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Cloning of Genes Involved in Carbazole Degradation of *Pseudomonas* sp. Strain CA10: Nucleotide Sequences of Genes and Characterization of *meta*-Cleavage Enzymes and Hydrolase

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The DNA fragment encoding *meta*-cleavage enzymes and the *meta*-cleavage compound hydrolase, involved in carbazole degradation, was cloned from the carbazole-utilizing bacterium *Pseudomonas* sp. strain CA10. DNA sequence analysis of this 2.6-kb *Sma*I-*Sph*I fragment revealed that there were three open reading frames (ORF1, ORF2, and ORF3, in this gene order). ORF1 and ORF2 were indispensable for *meta*-cleavage activity for 2′-aminobiphenyl-2,3-diol and its easily available analog, 2,3-dihydroxybiphenyl, and were designated *carBa* and *carBb*, respectively. The alignment of CarBb with other *meta*-cleavage enzymes indicated that CarBb may have a non-heme iron cofactor coordinating site. On the basis of the phylogenetic tree, CarBb was classified as a member of the protocatechuate 4,5-dioxygenase family. This unique extradiol dioxygenase, CarB, had significantly higher affinity and about 20-times-higher *meta*-cleavage activity for 2,3-dihydroxybiphenyl than for catechol derivatives. The putative polypeptide encoded by ORF3 was homologous with *meta*-cleavage compound hydrolases in other bacteria, and ORF3 was designated *carC*. The hydrolase activity of CarC for 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid, the *meta*-cleavage compound of 2,3-dihydroxybiphenyl, was 40 times higher than that for 2-hydroxy-6-oxohepta-2,4-dienoic acid, the *meta*-cleavage compound of 3-methylcatechol. Alignment analysis and the phylogenetic tree indicate that CarC has greatest homologies with hydrolases involved in the monoaromatic compound degradation pathway. These results suggest the possibility that CarC is a novel type of hydrolase.

Microorganisms which degrade xenobiotic compounds such as polycyclic aromatic hydrocarbons and heterocyclic aromatic compounds are widely spread throughout the environment and have been isolated on the basis of their ability to assimilate these compounds. In the general bacterial degradation system, the aromatic compounds are converted to the corresponding *cis*-dihydrodiol compounds and then to the corresponding catechol-type derivatives, after which they are cleaved into the *meta*- or *ortho*-ring fission compounds (10, 54). The degradation pathways and degradative genes of polycyclic aromatic hydrocarbons and biphenyl derivatives have been well studied (5, 14, 22, 26, 30, 34, 35, 51). On the other hand, the catabolic pathways of heterocyclic aromatic compounds such as dibenzothiophene are established (29, 40), but there is little information on the degradative genes of these compounds (11).

Carbazole (CAR) is a heterocyclic aromatic compound containing a dibenzopyrrole system, being derived from coal tar and shale oil (37). As CAR is known to possess mutagenic and toxic activities (3) and also to be a recalcitrant molecule (9), serious problems arise if it is released into the environment. Although several CAR-utilizing bacteria have been isolated (17, 18, 25, 32, 41), almost no information has been obtained on the genes involved in the degradation of CAR. The conversion of CAR by bacterial multicomponent dioxygenases such as naphthalene 1,2-dioxygenase or biphenyl 2,3-dioxygenase was examined by Resnick et al. (42), but these dioxygenses could not attack the angular position adjacent to the nitrogen atom.

Previously, we isolated *Pseudomonas* sp. strain CA10 from a sample of activated sludge as a microorganism with the ability to utilize CAR as a sole source of carbon, nitrogen, and energy (41). As shown in Fig. 1, the degradation pathway of CAR in strain CA10 was proposed on the basis of identification of metabolites during growth and comparison with other aromatic compound degradative pathways such as biphenyl and naphthalene. The initial step of degradation of CAR is considered to be dioxygenation at the angular position adjacent to the nitrogen atom to give the dihydroxylated intermediate, which is considered to be spontaneously converted to 2′-aminobiphenyl-2,3-diol. Then extradiol dioxygenase attacks the hydroxylated ring at the *meta* position. Hydrolysis of the *meta*-cleavage compound yields anthranilic acid, which is further converted to catechol. Catechol is considered to be metabolized through the β-ketoadipate pathway.

In a previous study (31), we isolated and characterized a transposon Tn*5* mutant of strain CA10 which is deficient in CAR catabolism, and we cloned the Tn*5* and its flanking sequences. We also reported that the cloned DNA fragment contained the *meta*-cleavage enzyme and that the genes involved in degradation of CAR were clustered. Recently, we succeeded in cloning the genes encoding the enzymes involved

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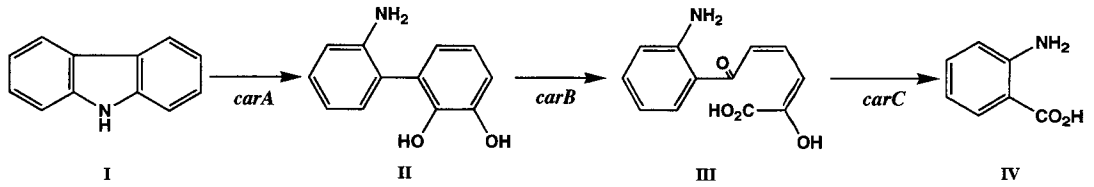

FIG. 1. Degradation pathway of carbazole by *Pseudomonas* sp. strain CA10. Gene designations: *carA*, carbazole 1,9a-dioxygenase; *carB*, 2′-aminobiphenyl-2,3-diol 1,2-dioxygenase; *carC*, 2-hydroxy-6-oxo-6-(2′-aminophenyl)-hexa-2,4-dienoic acid (*meta*-cleavage compound) hydrolase. Compounds: I, carbazole; II, 2′-aminobiphenyl-2,3-diol; III, 2-hydroxy-6-oxo-6-(2′-aminophenyl)-hexa-2,4-dienoic acid; IV, anthranilic acid.

in the carbazole degradation pathway from the wild-type strain CA10, and *meta*-cleavage enzyme and hydrolase were found to be contained in the DNA fragment (6.9-kb *Eco*RI fragment) corresponding to the above-mentioned DNA sequences with the Tn*5* insertion. We also found that the 6.9-kb *Eco*RI fragment contained the genes encoding carbazole 1,9a-dioxygenase (CARDO).

We herein describe the details of cloning and characterization of the genes encoding *meta*-cleavage enzyme and hydrolase involved in CAR degradation. The cloning and characterization of CARDO are reported in the accompanying report (46).

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Strain CA10 was grown as described previously (41). *Escherichia coli* JM109 (57) was used as the host strain for plasmids pUC118 and pUC119 (*lacZ* Ap^r, 3,162 bp [55]), pSTV29 (*lacZ* Cm^r, 2,999 bp [Takara Shuzo Co., Ltd., Kyoto, Japan]), and their derivatives. *E. coli* strains were grown on 2×YT medium (43) at 37°C. Ampicillin and chloramphenicol (final concentrations, 50 and 30 μg/ml, respectively) were used for liquid cultures and selection on agar plates. For plate cultures, the above-described media solidified with 1.6% (wt/vol) agar were used.

DNA manipulation. Plasmid DNA was prepared from the *E. coli* host strain by the alkaline lysis method (7). Total DNA of strain CA10 was prepared from cells grown on 2× YT medium at 30°C according to a protocol using hexadecyltrimethyl ammonium bromide (6). Restriction endonuclease, T4 DNA ligase, and a DNA blunting kit (Takara Shuzo) were used according to the manufacturer's instructions. DNA fragments were extracted by the glass powder method (Geneclean II kit; Bio 101 Inc., La Jolla, Calif.) as instructed by the manufacturer. Other DNA manipulations were performed according to standard methods (43).

Cloning of the genes encoding *meta*-cleavage enzymes. Total DNA of strain CA10 was digested with restriction endonuclease *Eco*RI or *Sph*I and ligated to pUC119 vector digested with *Eco*RI or *Sph*I, respectively. Transformation of *E. coli* JM109 was done as described by Hanahan (21), and ampicillin-resistant transformants were selected on 2×YT agar plates containing ampicillin. The activity of *meta*-cleavage enzymes was assayed by observing whether the color of the transformant colonies changed to yellow after the plates were sprayed with ethereal 2,3-dihydroxybiphenyl (0.5 mg/ml) (16). The solution of 2,3-dihydroxybiphenyl was prepared fresh daily.

Hybridization experiments. Southern blotting (50) was performed by using a Hybond-N^+ nylon membrane (Amersham Life Science, Tokyo, Japan) as recommended by the manufacturer. A nonradioactive digoxigenin DNA labeling and detection kit (Boehringer GmbH, Mannheim, Germany) was used according to the manufacturer's instructions. Hybridizations were carried out at 68°C in the presence of 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). After the hybridization, the membranes were washed with 0.1× SSC containing 0.1% sodium dodecyl sulfate (SDS) at 68°C for 30 min.

Resting cell reaction. *E. coli* JM109 cells harboring recombinant plasmids were cultivated in 100 ml of 2×YT medium at 37°C. When the optical density at 550 nm reached 0.8, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM, and the cells were incubated for another 5 h. Then the cells were harvested by centrifugation and washed twice with ice-cold 50 mM sodium phosphate buffer (pH 7.5), and the resultant cells were resuspended in 10 ml of the same buffer. A solution of 2,3-dihydroxybiphenyl (100 mg/ml) was prepared in ethanol, and then a 100-μl aliquot of this solution was added to each batch of resting cells. The resultant reaction mixture was incubated on a reciprocal shaker (300 strokes/min) at 30°C for 1 h. Metabolites were extracted with ethyl acetate after acidification to pH 3.0 with 1 N HCl. The ethyl acetate layer was dried with anhydrous sodium sulfate and then evaporated to dryness in vacuo at below 40°C. Each extract was methylated with ethereal diazomethane and subjected to gas chromatography-mass spectrometry (GC-MS).

GC-MS. For GC-MS, a model JMS-Automass 150 gas GC-MS system (JEOL, Ltd., Tokyo, Japan) fitted with a fused silica chemically bonded capillary column (DB-5; 0.25 mm [inside diameter] by 15 m, 0.25-μm film thickness; J & W Scientific Inc., Folsom, Calif.) was used. Each sample was injected onto the

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Bacterial strains		
Pseudomonas sp. strain CA10	Car^{+a}	41
E. coli JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*)/F′[*traD*36, *proAB*$^+$, *lacI*^{q} *lacZ*ΔM15]	57
Plasmids		
pUC118	Ap^r	55
pUC119	Ap^r	55
pSTV29	Cm^r	Takara Shuzo
pTD21	Ap^r, pUC119 with *Eco*RI insert of *Pseudomonas* sp. strain TD2 DNA	31
pUCA1	Ap^r, pUC119 with *Eco*RI insert of *Pseudomonas* sp. strain CA10 DNA	This study
pUCA121	Ap^r, pUC118 with *Sma*I-*Bgl*II insert from pUCA1 insert	This study
pUCA122	Ap^r, pUC118 with *Sma*I-*Sph*I insert from pUCA1 insert	This study
pUCA15	Ap^r, pUC119 with *Eco*RI-*Xho*I insert from pUCA1 insert	This study
pSCA16	Cm^r, pUC119 with *Xho*I-*Bgl*II insert from pUCA1 insert	This study
pUCA17	Ap^r, pUC119 with *Eco*RI-*Sac*II insert from pUCA1 insert	This study
pUCA18	Ap^r, pUC119 with *Eco*RI-*Bgl*II insert from pUCA1 insert	This study
pUCA123	Ap^r, pSTV29 with *Sma*I-*Kpn*I insert from pUCA1 insert	This study
pUCA191	Ap^r, pUC119 with *Hin*dIII-*Sph*I insert from pUCA1 insert	This study
pYN105	Ap^r, pUC119 with *Sph*I insert of *Pseudomonas* sp. strain CA10 DNA	This study

[a] Car^+ indicates the ability to grown on CAR as a sole source of carbon, nitrogen, and energy.

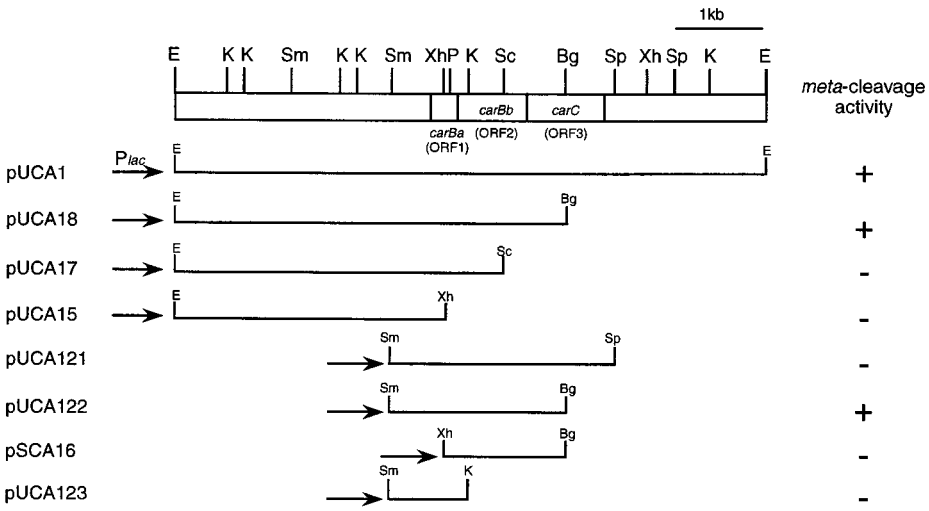

FIG. 2. (A) Restriction map of the 6.9-kb *Eco*RI insert of pUCA1 and relationship of the deleted plasmids. Only restriction sites of relevant enzymes are shown. The location and orientation of the *lac* promoter of the pUC118 and pUC119 vectors are indicated by arrows. The presence (+) or absence (−) of *meta*-cleavage activity, which was assayed by observing the change of color of the transformant colonies after being sprayed with ethereal 2,3-dihydroxybiphenyl, is shown at the right. Restriction site designations: E, *Eco*RI; Sm, *Sma*I; Xh, *Xho*I; Bg, *Bgl*II; Sp, *Sph*I; K, *Kpn*I; H, *Hin*dIII; P, *Pst*I; Sc, *Sac*II.

column at 60°C in the splitless mode. After a 2-min isothermal hold at 60°C, the column temperature was increased at 4°C/min to 72°C and then increased at 16°C/min to 280°C. The head pressure of helium carrier gas was 65 kPa.

Nucleotide sequence determination and phylogenetic analyses. Unidirectional deletion mutants were constructed by using a DNA deletion kit (Takara Shuzo) (24). Nucleotide sequence determination was carried out by the chain termination method of Sanger et al. (44), using an Applied Biosystems 373A DNA sequencer (Perkin-Elmer Japan Co. Ltd., Chiba, Japan), and the obtained nucleotide sequences were analyzed with DNASIS-Mac software (version 3.6; Hitachi Software Engineering Co. Ltd., Yokohama, Japan). Alignment of the CarBb, CarB2, and CarC sequences was performed with the CLUSTAL W package (53). Programs used to infer phylogenetic trees are contained in the PHYLIP package (version 3.5c; obtained from J. Felsenstein, University of Washington). PROTDIST with the Dayhoff PAM matrix option was used to calculate evolutionary distances. Phylogenetic trees were constructed from evolutionary distance data by the neighbor-joining method (45), implemented through the program NEIGHBOR. A total of 100 bootstrapped replicate resampling data sets for PROTDIST were generated with the program SEQBOOT, to provide confidence estimates for tree topologies (15).

Preparation of cell extract. *E. coli* harboring recombinant plasmid pUCA122, pYN105, or pUCA191 was grown to an optical density of 0.8 at 550 nm. After further incubation in the presence of 0.5 mM IPTG for 5 h at 37°C, the cells were harvested, washed twice with 50 mM sodium phosphate buffer (pH 7.5) containing 10% acetone, and resuspended in the same buffer. The resultant cell suspensions were sonicated and centrifuged at 17,000 × *g* at 4°C for 60 min, and their supernatants were used as cell extracts.

Measurement of enzymatic activities. The *meta*-cleavage activities for 2,3-dihydroxybiphenyl in the cell extracts were assayed by measuring the increase in A_{434} for 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA; the *meta*-cleavage compound of biphenyl) with a Beckman DU-7400 spectrophotometer equipped with a thermojacketed cuvette holder and a TAITEC circulating water bath. The assay was performed at 25°C by using 50 mM sodium phosphate buffer (pH 7.5) containing 10% acetone. The substrate was dissolved in ethanol, and the resultant stock solution was added to the reaction buffer at the desired concentrations. The reaction was initiated by the addition of 5 μl of appropriately diluted cell extracts to the reaction buffer. One unit of activity was defined as the amount of the enzyme required to form 1 μmol of *meta*-cleavage compound per min at 25°C. The molar extinction coefficient of HOPDA under this assay condition was taken to be 13,200 cm^{-1} M^{-1} (13).

The relative ring cleavage activities were determined from the extinction coefficients of the ring fission products formed from the following substrates: catechol (λ_{max}, 375 nm; *E*, 36,000 cm^{-1} M^{-1}), 3-methylcatechol (λ_{max}, 388 nm; *E*, 32,000 cm^{-1} M^{-1}), and 4-methylcatechol (λ_{max}, 382 nm; *E*, 17,000 cm^{-1} M^{-1}) (5). Specific activity was defined as the number of enzyme units per milligram of protein. 2′-Aminobiphenyl-2,3-diol was prepared from CAR by use of the resting cells of *E. coli* harboring pUCARA (46) and extracted as described for the resting cell reaction. The ethyl acetate extract from 5 ml of reaction mixture, which was redissolved in 500 μl of ethanol after the evaporation of ethyl acetate, was used as the substrate stock solution. The *meta*-cleavage activity for 2′-aminobiphenyl-2,3-diol was determined by monitoring the oxygen consumption during the dioxygenase reaction with a Clark-type oxygen electrode (Iijima Electronics Co., Aichi, Japan); 2 ml of 50 mM sodium phosphate buffer (pH 7.5) containing 10% acetone and supplemented with 200 μl of cell extract was used as the reaction mixture. Reactions were initiated by the addition of 20 μl of the substrate stock solution.

Protocatechuate 4,5-dioxygenase activity was determined by measuring the A_{410} of the reaction mixture, using the cell extract of *E. coli* harboring pUCA122, as described by Noda et al. (38).

The hydrolase activity of the cell extracts was assayed by monitoring the decrease in A_{434} and A_{388} for HOPDA and for 2-hydroxy-6-oxohepta-2,4-dienoic acid (HOHDA; *meta*-cleavage compound of toluene), respectively. HOPDA and HOHDA were prepared from 2,3-dihydroxybiphenyl and 3-methylcatechol by the resting cell reaction of *E. coli* harboring pUCA122 and pIP103 (2), respectively, as described above. After the conversion to *meta*-cleavage compounds, 5 ml of each reaction mixture was centrifuged to remove the cells, and the yellow supernatant was extracted with ethyl acetate. The ethyl acetate layer was evaporated to dryness at 25°C in vacuo. The residue was redissolved in 100 μl of ethyl acetate and used as the substrate for CarC. The assay was performed at 25°C by using 50 mM sodium phosphate buffer (pH 7.5) containing 10% acetone. The A_{434} and A_{388} of the reaction mixtures were adjusted to about 1.0 to 1.2 with ethyl acetate solutions of HOPDA and HOHDA, respectively. The reaction was initiated by the addition of 5 μl of appropriately diluted cell extracts to the reaction mixture. One unit of activity was defined as the amount of the enzyme required to degrade 1 μmol of *meta*-cleavage compound per min at 25°C.

The hydrolase activity for 2-hydroxy-6-oxo-6-(2′-aminophenyl)hexa-2,4-dienoic acid (HOADA; the *meta*-cleavage compound of 2′-aminobiphenyl-2,3-diol) was determined by observing the disappearance of the yellow *meta*-cleavage compound. Using the resting cells of *E. coli* harboring both pUCA18 and pSCA14 (46), we prepared HOADA from CAR as described above. The assay was performed under the conditions described above.

Determination of protein concentrations. The protein concentration was determined by the method of Bradford (8), with bovine serum albumin as a standard, using Protein Assay Kit II (Bio-Rad Laboratories, Richmond, Calif.).

SDS-PAGE. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (33), using 15% polyacrylamide gels. Protein staining of the gels was performed with Coomassie brilliant blue R-250.

Chemicals. CAR was purchased from Katayama Chemical Industries Co. Ltd., Osaka, Japan. Although this CAR was of the highest purity commercially available, recrystallization from ethanol was done for further purification. 2,3-Dihydroxybiphenyl was purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan, for use as an analogous compound of 2′-aminobiphenyl-2,3-diol. All other chemicals were of the highest purity commercially available.

Nucleotide sequence accession number. The nucleotide sequence data for the insert DNA fragments of pUCA1 and pYN105 are available from the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession no. D89064 and D89065, respectively.

RESULTS

Cloning of the genes encoding *meta*-cleavage enzymes. *E. coli* JM109 was transformed with the genomic library, and the

ampicillin-resistant colonies were selected and subsequently screened for production of the yellow compound after being sprayed with ethereal 2,3-dihydroxybiphenyl, which is an analog of 2′-aminobiphenyl-2,3-diol, a catabolic intermediate of CAR degradation. As a result of this screening, we obtained some clones which represented the *meta*-cleavage activity for 2,3-dihydroxybiphenyl, and plasmid DNAs were prepared from these clones. On the basis of restriction mapping analyses, these clones were classified into two types of plasmids, designated pUCA1 (6.9-kb *Eco*RI insert) and pYN105 (1.4-kb *Sph*I insert).

Hybridization analysis. To examine which of the cloned genes encoding the *meta*-cleavage enzymes were derived from strain CA10 and involved in the degradation of CAR, Southern blot analysis was carried out. We had previously obtained a Tn*5* mutant, strain TD2, which was deficient in the conversion from CAR to anthranilic acid, and cloned the Tn*5*-flanking region, the insert of pTD21, from strain TD2 (31). Therefore, pTD21 and the *Eco*RI-digested total DNA of strain CA10 were electrophoresed and transferred onto a nylon membrane, and then the membrane was hybridized with the insert DNA fragment of pUCA1 or pYN105. The labeled insert of pUCA1 hybridized to CA10 genomic DNA fragments and pTD21, while the labeled insert DNA of pYN105 hybridized to the CA10 genomic DNA fragment but not to pTD21 (data not shown). This result indicated that the insert DNA fragments of both pUCA1 and pYN105 were derived from strain CA10 and that the insert DNA fragment of pUCA1 encodes the gene for a *meta*-cleavage enzyme involved in the degradation of CAR.

Mapping of the gene encoding the *meta*-cleavage enzyme. Figure 2 shows the restriction map of the pUCA1 insert; the region constituting the structural gene for the *meta*-cleavage enzyme was identified by examination of the activities of a series of deletion mutants. Of the mutants carrying the deleted plasmids constructed from pUCA1, the transformant carrying pUCA122, which contained a 1.9-kb *Sma*I-*Bgl*II fragment, showed *meta*-cleavage activity. This result showed that the structural gene encoding the *meta*-cleavage enzyme is located in the 1.9-kb *Sma*I-*Bgl*II fragment. Nevertheless, no *meta*-cleavage activity was observed in the plate assay of the transformant carrying pUCA121, which contains the 1.9-kb *Sma*I-*Bgl*II fragment and its adjacent 0.7-kb *Bgl*II-*Sph*I fragment. This result implies the possibilities that the *Sma*I-*Sph*I fragment also encodes the *meta*-cleavage compound hydrolase and that the yellow *meta*-cleavage compound, which was produced by the *meta*-cleavage enzyme, was quickly converted to benzoic acid as in the study by Kimbara et al. (30).

Resting cell reactions. On the basis of the consideration noted above, 2,3-dihydroxybiphenyl was subjected to reaction with resting cells of *E. coli* JM109 harboring pUCA121, pUCA122, or pUC119 (as a control), and the metabolites were analyzed by GC-MS. In the strain carrying either pUC121 or pUCA122, the color of the reaction mixture turned yellow immediately after the addition of 2,3-dihydroxybiphenyl. However, benzoic acid was detected only in the reaction mixture with resting cells of *E. coli* JM109 harboring pUCA121 (data not shown). In the strain carrying pUC119, the color of the reaction mixture did not change, and benzoic acid was not detected as a metabolite. These results clearly indicated that pUCA121 insert DNA encodes not only the *meta*-cleavage enzyme but also the *meta*-cleavage compound hydrolase.

Nucleotide sequence analysis. After determining the nucleotide sequence of the 2,614-bp insert of pUCA121, we identified by computer analysis three open reading frames (ORF1, ORF2, and ORF3), which were linked tandemly and considered to be translated to the same direction, as shown in Fig. 3.

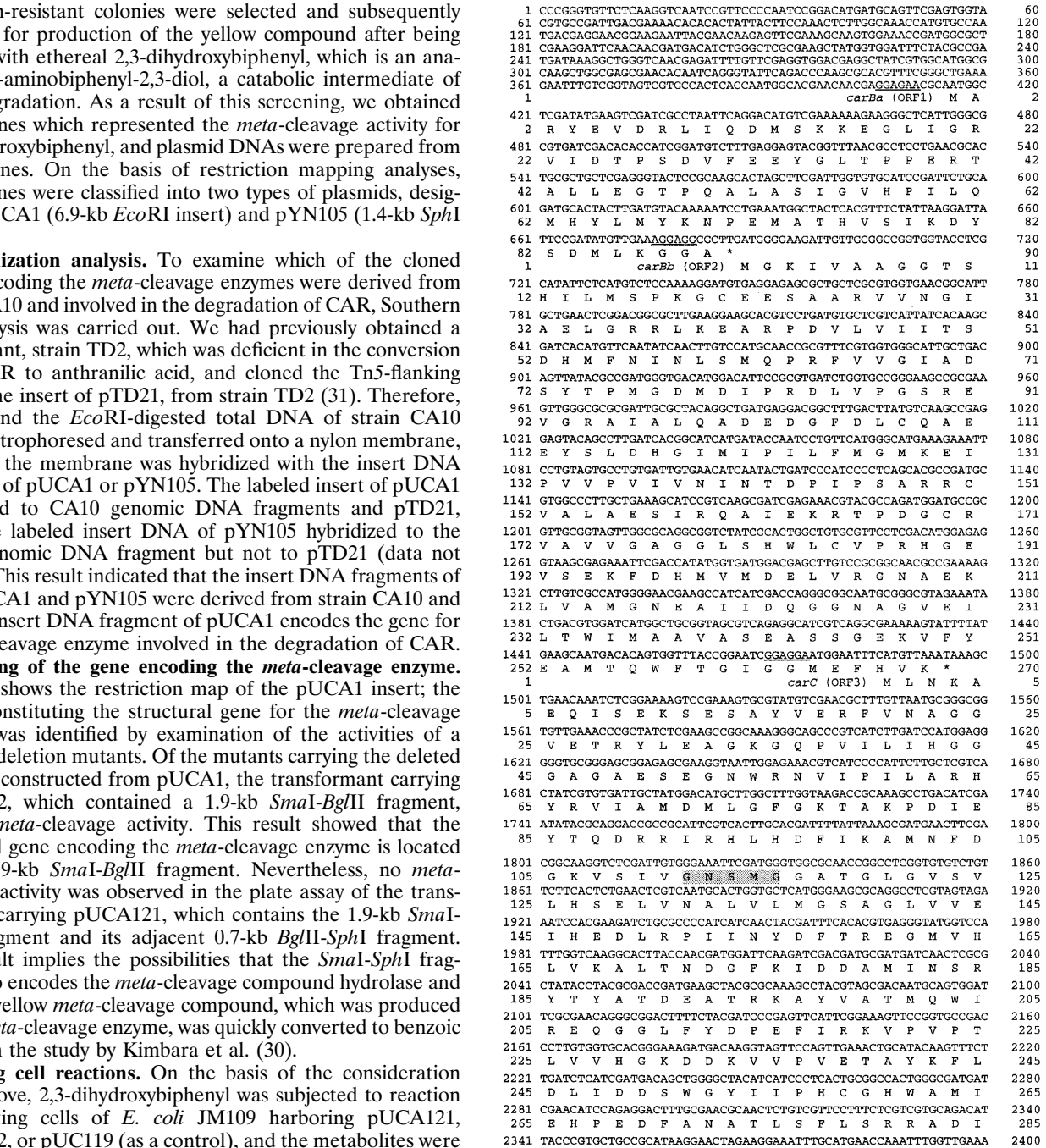

FIG. 3. Nucleotide and deduced amino acid sequences of the *Sma*I-*Sph*I fragment of pUCA121. The sense DNA strand is shown. The putative coding regions for ORF1, ORF2, and ORF3 span nucleotides 416 to 688, 688 to 1497, and 1487 to 2358, respectively. Putative SD sequences are underlined. Asterisks indicate stop codons. A Gly-Xaa-Ser-Xaa-Gly motif conserved in hydrolases (26) is indicated by a shaded box.

Each of three ORFs has a ribosomal binding site (Shine-Dalgarno [SD] sequence) (48) upstream of the putative initiation codon. Although no significant homologies of the nucleotide or amino acid sequence with ORF1 were found, the deduced

TABLE 2. Substrate specificities of two *meta*-cleavage enzymes

Substrate	Substrate concn (μM)	Activity with enzyme[a]	
		JM109 (pUCA122)	JM109 (pYN105)
2,3-Dihydroxybiphenyl	100	100	100
Catechol	300	1.2	0.5
3-Methylcatechol	300	4.4	5.5
4-Methylcatechol	300	5.9	2.3
2′-Aminobiphenyl-2,3-diol		+	−

[a] Activities measured in cell extracts of *E. coli* recombinant strains carrying plasmids pUCA122 and pYN105 for detection of CarB and CarB2 activities, respectively, are expressed as percentages of that measured with 2,3-dihydroxybiphenyl as a substrate, taken as 100%. The change of the absorbance of each product was monitored at the corresponding wavelength. Activity of 2′-aminobiphenyl-2,3-diol was determined with an oxygen electrode. +, rapid consumption of dioxygen and simultaneous production of yellow *meta*-cleavage compounds were observed; −, dioxygen consumption was almost same as for the negative control, using the cell extract of JM109(pUC119), and a *meta*-cleavage compound was not detected.

amino acid sequence of ORF2 showed 31.0% identity with LigB of protocatechuate 4,5-dioxygenase, which consists of two subunits, in *Pseudomonas paucimobilis* SYK6 (38). The deduced amino acid sequence of ORF3 showed 30.3, 31.3, and 31.8% identities with TodF (36), XylF (27), and DmpD (39), respectively, which are *meta*-cleavage compound hydrolases in other bacteria. The amino acid sequence Gly-Xaa-Ser-Xaa-Gly (Fig. 3), which was highly conserved in the hydrolases (1, 19, 26), was also observed in ORF3. These results indicate that ORF3 encodes the *meta*-cleavage compound hydrolase; thus, ORF3 was designated *carC*.

We also sequenced the about 1,351-bp insert of pYN105

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   1 GCATGCAGCGCACCGTAGGTATGGGTTGAGCGAAGCGATACCCATGCGGTCGGTATTGAT    60
  61 GGGTATCGCAAGCTCAACCCATCCTACGAAAAGCCGACCGTGCCGCGCCGTCCCCTCGCA   120
 121 ATGTGCCCCGCCACCGCCACAACCCCCGATTTCCGCGCCTTTCGCGCTAGTCCCATTGGA   180
 181 CGATGCCTCCCCTCCGGCCTGCCGATTAAATCCCTCCCCATGGAGCCCCGCGTACGACCT   240
 241 GCGCGGGGCCGATGACAATGAGGAGAACCGCATGGATATCCGTGGCCTGGGTTACGTCAC   300
   1                    carB2   M  D  I  R  G  L  G  Y  V  T    10
 301 CGTAGCCTCGACAGACCTGACGCGCTGGAGCGACTACGCCACCGGGGTGCTCGGCATGAT   360
  10   V  A  S  T  D  L  T  R  W  S  D  Y  A  T  G  V  L  G  M  M    30
 361 GGTGGATGCCGGCGCCCATGAGCGGCTCTACCTGAAGATGGACGAGCGCCCCTATCGCAT   420
  30   V  D  A  G  A  H  E  R  L  Y  L  K  M  D  E  R  P  Y  R  I    50
 421 CCTGGTGGAGCGCGCCGACCGCGACGGCTATGGCGCCTGCGGCTGGGAAGTGGCGGGCAA   480
  50   L  V  E  R  A  D  R  D  G  Y  G  A  C  G  W  E  V  A  G  K    70
 481 GGCGGCCTTCGAGCAGGCCATCGCCGAACTGCAGCAGGCCGATGTCGAGGTCCGCCGTGG   540
  70   A  A  F  E  Q  A  I  A  E  L  Q  Q  A  D  V  E  V  R  R  G    90
 541 CAGCGCCAGCGACGCCGCCTCGCGCAAGGTGCAGGAACTGGCCCTGTTCGCCGACCCGGA   600
  90   S  A  S  D  A  A  S  R  K  V  Q  E  L  A  L  F  A  D  P  D   110
 601 CGGCAACCGTCATGAGCTGTTCTGGGGCCCGCTGCAGGATTTCGCGCCATTCATTTCGCC   660
 110   G  N  R  H  E  L  F  W  G  P  L  Q  D  F  A  P  F  I  S  P   130
 661 GGCCGGCGTGTCCGGTTTCGTCACCAGCGCGCTGGGCATGGGCCATGCGGTCCTGCCGGC   720
 130   A  G  V  S  G  F  V  T  S  A  L  G  M  G  H  A  V  L  P  A   150
 721 GCCGTCCTTCGACCGCTGCCTGGACTTCTACCAGCGGGTCATGGGCTTCGGCCTCTCCGA   780
 150   P  S  F  D  R  C  L  D  F  Y  Q  R  V  M  G  F  G  L  S  D   170
 781 CCTGATGAAGGTGCGCTTCACCCCCGACCCCGCCGAGCCGGAAAAACGCATCCACTTCCT   840
 170   L  M  K  V  R  F  T  P  D  P  A  E  P  E  K  R  I  H  F  L   190
 841 CCATTGCAACAACGCCCGCCACCACTTCCCTGGCGTTGTTCGAATGCCCGATCCCTCCGG   900
 190   H  C  N  N  A  R  H  H  F  P  G  V  V  R  M  P  D  P  S  G   210
 901 TTGCGTGCACCTGATGGTGGAAGTGCGCGAGCTGGACGACGTCGGCCGCGCCTTGGACCG   960
 210   C  V  H  L  M  V  E  V  R  E  L  D  D  V  G  R  A  L  D  R   230
 961 CATGACGGCCCACGGCGTGAAGCTCTCCGCCACCTTCGGCCGCCACACCAACGACGACAT  1020
 230   M  T  A  H  G  V  K  L  S  A  T  F  G  R  H  T  N  D  D  M   250
1021 GACCAGCTTCTACATGCAGACCCCCGGCGGCTTCGACCTGGAGTACGGCTGCGGCGGCAA  1080
 250   T  S  F  Y  M  Q  T  P  G  G  F  D  L  E  Y  G  C  G  G  K   270
1081 GGTCATGGACTGGGAGGTCCACACCCCCTTCGAAAGCACGGTGGTCAGCCACTGGGGCCA  1140
 270   V  M  D  W  E  V  H  T  P  F  E  S  T  V  V  S  H  W  G  H   290
1141 TGACTTCAGCGTTGGCCGCCGCTAAGGAGCAGCACCATGGATAAACGCATGACCGCCGCC  1200
 290   D  F  S  V  G  R  R  *                                      299
1201 GACCTGGTCGGCCAGCTGCGCGATGGCATGACCATCGGCATCGGTGGCTGGGGCCCCAGG  1260
1261 CGCAAGCCGATGGCCCTGGTACGGGAGATACTCCGCTCCGACCTCAAGGACCTCACCGTG  1320
1321 GTCGCCTATGGCGGTGCCGATGTCGGCATGC                               1351
```

FIG. 4. Nucleotide and deduced amino acid sequences of the *Sph*I fragment of pYN105. The sense DNA strand is shown. The putative coding region spans nucleotides 272 to 1165. The putative SD sequence is underlined. Asterisks indicate stop codons.

TABLE 3. Kinetic parameters of two *meta*-cleavage enzymes cloned from strain CA10[a]

Enzyme	Substrate	K_m (μM)	V_{max} (nmol/min/mg)
CarB	2,3-DHBP	1.2 (0.1[b])	7,435
	3-MC	1,590.7 (171.4)	2,016
CarB2	2,3-DHBP	16.3 (1.2)	809
	3-MC	703.4 (93.2)	179

[a] Activities were measured in cell extracts of *E. coli* recombinant strains carrying plasmids pUCA122 and pYN105 for detection of CarB and CarB2 activities, respectively. 2,3-DHBP, 2,3-dihydroxybiphenyl; 3-MC, 3-methylcatechol.
[b] Standard error of the mean (n = 3).

(Fig. 4). Computer analysis revealed that there was one ORF, and its deduced amino acid sequence had 40.2 and 37.0% identities with 2,3-dihydroxybiphenyl dioxygenases (BphCs) of *P. pseudoalcaligenes* KF707 (51) and *Pseudomonas* sp. strain KKS102 (30), respectively. These results indicate that this ORF encodes the *meta*-cleavage enzyme; thus, we designated it *carB2*.

Deletion and complementation analysis of ORF1 and ORF2. The transformant harboring pUCA122 containing the *Sma*I-*Bgl*II fragment encoding complete ORF1 and ORF2 showed the *meta*-cleavage activity for 2,3-dihydroxybiphenyl (Fig. 2). This result indicated that either ORF1 or ORF2 or both are responsible for the *meta*-cleavage activity. To examine whether both ORFs are necessary for the *meta*-cleavage activity, we constructed additional deletion mutants and examined their *meta*-cleavage activities for 2,3-dihydroxybiphenyl and 2′-aminobiphenyl-2,3-diol by detection of the yellow *meta*-cleavage compound (16).

ORF1 and ORF2 were subcloned into vectors pUC119 and pSTV29, respectively, which could exist compatibly in *E. coli* cells, and the resultant plasmids were designated pUCA123 and pSCA16, respectively (Fig. 2). Although no yellow colony was observed in *E. coli* cells harboring either pUCA123 or pSCA16, the yellow pigmentation of colonies of transformant harboring both plasmids was observed after plates were sprayed with ethereal 2,3-dihydroxybiphenyl as well as 2′-aminobiphenyl-2,3-diol. Since these results strongly indicated that ORF1 and ORF2 are indispensable for the *meta*-cleavage activities for 2,3-dihydroxybiphenyl and 2′-aminobiphenyl-2,3-diol, ORF1 and ORF2 are designated *carBa* and *carBb*, respectively.

Enzymatic activities of two *meta*-cleavage enzymes. Both CarB and CarB2 showed *meta*-cleavage activity for 2,3-dihydroxybiphenyl, and their enzymatic activities for 2,3-dihydroxybiphenyl were 1.4 and 0.2 U/mg of protein in crude extracts from *E. coli* transformants harboring pUCA122 and pYN105, respectively. No activity was observed with the extract from JM109(pUC119). The activities of catechol, 3-methylcatechol and 4-methylcatechol were almost negligible (Table 2). The kinetic parameters of the two *meta*-cleavage enzymes for 2,3-dihydroxybiphenyl and 3-methylcatechol were calculated from Lineweaver-Burk plots (Table 3). CarB had the higher affinity for 2,3-dihydroxybiphenyl; its K_m value was about 14 times lower than that of CarB2. The maximum initial velocity (V_{max}) of CarB was two times higher than that of CarB2.

The *meta*-cleavage activities of these two enzymes for 2′-aminobiphenyl-2,3-diol, which is the intermediate of CAR degradation pathway, were analyzed by dioxygen consumption measured with an oxygen electrode. A rapid consumption of dioxygen and simultaneous production of yellow *meta*-cleavage compounds were clearly observed in a reaction mixture sup-

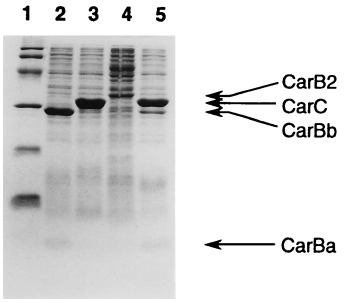

FIG. 5. Detection of the CarBa, CarBb, CarC, and CarB2 proteins. Total cellular proteins of strains were analyzed by SDS-PAGE. Lanes: 1, molecular mass standards of 94, 67, 43, 30, 20.1, and 14.4 kDa (top to bottom); 2, JM109 (pUCA122); 3, JM109(pUCA191); 4, JM109(pYN105); 5, JM109(pUCA121).

plemented with the cell extract of *E. coli* harboring pUCA122 (CarB). Although the expression level of CarB2 in the cell extract used in the determination of *meta*-cleavage activity was almost equivalent to that of CarBb as judged by SDS-PAGE (data not shown), the amount of dioxygen consumption was almost negligible, and the yellow *meta*-cleavage compound was not detected in a reaction mixture prepared with the cell extract of *E. coli* harboring pYN105 (CarB2) (Table 2).

Since the protein sequence of CarBb showed 31% homology with LigB, which is the subunit of protocatechuate 4,5-dioxygenase, the *meta*-cleavage activities of CarB and CarB2 for protocatechuate were measured. No *meta*-cleavage activities were detected in the reaction mixture prepared with the cell extract of *E. coli* harboring pUCA122 (CarB) or pYN105 (CarB2) (data not shown).

Enzymatic activity of CarC. We constructed pUCA191, which contains the *Hin*dIII-*Sph*I fragment encoding only *carC*, and analyzed the enzymatic activity of CarC in the cell extract. The enzymatic activities of the extract from JM109(pUCA191) for HOPDA and HOHDA were 1.20 and 0.03 U/mg, respectively. No hydrolase activity was observed with the extract from *E. coli* JM109 carrying pUC119. On the other hand, we detected rapid disappearance of the yellow color of HOADA in the reaction mixture (data not shown). These results indicated that the hydrolase activity of CarC for HOPDA was significantly higher than that for HOHDA and that CarC has hydrolase activity for HOADA.

Detection of *carBa*, *carBb*, *carC*, and *carB2* gene products. To examine if the observed ORFs could be translated to proteins of the predicted sizes in *E. coli* cells, cellular proteins were analyzed by SDS-PAGE (Fig. 5). Expression of genes contained in the *Sma*I-*Bgl*II insert of pUCA122 yielded two peptides with molecular masses of 29 and 10 kDa, which correspond to the predicted molecular masses of the *carBb* and *carBa* gene products, respectively (lane 2). Similar analysis of the *Hin*dIII-*Sph*I insert of pUCA191 and the *Sph*I insert of pYN105, containing the *carC* and *carB2* genes, respectively, revealed that these inserts encode proteins of 32 and 34 kDa, respectively (lane 3 and 4). These estimated molecular masses also correspond to the predicted molecular masses of the *carC* and *carB2* gene products, respectively. Expression of genes in

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CarBb          MGKIVAAGGTSHILMSPKGCEESAA------RVVNGIAELGRRLKEAR--PDVLVIITSD
LigB           MARVTTGITSSHIPALGAAIQTGTSDNDYWGPVFKGYQPIRDWIKQPGNMPDVVILVYND
MpcI           --MPIQLECLSHTPLHGYVDPAP----EVVAEVERVQAAARDRVRAFD--PELVVVFAPD
MhpB           --MHAYLHCLSHSPLVGYVDPAQ----EVLDEVNGVIASARERIAAFS--PELVVLFAPD
                         **                  *          .      *.....   *

CarBb          HMFNINLSMQPRFVVGIADSYTPMGDMDIPR--DLVPGSREVGRAIALQADEDGFDLCQA
LigB           HASAFDMNIIPTFAIGCAETFKPADEGWGPRPVPDVKGHPDLAWHIAQSLILDEFDMTIM
MpcI           HFNGFFYDVMPPFCIGAAA--TAIGDFKSLA--GKLPVPADLALSLAESVMAADIDVALS
MhpB           HYNGFFYDVMPPFCLGVGA--TAIGDFGSAA--GELPVPVELAEACAHAVMKSGIDLAVS
               *       . * * .*         .         .    ..    *        *..

CarBb          EEYSLDHGIMIPILFMG----MKEIPVVPVIVNINTDPIPSARRCVALAESIRQAIEKRT
LigB           NQMDVDHGCTVPLSMIFGEPEEWPCKVIPFPVNVVTYPPPSGKRCFALGDSIRAAVES-F
MpcI           HRMQVDHGCADALAALTGS--LHRYPVIPVFINSVAPPMATLRRARLLGDAVGRFLSR--
MhpB           YCMQVDHGFAQPLEFLLGG--LDKVPVLPVFNKAVATPLPGFQRTHMLGETIGRFTST--
                   .***    .  .          *.*      . *    .*.  * ...

CarBb          PDGCRVAVVGAGGLSHWLCVP-------------R-----------------HGEVSEKF
LigB           PEDLNVHVWGTGGMSHQLQGP-----------------------------RAGLINKEF
MpcI           -AGKRVLVVGSGGISHEPPVPELAGASEEVAERLIAGRN--PSPESAARQARTVAAAKSF
MhpB           -LNKRVLFLGSGGLSHQPPVPELAKADAHMRDRLLGSGKDLPASERELRQQRVISAAEKF
                    *   *.**.**    *                                      *

CarBb          D------H---------MVMDELVRGNAEKLVAMGNEAIID-QGGNAGVEILTWIMAAVA
LigB           DLNFIDKLISDPEELSKMPHIQYLRESGSEGVELVMWLIMRGALPEKVRDLYTFYHIPAS
MpcI           VAGDSHLHPLNPE-WNRAFLSLLASGELTAVDGMTNDAITR-DGGKSAHEIRTWVAAFGA
MhpB           VEDQRTLHPLNPI-WDNQFMTLLEQGRIQELDAVSNEELSA-IAGKSTHEIKTWVAAFAA
                                                  .    .          .. *      .

CarBb          SEASS---GEKVFYEAMTQWFTGIGGMEFHVK--
LigB           NTALGAMILQPEETAGTPLEPRKVMSGHSLAQA-
MpcI           LAAYGPYRASLDFYRAIPEWIAGFATMHAEPAAV
MhpB           ISAFGNWRSEGRYYRPIPEWIAGFGSLSARTEN-
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FIG. 6. Multiple amino acid sequence alignment of CarBb and other extradiol dioxygenases from *P. paucimobilis* SYK6 (LigB) (38), *A. eutrophus* JMP222 (MpcI) (28), and *E. coli* (MhpB) (49). Amino acid residues identical among all proteins are indicated by asterisks, and positions that are well conserved are indicated by dots. Histidine residues that are considered to form essential active sites are indicated by shaded boxes.

the *Sma*I-*Sph*I insert of pUCA121 yielded three proteins, CarBa, CarBb, and CarC (lane 5). As shown in lane 5, CarC was expressed more strongly than CarBa and CarBb. This result is in accordance with the observation that no yellow *meta*-cleavage product was detected from 2,3-dihydroxybiphenyl on the plate with *E. coli* harboring pUCA121 and that benzoic acid was identified by GC-MS as a metabolite of 2,3-dihydroxybiphenyl in the reaction mixture prepared with resting cells of JM109 harboring pUCA121.

DISCUSSION

In this study, we successfully cloned two different genes encoding *meta*-cleavage enzymes from the CAR-degrading bacterium *Pseudomonas* sp. strain CA10 into pUC119, and the resultant plasmids were named pUCA1 and pYN105. Nucleotide sequence analysis followed by assays of enzymatic activities of the gene products indicated that CarB and CarC encoded by pUCA121 were involved in the degradation of CAR. Many genes encoding *meta*-cleavage enzymes have been cloned from both gram-negative (2, 54) and gram-positive (4, 34, 35) bacteria, using color detection of *meta*-cleavage compounds as in our study. Although the protein sequence of CarBb could not be aligned to those shown in Fig. 7A (data not shown), alignment with the other *meta*-cleavage enzymes of the protocatechuate 4,5-dioxygenase family, protocatechuate 4,5-dioxygenase of *P. paucimobilis* SYK6 (LigB) (38), catechol 2,3-dioxygenase I of *Alcaligenes eutrophus* JMP222 (MpcI) (28), and 2,3-dihydroxyphenylpropionate 1,2-dioxygenase of *E. coli* (MhpB) (49), was successfully done (Fig. 6). The phylogenetic trees of these four *meta*-cleavage enzymes and other *meta*-cleavage enzymes were individually constructed (Fig. 7). As shown in Fig. 7, all *meta*-cleavage enzymes were classified to three families. The catechol 2,3-dioxygenase family constitutes most of the *meta*-cleavage enzymes involved in the degradation of several aromatic compounds. CarB2 is a member of this family. The *meta*-cleavage enzymes obtained from rhodococci (4, 5) and *Sphingomonas* sp. strain BN6 (23) consist of a short-chain dioxygenase family. CarBb was classified as a member of the protocatechuate 4,5-dioxygenase family by this phylogenetic tree.

The alignment of 12 *meta*-cleavage enzymes performed by Hofer et al. revealed that 18 amino acids were highly conserved (26). Recently, the three-dimensional structure of *meta*-cleavage enzymes (BphCs) from polychlorinated biphenyl-degrading pseudomonads were determined, and the locations of the active site, ferrous iron coordination system, and oxygen binding site were elucidated (20, 47). With respect to the protocatechuate 4,5-dioxygenase family, MpcI and MhpB show activities in a single protein (28, 49), and CarB and protocatechuate 4,5-dioxygenase (38) need the two subunits for *meta*-cleavage activity. As a result of the alignment of CarBb, LigB, MpcI, and MhpB (Fig. 6), the histidine residues, which may constitute a non-heme iron cofactor coordinating site (49), were found to be conserved (Fig. 6). The conserved His-12 of CarBb corresponds in position to His-146 of BphC in LB400, which is one of the iron(II) ligands (20). His-53 and His-117 of CarBb correspond in position to His-195 and His-241 of the LB400 enzymes, which are positioned close to the iron(II) cofactor (20). On the other hand, the substrate specificities of CarB and protocatechuate 4,5-dioxygenase are quite different. Protocatechuate 4,5-dioxygenase has no *meta*-cleavage activities for catechol, 4-methylcatechol, and so on, although the *meta*-cleavage activity for 2,3-dihydroxybiphenyl was not examined (58). However, CarB has *meta*-cleavage activities

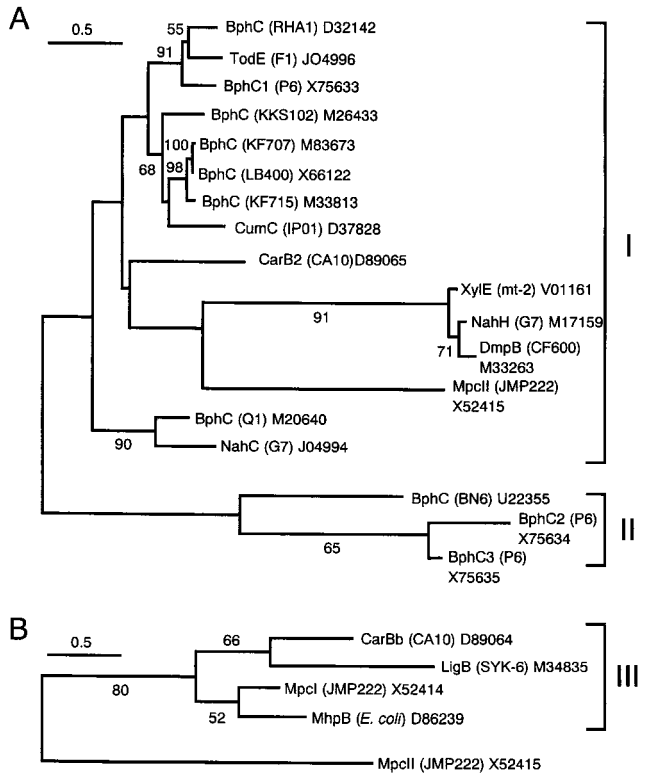

FIG. 7. Phylogenetic trees of *meta*-cleavage enzymes in the catechol-2,3-dioxygenase family or short-chain dioxygenase family (A) and those in the protocatechuate 4,5-dioxygenase family (B). Names of bacterial strains (in parentheses) and GenBank database accession numbers are indicated after the enzyme names. The *meta*-cleavage enzymes in the short-chain dioxygenase family and MpcII were used as outgroups in the phylogenetic trees in panels A and B, respectively. The scale bar denotes 0.5 substitution per site. The trees were constructed by the neighbor-joining method, and bootstrap values above 50 from 100 resamplings are shown for each node. Groups: I, catechol 2,3-dioxygenase family; II, short-chain dioxygenase family; III, protocatechuate 4,5-dioxygenase family.

for 2,3-dihydroxybiphenyl and 4-methylcatechol (Table 2) but not protocatechuate 4,5-dioxygenase (data not shown). Given these observations, it is quite possible that the active site of CarB is located in the CarBb subunit. However, because there is little information on *meta*-cleavage enzymes of the protocatechuate 4,5-dioxygenase family, locations of the active site, oxygen binding site, and substrate recognition site of each of these *meta*-cleavage enzymes are still undetermined. The accumulation of information on *meta*-cleavage enzymes classified into this family and the determination of three-dimensional structures will help us to understand what structure of CarB is involved in the recognition of substrates and what amino acid residues are located in the active site of CarB.

The nucleotide sequence of *carB2* showed 50.6, 53.2, and 53.8% homologies at the DNA level with *nahC* of the NAH7 plasmid (22), *bphC1* of *Rhodococcus globerulus* P6 (4), and *bphC* of *Pseudomonas* sp. strain KKS102 (30), respectively. Because the genes encoding these previously cloned *meta*-cleavage enzymes are known to be clustered with the other degradative genes, we carried out sequence analysis of the upstream and downstream flanking regions of *carB2*; however, we could find no other genes exhibiting homologies with the other degradative genes. This result indicates the possibility that *carB2* does not form a cluster as is the case with *bphC2* and *bphC3* of *R. globerulus* P6 (4). Multiple genes encoding differ-

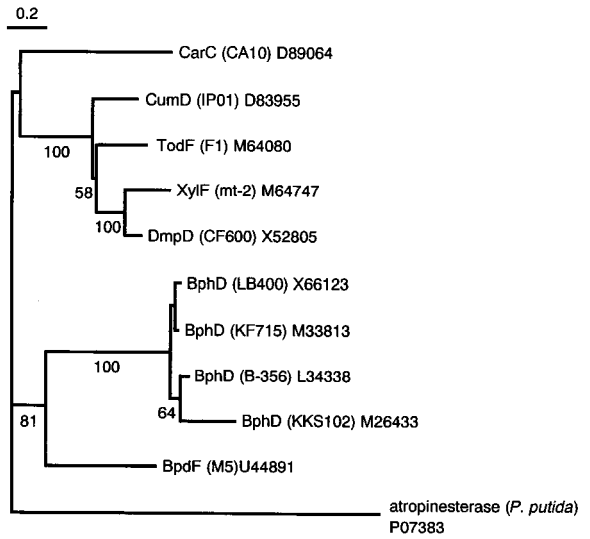

FIG. 8. Phylogeny of the *meta*-cleavage compound hydrolase. The atropinesterase protein sequence was used as an outgroup. GenBank database accession numbers are indicated after the names of enzymes and bacterial strains (in parentheses) except for atoropinesterase, whose sequence was from the SwissProt protein database. The scale bar denotes 0.2 substitution per site. The trees were constructed by the neighbor-joining method, and bootstrap values above 50 from 100 resamplings are shown for each node.

ent *meta*-cleavage enzymes were cloned from gram-positive polychlorinated biphenyl-degrading rhodococci (4, 5, 34) and gram-negative bacteria (23, 28), although its significance is not clear.

As shown in Fig. 8, the phylogenetic tree of hydrolase showed that hydrolase involved in the degradation of monocyclic aromatic compounds was distinct from that involved in degradation of biphenyl. According to the alignment and this phylogenetic tree, CarC has more homologies with the hydrolases involved in the degradation of monocyclic aromatic compounds than that involved in the degradation of biphenyl. However, CarC showed 40-times-higher activity for HOPDA than for HOHDA. Because the bootstrap value of the node that involves CarC and monocyclic aromatic compounds was 47 from 100 resamplings, it is also possible that CarC is another type of hydrolase. Thus, it is of interest to clarify the substrate recognition site of CarC.

The G+C contents of the *carBC* gene cluster and *carB2* gene were 52 and 67%, respectively. The G+C content of *carBC* is less than that of the chromosome of strain CA10 (64%) (data not shown). Such a low value is partly due to the equality of codon usage for the third base of several amino acids (52). The difference of G+C content and associated codon usage may suggest that the *carBC* gene cluster was transferred from a different microorganism.

Although sequence analysis of the upstream region of *carBa* suggested the existence of a 3′ terminus of a putative ORF, the nucleotide sequence of this region showed no homology with any other degradative genes. On the other hand, it was found that there was a 5′ terminus of a putative ORF in the downstream region of *carC*. The nucleotide sequence of this putative ORF showed 58.0, 57.1, and 57.1% homologies with ferredoxin components of chlorobenzene dioxygenase (56), biphenyl dioxygenase (35), and toluene dioxygenase (59), respectively. In this study, we observed that *E. coli* harboring pUCA1 forms indigo on 2× YT plates. This phenomenon was reported in the case of naphthalene dioxygenase (14) and cumene dioxygenase (2, 12), suggesting that the pUCA1 insert contains genes encoding CARDO. Further sequence analysis of the upstream and downstream regions of *carBC* will be done to obtain information on the genes encoding CARDO.

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