CA10 (DDBJ/EMBL/GenBank accession no. D89064) respectively. Following the nomenclature of strain CA10, these 7 ORFs were designated carAa, carBa, carBb, carC, carAc, ORF7, and carAd in strain CA10 (DDBJ/EMBL/GenBank accession no. D89064) respectively. The following nomenclature of strain CA10, these 7 ORFs were designated carAa13, carBa13, carBb13, carC13, carAc13, ORF713, and carAd13 respectively. Comparison of the car gene cluster of strain J3 (carJ3 gene cluster containing carAaBbCa ORF7Ad) and the corresponding region of strain CA10 showed that the overall identity in nucleotide sequence level was 94%.

(2) Genetic organization of the flanking regions of the carJ3 gene cluster
To compare the genetic structures of the flanking regions of the carCA10, carK23, and carJ3 gene clusters, a genomic cosmid library of strain J3 was constructed as in the case of strain K23, and a clone with the carJ3 gene cluster was selected, as described in “Materials and Methods.” From a positive clone (pSCosJ3003), we constructed deletion plasmids pBJ3101 and pBJ3102, which contained the 11.0-kb upstream region of carAa13

Table 2. The Properties of ISs Observed in the Flanking Regions of the car Gene Cluster

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a Copy numbers of the IS in source strain cells determined by Southern hybridization analysis are shown.
b Left and right follow the nucleotide sequence direction in Fig. 2.

Genetic structure of the car gene cluster and its flanking region in strain J3

in the case of strain K23, and a clone with the carJ3 gene cluster was selected, as described in “Materials and Methods.” From a positive clone (pSCosJ3003), we constructed deletion plasmids pBJ3101 and pBJ3102, which contained the 11.0-kb upstream region of carAa13

In conclusion, the genetic structures of the car gene cluster and its flanking region in strain J3

Operons 1473

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<td>TGAACCGCCCAGGGTGAACCGCCCAGGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ISSsp1</td>
<td>IS5</td>
<td>1</td>
<td>1,526</td>
<td>4</td>
<td>Sphingomonas sp. strain KA1</td>
<td>GATACCGTTGAAGAAAGATCCGATACCGTTGAAGAAAGATCCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Putative IS</td>
<td>IS3</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Sphingomonas sp. strain KA1</td>
<td>TGTCGCGGTCAGCAAACAT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Copy numbers of the IS in source strain cells determined by Southern hybridization analysis are shown.
b Left and right follow the nucleotide sequence direction in Fig. 2.
and the 5.2-kb downstream regions of the carAd3 gene respectively (Fig. 2). Restriction maps created for the above carAd3-flanking region showed a restriction pattern unrelated to those of the carCA10-flanking regions, and these flanking regions were not hybridized by using the carCA10-flanking regions as probes (data not shown). Therefore, we determined the nucleotide sequences of the inserts of pBJ3101 and pBJ3102.

In the pBJ3101 insert, 13 ORFs (ORFU1J3 to ORFU13J3) were identified (Fig. 2). By comparing the nucleotide sequence of the upstream region of the carJ3 gene cluster with the corresponding region in strain CA10, we found that the 1,096-bp-long upstream region of the carAaJ3 gene showed 92.2% identity with that of the carCA10 gene cluster. This region corresponds to the 3'-half of ORF9CA10, which was created by gene fusion (Figs. 2 and 3A). A previous study indicated that a 698-bp 5'-portion of ORF9CA10 was derived from the antA gene (the gene encoding the large subunit of the terminal oxygenase component of anthranilate 1.2-dioxygenase), originally located 21-kb upstream of the carCA10 gene cluster on plasmid pCAR1, and the partial DNA region of antA was fused to the 3'-portion of ORF9CA10 accompanied by transposition of ISPre1 (Fig. 3A). In the upstream region of the carCA10 gene cluster, the 5'-portion of the antA gene appears to have transposed immediately upstream of the initiation codon of the ORFU13J3-like ancestral gene, resulting in the present fusion structure of ORF9 in strains CA10 and K23 (Fig. 3A).

The deduced amino acid sequence of ORFU11J3, identified 1,513-bp upstream of the carAaJ3 gene in the opposite direction, showed 27% identity with that of the transcriptional regulator found by genome sequencing of Mesorhizobium loti strain MAFF303099 (no. BAB53175). Moreover, the ORFU11J3 product showed 37% amino acid identity with the ORF6KAI product from Sphingomonas sp. strain KA1 (see “Genetic

\[\text{Fig. 3. Genetic Organizations of the car Gene Clusters and Their Flanking Regions of Pseudomonas resinovorans Strain CA10 and Janthinobacterium sp. Strain J3.}\]

Comparison of the Nucleotide Sequence of the Left (immediately upstream of ORFU13J3) and Right (intergenic region of ORFL2J3) Ends of the carJ3-Flanking Region Homologous with the Corresponding Region from Strain CA10 (A). The Genetic Structures of the Putative Ancestral Form of ORFL2J3, the ORF34-carF Region on the Strain CA10 Genome, and ISJsp2-ORFL2J3 on the Strain J3 Genome are Compared (B).

The pentagons indicate the sizes, locations, and directions of transcription of the ORFs. Homologous DNA regions are shown by shading. Black pentagons and black box represent the car genes and ISJsp2 respectively.
structure of the car gene cluster and its flanking region in strain KA1”). Several deduced amino acid sequences of the proteins showing homology with ORFU11J3 were the GntR family transcriptional regulators.22) The helix-turn-helix motif, which may be involved in DNA-binding for transcriptional regulation,22) was found in the N-terminal region of the ORFU11J3 product (data not shown). Because transcriptional regulator genes are frequently located in the immediate vicinity of the catabolic operon, ORFU11J3 was tentatively designated *carR*J3, as the putative transcriptional regulator of the *car*J3 operon.

No other genes possibly involved in CAR-degradation were observed further upstream of ORFU11J3, although a putative transposase gene was found. The deduced amino acid sequence of ORFU3J3 showed 76% identity with transposase from *Xanthomonas axonopodis* pv. citri strain 306 (no. AAM37283). The ORFU4J3 product showed 75% identity with the hypothetical protein from *Azotobacter* sp. strain FA8 (no. CAD42760). Also, in both ORF products, rather low but significant homologies were observed with the transposases belonging to the IS3 family, such as IS401 located on pTGL6 from *Burkholderia cepacia* ATCC17616.23) As a result of comparison with the IR of IS401 and alignment analysis of the flanking region of these ORFs, a 13-bp perfect IR was detected (Table 2). This 1,312-bp-long DNA region between 13-bp IR was designated ISJsp1. Southern hybridization analysis revealed that strain J3 had 2 copies of ISJsp1 (Table 2). The amino acid sequence of ORFU2J3, on the left next to the ISJsp1 in Fig. 1, showed 42% identity with that of the *tral* gene, which may be involved in the transfer of plasmid pADP-1 of *P. putida* strain ADP.23) implying the possibility that the sequenced region of strain J3 in this study is located on a transferable plasmid.

Also, we determined the nucleotide sequence of the 5.2-kb downstream region of the *carAd*J3 gene (pBJ3102 insert) and 4 ORFs and the 3’-terminal of ORF were identified (Fig. 2). The nucleotide sequence of the 3,444-bp-long DNA region located immediately downstream of the *carAd*J3 gene showed 90% identity with the corresponding region of the *car*CA10 gene cluster, except for the unrelated 1,045-bp DNA region 1,759-bp downstream of the termination codon of the *carAd*J3 gene (Fig. 3A). This 1,045-bp DNA region had an ORF (ORFL1J3) whose product showed 41% identity with the transposase of IS903,25) classified in the IS5 family.25) Alignment analysis using IR of IS903 showed the presence of imperfect 18-bp-long IR in both termini of the inserted 1,045-bp DNA region (Table 2). This DNA region, moreover, was flanked by a 9-bp DR. Hence, we identified this DNA region as an IS element and designated ISJsp2 (Table 2). As a result of Southern hybridization analysis, 3 copies of ISJsp2 were detected in strain J3 genome (Table 2). Immediately downstream of the *carAd*J3 gene, there was an ORF (ORF32J3) showing 93% nucleotide sequence identity with the *carD* gene, and another (ORF33J3) showing 93% identity with ORF33 in the *car*CA10 gene cluster (Figs. 2 and 3A). While ORF34 is located immediately downstream of ORF33 in the *car*CA10 gene cluster,25) an ORF34-like gene was disrupted by ISJsp2 (Fig. 3B). Based on this result, although the initiation codon of ORFL2J3 was at first annotated to be located 93-bp downstream of the ORFL1J3 termination codon, it is possible that the insertion of ISJsp2 disrupted the actual reading frame of ORFL2J3. In fact, the coding region of ORFL2J3 can be extended to the putative initiation codon observed upstream of the ISJsp2 insertion, which corresponded to that of ORF34CA10 (Fig. 3B). The extended ORFL2J3, shown as “Former form of ORFL2J3” in Fig. 3B, encoded a 533-amino acid polypeptide, and the entire region of this peptide showed significant homology with the putative methyl-accepting chemotaxis proteins reported in *Rhizella aquatilis* (41%) (no. AAK83219). As shown in Fig. 3A and 3B, the 5’-half of ORFL2J3 showed high identity with nine tenth from the 5’-terminal of ORF34CA10, and the similarity was similarly lost 39-bp upstream of the initiation codon of the *carFC*CA10 gene (Fig. 3A). The 3’-portion of ORFL3J3 was found further downstream of ORFL2J3 in the opposite direction (Fig. 2), and the product had a putative helix-turn-helix motif of a transcriptional regulator protein of the MatR family.

Genetic structure of the car gene cluster and its flanking region in strain KA1

We constructed the appropriate deletion plasmids, pBKA101, pBKAI02, and pBKA103, from the genomic cosmid clone pSCKA002 containing a *car*KA1 gene cluster, and determined the nucleotide sequence of the respective inserts. Consequently, the nucleotide sequence of a 13,651-bp *HindIII*-XhoI region (Fig. 2) containing the previously reported 1,110-bp DNA region22) became apparent. As shown in Fig. 2, we found 13 complete ORFs (ORF1KA1 to ORF13KA1) in this region.

*(1) The *car*KA1 gene cluster*

The nucleotide sequence of the 4,126-bp DNA region, positions 4,569 to 8,696 in accession no. AB095953, containing the previously reported PCR-amplified partial *carA*BA gene region,22) was identical with that of the CAR-catabolic operon isolated from *Sphingomonas* sp. strain GTIN11.11) Following the nomenclature of strain GTIN11, 4 complete ORFs (ORF1KA1 to ORF4KA1) detected in this region were designated *carA*KA1, *carB*KA1, *carB*KB1, and *carC*KA1. Because the products of the *car*KA1 genes (CarAa, CarBa, CarBb, and CarC) also showed moderate homology with the respective Car proteins from strain CA10, whose functions in CAR-metabolism have been well-characterized,9,10) the function of each gene product in CAR metabolism by strain KA1 was predicted as follows: CarAaKA1, the terminal oxygenase component of CAR 1,9a-dioxygenase; CarB*KA1, and CarB*KA1, meta-cleav-
age enzyme subunits; CarC_{KA1}, meta-cleavage compound hydrolase. In fact, it has been reported that disruption of the carAa gene of *Sphingomonas* sp. strain GTIN11 resulted in a deficiency in CAR degrading capacity.\(^{(1)}\) On the other hand, homology search and alignment analysis indicated that the ORF5_{KA1} product and CarA_{GTIN11} showed no relation to the CarA_{CA10} protein (~9% identity). The ORF5_{KA1} product, however, showed 56% amino acid identity with ferredoxin from *Brucella melitensis* strain 16M (no. AAL52140), and the ferredoxins showing similarity with the ORF5_{KA1} product had a [2Fe-2S] cluster coordination sequence (CXXCXXC) conserved in the putidaredoxin-type or adrenodoxin-type ferredoxins.\(^{(26)}\) Because the function of carA_{GTIN11}, identical to ORF5_{KA1}, in CAR metabolism is still unknown, we examined whether the ORF5_{KA1} product can function in electron transport to the terminal oxygenase CarA_{KA1} protein by resting cell reaction. The pBR1MCS-2-based plasmids pBBKA1K\(^{(17)}\) and pBBKA2K, carrying carA_{KA1} and carA_{KA1} + ORF5_{KA1} respectively under the constitutive nptII promoter, were constructed and then transformed into *E. coli* strain JM109. Resting cells of *E. coli* that contained pBBKA1K, pBBKA2K, or plasmid vector without insert DNA were provided with 0.1% (w/v) carbazole. After 18 h of incubation, the cells were removed and the resultant supernatants were extracted with ethyl acetate. The formation of dioxygenating product in the ethyl acetate extracts was investigated with ethyl acetate. The formation of dioxygenating product can function in electron transport to the terminal oxygenase CarA_{KA1} protein by resting cell reaction. The pBR1MCS-2-based plasmids pBBKA1K\(^{(17)}\) and pBBKA2K, carrying carA_{KA1} and carA_{KA1} + ORF5_{KA1} respectively under the constitutive nptII promoter, were constructed and then transformed into *E. coli* strain JM109. Resting cells of *E. coli* that contained pBBKA1K, pBBKA2K, or plasmid vector without insert DNA were provided with 0.1% (w/v) carbazole. After 18 h of incubation, the cells were removed and the resultant supernatants were extracted with ethyl acetate. The formation of dioxygenating product in the ethyl acetate extracts was investigated with ethyl acetate.

The mass-fragmentation pattern of this compound is as follows: 345 (M\(^{+}\), 2), 313 (69), 269 (4), 201 (9), 145 (48), 117 (94), 73 (100) (the parenthetic numbers present % base peak). Such GC-MS data were consistent with those for 2-amino-3-biphenyl-2,3-diol,\(^{(27)}\) clearly suggesting that angular dioxygenation of CAR occurred only in the presence of both CarA_{KA1} and the ORF5_{KA1} product. This indicates that the ORF5_{KA1} product can function as an electron mediator to CarA_{KA1} protein. Hence, we designated ORF5_{KA1}, carA_{KA1}.

In the upstream region of the carA_{KA1} gene, the 681-bp-long ORF6_{KA1} was located in the direction opposite to the transcription of the carA_{KA1} gene (Fig. 2). The deduced amino acid sequence of this ORF showed 28% identity with transcriptional regulators from *Vibrio cholerae* strain N16961 (no. AAF94493). The ORF6_{KA1} product can be aligned with several GntR-family transcriptional regulators, including CarR_{J3}, and the DNA-binding motif\(^{(28)}\) was also conserved in the N-terminal region (data not shown). Based on these results, the gene product of ORF6_{KA1} might be the transcriptional regulator for the carA_{KA1} operon, and hence this ORF was tentatively designated carR_{KA1}. The carR_{KA1}-like gene (547-bp) was also observed in the reported DNA sequence of the carR gene cluster from strain GTIN11.\(^{(13)}\) However, although the nucleotide sequence of only the 5’-portion of the carR_{KA1}-like gene of the strain GTIN11 genome was available, the terminal 27-bp region of the reported sequence did not match the nucleotide sequence of the carR_{KA1} gene, as shown in Fig. 4. BLAST search analysis showed that the 27-bp mismatched sequence from strain GTIN11 was identical with the 27-bp terminal of the left end of IS6100, which included 14-bp IR (Fig. 4).\(^{(28)}\) This suggests that the carR_{GTIN11} gene was disrupted by the insertion of IS6100, and that the constitutive expression of CAR-degrading capacity reported in strain GTIN11\(^{(13)}\) was derived from the disruption of the regulator protein.

(2) Genetic organization of the flanking regions of the carA_{KA1} gene cluster

No other genes in the flanking region of the carA_{KA1} locus were expected to be involved in CAR degradation, and some gene products showed homology with the transposases. The deduced amino acid sequence of ORF12_{KA1} showed 57% identity with TnpA from *Pseudomonas* sp. strain ADP.\(^{(24)}\) The flanking region of ORF12_{KA1} had a 19-bp IR flanked with a 4-bp DR-like sequence (Table 2). Based on this feature, the 1,526-bp DNA region between the IR was considered to be IS and was classified in the IS5 family. Hence the region was designated ISShp1. Southern hybridization analysis using the internal region of IS as probe revealed that strain KA1 has only one copy of this ISShp1 in its genome (Table 2). ORF13_{KA1} also showed significant amino acid identity (87%) with transposase Orf\(A\) of ISCc3 from *Caulobacter crescentus* strain CB15 (no. AAK24693). A search for a sequence similar to the IR of ISCc3 revealed a 20-bp region that had 19 nucleotides identical with that of ISCc3 (Table 2). These findings suggested that ORF13_{KA1} and its flanking non-sequenced region are a portion of the IS.

**Fig. 4.** Alignment of the Internal Sequences of *carR* Genes of *Sphingomonas* sp. Strains GTIN11 (13) and KA1, and the Left End of IS6100 (15).

The 3’-terminal of the strain GTIN11 sequence is the terminal of the reported sequence. The 14-bp IR of IS6100 is underlined. Identical nucleotides are shown by asterisks. Numbers refer to nucleotide numbering in the original GenBank accessions as indicated.
Inducible expression of CAR-catabolic capacity of strain KA1

To determine the inducibility of the CAR-degrading capacity in strain KA1 cells, we examined the depletion of CAR using strain KA1 cells grown on CAR or nutrient broth. After 10 min of incubation, about 96% of CAR was degraded by KA1 cells grown on CAR, though only about 52% of CAR was degraded by KA1 cells grown on nutrient broth (data not shown). Although KA1 cells grown on nutrient broth retained the CAR-catabolic capacity, the cells grown on CAR had higher CAR-catabolic capacity. These results indicated that the CAR-degrading capacity of strain KA1 was expressed constitutively or induced during the first 10 min of incubation. In any case, it was concluded that the expression of CAR-degrading capacity of strain KA1 was enhanced inducibly when the cells were grown on CAR.

Discussion

The operonic structure of bacterial degradative genes is considered to form during the adaptation process to exposure to toxic or xenobiotic compounds. The differences in the genetic organization of car gene clusters and their flanking regions in *P. resinovorans* strains CA10, *Pseudomonas* sp. strain K23, *Janthinobacterium* sp. strain J3, and *Sphingomonas* sp. strain KA1 showed not only variation in the alleles of the *car* gene cluster, but also evolutionary trails, namely traces of gene recruitment and rearrangement. Figure 5 shows a hypothetical evolutionary process for CA10-type *car* gene clusters in several bacterial strains. The *car* gene clusters analyzed so far, including the *car*CA10 gene cluster, were supposed to have formed from the common genetic parts *carAaBaBbC* and other accessory parts. CA10-type *car* gene clusters probably evolved from a common prototype having a genetic structure similar to that of the *car*J3 gene cluster (Fig. 5). The termini of the strain J3 DNA region showing homology with the *car*CA10 gene cluster and flanking region were quite clear (Figs. 2 and 3A). The left terminus of the homologous region was located 24-bp upstream of the start codon of ORFU13J3, which corresponds to the center of ORF9CA10 (Fig. 3A). In the genome of strains CA10 and K23, ORF9 is thought to be a fusion gene generated by the one-ended transposition of ISPre1 with the 5'-portion of the *antA* gene encoding large subunit of the terminal oxygenase component of anthranilate 1,2-dioxygenase (Fig. 3A). Interestingly, alignment analysis revealed that the alignment of the left terminal of the homologous region of strain J3 with that of strain CA10 is very close to the
terminals of the one-ended transposition observed within ORF9 (Fig. 3A). There is only a 7-bp-long sequence overlap (5′-GCCGCGC-3′) between the two regions (Fig. 3A). The significance of this overlapped sequence is unknown. However, the deduced amino acid sequence of ORFU13J3 showed overall length-wise homology with several reported (hypothetical) proteins with similar amino acid length (data not shown), suggesting that ORFU13J3 may be not an artifact of recombination processes. Thus it is likely that the transposition of ISPre1 and the flanking 5′-portion of the antA gene into the ORFU13J3-like ancestral gene resulted in the present structure of ORF9 and its upstream region observed in strains CA10 and K23 (Fig. 5). In the right terminal of the ancestral form (the prototype of the CA10-type car gene cluster), the ORF2J3-like gene without disruption by ISJsp2 is thought to be located immediately downstream of ORF3J3 (Figs. 3B and 5). It is also supposed that, in the genome of strains CA10 and K23, the ancestral ORF2J3-like gene has been destroyed by fusion with the DNA region containing carFE genes through an unknown mechanism (Fig. 5) to form the truncated ORF34CA10/K23. This prediction is in accordance with previous speculation on the evolutionary process of the carCA10 gene cluster based on the results of homology search.31

The carKA1 gene cluster did not even include carAdD genes (Fig. 2). As reported previously, the adenine base of the termination codon of carAaKA1 overlapped with that of the initiation codon of carBbKA1, though there is a 57-bp intergenic space between the corresponding genes in the carCA10 gene cluster.12 Moreover, although carAc genes encoding ferredoxin were similarly located immediately downstream of the carC gene in both the carCA10 and carKA1 gene clusters, the types of each CarAc were different. There are a few reports that putidaredoxin-type ferredoxin is an electron donor to the terminal oxygenase component involved in the catabolism of aromatic compounds.13,29-31) These results clearly indicated that the carKA1 gene cluster is another prototype of the car gene cluster, although common genetic parts were also used as material. In this study, we found that the genetic parts involved in CAR-degradation, carR, carAaBaBbC, carAc, carAdD, and carFE, existed separately, and that various car genes were assembled from such genetic regions. Even in the carAaBaBbC gene, there was discontinuity in homology to the reported polypeptide. For example, CarAaCA10 and CarAaKA1 have less than 10% identity with the catalytic subunits of oxygenase components of known oxygenase systems, although the identities with the OxoO protein in the 2-oxo-1,2-dihydroquinoline 8-monooxygenase system32) were 39% and 34%, respectively. On the other hand, CarBaB and CarC had about 30% identity with known isofunctional enzymes. These facts might indicate that carAaBaBbC was also assembled. More detailed screening for the different alleles of car genes and subsequent comparison will provide more information about the construction process of the genetic structure of the carAaBaBbC gene cluster and the origin of the novel gene encoding the angular dioxygenase component, CarAa.

The ferredoxin of the CAR 1,9a-dioxygenase system of strain KA1, CarAcKA1, was a putidaredoxin-type, differing from that of strains CA10 and J3. On the other hand, the amino acid sequence of CarAaKA1 is 60% identical with that of CarAaJ3. From these facts, it was inferred that the difference in CarAa structures results in a change in electron recipient capability depending on the difference of the ferredoxins. Hence, it would be interesting to quantify the electron transport capacities among the components of the carbazole dioxygenase (CARDO) systems of strains CA10, J3, and KA1. Presently, we are examining the activity of CARDO using purified forms of each component from strains CA10, J3, and KA1. Information on the three-dimensional structures of the CARDO system components will be also helpful to understand evolutionary change(s) in the functions of electron transport proteins.

The nucleotide sequence of the 4,126-bp DNA region containing carAaBaBbBcAc genes from strain KA1 is identical with that of strain GTIN11.13) However, the nucleotide sequences of the 16S rRNA genes from the two strains were 95.2% identical, suggesting that the car gene clusters of strains KA1 and GTIN11 were located on the mobile genetic element that can mediate the lateral gene transfer. In fact, the carKA1 gene cluster is known to be located on the large plasmid pCAR3.12) As described in Fig. 2, the car gene cluster flanking region showed high divergence between strains KA1 and CA10. In addition, the probe mixture prepared from the entire region of plasmid pCAR1 did not hybridize with restriction enzyme-digested pCAR3, suggesting that the total genetic structure of pCAR3 is different from that of pCAR1 (data not shown). We are now determining the entire nucleotide sequence of pCAR3. It will provide us useful information on the mechanism of lateral transfer of the carKA1 gene cluster in nature. In addition, it is also interesting to investigate how the carKA1 gene cluster has been recruited on pCAR3.

As a result of sequencing analysis, the putative GntR family transcriptional regulators, CarR proteins, were found in the upstream regions of the carT3 and carKA1 gene clusters (Fig. 2). The amino acid sequence alignment of CarRJ3, CarRK1, and other GntR family regulatory proteins showed that these two proteins were not well-aligned with other GntR family regulatory proteins except for the helix-turn-helix motif, although two CarRs shared the highest identity with each other (37%) throughout the entire peptide (data not shown). This might result from the difference in the effector molecules between the two CarRs and other GntR family regulators.

In sharp contrast with our isolated strain CA10, strain GTIN11 was reported to express the CAR-metabolic enzymes constitutively.13) As shown above, the constit-
utive expression of the CAR catabolic capacity of strain GTIN11 was thought to be derived from the disruption of carR. This implies that the CarRKA1 protein functions as a repressor protein in the absence of the effector molecule, and that the effector molecule binds to the CarR protein, resulting in derepression. The fact that the capacity of strain KA1 cells to degrade CAR was enhanced by the presence of CAR (data not shown) supports this speculation. We are now analyzing the detailed function of CarRKA1 to clarify the regulatory mechanisms of the carRKA1 gene clusters.

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