The *Sphingomonas* Plasmid pCAR3 Is Involved in Complete Mineralization of Carbazole^{▽†}

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We determined the complete 254,797-bp nucleotide sequence of the plasmid pCAR3, a carbazole-degradative plasmid from *Sphingomonas* sp. strain KA1. A region of about 65 kb involved in replication and conjugative transfer showed similarity to a region of plasmid pNL1 isolated from the aromatic-degrading *Novosphingobium aromaticivorans* strain F199. The presence of many insertion sequences, transposons, repeat sequences, and their remnants suggest plasticity of this plasmid in genetic structure. Although pCAR3 is thought to carry clustered genes for conjugative transfer, a filter-mating assay between KA1 and a pCAR3-cured strain (KA1W) was unsuccessful, indicating that pCAR3 might be deficient in conjugative transfer. Several degradative genes were found on pCAR3, including two kinds of carbazole-degradative gene clusters (*car-I* and *car-II*), and genes for electron transfer components of initial oxygenase for carbazole (*fdxI*, *fdrI*, and *fdrII*). Putative genes were identified for the degradation of anthranilate (*and*), catechol (*cat*), 2-hydroxypenta-2,4-dienoate (*carDFE*), dibenzofuran/fluorene (*dbf/fln*), protocatechuate (*lig*), and phthalate (*oph*). It appears that pCAR3 may carry clustered genes (*car-I*, *car-II*, *fdxI*, *fdrI*, *fdrII*, *and*, and *cat*) for the degradation of carbazole into tricarboxylic acid cycle intermediates; KA1W completely lost the ability to grow on carbazole, and the carbazole-degradative genes listed above were all expressed when KA1 was grown on carbazole. Reverse transcription-PCR analysis also revealed that the transcription of *car-I*, *car-II*, and *cat* genes was induced by carbazole or its metabolic intermediate. Southern hybridization analyses with probes prepared from *car-I*, *car-II*, *repA*, *parA*, *traI*, and *traD* genes indicated that several *Sphingomonas* carbazole degraders have DNA regions similar to parts of pCAR3.

In the last 30 years, many plasmids have been isolated that carried genes encoding enzymes for degrading organic xenobiotics. Some of these plasmids identified in *Pseudomonas* bacteria are classified into incompatibility (Inc) group P, such as IncP-1, IncP-2, IncP-7, and IncP-9 (13, 44, 45, 68). Among the *Pseudomonas* degradative plasmids, whole nucleotide sequences of pADP-1 (37), pEST4011 (65), pJP4 (62), pUO1 (IncP-1 [56]), pCAR1 (36, 54), pND6-1 (IncP-7 [35]), NAH7 (57), pDTG1 (14), and pWW0 (IncP-9 [18]) have been determined and analyzed in detail. Various *Sphingomonas* strains have also been identified as xenobiotic degraders and have been shown to have diverse catabolic capabilities toward acenaphthene, biphenyl, naphthalene, carbazole (CAR), dibenzop-dioxin, and many other compounds (2, 12, 21, 34). The sequences and organization of degradative genes are remarkably similar in several sphingomonads but differ from those in pseudomonads (46). In addition, large plasmids are ubiquitous in xenobiotic-degrading sphingomonads, as well as in pseudomonads (5, 6). Although it is thought that these plasmids are

important for the degradative abilities of these organisms, few studies have reported on whole sequences of plasmids isolated from sphingomonads. The only example is seen in pNL1 identified in a naphthalene- and biphenyl-degrading bacterium, *Novosphingobium aromaticivorans* F199 (58), in which the entire sequence was determined (48). Thus, it is necessary to investigate other sphingomonad plasmids to better understand their function, diversity, and molecular evolution.

Sphingomonas sp. strain KA1 was isolated from activated sludge. It is able to grow on CAR as a sole carbon, nitrogen, and energy source, and its CAR-degradative (*car*) genes are localized on the large plasmid pCAR3 (21). Different CAR-degrading *Sphingomonas* bacteria possess *car* gene homologues (28, 29). This finding also suggests that sphingomonad strains may be considered important degraders in CAR-contaminated environments.

Our group has studied the CAR degradation system in bacteria for 10 years, and it appears that the most important enzyme for CAR breakdown is carbazole 1,9a-dioxygenase (CARDO), which has three components: a terminal oxygenase, ferredoxin, and ferredoxin reductase (42, 51). As recently reported, the partial DNA sequence of pCAR3 has been determined (28, 29, 64), and it appears that pCAR3 has several genes encoding the components of CARDO: two *car* genes, *carAaIAcIAdI* and *carAaIIAcIIAdII*, designated the *car-I* and *car-II* gene clusters, and genes encoding ferredoxin and ferredoxin reductase (*fdxI* and *fdrI/fdrII*, respectively [64]). Each gene product functions in the degradation of CAR, and thus pCAR3 possesses genes encoding the multiplex CARDO (64).

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However, it remains unclear what types of genes for replication, partition, and conjugative transfer are on pCAR3 or whether pCAR3 possesses other degradative genes, such as those for the degradation of CAR intermediates. In the present study, we determined the whole nucleotide sequence of pCAR3 and analyzed its catabolic genes. We compared the pCAR3 genes with analogous plasmid or chromosomal genes in other *Pseudomonas* and *Sphingomonas* bacteria and discuss the degradative functions of pCAR3-borne genes.

MATERIALS AND METHODS

Preparation of the plasmid pCAR3 from *Sphingomonas* sp. strain KA1 cells. *Sphingomonas* sp. strain KA1 cells cultivated in L broth (50) for 15 h at 30°C were used for extraction of the pCAR3 plasmid. The cells were harvested by centrifugation ($6,000 \times g$, 15 min, 4°C), and plasmid DNA was isolated by using a Large-Construct kit (QIAGEN, Santa Clarita, CA) according to the manufacturer's recommendations.

Sequencing of pCAR3. Shotgun sequencing of pCAR3 was performed by Dragon Genomics Co., Ltd. (Shiga, Japan). Sheared and blunt-ended pCAR3 DNA was ligated to the SmaI site of pUC18. Nucleotide sequence determination was carried out by the chain termination method. One gap region was very difficult to sequence using these methods. To close the gap region, DNA fragments containing the gap region (about 0.4 kb, BamHI–NotI fragment) from the total DNA of strain KA1 was cloned into pBluescript II SK(–) (Toyobo, Tokyo, Japan), yielding pBKA302. The pBKA302 insert was sequenced by the dye-primer method using a Li-Cor model 4200L-2 auto-DNA sequencer (Li-Cor, Inc., Lincoln, NE) with M13 primers (see Table S1 in the supplemental material). Then we determined the sequence from positions 254,449 to 254,511, which contained a large DNA loop structure with high GC content (5'-ATGCCGGGGGGAGTGGCCTTCGGGCTACGCCCTACGCCACTCCCCCGGCAT-3'; the inverted repeat sequences are underlined). The complete sequence of pCAR3 was deposited in the DDBJ/EMBL/GenBank DNA databases (accession no. AB270530).

Annotation. The identification of open reading frames (ORFs) and calculation of the G+C content were performed with computer software as described previously (36). The homology search and alignment of deduced amino acid sequences of observed ORFs were performed with BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>) and CLUSTAL W (<http://align.genome.jp/>).

Mating techniques. Filter mating was performed by previously described methods (55). Before the mating experiment, we selected spontaneous rifampin (Rif)-resistant derivatives of *Sphingomonas* sp. strain KA1W (21). A mini-Tn5 transposon with the gentamicin (Gm)-resistance gene of pBSL202 (1) was introduced into the strain by conjugation, and combined resistance to Rif and Gm for strain KA1W was used for counterselection against the donor strain KA1.

Comparison of KA1 and KA1W growth on different aromatic compounds. To compare the growth of KA1 and KA1W on several aromatic compounds, both strains were grown on L-broth for 15 h at 30°C (preculture). Then cells were harvested and washed with CF buffer (Na_2HPO_4 , 2.2 g liter⁻¹; KH_2PO_4 , 0.8 g liter⁻¹; NH_4NO_3 , 3.0 g liter⁻¹), and about 10^5 cells were transferred into fresh NFMM-4, which is CF buffer with added minerals ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g liter⁻¹; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.01 g liter⁻¹; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.01 g liter⁻¹) containing 0.1% (wt/vol) of one of several aromatic compounds (except for catechol, it was included 0.01%) (see Table 2). After 14 days of growth, the resultant cultures were diluted appropriately and spread onto an L-Broth plate. Each CFU was counted, and the densities of two strains became at least 2×10^5 CFU/ml after growing. A sample showing more than 10^8 CFU/ml was assessed as capable of growing on the substrate because there were more than 100-fold gap between the growth substrates and other nongrowth substrates.

RNA and DNA manipulation. Total RNA samples were isolated from *Sphingomonas* strains by using NucleoSpin RNA II (Macherey-Nagel, Sudbury, MA) according to the manufacturer's instructions. The culture conditions for each strain were identical. A 4-ml preculture of each strain was harvested and washed twice with CF buffer and then resuspended in 4 ml of CF buffer. Cells were starved by incubation at 30°C with reciprocal shaking at 300 strokes/min for 3 h. Afterward, 500 μl of starved-cell suspension were transferred into 5 ml of succinate mineral medium, which was composed of NFMM-4 containing 0.1% (wt/vol) sodium succinate. To assess whether some aromatic compounds would induce the transcription of each of the degradative genes on pCAR3, 0.02% anthranilate, benzoate, CAR, phthalate, or protocatechuate or 0.01% catechol was added to the succinate mineral medium with each cell strain. After digestion

of contaminating DNA with 1 U of RQ1 RNase-free DNase (Promega, Madison, WI), 0.1 μg of RNA was used as the template for reverse transcription-PCR (RT-PCR). RT-PCR was performed with the One-Step RNA PCR kit (Takara Bio, Tokyo, Japan) under the following conditions: 60°C for 30 min, 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, 60 to 62°C for 1 min, and 72°C for 1 min. For the negative control, the reverse transcriptase was not included in the reaction mixture. Primers used in the present study are listed in Table S1 in the supplemental material. Total DNA extraction from various *Sphingomonas* strains and Southern hybridization analyses were performed as previously described (29, 53).

RESULTS AND DISCUSSION

Nucleotide sequence overview. Annotation of the complete 254,797-bp sequence of the circular plasmid revealed that pCAR3 contained 263 ORFs, including 26 ORFs (ORF1 to ORF8, ORF35 to ORF44, ORF84 to ORF87, and ORF114 to ORF116) previously reported (28, 29, 64) (Fig. 1). The average G+C content of pCAR3 was 62.5%, a value similar to that of other sphingomonad genomes (61.6–67.8% [3]). The average G+C content of the 5-kb region including the *car-II* operon (from ORF264 to ORF6) was high (70%), whereas that of the 5-kb region from ORF174 to ORF180 was low (53%). The sequences and organization of ORF116 to ORF191 (120,339–179,786 in GenBank/EMBL/DBJ database accession no. AB270530) and ORF210 to ORF216 (199,675–205,443 in accession no. AB270530) showed homologies with those of pNL1 from *N. aromaticivorans* F199 (39–95% identity at the amino acid sequence level for each gene product; Fig. 1). The 263 ORFs found on pCAR3 were classified into nine groups according to their predicted functions (including unknown functions) and characteristics (Table 1).

Plasmid replication and partition. The deduced amino acid sequences of ORF212, ORF213, and ORF214 showed 92, 95, and 88% overall lengthwise identities, respectively, with the putative replication initiator proteins RepAb, RepAa, and RepB of the plasmid pNL1 from *N. aromaticivorans* F199 (48), whereas their functions for replication of the pNL1 were not clear (see Fig. S1 in the supplemental material). Although ORF212 did not share a high identity with any other protein except for the RepAb gene of pNL1, it had a conserved RepA_C domain (Conserved Domain Database ID pfam04796 [<http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=pfam04796>]), and this domain was found in some RepA proteins of plasmids or genomic islands, such as R388 from *Escherichia coli* (IncW [59]), which showed 17% identity with ORF212 product pSB102 from *Sinorhizobium meliloti* FP2 (15% identity); pIPO2T from the wheat rhizosphere (14% identity [61]); and the genomic island pKLC102 from *Pseudomonas aeruginosa* (19% identity [33]). Thus, we tentatively named ORF212 as *repA*.

The origin of vegetative replication (*oriV*) on pCAR3 was predicted to be located between *repA* and ORF213 (positions 201898 to 202395). An A+T-rich region with five copies of a 12-bp repeat sequence [5'-AATCG(G/C)(T/A)TTTTT-3'] and seven copies of a 16-bp repeat sequence [5'-CCGATGATCTCGG(A/T)GC-3'] (Fig. 2A) was found. The nucleotides of these repeat sequences were conserved (>75%) compared to the consensus sequences (Fig. 2B and C). A DnaA box-like sequence was also found in that region (5'-TTGTCCACA-3' [8]). Although the importance of these repeats is not currently clear, we tentatively named the 12-bp repeat sequences as "12

the amino acid sequence of RepA and sequences of other plasmids, including well-known degradative plasmids or plasmids carrying the above RepA_C domains. Although the Inc groups of plasmids isolated from non-*Pseudomonas* or non-

TABLE 1. Classification of ORFs identified on pCAR3

Putative function	Homology observed	ORF no. (gene name) ^a
Maintenance and DNA processing	Yes	189, 199, 200, 205, 206, 210, 212 (<i>repA</i>), 213 (<i>parA</i>), 214 (<i>parB</i>)
Conjugative transfer	Yes	120 (<i>traL</i>), 121 (<i>traE</i>), 122 (<i>traK</i>), 123 (<i>traB</i>), 126 (<i>traC</i>), 128, 129, 130 (<i>traW</i>), 131 (<i>traU</i>), 132 (<i>trbC</i>), 133 (<i>traN</i>), 135 (<i>traF</i>), 136 (<i>traH</i>), 137 (<i>traG</i>), 143 (<i>trwC</i>), 144 (<i>traD</i>)
Transposition and integration	Yes	7 (<i>tnpR</i>), 8 (<i>tnpA</i>), 9, 10 (<i>orfB</i>), 11 (<i>orfA</i>), 12, 13, 14, 15, 16, 32, 33, 34, 44, 45, 46 (<i>orfB</i>), 47 (<i>orfA</i>), 48, 49, 61 (<i>tnpA</i>), 62 (<i>tnpR</i>), 73, 74, 75, 77, 79, 80, 81, 82, 83, 84, 94, 95, 96, 97, 190, 207, 208, 209, 217 (<i>tniA</i>), 218 (<i>tniB</i>), 219, 220, 221, 253, 254, 259 (<i>istB</i>), 260 (<i>istA</i>), 261, 262 (<i>istA</i>), 263 (<i>istB</i>)
Degradation	Yes	1 (<i>carAaII</i>), 2 (<i>carBaII</i>), 3 (<i>carBbII</i>), 4 (<i>carCII</i>), 5 (<i>carAcII</i>), 17 (<i>andAa</i>), 18 (<i>andAb</i>), 19 (<i>andAd</i>), 20 (<i>andAc</i>), 22 (<i>catF</i>), 23 (<i>catJ</i>), 24 (<i>catI</i>), 27 (<i>catB</i>), 28 (<i>catC</i>), 29 (<i>catA</i>), 30 (<i>catD</i>), 36 (<i>carAcl</i>), 37 (<i>carCI</i>), 38 (<i>carBbl</i>), 39 (<i>carBaI</i>), 40 (<i>carAaI</i>), 64, 85 (<i>fdxI</i>), 86 (<i>fdxI</i>), 88, 89, 90, 91, 92, 93, 99 (<i>ligI</i>), 100, 102 (<i>ligK</i>), 103a (<i>ligBII</i>), 103b (<i>ligAII</i>), 104, 105 (<i>ligJ</i>), 106 (<i>ligA</i>), 107 (<i>ligBI</i>), 108, 109 (<i>ligC</i>), 110, 111, 112, 113, 114, 115 (<i>fdxII</i>), 222, 224 (<i>ophA2</i>), 225, 226 (<i>ophB</i>), 227 (<i>ophC</i>), 231 (<i>phe</i>), 238, 239, 240 (<i>dbfA1</i>), 241 (<i>dbfA2</i>), 242 (<i>flnE</i>), 243 (<i>dbfA3</i>), 244 (<i>flnD1</i>), 245 (<i>flnB</i>), 246, 249
Transport	Yes	35, 51 (<i>czcD</i>), 52, 53 (<i>czcC</i>), 54 (<i>czcB</i>), 55 (<i>czcA</i>), 58, 59, 234, 237,
Regulation	Yes	6 (<i>carRII</i>), 21 (<i>andR</i>), 25 (<i>catRII</i>), 26 (<i>catRI</i>), 31, 41 (<i>carRI</i>), 50, 70, 71, 72, 87, 103 (<i>ligR</i>), 139, 158, 179, 198, 202, 223, 229, 230, 232, 233, 248 (<i>flnR</i>), 264
Others	Yes	66, 67 (<i>vceB</i>), 68 (<i>vceA</i>), 69 (<i>vceC</i>), 98, 115a, 124, 160 (<i>ssbR</i>), 168, 177, 178, 194, 195, 196, 197, 204, 228, 236, 247, 251, 257, 141a
Unknown	Yes	35, 42, 43, 56, 57, 60, 63, 65, 78, 116, 117, 118, 119, 125, 127, 134, 138, 140, 141, 142, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 159, 162, 164, 165, 166, 167, 169, 170, 171, 172, 173, 174, 175, 176, 180, 181, 182, 184, 185, 186, 187, 188, 191, 192, 193, 201, 203, 211, 215, 216, 252, 255, 258
	No	163, 218a

^a ORF numbers of 76, 101, 161, 183, 250, and 256 did not exist on pCAR3.

Enterobacteriaceae bacteria were not analyzed in detail, the RepA protein was completely different from those of other well-known IncP degradative plasmids (IncP-1 β , IncP-7, and IncP-9) (Fig. 3). As for other plasmids from strains of α -proteobacteria (pGOX2 or pRhico) or γ -proteobacteria (R388,

pXAC64 or pKLC102), it was implied that Rep proteins from them could make different Inc groups and, especially, it appears that pCAR3 and pNL1 may belong to the same novel, unidentified Inc group (Fig. 3).

The products of ORF213 and ORF214 showed 27 and 16%

FIG. 2. Putative *oriV* region of plasmid pCAR3. (A) Genetic map of the *parBA-oriV-repA* region. A physical map of the 497-bp putative *oriV* region between the *parA* and *repA* initiation codons of pCAR3 is shown (nucleotides 201898 to 202395 in accession no. AB270530). The shaded rectangle and black circles indicate the A+T-rich region and a DnaA box, respectively. The white triangles and black arrowheads represent the 12- and 16-bp repeat sequences designated "12-mer a" to "12-mer e" and "iteron 1" to "iteron 7," respectively. (B) Alignment of the pCAR3-borne five 12-mer repeat sequences and the *E. coli* *oriC* 13-mer sequence. (C) Alignment of the pCAR3-borne seven iterons. In panels B and C, the asterisks indicate that the direction of each sequence is inverted; conserved nucleotide and consensus sequences are shaded.

FIG. 3. Phylogenetic tree of RepA protein showing the position of pCAR3, other plasmids, and genomic islands. The GenBank accession numbers of the RepA proteins used for phylogenetic analysis are: YP_534792 (NAH7), NP_863059 (pDTG1), NP_542798 (pWW0), NP_444546 (pIPO2T), NP_361015 (pSB102), AAW59757 (pGOX2), AAS82991 (pRhico), BAF03441 (pCAR3), AAD03875 (pNL1), AAP22621 (pKLC102), NP_644784 (pXAC64), NP_943141 (pND6-1), NP_758604 (pCAR1), YP_025419 (pJP4), AAS49458 (pEST4011), NP_862528 (pADP-1), BAC81982 (pUO1), and FAA00060 (R388). The scale bar denotes 0.1 substitution per site. The tree was constructed by the neighbor-joining method, and bootstrap values of 1,000 resamplings are shown for each node.

identity with SopA and SopB proteins of the F plasmid (IncFI, accession no. AVECAF), respectively. Each conserved domain of the ParA/SopA or ParB/SopB protein (Conserved Domain Database ID pfam01656 and pfam02195) also existed in the products of both ORFs on pCAR3 (data not shown). Thus, we designated ORF213 and ORF214 as *parA* and *parB*.

Conjugative transfer. The deduced amino acid sequences of 16 ORFs (ORF120 to ORF123, ORF126 to ORF133, ORF135 to ORF137, ORF143, and ORF144) showed significant homology (71 to 90% overall lengthwise identity) to *tra* or *trw* gene products on the pNL1 plasmid (see Fig. S2 in the supplemental material) (48). These ORFs showed very low homology (15 to 27% identity) to those of the F plasmid. The products of ORF143 and ORF144 showed 23 and 22% identity, respectively, with TrwB and TrwC of the R388 plasmid, which are thought to be involved in DNA metabolism during conjugative transfer. Since the pNL1

was reported to be a conjugative plasmid (5, 48), we assessed the transferability of pCAR3 by filter mating with *Sphingomonas* sp. strain KA1W as a recipient strain (obtained as a pCAR3-cured strain [21]). As for KA1W, we confirmed that KA1W completely lost the pCAR3 as determined by PCR with primers designed for amplification of the inner region of *carAaII*, *carAcII*, *repA*, *parA*, *traI*, and *traD* genes. As a result, no PCR products were obtained using the total DNA of KA1W as a template, whereas we could detect the amplicon for each gene with that of KA1 (data not shown). To obtain transconjugants, CAR degradative ability and Rif and Gm resistance were used as selective markers. However, we were unable to detect any transconjugants on selective media (the frequency of conjugation was below 10^{-9} per donor cell). Considering that strain KA1W was a derivative of KA1 and their inner cell environments were identical and that KA1W would be completely lost the pCAR3, these results suggest that pCAR3 is

TABLE 2. ISs and Tns found on pCAR3

Name (position on pCAR3)	Size (bp)	DR (bp) ^a	IR (bp)	ORF	Homologous protein				Accession no.
					Protein	Putative function(s)	aa identity (%) ^b	Source	
ISSsp1 (6595–7823)	1,229	3	28	ORF10 ORF11	SKA58_17028 (264) ORFB (285) SKA58_17033 (90)	IS3 family element, transposase OrfB Transposase Helix-turn-helix, Fis-type transposase, IS3/IS911 family protein	94 66 91	<i>Sphingomonas</i> sp. strain SKA58 <i>Erwinia amylovora</i> strain (pEA34) <i>Sphingomonas</i> sp. strain SKA58	EAT07716 AAA98413 EAT07717
					ORFD (91)	Transposase	62	<i>Erwinia amylovora</i> (pEA34)	AAA98412
					SKA58_17033 (90)	Helix-turn-helix, Fis-type transposase, IS3/IS911 family protein	91	<i>Sphingomonas</i> sp. strain SKA58	EAT07717
ISSsp1 (86903–88131)	1,229	3	28	ORF82 ORF83	ORFD (91) SKA58_17028 (264) ORFB (285)	Transposase IS3 family element, transposase OrfB Transposase	62 94 66	<i>Erwinia amylovora</i> (pEA34) <i>Sphingomonas</i> sp. strain SKA58 <i>Erwinia amylovora</i> (pEA34)	AAA98412 EAT07716 AAA98413
					ORFB (326) ORFB (326) ORF13 (124)	ISCC3, transposase OrfB Transposase OrfB (ISCC3) Transposase	87 77 100	<i>Erythrobacter litoralis</i> HTCC2594 <i>Caulobacter crescentius</i> CB15 <i>Sphingomonas</i> sp. strain KA1	YP_459021 NP_421526 BAC56766
					ORFA (124) ORFA (125)	ISCC3, transposase OrfA Transposase OrfA (ISCC3)	90 85	<i>Erythrobacter litoralis</i> HTCC2594 <i>Caulobacter crescentius</i> CB15	YP_459022 NP_421525
ISSsp2 (28488–29998)	1,511	-	14	ORF32 ORE33	ORFB (326) ORFB (326) ORF13 (124)	ISCC3, transposase OrfB Transposase OrfB (ISCC3) Transposase	87 77 100	<i>Erythrobacter litoralis</i> HTCC2594 <i>Caulobacter crescentius</i> CB15 <i>Sphingomonas</i> sp. strain KA1	YP_459021 NP_421526 BAC56766
					ORFA (124) ORFA (125)	ISCC3, transposase OrfA Transposase OrfA (ISCC3)	90 85	<i>Erythrobacter litoralis</i> HTCC2594 <i>Caulobacter crescentius</i> CB15	YP_459022 NP_421525
					ORFA (125)	Transposase B Transposase OrfB	96 78	<i>Sphingopyxis alaskensis</i> RB2256 <i>Sphingomonas</i> sp. strain SKA58	BAE48286 ZP_01304205
ISSsp3 (42994–44514)	1,521	2	12	ORF46 ORF47	SKA58_04631 (241) tISRso12b (234) Orf2 (92)	ISRSO12-transposase ORFB protein Transposase A Transposase and inactivated derivative	34 100 100	<i>Rakstonia solanacearum</i> GMI1000 <i>Sphingopyxis alaskensis</i> RB2256 <i>Sphingomonas</i> sp. strain SKA58	NP_522065 BAE48287 ZP_01304206
					SKA58_04636 (92) tISRso12a (88)	ISRSO12-transposase ORFA protein	39	<i>Rakstonia solanacearum</i> GMI1000	NP_522064
					SalaDRAFT_2155 (242) IstB (247) SalaDRAFT_2154 (504) IstA (501)	IstB-like ATP-binding protein Putative transposase Integrase, catalytic region Putative transposase	83 54 74 46	<i>Sphingopyxis alaskensis</i> RB2256 <i>Pseudomonas aeruginosa</i> SG17M <i>Sphingopyxis alaskensis</i> RB2256 <i>Pseudomonas aeruginosa</i> SG17M	ZP_00578111 AAÑ62229 ZP_00578110 AAÑ62228
ISSsp5 (252124–254540)	2,417	-	12	ORF262 ORF263	IstA (498) IstB (252)	IS21 family transposase IS21 family transposase	77 87	<i>Agrobacterium tumefaciens</i> C58 <i>Agrobacterium tumefaciens</i> C58	NP_535340 NP_535339
					SKA58_18960 (964) TnpA (959) TnpA (988) SKA58_18955 (199)	Transposase Tn3 Putative transposase Transposase Tn4653 Putative resolvase	99 92 38 100	<i>Sphingomonas</i> sp. strain SKA58 <i>Acetobacter aceti</i> (pAC5) <i>Pseudomonas putida</i> mt-2 (pWW0) <i>Sphingomonas</i> sp. strain SKA58	ZP_01303724 NP_052127 CAC86846 ZP_01303723
					TnpR (182) TnpR (186)	Resolvase Resolvase (Tn4653)	85 30	<i>Acetobacter aceti</i> (pAC5) <i>Pseudomonas putida</i> mt-2 (pWW0)	NP_052128 CAC86845
TnsSsp1 (62141–66397)	4,257	2	38	ORF61 ORF62	TnpA (541) TniA (559) TniB (289) TniB (226)	Putative TniA-like transposition protein Transposase Putative TniB-like transposition protein TniBdelta1	22 23 23 24	<i>Rakstonia eutropha</i> H16 (pHG1) Plasmid R100 <i>Rakstonia eutropha</i> H16 (pHG1) Plasmid R100	NP_943042 NP_052889 NP_943043 NP_052890

^a -, Direct repeats (DRs) were not found.^b Overall lengthwise amino acid (aa) identities aligned by CLUSTALW are shown.

deficient in conjugative transfer. When we compared the pNL1 and pCAR3 DNA regions related to conjugative transfer, ORF851 on pNL1 existed as two ORFs on pCAR3: ORF128 and ORF129. Their products showed homology to the N-terminal and C-terminal regions of the ORF851 product, respectively (see Fig. S2 in the supplemental material). It does not appear that the ORF851 product functions in conjugative transfer, although its C-terminal region showed homology with the signal peptidase of the F plasmid (TraF). It is possible that such a difference between the two plasmids may affect plasmid transferability, but other causes were not excluded.

Transposons and their putative remnants. Several insertion sequences (ISs), which comprised five different kinds, and transposons (Tns) were found on pCAR3 (Fig. 1 and Table 2). Its candidates of inverted repeats of each of them are shown in Fig. S3 in the supplemental material. We tentatively designated these ISs and Tns as ISSsp1 to ISSsp5 and TnSsp1 and TnSsp2 (Table 2), and their sizes, putative genes, homologous proteins are listed in Table 2. From genetic structures, ISSsp1, ISSsp2, and ISSsp3 were predicted to belong to the IS3 family, whereas it was predicted that ISSsp4 and ISSsp5 belong to the IS21 family. pCAR3 possessed two copies of ISSsp1 (Fig. 1), and putative gene products of this IS showed high identity with those on *Sphingomonas* sp. strain SKA58 (Table 2). The putative gene products of ISSsp2 showed low identity to those of ISCc3 from *Caulobacter crescentus* CB15 (Table 2), which is the original host strain of ISCc3 (41). ISSsp3 had two ORFs, and their products showed high identity (>95%) with two putative transposases of *Sphingopyxis macrogoltabida* or *Sphingomonas* sp. strain SKA58 and also showed 34 to 35% identity with those of ISRs012 isolated from *Ralstonia solanacearum* GMI1000 (Table 2) (49). As for ISSsp4 and ISSsp5, each of them carried two ORFs, and their products showed identity with IstA and IstB proteins of IS from *P. aeruginosa* SG17M (7), or with IstA and IstB of IS from *Agrobacterium tumefaciens* C58 (17) (Table 2).

TnSsp1 is a putative class II transposon, and it carried two ORFs showing identity with TnpA (transposase, 91%) and TnpR (resolvase, 77%) of Tn2815 on the plasmid pAC5 from *Acetobacter aceti* (Table 2). Tn2815 is a class II transposon that carries no other accessory genes (19). Another putative transposon, TnSsp2, was observed around ORF217 and ORF218, whose products showed low identity with TniA and TniB (22 to 23%; Table 2) of the putative Tn21-family transposon on the plasmid pHG1 from *Ralstonia eutropha* H16 (52).

Many putative transposon remnants were locally found on pCAR3 (Table 3). One locus was around the *car*-II gene cluster (Fig. 1), and some remnants of the putative Tn3-family transposon were found (Table 3). Although ORF8 was disrupted by ORF9, and no putative remnants of the transposase gene (3'-terminal of ORF8) were observed on pCAR3, ORF7 and ORF8 products had high identity with transposase and resolvase of on pPDL2 on *Flavobacterium* sp. strain ATCC 27551 (Table 3). ISSsp1 was inserted in ORF9 and ORF12 (also ORF84 and ORF81; Table 3), whose products showed high identity with the putative resolvase on pMLa from *Mesorhizobium loti* MAFF303099 (31). This gene was predicted to encode the resolvase of the Tn3 family transposon, and the gene for the corresponding transposase was found downstream of ORF12, although the frameshift mutation disrupted the putative transposase gene to generate two ORFs (ORF13 and

TABLE 3. Putative transposon remnants on pCAR3

Remnant position on pCAR3 ^a	ORF	Size (bp)	Putative functions	aa identity ^b (%)	Homologous protein	Source	Accession no.
Tn3 family transposon remnants							
4471–5040 (c)	ORF7	570	Resolvase	100 (189/189)	<i>Sphingomonas</i> sp. strain KAI		BAE75870
5181–6083 (n)	ORF8 (disrupted by ORF9)	903	Resolvase Transposase	95 (181/189) 100 (300/300)	<i>Flavobacterium</i> sp. strain ATCC 27551 (pPDL2)		CAD13184
6050–6583 (c)	ORF9 (inserted by ISSsp1)	534	Transposase	93 (155/165)	<i>Sphingomonas</i> sp. strain KAI		BAE75871
7813–8148 (c)	ORF12 (inserted by ISSsp1)	336	Resolvase/Integrase	100 (177/177)	<i>Sphingomonas</i> sp. strain KAI		CAD13183
8291–9148 (c)	ORF13	858	Resolvase/Integrase	76 (136/178)	<i>Mesorhizobium loti</i> MAFF303099 (pMLa)		NP_085611
8982–11201 (c)	ORF14	2,220	Resolvase/Integrase	92 (101/109)	<i>Mesorhizobium loti</i> MAFF303099 (pMLa)		NP_085610
11447–11794 (c)	ORF15	348	Transposase	90 (256/282)	<i>Mesorhizobium loti</i> MAFF303099 (pMLa)		NP_085610
11963–14890 (n)	ORF16	2,928	Invertase/recombinase protein Transposase	81 (563/687) 64 (56/87)	<i>Xanthomonas campestris</i> pv. <i>campestris</i>		NP_637000
Others				76 (745/974)	<i>Glucanobacter oxydans</i> 621H		AAW59709
210913–212118 (n)	ORF219	1,205	Integrase-like protein	40 (133/330)	<i>Sphingomonas</i> sp. strain SKA58		EAT07252
212122–212445 (n)	ORF220	324	Transposase N terminus	42 (38/90)	<i>Novosphingobium aromaticivorans</i> F199 (pNL1)		AAD03944
212532–213005 (n)	ORF221	474	Transposase C terminus	94 (148/157)	<i>Novosphingobium aromaticivorans</i> F199 (pNL1)		AAD03945

^a The direction of transcription is indicated: n, normal; and c, complementary.
^b Values in parentheses refer to numbers of identical amino acids (aa) per number of amino acids examined.

FIG. 4. Repeat sequences (repeat I and repeat II) and highly conserved DNA regions found on pCAR3. Rectangle color indicates the kind of DNA region: orange, repeat-I; yellow, repeat-II; pink, 172-bp DNA region; purple, 1.1-kb DNA region. (A) The circular map of pCAR3 showing the position of each repeat sequence. Blue arrowheads indicate the position of repeat I-a and repeat I-b, and green arrowheads denote those of repeat II-a to repeat II-c. (B and C) Comparison of the genetic structures of repeat I-a and repeat I-b (B) and repeat II-a, II-b, and II-c and highly conserved DNA on the flanking regions (C). The position of each region is presented. Pentagons indicate the open reading frames on each repeat sequence. The sequences of the putative inverted repeats are shown, and complementary nucleotides are given in capital characters. The identity between two sequences is denoted by red numbers.

ORF14; Table 3). We found two copies of 5,337-bp repeat sequences including ORF9 to ORF14 (and ORF79 to ORF84) on pCAR3 and designated them as repeats I-a and I-b (Fig. 1 and 4). Interestingly, we found 45-bp inverted repeat sequences on the terminal end of repeats I-a and I-b (Fig. 4). Considering that these sequences included putative transposases and resolvases of the Tn3-family transposon, it appears that these sequences might function as a transposon, although the putative ORFs were disrupted or mutated as described above. Around repeat I, ORF15 and ORF16 also showed identity with genes encoding a putative invertase and transposase of the Tn3 family transposon of *Sulfitobacter* sp. strain NAS-14.1 (Table 3).

The second putative transposon locus was around ORF45, whose product showed identity with the putative insertion sequence of an ATP-binding protein on p42d from *Rhizobium etli* CFN42 (Table 3) (16). Including ORF45, a 2,637-bp sequence was completely conserved at three positions on pCAR3, designated as repeat II-a to repeat II-c (repeat II-b was inversely positioned, Fig. 1 and 4). The 172-bp DNA sequences flanking repeat II-a (positions 40264 to 40435) were highly conserved with the same-length DNA sequences as the repeat II-c flanking region (97236 to 97407) and those of the ORF258 flanking region

(248573 to 248708) (Fig. 4). The DNA sequences of these three regions showed more than 79% identity to one another (Fig. 4). Similarly, the 1,103-bp DNA region flanking repeat II-a (43067 to 44169) showed 83% identity with a 1,097-bp region flanking repeat II-b (inversely, 78739 to 79835; Fig. 4). Around repeat II-a, including the above-mentioned 172- and 1,103-bp DNA regions, we found a 12-bp inverted repeat (40264 to 40274 and 44158 to 44169; Fig. 4), whereas we were unable to detect any other repeat II sequences. These facts suggest that repeat II-a might be a remnant of the DNA regions that have recruited a transposable element from other replicons.

ORF208, ORF209, and ORF210 showed identity with genes encoding putative tyrosine recombinase genes, which conserved the characteristic RKHRKY signature of the putative active site residue (4; data not shown), although their functions are not apparent. They were inserted in the putative helicase/methylase gene of pCAR3 (ORF206 and ORF210), which showed identity with a single ORF on pNL1 (Table 3).

Degradative genes. Several catabolic genes related to the degradation of xenobiotic compounds were detected on pCAR3 (Fig. 1 and Table 1). As already reported, CAR-degrading enzymes of KA1 were encoded by two kinds of carbazole degradative gene clusters (*car-I* and *car-II* gene clusters in Fig. 1) with separately

FIG. 5. Putative degradative pathway of carbazole by products of each gene on pCAR3.

located genes for the electron transfer systems of CAR 1,9a-dioxygenase (*fdrI*, *fdrII*, and *fdxI* [64]). The products of ORF88, ORF89, and ORF90 showed 52, 67, and 81% identities, respectively, with three enzymes of the lower pathway of xylene degra-

dation, XylJQK, found on pNL1 (48). These enzymes convert 2-hydroxypenta-2,4-dienoic acid, the intermediate of CAR degradation, to acetyl-coenzyme A (CoA) (Fig. 5), and thus we tentatively named these genes *carDFE* (Fig. 1).

The deduced amino acid sequences of ORF17 to ORF21 showed 35 to 73% identity with And proteins identified in *Burkholderia cepacia* DBO1 for the degradation of anthranilate (see Fig. S4 in the supplemental material) (9), which is a ubiquitous intermediate of bacterial CAR degradation pathways (42, 51). These genes were designated *andAaAbAdAc* (Fig. 1). In the case of DBO1, it appears that the And enzyme consists of a three-component system (9), and it is largely different from the two-component anthranilate dioxygenase encoded by the *ant* operon on another CAR-degradative plasmid, pCAR1 (43, 63). Similar to the *and* operon of DBO1, we also found a gene encoding a putative transcriptional activator of the operon on pCAR3, although the direction was opposite to that of DBO1, and it was designated *andR* (see Fig. S4 in the supplemental material).

We found ORFs whose products showed homologies with the enzymes of the β -ketoadipate catabolic pathway. The products of ORF22 to ORF30 showed identity with the enzymes of the catechol-catabolic pathway found in *Pseudomonas* strains. The ORF26, ORF27, ORF28, and ORF29 products showed 39, 59, 66, and 43% identities with the catechol-catabolic (*cat*) gene products CatR, CatB, CatC, and CatA of *P. putida* KT2440, respectively, and the order and direction were conserved as described by Jiménez et al. (30). The putative catechol catabolic genes on pCAR3 were designated *catBCA*, and ORF26 was named *catRI* (see Fig. S4 in the supplemental material). The products of *catABC* genes could convert catechol to β -ketoadipate enol-lactone as shown in Fig. 5. The products of ORF24, ORF23, ORF22, and ORF30 showed 71, 71, 69, and 42% identities with those of the *pcaF*, *pcaJ*, and *pcaI* gene cluster and the *pcaD* gene of *P. putida* KT2440 (Fig. S4 in the supplemental material), respectively; however, their order was similar to that seen in *P. fluorescens* Pf0-1 (Fig. S4 in the supplemental material) (30). The products of these ORFs would catalyze the conversion of the β -ketoadipate enol-lactone formed from catechol by CatABC into the Krebs cycle intermediates succinyl-CoA and acetyl-CoA (Fig. S4 in the supplemental material) (26, 30). They were designated *catF*, *catJ*, *catI*, and *catD*. We also found that ORF25 showed 42% identity with the *pcaR* product of KT2440, and its product would regulate the expression of *catFJI* genes. It was designated *catRII* (Fig. S4 in the supplemental material). Given the two kinds of Car and And proteins produced by pCAR3, host cells would be able to convert CAR to catechol. These facts suggest that pCAR3 might carry a complete series of genes for the degradation of CAR (Fig. 5) in contrast to pCAR1, which carries only genes for carbazole conversion to catechol (36).

The deduced amino acid sequences of ORF240 and ORF241 showed low identity (34 and 35%, respectively) with the large and small subunits of the terminal oxygenase of dibenzofuran 4,4a-dioxygenase (DFDO), DbfA1A2, of *Terrabacter* sp. strain DBF63 (see Fig. S5 in the supplemental material) (32). In DBF63, the DFDO is thought to be composed of a multicomponent system with a terminal oxygenase, ferredoxin, and ferredoxin reductase, although the specific ferredoxin reductase has not been identified in the DBF63 genome (60). The *dbf* genes are localized on a linear plasmid pDBF1 in DBF63 clustered with other fluorene-catabolic (*fln*) genes, phthalate-catabolic (*pht*) genes, and protococatechuate-catabolic (*pca*) genes, and their products can convert fluorene into tri-

carboxylic acid cycle intermediates (22, 23, 25). From the pCAR3 sequence, we found a gene encoding a candidate ferredoxin component of the DFDO; the product of ORF243 showed 33% identity with CumA3 identified in *P. fluorescens* IP01 (24), and it had a putative Rieske-type [2Fe–2S] binding motif (data not shown). Although the ferredoxin component DbfA3 of the DFDO system of DBF63 is a [3Fe–4S] ferredoxin (60), we tentatively designated the ORF243 of pCAR3 as *dbfA3* (see Fig. S5 in the supplemental material). In contrast, we failed to detect the gene encoding a putative ferredoxin reductase component. The product of ORF242 showed 41 or 25% identity with BphD of *P. pseudoalcaligenes* KF707 (66) or FlnE of pDBF1 (Fig. S5 in the supplemental material) (23) known as meta-cleavage product hydrolase. ORF245 product showed 62% identity with short-chain dehydrogenase/reductase FlnB of DBF63 (Fig. S5 in the supplemental material) (23). ORF244 product showed 20 or 18% identity with the large subunit of extradiol dioxygenase CarBb (pCAR1) or FlnD1 (Fig. S5 in the supplemental material; pDBF1). In the case of the fluorene-degradative system of DBF63, the small subunit of the enzyme is encoded by a fusion gene with the ORF encoding the ferredoxin component of DFDO (ORF16a and ORF16b shown in Fig. S5 in the supplemental material [60]). However, we were unable to identify the corresponding gene(s) on pCAR3. Although the ferredoxin reductase component of DbfA1A2A3 and the small subunit of the meta-cleavage enzyme (passively FlnD2) were not found, other Fln and Dbf enzymes could provide the ability to degrade fluorene to phthalate. Although we cannot detect the ability of these gene products for degradation of fluorene, we tentatively named these genes after the *dbf* and *fln* gene clusters of DBF63 (Fig. S5 in the supplemental material).

The deduced amino acid sequence of ORF224 showed 50% identity with the oxygenase component of phthalate dioxygenase OphA2 identified in *B. cepacia* DBO1 (Fig. S6 in the supplemental material) (10). We also found that products of ORF226 and ORF227 showed 45 and 41% identity with the OphB (phthalate dihydrodiol dehydrogenase) and OphC (4,5-dihydroxyphthalate decarboxylase) of DBO1 (Fig. S6 in the supplemental material). However, the corresponding gene encoding OphA1, the reductase component of phthalate dioxygenase, was not found on pCAR3, although the possibility that other gene products could function as an electron donor to the oxygenase component (OphA2) was not excluded. If the products of these genes function as phthalate-degrading enzymes in the host strain of pCAR3, phthalate would be converted into protococatechuate.

Nine ORFs around ORF106 whose products were predicted to catalyze protococatechuate degradation were identified. They showed homologies with the *lig* genes of *S. paucimobilis* SYK-6 (38) and the *fld* genes of *Sphingomonas* sp. strain LB126 (Fig. S7 in the supplemental material) (67). Their order and direction on pCAR3 were the same as those of the aforementioned strains. We found two copies of the *ligAB* genes encoding protococatechuate 4,5-dioxygenase on pCAR3, ORF106 and ORF107, or ORF103a and ORF103b (see Fig. S7 in the supplemental material). These genes were designated *ligI* (ORF99), *ligK* (ORF102), *ligR* (ORF103), *ligBII* (ORF103a), *ligAII* (ORF103b), *ligJ* (ORF105), *ligAI* (ORF106), *ligBI* (ORF107), and *ligC* (ORF109) (Fig. S7 in the supplemental material).

TABLE 4. Growth by KA1 and KA1W on aromatic compounds

Aromatic compound	Growth on strain ^a :	
	KA1	KA1W
Anthranilate	+	+
Benzoate	+	–
Biphenyl	–	–
Carbazole	+	–
Catechol	+	+
Dibenzofuran	–	–
Dibenzothiophene	–	–
Dibenzothiophenesulfone	–	–
Fluorene	–	–
9-Fluorenone	–	–
Gentisate	+	+
Naphthalene	–	–
Phenol	–	–
Phthalate	+	+
Protocatechuate	+	+
Salicylate	–	–
Toluene	–	–
Xylene	–	–

^a A “+” indicates that the number of the cells on LB plates was more than 10⁸ CFU/ml after transfer from 14-day cultures of NMM-4 with each aromatic hydrocarbon, whereas a “–” indicates that the number was below 10⁶ CFU/ml.

Comparison of aromatic-degradative functions between strains KA1 and KA1W. To examine the contribution of putative degradative gene products from pCAR3 to the whole metabolic function of KA1, we compared the catabolic ability of KA1 and KA1W (KA1 derivative strain cured of pCAR3) using different aromatic compounds as a carbon source (shown in Table 4). We found that KA1W was able to grow on medium supplemented with anthranilate, catechol, gentisate, phthalate, or protocatechuate (Table 4). KA1 can grow on benzoate and CAR, in addition to all of the substrates utilized by KA1W. This suggests that KA1W would be able to convert benzoate and CAR into each substrate intermediate if it possessed pCAR3 and that the two kinds of *car* genes on pCAR3 are functional and essential to metabolizing CAR. We performed PCR with total DNAs from KA1 and KA1W using primers for degradative genes shown in Table 5. As a result, no products were obtained with KA1W, whereas each amplicon was detected with KA1 (data not shown). It was suggested that KA1 did not possess the identical genes with those on pCAR3. Since KA1W could grow on the intermediate compounds of CAR, anthranilate and catechol, it appears that another kind of the

degradative genes for these substrates may be located on the chromosome of KA1.

To assess the transcription of each gene for the degradation of CAR on pCAR3, we performed RT-PCR analyses with total RNA extracted from KA1 growing on the succinate-containing minimum medium supplemented with anthranilate, benzoate, catechol, CAR, phthalate, protocatechuate, or succinate. Every gene predicted to be necessary for the degradation of the metabolic intermediates of CAR (*carDFE*, *and*, and *cat* genes) was transcribed when grown with CAR as a sole carbon source (Table 5). Although *carAaI*, *carAaII*, *carAaIII*, and *carAcII* genes were transcribed at a low level when grown with succinate (64), we were unable to detect transcription products of *carDFE* and *cat* genes when KA1 was grown on succinate as a sole carbon source. When KA1 was exposed to CAR, transcription of *car-I*, *car-II*, *carDFE*, and *cat* gene clusters was clearly induced, although transcription of the *and* genes seemed to remain the same. Together with the results of the growth comparison on various substrates, these findings indicate that elevated gene expression in response to CAR exposure reveals those genes that function in CAR metabolism.

Although pCAR3 was indispensable for growth on benzoate, we were unable to find the genes showing highest homology to the benzoate degradative *ben* genes. One possibility is that benzoate might be converted into catechol by the And enzyme, although in the case of *B. cepacia* DBO1, the activity of the three-component anthranilate dioxygenase (*and* genes products) system toward benzoate is only 10% of its activity toward anthranilic acid (9).

As mentioned above, neither strain KA1 nor strain KA1W could grow on dibenzofuran or fluorene (Table 4), suggesting that the *dbf* and *fln* genes on pCAR3 (or their products) may not provide the ability to degrade these substrates. From the results of transcriptional analyses, these genes were transcribed with all substrates used in the present study (Table 5). Several reasons may explain why these genes or their products were not able to degrade all substrates. First, the substrates may not induce sufficient transcription to elevate their expression to a functional level. Second, although expression may be high enough, some components may be defective and result in failure of the complete enzyme degradation system. Third, the gene products might function as degrading enzymes for other compounds not used in the present study. As for phthalate and protocatechuate, one of the intermediate compounds of dibenzofuran or fluorene (22, 23, 25, 42), both strains KA1 and

TABLE 5. RT-PCR analyses with primers prepared from degradative genes on pCAR3

Growth substrate	Result obtained with degradative gene ^a :										
	<i>carAaI</i>	<i>carAcI</i>	<i>carAaII</i>	<i>carAcII</i>	<i>carDFE</i>	<i>andA</i>	<i>catBCAD</i>	<i>catIJF</i>	<i>ligAB</i>	<i>ophA</i>	<i>dbfA1A2</i>
Anthranilate	ND	ND	ND	ND	–	+	+	+	+	+	+
Benzoate	ND	ND	ND	ND	–	+	+	+	+	+	+
Carbazole	++	++	++	++	+	+	+	+	+	+	+
Catechol	ND	ND	ND	ND	+	+	+	+	+	+	+
Phthalate	ND	ND	ND	ND	ND	+	ND	ND	+	+	ND
Protocatechuate	ND	ND	ND	ND	ND	+	ND	ND	+	+	ND
Succinate	+	+	+	+	–	+	–	–	+	+	+

^a ND, not determined. “++” indicates that the amount of mRNA for each gene and condition was more abundant than those determined to be “+” as confirmed by quantitative RT-PCR. A “–” indicates that no amplicons were detected. The results for *carAaI*, *carAcI*, *carAaII*, and *carAcII* are from Urata et al. (64).

KA1W were able to grow on these substrates. Therefore, it is not clear if the products of *oph* or *lig* genes on pCAR3 functioned as degrading enzymes (Table 4). Transcription of *oph* or *ligAB* genes seemed to be constitutive (Table 5), and if these products do not give the ability to degrade phthalate or protocatechuate, several reasons could be considered as those for the *dbf* and *fln* genes as described above.

Putative functions of other gene products. The products of ORF69, ORF68, and ORF67 showed 40, 54, and 53% identities with the tripartite drug efflux pumps composed of VceCAB isolated from the pathogen *Vibrio cholerae* (11, 15, 69). VceCAB can complement the multidrug resistance phenotype in *E. coli* null mutants, and the complemented strain shows resistance against the uncoupler cyanide *m*-chlorophenylhydrazine, the detergent sodium deoxycholate, phenylmercuric acetate, and several antibiotics such as chloramphenicol (Cm), nalixidic acid (Nal), erythromycin, and Rif (11, 15, 69). To assess whether the products of these genes would be functional, we compared resistance of KA1 and KA1W to the antibiotics Cm, Nal, and Rif. Although both strains showed resistance to each antibiotic, we could not detect a difference between the two strains (data not shown).

Some putative genes for heavy metal resistance were also found on pCAR3. Putative efflux pump components were detected (ORF53, ORF54, and ORF55) that are similar to the *czrCBA* gene cluster of *P. aeruginosa* PAO1 (27) or the *czcCBA* gene cluster of *Ralstonia metallidurans* (*Alcaligenes eutropha*) CH34 (20, 47) for resistance to heavy metals such as Co, Zn, or Cd. The products of ORF51 and ORF58 showed identity with the CzcD protein (a putative membrane-bound protein necessary for recognition of metals) of *R. metallidurans* CH34 (Fig. 1). To assess whether the *czc* genes products would be functional, we compared resistance of KA1 and KA1W to the heavy metals, Co and Zn by using the salts of them as described by Rensing et al. (47). Although both strains showed resistance to 1 μ M ZnCl₂, we could not detect a difference between the two strains with higher concentration of Zn or with CoCl₂ (data not shown). ORF194, ORF195, ORF197, and ORF198 showed identity with putative genes for arsenic resistance. However, it is possible that the total gene set for arsenic resistance might not be present (39). Although their functions are not currently clear, these gene products may provide an advantage to the plasmid host strain.

Distribution of pCAR3-like plasmid in other CAR-degrading bacteria. Basta et al. (5) showed that the *repAa* and *repAb* genes of pNL1 existed in other plasmids isolated from two *Sphingomonas* strains: *S. subterranea* and *S. aromaticivorans*. This indicates that these plasmids may be members of a novel incompatibility group found in the genus *Sphingomonas*. Previously, Inoue et al. (28) isolated 27 CAR-degrading bacteria from various sites in Japan, which were different than that of plasmid pCAR3 isolated, and Southern analyses indicated that 17 of them have a *carAaI*-homologous gene. To assess the distribution of pCAR3-like plasmids in previously isolated CAR-degrading *Sphingomonas* strains (Table 6) (28, 29), Southern hybridization analyses were performed on total DNA from the degraders with probes prepared from *carAaII*, *carAcII*, *repA*, *parA*, *traD*, or *traI* genes on pCAR3 (designated *carAaI* probe, *carAcII* probe, *repA* probe, *parA* probe, *traI*

TABLE 6. Southern hybridization analyses with probes prepared from DNA of pCAR3 for various *Sphingomonas* CAR degraders

<i>Sphingomonas</i> sp. strain	Result obtained with probe ^a :						
	<i>carAaI</i>	<i>carAaII</i>	<i>carAcII</i>	<i>repA</i>	<i>parA</i>	<i>traI</i>	<i>traD</i>
KA1	+	+	+	+	+	+	+
IC074	+	–	–	–	–	–	–
IC075	+	+	+	–	–	–	–
IC081	+	+	+	+	+	+	+
IC097	+	+	+	–	–	–	–
IC193	+	–	–	–	–	–	–
IC273	+	+	+	+	+	+	+
IC291	+	+	–	–	–	–	–
IC321	+	+	+	+	+	+	+

^a The results of Southern hybridization with *carAaI* are from Inoue et al. (28).

probe, and *traD* probe) under highly stringent conditions (hybridization performed under 60°C).

The *carAaII* and *carAcII* probes hybridized with DNA from IC075, IC081, IC097, IC273, and IC321, although the IC291 genome seemed to lack the *carAaII* gene (Table 6). We also detected signals with *repA* and *parA* probes using the DNA of IC081, IC273, and IC321 and with *traI* and *traD* probes using the DNA of IC081, IC273, and IC321 (Table 6). These facts suggest that IC081, IC273, and IC321 might carry pCAR3-like plasmids and that other strains (IC074, IC075, IC193, and IC291) may have their *car-I* genes (and *car-II* genes for IC075 and IC291) on different replicons from pCAR3, including the chromosome. Transfers of these pCAR3-derivative plasmids may distribute *car-I* and *car-II* gene homologues, and some genes on the plasmids might be lost by homologous recombination or genetic rearrangement with the repeat sequences, transposons, or their remnants on pCAR3. Indeed, the DNA region of pCAR3 including the *car-I* gene cluster was deleted after transfer into fresh L media (Y. Saiki, unpublished data). In conclusion, although pCAR3 itself did not show a conjugative transfer function, similar plasmids may have an important role in distributing *car-I* or *car-II* genes in nature.

Conclusions. pCAR3 carried genes involved in the degradation of CAR into Krebs cycle intermediates. There are few reports of a single plasmid possessing genes for the mineralization of a polyaromatic compound except for the NAH7 plasmid and its derivatives and, to the best of our knowledge, this is the first demonstration in the genus *Sphingomonas*. Interestingly, the products of *car-I* and *car-II* genes on pCAR1 can use the products of *fdxI*, *fdxI*, or *fdxII* on pCAR3 as an electron transfer component (64).

Studies on the degradative genes of *Sphingomonas* and related strains suggest that each of these genes are dispersed in their genomes (2, 40, 46, 48). In the case of pCAR3, the CAR degradation upper pathway genes were located separately (*fdxI*, *fdxI*, and *fdxII* were apart from *car-I* and *car-II* gene clusters; see Fig. S8 in the supplemental material) (64). However, other degradative genes such as *and*, *cat*, and *lig* were clustered on pCAR3 (Fig. 1 and see Fig. S4 to S7 in the supplemental material). In addition, *and*, *cat*, *dbf*, and *oph* genes showed homologies to genes in different genera, such as *Pseudomonas*, *Burkholderia*, or *Terrabacter*. The observations that some genes (*and*, *cat*, *carDFE*, and *car-I*) were encompassed by two kinds of repeat sequences (repeat I and repeat

II) and that a *car-I* gene cluster can be deleted suggest that the genes between the repeat sequences may be inserted into other genes or be related to the reorganization of pCAR3 genetic structure (see Fig. S8 in the supplemental material). Detailed analyses comparing pCAR3 with other CAR-degradative plasmids isolated from *Sphingomonas* or comparing the genetic structures of *car-I* and *car-II* gene clusters on other replicons will be important to understanding the evolution of pCAR3.

pCAR3 had a core region similar to that of pNL1 (Fig. 1, S8). This region is necessary for replication, partition, and conjugative transfer of the plasmid. Some genes in this region were conserved with other *Sphingomonas* CAR degraders (Table 6), as seen in the case of pNL1 (5, 6). This indicates that plasmids carrying core regions similar to those of pCAR3 or pNL1 are widely distributed among *Sphingomonas* spp. Therefore, although more detailed analyses of pCAR3 plasmid features are necessary, the present study gives important information characterizing a novel *Sphingomonas* plasmid group.

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