

Transcriptional Regulation of the *ant* Operon, Encoding Two-Component Anthranilate 1,2-Dioxygenase, on the Carbazole-Degradative Plasmid pCAR1 of *Pseudomonas resinovorans* Strain CA10

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The carbazole-degradative plasmid pCAR1 of *Pseudomonas resinovorans* strain CA10 has two gene clusters, *carAaAaBaBbCacAdDFE* and *antABC*, which are involved in the conversions of carbazole to anthranilate and anthranilate to catechol, respectively. We proved that the *antABC* gene cluster, encoding two-component anthranilate 1,2-dioxygenase, constitutes a single transcriptional unit through Northern hybridization and reverse transcription-PCR (RT-PCR) analyses. The transcription start point of *antA* was mapped at 53 bp upstream point of its translation start point, and the -10 and -35 boxes were homologous to conserved σ^{70} recognition sequence. Hence the promoter of the *ant* operon was designated P_{ant} . 5' Deletion analyses using luciferase as a reporter showed that the region up to at least 70 bp from the transcription start point of *antA* was necessary for the activation of P_{ant} . Luciferase expression from P_{ant} was induced by anthranilate itself, but not by catechol. Two probable AraC/XylS-type regulatory genes found on pCAR1, open reading frame 22 (ORF22) and ORF23, are tandemly located 3.2 kb upstream of the *antA* gene. We revealed that the product of ORF23, designated AntR, is indispensable for the stimulation of P_{ant} in *Pseudomonas putida* cells. Northern hybridization and RT-PCR analyses revealed that another copy of P_{ant} , which is thought to be translocated about 2.1 kb upstream of the *carAa* gene as a consequence of the transposition of ISPre1, actually drives transcription of the *carAa* gene in the presence of anthranilate, indicating that both *ant* and *car* operons are simultaneously regulated by AntR.

Among the toxic and persistent compounds that have been released in the environment by human action, the aromatic compounds are of major concern. In spite of their recalcitrance to degradation by most organisms, bacterial strains have adapted to them in consequence of the acquisition of the necessary catabolic abilities to utilize aromatic compounds, and thus rapidly adapting bacteria are regarded as the experimental systems of choice in understanding how catabolic genes end up with regulated expression (8).

Carbazole is a recalcitrant N-heterocyclic aromatic compound derived from coal tar and shale oil and possesses mutagenic and toxic activity (1). *Pseudomonas resinovorans* strain CA10, a gram-negative bacterium isolated from activated sludge, degrades carbazole as the sole source of carbon, nitrogen, and energy (27). In strain CA10, carbazole is initially converted into anthranilate and 2-hydroxypenta-2,4-dienoate through a three-step upper pathway catalyzed by carbazole 1,9a-dioxygenase (CarA), the *meta*-cleavage enzyme (CarB), and the *meta*-cleavage compound hydrolase (CarC) (Fig. 1A) (32, 33). CarA is a multicomponent dioxygenase system composed of terminal oxygenase component CarAa, ferredoxin component CarAc, and ferredoxin reductase component

CarAd. *meta*-Cleavage enzyme CarB is a heterotetrameric enzyme ($\alpha_2\beta_2$) composed of CarBa and CarBb subunits. The resulting metabolite, anthranilate, is a naturally occurring compound formed through tryptophan degradation (19) and is also known as an important intermediate in the metabolism of many N-heterocyclic compounds, such as indole (15, 21, 23), *o*-nitrobenzoate (5, 10), and quinaldine (28). Via dioxygenation catalyzed by class IB (2) two-component anthranilate 1,2-dioxygenase (AntDO) composed of the terminal oxygenase component (AntAB) and the reductase component (AntC), followed by spontaneous deamination and decarboxylation, anthranilate is converted to catechol, which is further degraded to tricarboxylic acid (TCA) cycle intermediates (25, 26). On the other hand, 2-hydroxypenta-2,4-dienoate is metabolized by the *meta*-cleavage pathway enzymes CarD, CarE, and CarF to form TCA cycle intermediates (26).

As shown in Fig. 1B, the genes encoding components (or subunits) of each upper catabolic enzymes constitute the *carAaBaBbCacAd* gene cluster, although the *carAa* gene is tandemly duplicated (32, 33). Just downstream of the upper *car* gene cluster, there is the lower *carDFE* gene cluster (26). On the other hand, the *antABC* gene cluster of strain CA10 is located in the region 21 kb upstream of the *car* gene cluster, and interestingly, the 5' portion of the *antA* gene has been transposed onto the region immediately upstream of the *carAa* gene along with ISPre1 to form a fusion open reading frame (ORF), ORF9, implying the developmental scheme of the novel genetic structure of the *car* gene cluster

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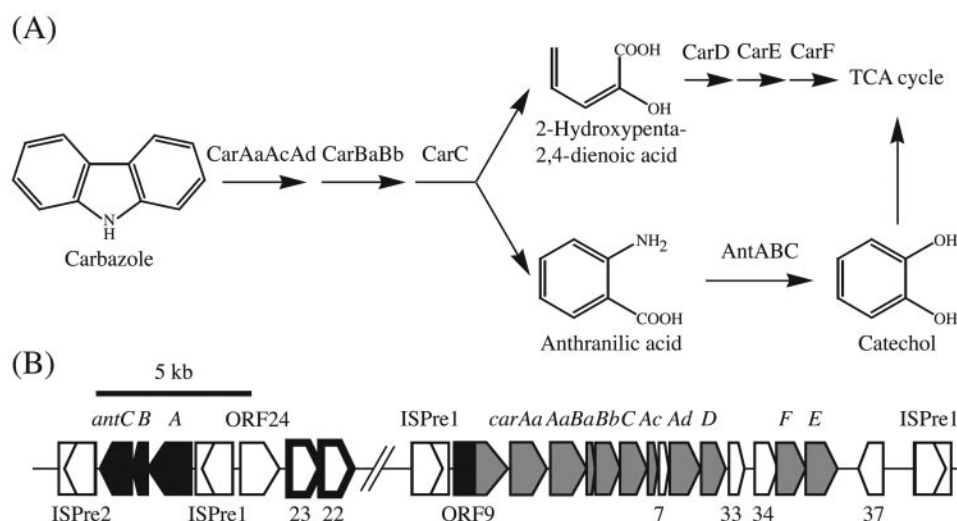


FIG. 1. (A) Carbazole degradation pathway via anthranilate in *P. resinovorans* strain CA10. (B) Genetic organization of *car* and *ant* gene clusters located on carbazole-degradative plasmid pCAR1. Black and gray pentagons represent the genes and their transcriptional directions of the *ant* and *car* gene clusters, respectively. Pentagons in ISPre1 and ISPre2 represent the transposase genes. Unknown ORFs are shown as a white pentagon. The black box located in the 5' region of ORF9 represents the transposed 5' portion of *antA* as described previously (26). ORF22 and ORF23 encoding probable AraC/XylS-type transcriptional regulators are represented by pentagons with thick lines.

and its flanking region (Fig. 1B) (26). The *ant* and *car* genes of strain CA10 exist on carbazole-degradative plasmid pCAR1 (26). Recently, the nucleotide sequence of pCAR1 was completely determined, and both *ant* and *car* gene clusters were found within the 73-kb transposon Tn4676 (24). On pCAR1, three ORFs encoding putative transcriptional regulators were found, and two of them (ORF22 and ORF23), both of whose products belong to AraC/XylS family, were also located within Tn4676 (24).

It has been known that the expression of *antABC* genes encoding two-component AntDO is induced in the presence of anthranilate itself in *Acinetobacter* sp. strain ADP1 (4), but the transcriptional regulation of the *antABC* genes has not been elucidated yet. In this report, we describe that the *ant* genes located on pCAR1 are transcribed as a single mRNA that originated from the P_{ant} promoter and that the product of ORF23, designated AntR, is required for the expression from P_{ant} induced by anthranilate. We also describe that another copy of P_{ant} within the transposed region upstream of the *carAa* gene is functional and that the transcription of *carAa* is also induced by anthranilate, indicating that transposition of the P_{ant} promoter has made the transcription of *car* genes induced by anthranilate and regulated by AntR.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Pseudomonas* strains were routinely grown on nutrient broth (Eiken Chemical Co., Ltd., Tokyo, Japan) or carbon- and nitrogen-free mineral medium (CNFMM) supplemented with carbazole or sodium anthranilate as the sole source of carbon, nitrogen, and energy at a final concentration of 1 mg/ml at 30°C as described previously (26). *P. resinovorans* strains CA10dm1 and CA10dm3 were obtained from successive cultures of strain CA10 on nutrient broth. Strain CA10dm1 harbors plasmid pCAR1Δ1, which lacks the 13-kb DNA region spanning ORF9 and ORF37 (between two copies of ISPre1, including only *car* genes), whereas strain CA10dm3 harbors plasmid pCAR1Δ3, which lacks the 120-kb DNA region spanning ORF136 and ORF50

(between two copies of ISPre3, including *ant* genes, *car* genes, and the putative AraC/XylS-type regulatory genes); please see the physical map of pCAR1 (24).

Escherichia coli strain DH5α (TOYOBO Co., Ltd., Tokyo, Japan) used for cloning procedures as a host strain was grown on 2× YT medium (31) at 37°C. Ampicillin (50 μg/ml), gentamicin (15 μg/ml), or kanamycin (50 μg/ml) was added for selective media. For plate cultures, the above media solidified with 1.6% (wt/vol) agar were used.

DNA manipulation. The plasmid was prepared from the *E. coli* host strain by the alkaline lysis method. DNA fragments were extracted from agarose gel with an E.Z.N.A. gel extraction kit (Omega Bio-tek, Inc., Doraville, Ga.). Other DNA manipulations were done according to standard protocols (31).

Northern hybridization analysis. According to the method described previously (26), total RNA was prepared from strain CA10 cells just after starvation on CNFMM and after growth on nutrient broth or CNFMM supplemented with carbazole or anthranilate. Probes for the *antA*, *antB*, and *antC* genes were prepared from a 200-bp SmaI fragment of pBCA711 (3' portion of *antA* gene) (26), a 953-bp SmaI fragment of pBCA731 (3' portion of the *antB* gene), and a 553-bp SmaI fragment of pBCA731 (3' portion of the *antC* gene), respectively. A probe for the *carAa* gene was prepared from a 359-bp fragment amplified by PCR using pUCA1 as a template with the primer set CARAA-F (5'-TTATTG GCGAACATGGGGTC-3') and CARAA-R (5'-GCCTTTCTCATCGGCGTA GA-3'). The ³²P-labeling reaction, prehybridization, hybridization, washing, and detection were performed as described previously (26).

RT-PCR analysis. As a template, 0.1 μg of total RNA of strain CA10 prepared as described above was used. The primer set used was ANTA-F (5'-TGCGCA ACCTGAACATATAC-3') and ANTC-R (5'-GGCAGCAAACGGATCAAGC C-3') or ORF9-F (5'-AGGTCAACAGCGAAAAGGC-3') and CARAA-R. Reverse transcription-PCR (RT-PCR) was performed with a One Step RNA PCR kit (Takara Shuzo Co., Ltd., Kyoto, Japan). When ANTA-F and ANTC-R were used, after the RT reaction at 50°C for 30 min, PCR was performed according to the following conditions: 94°C for 10 min and 20 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 5 min. When ORF9-F and CARAA-R were used, the RT-PCR conditions were the same as described above, except there were 25 cycles and the temperature of 72°C lasted for 10 min. For a negative control, reverse transcriptase was not mixed in the reaction mixture. The RT-PCR product was subjected to electrophoresis with 0.9% agarose gel.

Primer extension analysis. Primer extension was performed with the Li-Cor model 4200L-2 auto-DNA sequencer (Li-Cor, Inc., Lincoln, Nebr.) according to the manufacturer's instructions. Oligonucleotides ANTA-1 (5'-TCCGGCTCGG TGAATATAT-3') and ANTA-2 (5'-GCGGTAATAATCATCGGCT-3') were 5' end labeled with IRD₈₀₀ (Aloka, Ltd., Tokyo, Japan). The ANTA-1 primer was a complement only to the DNA region of *antA* gene, and its 5'-end thymine

TABLE 1. Bacterial strains and plasmids used in this study

Strains or plasmid	Relevant characteristics ^a	Source or reference
Strains		
<i>P. resinovorans</i> strain CA10	pCAR1 CAR ⁺ AN ⁺	27
<i>P. resinovorans</i> strain CA10dm1	pCAR1Δ1 CAR ⁻ AN ⁺	This study
<i>P. resinovorans</i> strain CA10dm3	pCAR1Δ3 CAR ⁻ AN ⁺	This study
<i>P. putida</i> strain DS1	CAR ⁻ AN ⁻	14
<i>P. putida</i> strain HS01	pCAR2 CAR ⁺ AN ⁺	Shintani et al., submitted
Plasmids		
pBBR1MCS-5	Gm ^r <i>lacZα mob</i> ; compatible with IncP IncQ IncW	22
pBCA711	Ap ^r , pBluescript II SK(-) containing 4.4-kb EcoRI insert of strain CA10 DNA	26
pBCA721	Ap ^r , pBluescript II SK(-) containing 11.3-kb XhoI insert of strain CA10 DNA	26
pBCA731	Ap ^r , pBluescript II SK(-) containing 7.5-kb EcoRV- <i>Clal</i> insert of strain CA10 DNA	26
pBluescript II SK(-)	Ap ^r , <i>lacZ</i>	Stratagene
pBRCantA103	Gm ^r , pBBR1MCS-5 containing the region -50 to +53 from <i>antA</i> start codon and <i>luc</i> +NF	This study
pBRCantA113	Gm ^r , pBBR1MCS-5 containing the region -60 to +53 from <i>antA</i> start codon and <i>luc</i> +NF	This study
pBRCantA123	Gm ^r , pBBR1MCS-5 containing the region -70 to +53 from <i>antA</i> start codon and <i>luc</i> +NF	This study
pBRCantA133	Gm ^r , pBBR1MCS-5 containing the region -80 to +53 from <i>antA</i> start codon and <i>luc</i> +NF	This study
pBRCantA143	Gm ^r , pBBR1MCS-5 containing the region -90 to +53 from <i>antA</i> start codon and <i>luc</i> +NF	This study
pBRCantA153	Gm ^r , pBBR1MCS-5 containing the region -100 to +53 from <i>antA</i> start codon and <i>luc</i> +NF	This study
pBRCantA203	Gm ^r , pBBR1MCS-5 containing the region -150 to +53 from <i>antA</i> start codon and <i>luc</i> +NF	This study
pBRCantA253	Gm ^r , pBBR1MCS-5 containing the region -200 to +53 from <i>antA</i> start codon and <i>luc</i> +NF	This study
pMK	Km ^r , pMMB66HE inserted into DraI restriction site with 1.3-kb HincII fragment of pUC4K	This study
pMK22	Km ^r , pMK with 1.0-kb Sall-BamHI insert containing ORF22	This study
pMK23	Km ^r , pMK with 1.1-kb Sall-BamHI insert containing ORF23	This study
pMK2322	Km ^r , pMK with 2.1-kb Sall-BamHI insert containing ORF22 and ORF23	This study
pMMB66HE	Ap ^r IncQ	16
pSP- <i>luc</i> +NF	Ap ^r <i>luc</i> +NF	Promega
pUC4K	Ap ^r Km ^r	37
pUCA1	Ap ^r , pUC19 with 6.9-kb EcoRI insert of strain CA10 DNA	33
pUCA741	Ap ^r , pUC119 with 8.9-kb Sall insert of strain CA10 DNA	26

^a CAR⁺/CAR⁻ or AN⁺/AN⁻ represents ability/inability to grow on carbazole or anthranilate as a sole source of carbon, nitrogen, and energy. Ap^r, Gm^r, and Km^r represent resistance to ampicillin, gentamicin, and kanamycin, respectively.

base corresponded to position +113 from the translation start point of *antA*. The 5'-end guanine base of the ANTA-2 primer corresponded to both position +243 from the *antA* translation start point and position +54 from the translation start point of ORF9. The primer extension reaction was done with 1.5 pmol of labeled primer and 20 μg of total RNA of strain CA10 prepared as described above. The sequence ladder for the upstream region of *antA* or ORF9 was obtained with pBCA731 or pBCA721 as a template, respectively.

Construction of transcriptional fusions. Transcriptional fusions for luciferase reporter analyses were prepared by amplifying appropriate DNA fragments corresponding to the upstream region of *antA* gene by PCR using adequately designed primer sets and pUCA741 as a template. Forward primers ANTA-F-BamHI-103 (5'-GGATCCCACCGATAAAGCGGATAGA-3'), ANTA-F-BamHI-113 (5'-GGATCCGCTGTGTTCCACCGATAA-3'), ANTA-F-BamHI-123 (5'-GGATCCC CAATTTTATGGCTGTGTTCA-3'), ANTA-F-BamHI-133 (5'-GGATCCCG CCGGGCGGCCAATTTTATG-3'), ANTA-F-BamHI-143 (5'-GGATCCGCC AGGGCCGCGCCGGCGGCCAATTTT-3'), ANTA-F-BamHI-153 (5'-GGA TCCATCGCCCACGGCCAGGGCCGCGGCCGCGGCCAAT-3'), ANTA-F-BamHI-203 (5'-GGATCCGACCATCCCTAGCCTGTTA-3'), and ANTA-F-BamHI-253 (5'-GGATCCTTTTAAATCGGCAGGGGC-3') and reverse primer ANTA-R-HindIII (5'-AAGCTTCCTTTAATCGATACCTCG-3') were designed to introduce artificial BamHI and HindIII restriction sites, the ribosomal binding site, and a stop codon preventing the fusion with the reporter gene (indicated by an underline, boldface type, and double underline, respectively). The amplified DNA fragments were sequenced and confirmed to be accurate.

The resultant fragments were digested with BamHI and HindIII and then inserted into the broad-host-range vector pBBR1MCS-5 together with the HindIII-EcoRI fragment containing the engineered firefly luciferase gene, *luc*+NF, from pSP-*luc*+NF fusion vector (Promega, Madison, Wis.).

Luciferase reporter assay. *Pseudomonas* strains were grown on 100 ml of appropriate medium overnight, and the resultant cells were gathered by centrifugation (3,300 × g), washed twice with chilled sterile water, and then resuspended with 500 μl of chilled sterile water containing 10% (vol/vol) glycerol. To electrotransform 50 μl of the cells with appropriate plasmids, GENE PULSER II (Bio-Rad, Hercules, Calif.) was used under the following conditions: 200 Ω, 25 μF, and 1.8 kV for 4.7 to 5.0 ms in a 0.1-cm cuvette.

The resultant transformants were grown on nutrient broth overnight, and cells from 4 ml of the culture were washed twice with CNF buffer (26). The washed cells were suspended in 2 ml of the same buffer and then starved by incubation at 30°C with reciprocal shaking at 300 strokes/min for 3 h. Five milliliters of succinate mineral medium (SMM) containing 2.2 g of Na₂HPO₄, 0.8 g of KH₂PO₄, 3.0 g of NH₄NO₃, 2.7 g of Na₂C₄H₄O₄ · 6H₂O, 0.2 g of MgSO₄ · 6H₂O, 0.01 g of FeSO₄ · 7H₂O, 0.01 g of CaCl₂ · 2H₂O, and 0.05 g of yeast extract per liter, supplemented with sodium anthranilate or catechol (at a final concentration of 1 mg/ml), was inoculated with 500 μl of cell suspension after starvation. If necessary, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to SMM at a final concentration of 1 mM. After 2 h of incubation under the above-mentioned condition, the resultant cells from 2 ml of incubation mixture were harvested by centrifugation (2,400 × g, 5 min, 4°C). The pellets obtained were

resuspended in sonication buffer containing 25 mM Tris-HCl (pH 8.0), 2 mM EDTA, and 10% (vol/vol) glycerol, and then the cells were broken by ultrasonic treatment. The crude extract was collected after centrifugation (21,600 × g, 10 min, 4°C). On a 96-well microtiter plate, 10 µl of crude extract (diluted with sonication buffer to a 0.1-µg/µl protein concentration) and 10 µl of Picagene LT2.0 (Toyo Ink Co., Ltd., Tokyo, Japan) were added, the mixture was shaken for 10 s, and then luciferase activities were measured for 10 s with Centro LB960 (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). Each sample was assayed at least three times independently.

Construction of the expression vectors of ORF22 and ORF23. To express the putative regulatory genes from the *tac* promoter induced by the addition of IPTG, pMK derived from the expression vector pMMB66HE was used. ORF22 and ORF23 were separately amplified by PCR using pUCA741 as a template with primer sets ORF22-F (5'-GTCGACGAATTCAAGGAGATTGAGCCATG GCCGGTTAGCGGGGG-3') and ORF22-R (5'-GGATCCTTACTGGGCA ATAGTCTGGTGGCGCAGCTCGCCGAAGCG-3') and ORF23-F (5'-GTCG ACGAATTCAAGGAGAGTGCCCCGTGATGAGTACAAGCC-3') and ORF23-R (5'-GGATCCTCAAAGCGACCGGTTGCGGCGGGCTTCGTTGCGTTG C-3'), respectively. Primers were designed to introduce artificial restriction sites for SalI, EcoRI, and BamHI (indicated by underline) and ribosomal binding site (indicated by boldface type). Furthermore, a DNA fragment containing both ORF23 and ORF22 was similarly generated by PCR using ORF23F and ORF22R. These fragments were sequenced and confirmed to have precise sequences. The SalI-BamHI-digested fragment containing ORF22, ORF23, or both ORF22 and ORF23 was ligated into the corresponding site of pMK to form pMK22, pMK23, or pMK2322, respectively.

Alignment of amino acid sequences. Alignment of amino acid sequences of AraC/XylS family regulatory proteins was performed by the CLUSTAL W multiple alignment program (35).

RESULTS

Transcriptional analyses of the *antABC* gene cluster. Transcription of the *antA* gene is induced when *P. resinovorans* strain CA10 is grown on carbazole (26). Using the same probe used previously (26), we detected the specific transcript of *antA* from the total RNA of anthranilate-grown strain CA10 cells, as well as carbazole-grown cells (Fig. 2A, lanes 3 and 4). No hybridization was detected when total RNA extracted just after the starvation on CNFMM or after the growth on nutrient broth was used (Fig. 2A, lanes 1 and 2). Similar hybridization patterns were detected when the probes prepared from *antB* and *antC* genes were used (data not shown). These results clearly showed that the transcription of *antABC* genes is induced at least when strain CA10 is grown on anthranilate. Although the hybridized band was smeared, the maximum size of the transcript was estimated at about 3 kb.

In order to confirm that the *antABC* genes are cotranscribed, RT-PCR analysis was performed with a primer set designed to amplify across the *antABC* genes. As shown in Fig. 2B, amplification of the DNA fragments with the expected size (1,506 bp) was observed, using the RNA template prepared from the cells grown on anthranilate or carbazole. No amplified bands were detected when the template prepared from the cells just after the starvation or grown on nutrient broth was used (data not shown). Along with the results of Northern hybridization analyses, we concluded that *antABC* genes are transcribed as a single transcriptional unit.

Determination of the transcription start point of the *antA* gene. We performed primer extension using 5'-end-labeled ANTA-1 primer and the same RNA samples used in Northern hybridization and RT-PCR analyses. The ANTA-1 primer anneals to a specific site inside the *antA* gene. A common single transcription start point was found 53 bp upstream of the *antABC* translation start point when anthranilate or carbazole

was supplied as the growth substrate (Fig. 3, lanes 1 and 2). As shown in Fig. 3, the -35 and -10 regions corresponding to the transcription start point (TAGACC-N₁₇-TTTAAT) were highly consistent with the conserved sequence of σ^{70} promoter of *E. coli* (TTGACA-N₁₆₋₁₈-TATAAT) (30): 4 out of 6 and 5 out of 6 matches, respectively. Therefore the promoter playing a central role in the inducible transcription of *antABC* of strain CA10 was designated P_{ant}.

The DNA region required for the inducible expression of the *ant* operon. Deletion analyses of the region upstream of P_{ant} promoter were performed with luciferase as a reporter. Strain CA10 was transformed by each pBRC plasmid harboring transcriptional fusions (Fig. 4), and the luciferase activity of the resultant transformants was measured in the presence or absence of anthranilate as described in Materials and Methods.

In the reaction mixtures without anthranilate, the luciferase activities of transformants were generally low (Fig. 4). On the other hand, in the presence of anthranilate, the transformants having the DNA fragments larger than (or equal to) 123 bp showed significantly higher luciferase activities than that having pBRCantA113 and pBRCantA103, although luciferase activity was somehow decreased with pBRCantA203 and pBRCantA253. This result clearly indicated that the *cis*-activating region necessary for the inducible expression of *ant* operon in strain CA10 is located within the region up to at least 70 bp from the transcription start point of *antA*.

Identification of the inducer of the *ant* operon. Using strain CA10 harboring pBRCantA253, we investigated the inducer of the *ant* operon. Because the P_{ant} promoter governs the expression of anthranilate 1,2-dioxygenase, which catalyzes the conversion of anthranilate to catechol (Fig. 1A), the effect of the presence of anthranilate (substrate) or catechol (product) on the induction of P_{ant} promoter was determined. The level of luciferase activity was clearly increased by the presence of anthranilate, while the activity detected in the presence of catechol was nearly negligible, as was found in the absence of either compound (Fig. 5). This result strongly indicated that anthranilate is the inducer for transcription of the *ant* operon in strain CA10.

Identification of the transcriptional regulator of the *ant* operon. The nucleotide sequence of carbazole-degradative plasmid pCAR1 containing both *ant* and *car* genes has been completely determined (24). On pCAR1, there exist only three ORFs whose deduced amino acid sequences show homologies to known transcriptional regulators. Two of them, ORF22 and ORF23, encode putative AraC/XylS family transcriptional regulators containing the conserved helix-turn-helix DNA binding motif in their C termini (18). These ORFs are tandemly located with each other in the same orientation at the region 3.2 kb upstream of *antA*, although ISPre1 and ORF24 are interposed (Fig. 1B). In deduced amino acid sequences, ORF23 showed 58.6% identity to PA2511 (accession no. 251201), a probable AraC/XylS-type regulatory gene located divergently just upstream of the *antA* gene of *P. aeruginosa* strain PAO1, but the overall lengthwise identity to other members of AraC/XylS was below 30%. On the other hand, ORF22 did not have any particularly close relatives (<39%) within the AraC/XylS family.

To determine whether the product of ORF23 or that of ORF22 takes part in the inducible expression of the *ant*

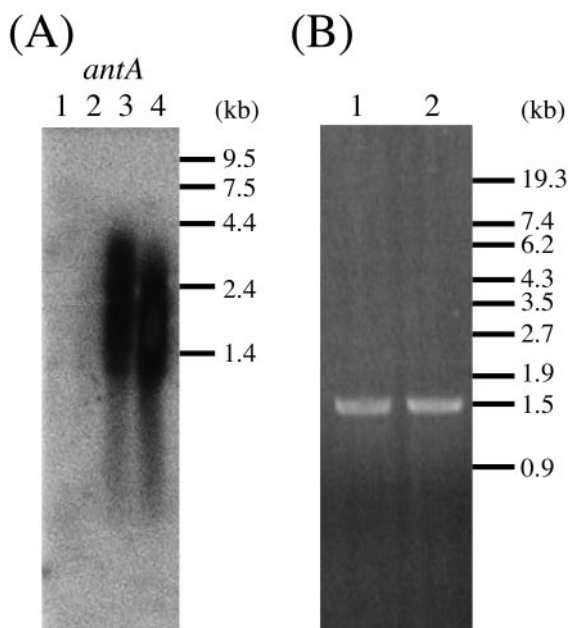


FIG. 2. (A) Northern hybridization analysis using a 200-bp probe inside of the *antA* gene. Total RNA was prepared from strain CA10 cells grown on nutrient broth (lane 2), anthranilate (lane 3), and carbazole (lane 4). As a control, total RNA was similarly extracted from the cells just after starvation on CNFMM (lane 1). (B) RT-PCR amplification of the region spanning *antA* to *antC*. Total RNA extracted from the strain CA10 cells grown on anthranilate (lane 1) or carbazole (lane 2) was used as a template.

operon, we performed reporter gene analyses using the transcriptional fusion plasmid pBRCantA253 in the first two types of Δcar mutants of strain CA10, designated *P. resinovorans* strains CA10dm1 and CA10dm3, as host strains. Strains

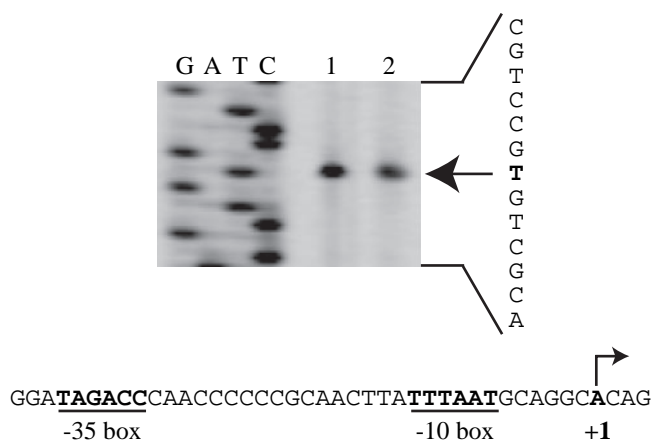


FIG. 3. Determination of the transcription start point of *antA* by primer extension. Total RNA was isolated from strain CA10 grown on anthranilate (lane 1) and carbazole (lane 2). The other lanes correspond to a sequence ladder obtained with pBCA731 as a template, and the sequence pattern is shown on the right. The arrows indicate the primer extension product and the corresponding transcription start point in the nucleotide sequence shown below. The nucleotide sequence of the P_{ant} promoter is shown with the -35 and -10 boxes underlined.

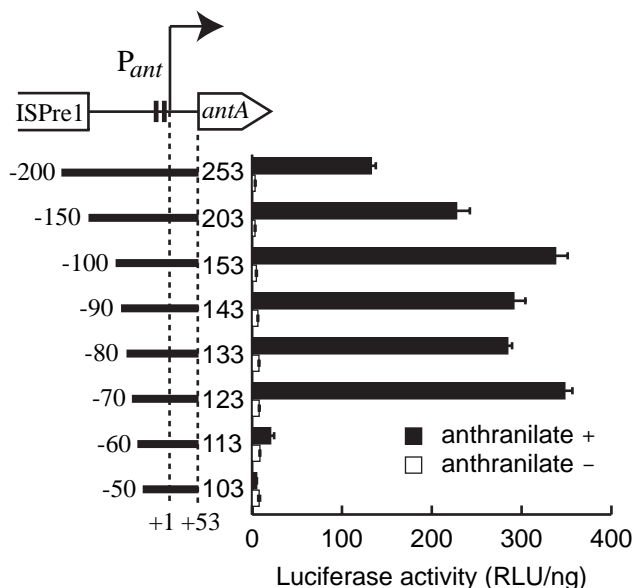


FIG. 4. Deletion analysis of the 5' region upstream of the P_{ant} promoter. The DNA region fused with the *luc* gene is shown to the left. The numbers indicate the position of the 5' termini of the transcriptional fusions relative to the transcription start point of *antA* (+1). Black boxes upstream of *antA* indicate -35 and -10 boxes of the P_{ant} promoter, respectively. Strain CA10 was transformed by the reporter plasmids of pBRC series indicated at the middle. Luciferase activity of the cells incubated on SMM with (black bars) or without (white bars) anthranilate is shown to the right. Values and error bars represent averages and standard deviations of at least three independent experiments. RLU, relative light units.

CA10dm1 and CA10dm3 harbor pCAR1-derived plasmids pCAR1 Δ 1 and pCAR1 Δ 3, respectively. While both ORF22 and ORF23 were located on pCAR1 Δ 1, pCAR1 Δ 3 lost both ORFs. In the absence of anthranilate, both of these strains harboring pBRCantA253 showed significantly low activity of luciferase as well as strain CA10 (Table 2). In the presence of inducer, while strain CA10dm1(pBRCantA253) expressed almost the same level of luciferase as strain CA10, the expression level in strain CA10dm3(pBRCantA253) was decreased to about 76% of that in strain CA10 cells (Table 2).

Second, *P. putida* strains HS01 (Shintani et al., submitted) and DS1 (14) were used as host strains in reporter gene analyses. Strain HS01 was generated by the mating of carbazole-degrader *Pseudomonas* sp. strain K23 (20) with *P. putida* strain DS1 and harbors carbazole-degradative plasmid pCAR2, the genetic structure of which is similar to that of pCAR1 and which contains not only *ant* and *car* catabolic genes but also ORF22 and ORF23 (Shintani et al., submitted). Strain HS01 can grow on carbazole and anthranilate as the sole source of carbon, nitrogen, and energy, but strain DS1 cannot. We detected a significant induction of luciferase expression by anthranilate in strain HS01 harboring pBRCantA253, although the expression of the reporter was not induced in strain DS1 harboring pBRCantA253 (Table 2). This clearly indicated that the putative transcriptional regulator for the promoter P_{ant} was encoded on plasmid pCAR2 (or pCAR1) but not on the genome of strain DS1.

Then, to elucidate whether the product of ORF22, ORF23,

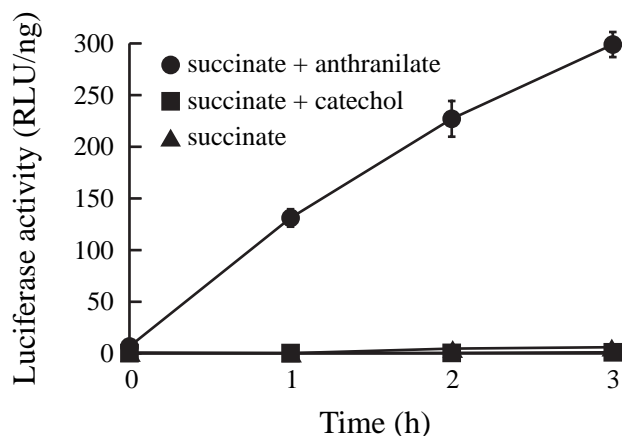


FIG. 5. Expression profile of the P_{ant} promoter. Strain CA10 harboring pBRCantA253 was inoculated on SMM containing succinate plus anthranilate (●), succinate plus catechol (■), or succinate (▲) at time zero. Luciferase activity of the cells harvested periodically was measured. Values and error bars represent averages and standard deviations of at least three independent experiments. RLU, relative light units.

or both can activate the expression from P_{ant} , we used *P. putida* strain DS1 transformed by a pMK-based expression vector for ORF22, ORF23, and both ORFs. Expression of these ORFs was induced by the addition of IPTG. Reporter plasmid pBRCantA253 was simultaneously introduced into strain DS1 with each pMK series, and the luciferase activities of respective transformants were determined. When both were expressed using pMK2322, the luciferase activity in response to inducer was significantly increased (Table 2). The expression of luciferase from the P_{ant} promoter was clearly induced by anthranilate in strain DS1 expressing only the ORF23 product, and the luciferase activity detected was as high as that of strain DS1 expressing both ORF22 and ORF23 products. On the other hand, no induction was observed in strain DS1 expressing only ORF22 (Table 2). Hence, it was concluded that the product of ORF23, designated AntR, is required for the stimulation of expression from P_{ant} , but that ORF22 is not involved in the regulation of anthranilate degradation.

Another copy of the P_{ant} promoter drives transcription of the upper *car* gene cluster. ORF9, which is located upstream of the upper *car* gene cluster (Fig. 1B), is proposed to be formed via the transposition of ISPre1 along with the 5' portion of the *antA* gene (26). Because the intergenic region between ISPre1 and *antA* is identical in nucleotide sequence to that between ISPre1 and ORF9 (Fig. 6A), the second P_{ant} promoter upstream of ORF9 (about 2.1 kb upstream of the *carAa* gene) was assumed to be functional. Here we used the 5'-end-labeled ANTA-2 primer that is specific to both *antA* and ORF9 for primer extension analysis. Two primer extension products were detected with total RNA prepared from the cells grown on anthranilate or carbazole. The larger band represented the primer extension product transcribed from the *antA* transcription start point as determined by the sequence ladder made from pBCA731 containing ISPre1-*antA* intergenic region (data not shown). On the other hand, the position of the shorter band corresponded to a thymine base 79 bp upstream of the

TABLE 2. Transcription from the promoter P_{ant} in several strains

Host strain ^a	Genetic characteristics	Luciferase activity (RLU/ng) ^b	
		Without anthranilate	With anthranilate
CA10	ORF22 ⁺ ORF23 ⁺	4.21 ± 0.34	289.63 ± 25.08
CA10dm1	ORF22 ⁺ ORF23 ⁺	5.79 ± 0.94	279.45 ± 13.56
CA10dm3	ORF22 ⁻ ORF23 ⁻	2.94 ± 0.91	220.75 ± 13.06
HS01	ORF22 ⁺ ORF23 ⁺	0.33 ± 0.08	316.90 ± 30.94
DS1	ORF22 ⁻ ORF23 ⁻	0.48 ± 0.09	0.30 ± 0.03
DS1(pMK2322)	ORF22 ⁺ ORF23 ⁺	2.78 ± 0.98	563.78 ± 7.97
DS1(pMK23)	ORF22 ⁻ ORF23 ⁺	2.70 ± 0.48	546.05 ± 61.98
DS1(pMK22)	ORF22 ⁺ ORF23 ⁻	0.79 ± 0.07	0.56 ± 0.06
DS1(pMK)	ORF22 ⁻ ORF23 ⁻	1.03 ± 0.22	0.66 ± 0.15

^a *P. resinovorans* strains CA10, CA10dm1, and CA10dm3, and *P. putida* strains HS01 and DS1 were transformed by pBRCantA253. Strain DS1 was transformed by pBRCantA253 along with expression plasmids shown in parentheses. Luciferase activity was determined from the cells grown on SMM with or without anthranilate for 2 h. The values shown represent averages ± standard deviations of at least three independent experiments.

^b RLU, relative light units.

translation start point of ORF9, as determined by the sequence ladder obtained from pBCA721 containing the ISPre1-ORF9 intergenic region (Fig. 6B). This transcription start point is definitely consistent with that formerly found upstream of the *antA* gene in the nucleotide sequence (Fig. 3 and 6B). This result clearly indicated that the second copy of P_{ant} promoter upstream of ORF9 is functional.

Then, we proceeded to confirm that the upper *car* gene cluster is transcribed from P_{ant} promoter. The specific transcript of *carAa* from the total RNA of anthranilate-grown cells, as well as carbazole-grown cells, was detected through Northern hybridization, while a weak hybridization was detected with total RNA extracted from cells just after starvation or grown on nutrient broth (Fig. 7A). This result indicated that transcription of the *carAa* gene is induced when strain CA10 was grown on carbazole or anthranilate, but the lower level of constitutive expression was detected when the strain was starved or grown on nutrient medium. Next, we performed RT-PCR with primer set ORF9-F and CARAA-R. Amplification of the DNA fragments of the expected size (2,359 bp) was observed with total RNA prepared from the cells grown on anthranilate or carbazole as a template. This result revealed that *carAa* and ORF9 are cotranscribed. These facts indicated that the upper *car* gene cluster is transcribed from the second copy of the P_{ant} promoter and that AntR simultaneously regulates the transcription of both the *ant* operon and the upper *car* gene cluster, while it was suggested that the other constitutive promoter is also involved in the transcription of the upper *car* gene cluster.

DISCUSSION

In this study, we showed that *antABC* genes of pCAR1 encoding two-component anthranilate 1,2-dioxygenase are cotranscribed in strain CA10. It was proven that transcription of the *ant* operon originates from the σ^{70} -dependent promoter P_{ant} in the presence of anthranilate itself and that an AraC/XylS family transcriptional regulator, AntR, encoded on pCAR1 is required for inducible expression from the P_{ant} promoter. In strain CA10, the 5' deletion reporter assay indicated

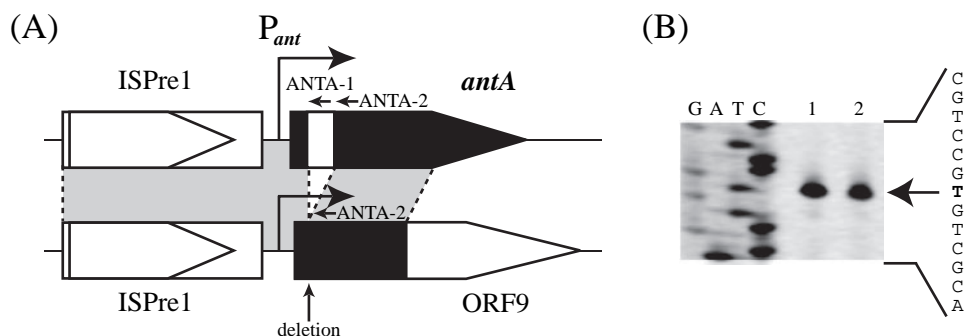


FIG. 6. (A) Comparison of the downstream regions of two ISPre1s on *ant* and *car* loci. The white box in the *antA* gene represents the deleted region in the sequence of ORF9 as described previously (26). Nearly identical regions are shown by shading. Small arrows represent the primers used in primer extension analyses. Transcription start points of two copies of P_{ant} promoter are marked by arrows. (B) Transcription start point at the ORF9 locus determined by primer extension analysis. Total RNA was isolated from strain CA10 cells grown on anthranilate (lane 1) and carbazole (lane 2). The other lanes correspond to a sequence ladder obtained with pBCA721 as a template, and the sequence pattern is shown on the right.

that the region up to at least 70 bp from the transcription start point of *antA* is necessary for the anthranilate-dependent activation of P_{ant} (Fig. 4). This result suggests that AntR binds at its binding sequence that exists just upstream of the -35 box to activate RNA polymerase as reported for many AraC/XylS family members (18), although a further experiment on the binding ability of AntR to P_{ant} promoter region is needed. Identification of the binding sequence of AntR protein will provide further information about the activation mechanism of the P_{ant} promoter. On the other hand, the anthranilate-dependent transcription from P_{ant} was still observed in the strain CA10dm3 environment lacking both *antABC* and *antR* genes (Table 2). This fact suggests the possibility that an AntR homologue is encoded on the chromosome of strain CA10 and substitutes for AntR in activating the P_{ant} promoter. This hypothesis is also supported by the facts that strain CA10dm3 lacking *antABC* genes can still grow on anthranilate as the sole source of carbon, nitrogen, and energy; that *P. resinovorans* is taxonomically classified into the *P. aeruginosa* group; and that *P. aeruginosa* strain PAO1 has the PA2511 (*antR* homologue)-*antABC* gene cluster on its chromosome (accession no. NC_002516). It was also likely that the putative second anthranilate-dependent activator of strain CA10 is common to AntR in its manner of binding to specific DNA sequence. In fact, the 5' deletion profile of the P_{ant} promoter was the same in strain HS01 as that in strain CA10 (data not shown).

Since Hayaishi and Stanier first reported the oxidation of anthranilate by the cell extracts from *Pseudomonas* (19), the *antABC* genes encoding two-component AntDO have been found for strain CA10 (26), *Acinetobacter* sp. strain ADP1 (4), and *P. aeruginosa* strain PAO1 (accession no. NC_002516). Although any homologous genes were not found in the whole genome of *Pseudomonas putida* strain KT2440, *antABC* genes, which were nearly identical to those of *P. aeruginosa* strain PAO1, were isolated in *P. putida* strain P111 (accession no. AY026914). These facts suggest that similar *antABC* gene clusters have been dispersed in some part of gram-negative bacteria, mainly *Pseudomonas*.

The enzymatic activity and biochemical property of two-component AntDO of *Acinetobacter* sp. strain ADP1 have been characterized precisely (3, 4, 11, 13). In strain ADP1, the

expression of the *antA::lacZ* transcriptional fusion is also induced by anthranilate, while any regulators of *antABC* genes have not been identified yet (4). Downstream of the *antC* gene on strain ADP1 chromosome there exists ORF3, the product of which contains the AraC/XylS-type helix-turn-helix motif in the C terminus. However its nucleotide sequence deposited in databases is truncated in this ORF. The ORF3 disruptant of strain ADP1 remained capable of growing on anthranilate as the sole carbon source (4). In addition, expression of the *antA::lacZ* transcriptional fusion remained induced by anthranilate in the ORF3 disruptant, but its expression level was

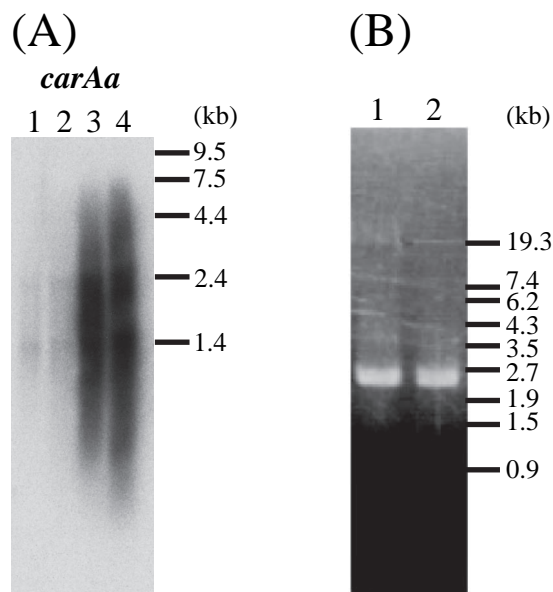


FIG. 7. (A) Northern hybridization analysis using a 359-bp probe inside of the *carAa* gene. Total RNA was prepared from strain CA10 cells grown on nutrient broth (lane 2), anthranilate (lane 3), and carbazole (lane 4). As a control, total RNA was similarly extracted from the cells just after starvation on CNFMM (lane 1). (B) RT-PCR amplification of the region spanning ORF9 and *carAa*. Total RNA extracted from the strain CA10 cells grown on anthranilate (lane 1) or carbazole (lane 2) was used as a template.

reduced to 70% of that in the wild-type strain (4). This phenomenon bears a close resemblance to the reduced induction of the P_{ant} promoter in strain CA10dm3 lacking the *antR* gene and suggests the possibility that the product of ORF3 can regulate transcription of the *ant* operon in strain ADP1, although at least one functional homologue of *antR* exists in the strain ADP1 chromosome.

Both *P. aeruginosa* strain PAO1 and *P. putida* strain P111 have the *antABC* gene cluster on their chromosome flanked by *cat* and *ben* gene clusters, which encode benzoate and catechol catabolic genes, respectively, and the DNA regions containing the *cat*, *ant*, and *ben* genes of both strain genomes are almost identical at the nucleotide sequence level. Upstream of each *antA* gene, there is a probable AraC/XylS-type regulatory gene, PA2511 or ORFAN, in divergent directions, although the function of their products has not been characterized yet. Within the AraC/XylS transcriptional regulators, AntR was the closest protein to the product of PA2511 (58.6% of amino acid identities in overall length). Therefore, the possibility that the product of PA2511 (or ORFAN) is involved in anthranilate degradation is high.

Anthranilate is also known as a precursor for 2-heptyl-3-hydroxy-4-quinolone, alias *Pseudomonas* quinolone signal (PQS), which functions as a central quorum-sensing signal in *P. aeruginosa* strain PAO1 along with *N*-(3-oxododecanoyl)homoserine lactone (3-oxo-C₁₂-HSL) and *N*-butyrylhomoserine lactone (C₄-HSL) (6, 12, 29). PQS synthetic genes (*pqsABCDH*), a LysR-type regulatory gene (*pqsR*) which controls the expression of the *phnAB* operon encoding anthranilate synthase (7), and a response effector (*pqsE*) were identified, and the transcription of *pqsH* was found to be regulated by the LasR-LasI quorum-sensing system (17). In this quorum-sensing system, two-component AntDO may play an important role because it degrades the precursor of PQS. In fact, the expression of the *antABC* of strain PAO1 is induced in the presence of 3-oxo-C₁₂-HSL and C₄-HSL, as shown by the results of transcriptome analysis (34), and recently, Lee et al. reported that the *antA* gene of strain PAO1 is transcribed from two promoters: one is constitutive and another (designated *antA_p*) is up-regulated by RhIR (J. H. Lee, M. Schuster, and E. P. Greenberg, Abstr. *Pseudomonas* 2003, abstr. 69, 2003).

The expression of *andAcAdAbAa* genes, encoding class IIB (2) three-component AntDO, which is located on the *Burkholderia cepacia* strain DBO1 chromosome, is also induced in the presence of anthranilate and regulated by an AraC/XylS family transcriptional regulator, AndR (9). Because the DNA-binding motif is located on the C-terminal region of the AraC/XylS family transcriptional regulators (18), the homology observed among the N-terminal regions is generally lower than that observed in C-terminal regions. This tendency is also related to the fact that the motif responsible for specific binding with the respective effector molecule is located at the N-terminal regions of AraC/XylS family regulators (18). In spite of their low identities (24.7%), both AntR and AndR are involved in anthranilate catabolism, and it has been found that the inducer molecules of the genes regulated by the two transcriptional regulators are common. Because several conserved residues observed in N-terminal regions between AntR and AndR could be involved in the recognition of anthranilate, further investigation on effector binding will be necessary to

understand the mode of action of AntR, such as effector binding and subsequent conformational change.

The second copy of P_{ant} on pCAR1 recruited by the transposition of ISPre1 promotes the transcription of the *carAa* gene encoding the terminal oxygenase component of carbazole 1,9a-dioxygenase, which catalyzes the initial step of carbazole degradation and disrupts the planar structure of carbazole to reduce its toxicity. From the result of Northern hybridization using the *carAa* probe, the maximum size of the transcript originated from the second P_{ant} promoter induced by anthranilate is estimated at about 7 kb, although the hybridized band was smeared (Fig. 7A). It was suggested that this length of transcript covers the genes from *carAa* to at least *carD*. Although P_{ant} promoter-mediated transcription is specific to the anthranilate catabolic pathway, both of the *ant* and *car* genes involved in the regulation of carbazole degradation are transcribed from P_{ant} promoters and are simultaneously regulated by AntR in consequence of the transposition of ISPre1. Thus, the transposition of P_{ant} is suggested to have directed the xenobiotic compound-degradative gene cluster to be induced by a biotic compound. However, the ancestral mechanism of transcriptional regulation of the *car* operon before transposition of P_{ant} is unknown. Further analysis of the transcriptional regulation of the upper *car* gene cluster of strain CA10 or its ancestral gene cluster will provide an important implication about the evolution of catabolic operons. Furthermore, because knowledge on the expression range of catabolic genes carried on mobile genetic elements is still limited (36), more detailed analyses of the global regulatory mechanisms of *car* and *ant* operons in strain CA10 and also in several other recipient strains of pCAR1 will be needed.

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