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Evidence for Autoregulation of *camR*, Which Encodes a Repressor for the Cytochrome P-450cam Hydroxylase Operon on the *Pseudomonas putida* CAM Plasmid

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The regulatory gene *camR* on the CAM plasmid of *Pseudomonas putida* (ATCC 17453) negatively controls expression of the cytochrome P-450cam hydroxylase operon (*camDCAB*) for the camphor degradation pathway and is oriented in a direction opposite to that of the *camDCAB* operon. In this study, we examined expression of the *camR* gene by monitoring the β-galactosidase activity of *camR-lacZ* translational fusions in *P. putida camR* and *camR*$^+$ strains. We found that the *camR* gene was autogenously regulated by its own product, CamR. To search for an operator site of the *camR* gene, a *cam* repressor (CamR)-overproducing plasmid, pHAOV1, was constructed by placing the *camR* gene under the control of a p_L promoter. The translational initiation codon of CamR was changed by site-directed mutagenesis from GTG to ATG to improve translation efficiency. Judging from sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis, the CamR protein was expressed up to about 10% of the soluble protein of CamR-overproducing *Escherichia coli* JM83/pHAOV1 cells. Results of DNase I footprinting assays using the cell lysate indicated that the CamR repressor covered a single region between the *camR* gene and the *camDCAB* operon. Our findings also suggest that the *camR* gene autogenously regulates its own expression by binding of the gene product, CamR, to the operator, which also serves as an operator of the *camDCAB* operon.

Pseudomonas spp. utilize many aromatic compounds as carbon and energy sources, and some functions depend on enzymes encoded by plasmids (4). *Pseudomonas putida* PpG1 (ATCC 17453) was originally isolated by enrichment culture on D-camphor as the sole carbon source. As shown in Fig. 1, the CAM plasmid of *P. putida* PpG1 encodes oxidative enzymes for a pathway for catabolism of camphor to isobutylate (25, 27). The initial step of camphor degradation is oxidation of camphor to 5-*exo*-hydroxycamphor by a monooxygenase system, which consists of the enzymes NADH-putidaredoxin reductase (45 kDa; encoded by the *camA* gene), putidaredoxin (12 kDa; encoded by the *camB* gene), and cytochrome P-450cam (47 kDa; encoded by the *camC* gene) (8, 14). The second step of degradation is conversion of the alcohol to 2,5-diketocamphane by 5-*exo*-hydroxycamphor dehydrogenase (80 kDa; encoded by the *camD* gene) (9). Genes of these four enzymes form an operon called the cytochrome P-450cam hydroxylase operon (*camDCAB*) (15, 16). The *camDCAB* operon is under negative control by *camR*, which is located immediately upstream of the *camD* gene, and shows maximal expression in the presence of D-camphor (15, 16). The *camR* gene is transcribed in the direction opposite to that of the *camDCAB* operon (2). The regulatory mechanism of expression of the *camR* gene remains to be clarified.

We report here that expression of *camR* is negatively controlled by its own product. The operator site of *camR* was determined both in vivo by measurement of β-galactosidase activity resulting from *camR-lacZ* translational fusions in a *P. putida camR*$^+$ strain and in vitro by a DNase I footprinting assay using the cell lysate of the *cam* repressor (CamR) overproducer. CamR binds to only a single region between the *camR* gene and the *camDCAB* operon, indicating that the binding region serves as a common operator for the promoters of both the *camR* gene and the *camDCAB* operon. On the basis of these findings, a putative mechanism for *cam* gene expression is proposed.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are given in Table 1.

Media. L broth was used for cell growth. L agar containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside was used to visualize β-galactosidase activity resulting from *camR-lacZ* translational fusions in cells on the agar plates. When needed, streptomycin (500 μg/ml) and/or kanamycin (50 μg/ml) were added for the culture of *P. putida*. Terrific broth (29) was used for the CamR protein-overproducing culture.

Plasmid constructions. To search the promoter-operator regions of the *camR* gene, DNA fragments containing sequences from various positions in the 5′-flanking region to the *Bgl*II site (+132) located in the *camR* gene were ligated to *lacZ* (Fig. 2A). To express the *camR-lacZ* translational fusions only from the *camR* promoter, the terminator gene (*terS*, reference 10) was inserted upstream of the 5′-flanking region of *camR*. The fusion gene was constructed by connecting the amino-terminal 45 codons of *camR* to codon 8 of *lacZ* (Fig. 2B) so that the β-galactosidase activity of the *camR-lacZ* translational fusion would be conserved. Plasmid pJP122 (Fig. 2A) was constructed as follows. The *Pst*I (−118)-*Bgl*II (+132) fragment of *camR* from pJP701 and the *Bam*HI-*Sal*I fragment containing *lacZ* from plasmid pMC1403 were coligated into the *Pst*I-*Sal*I sites of low-copy-number vector pTS1210, and the resulting plasmid was designated pJP115. Plasmid pSY115 was

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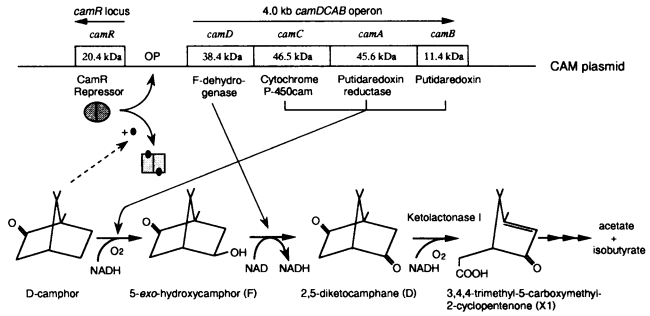

FIG. 1. Organization of the *camR* gene, the *camDCAB* operon, and the D-camphor oxidation pathway in *P. putida*. OP, operator promoter.

constructed by cloning the *Bam*HI-*Sal*I fragment containing *terS* from pTerS3 into the *Bam*HI-*Sal*I polylinker sites of pUC18. The *Eco*RI-*Pst*I fragment containing *terS* from pSY115 was then inserted at the *Eco*RI-*Pst*I sites of pJP115, yielding plasmid pJP122. To construct other plasmids containing various lengths of the 5′-flanking region of *camR*, DNA fragments from *Pst*I (−118) to *Hin*cII (−201), *Bal*I (−218), *Nae*I (−288), *Sph*I (−326), and *Pst*I (−266) were isolated from pJP701 and then each pair of DNA fragments with the *terS* fragment was inserted into the *Eco*RI-*Pst*I sites of pJP115, yielding pJP311, pJP1013, pJP411, pJP1001, and pJP913, respectively (Fig. 2A). These *camR-lacZ* translational fusions were under the same control as the *camR* gene, except that plasmid pJP902 was constructed by ligating the *Pst*I (−118)-*Pst*I (−266) fragment of pJP913 in the opposite direction (Fig. 2A). Restriction sites *Pst*I (−266) and *Bal*I (−218) were prepared by site-directed mutagenesis on pMA3 containing the *Pst*I (−118)-*Sph*I (−326) fragment of pJP705. The oligonucleotides used for the mutation were 5′-ACAACTGCAGGAG GCGGG-3′ for *Pst*I (−266) and 5′-ACTCCTTCTGGCCA ATAT-3′ for *Bal*I (−218) (mismatches are underlined).

To determine the operator site of *camR* in vitro, we constructed a plasmid for overproduction of CamR. The translational initiation codon of CamR was changed from GTG to ATG by site-directed mutagenesis to introduce an *Nco*I site. An *Eco*RI site was also introduced into the 3′-flanking region of the *camR* gene so that the termination codon (TAG) of CamR would be located immediately upstream of the terminator (*trpA*). These two silent mutations were introduced as follows. The *Pst*I-*Sma*I fragment containing the *camR* gene from pJP705 was ligated to the *Pst*I-*Sma*I sites of the M13mp19 replicative form, and then *Nco*I and *Eco*RI restriction sites were introduced adjacent to the 5′ and 3′ ends of *camR*, respectively. The oligonucleotides used for the site-directed mutagenesis were 5′-GCCGGGCACCATGGAC ATC-3′ for *Nco*I and 5′-AGTAGGCGAATTCGTGCCGA-3′ for *Eco*RI (mismatches are underlined). The plasmid, isolated from *Escherichia coli* MV1190, was digested with *Nco*I and *Eco*RI and then blunt ended by a large fragment of DNA polymerase I. The 560-bp fragment containing the entire sequence of the *camR* gene was isolated by electroelution from a 5% acrylamide gel. Expression vector pUC-p_L-cI was di-

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
P. putida		
TN1126	*met trp*	12
E. coli		
JM83	F′ *ara* Δ(*lac proAB*) *rpsL* (ϕ80*lacZ*ΔM15)	21
CJ236	*dut-1 ung-1 thi-1 recA1*/pCJ105 (F′, Cm^r)	13
MV1190	Δ (*lac proAB*) Δ(*stl recA*)*306*::Tn*10 thi supE* (F′ *proAB lacI*^{q} *lacZ*MΔ15 *traD36*)	32
Plasmids		
pUC18	Cloning vector, Ap^r	33
pUC19	Cloning vector, Ap^r	33
M13mp19	Cloning vector	33
pTS1036	High-copy-number vector, Ap^r Km^r Su^r Sm^r	T. Nakazawa[a]
pTS1210	Low-copy-number vector, Ap^r Km^r	T. Nakazawa
pUC-pL-cI	Expression vector, Ap^r	18
pTerS3	*terS* DNA fragment in pBR322	10
pMC1403	Prototype hybrid β-galactosidase vector, Ap^r	31
pJP701	1.12-kb CAM *Pst*I-*Eco*RI fragment in pUC9	2
pJP705	0.71-kb CAM *Pst*I-*Sma*I fragment in pUC9	2
pJP4	*camR*$^+$ Ap^r Sm^r Su^r	15
pJP115	*camR* (*Pst*I-*Bgl*II)-*lacZ*	This work
pSY121	*terS* DNA fragment in pUC18	This work
pJP122	*ter-camR* (*Pst*I-*Bgl*II)-*lacZ*	This work
pJP311	*ter-camR* (*Hin*cII-*Bgl*II)-*lacZ*	This work
pJP1013	*ter-camR* (*Bal*I-*Bgl*II)-*lacZ*	This work
pJP411	*ter-camR* (*Nae*I-*Bgl*II)-*lacZ*	This work
pJP1001	*ter-camR* (*Sph*I-*Bgl*II)-*lacZ*	This work
pJP913	*ter-camR* (*Pst*I-*Bgl*II)-*lacZ*	This work
pJP902	Same as pJP913, but *Pst*I fragment in opposite orientation	This work
pMA3	0.71-kb CAM *Pst*I-*Sma*I fragment in M13mp19	This work
pHA37-1	*camR Sph*I-*Sma*I fragment in pUC19	This work
pHAOV1	*cam* repressor-overproducing plasmid	This work

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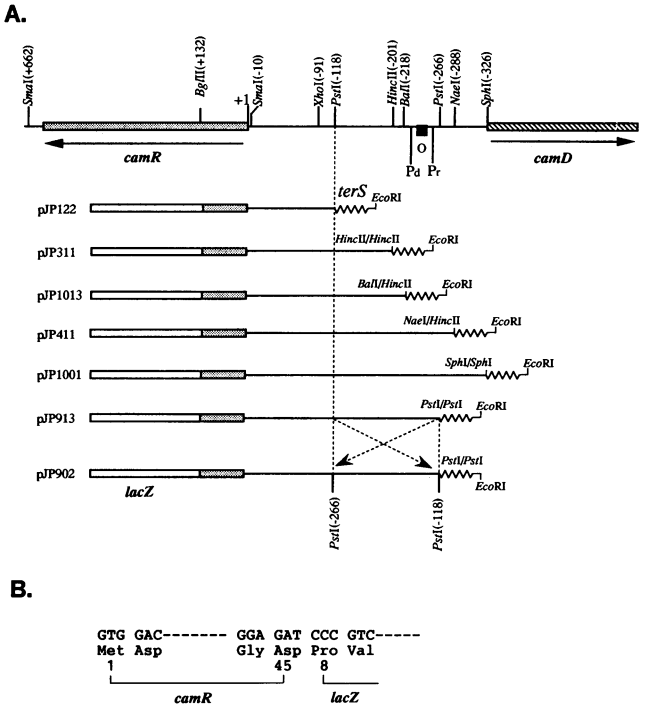

FIG. 2. Construction of *camR-lacZ* translational fusions. (A) At the top is a restriction map of the *camR* gene and the surrounding region. The positions of several restriction sites are based on the translational initiation site (+1) and are shown in parentheses. The operator (O) common to the *camR* gene and the *camDCAB* operon is indicated by the closed box. Pr and Pd indicate the putative promoters for the *camR* gene and the *camDCAB* operon, respectively. The *camR* and *camD* coding regions are also indicated. The lower part shows the compositions of *camR-lacZ* translational fusions. The shaded and open boxes represent *camR* and *lacZ* coding regions, respectively. The jagged lines indicate the terminator gene, *terS*. (B) Nucleotide and protein sequences of the junction of a *camR-lacZ* translational fusion. The amino-terminal region of CamR, residues 1 to 45, was connected to residue 8 of β-galactosidase.

gested with *Eco*RI and *Sph*I and then blunt ended by T4 DNA polymerase. The 4-kb fragment was isolated by electroelution from a 1% agarose gel. The mutated *camR* gene was then ligated downstream of the p_L promoter in pUC-p_L-cI by using T4 DNA ligase; the plasmid constructed was designated pHAOV1. By DNA sequencing analysis (30) of pHAOV1, we verified that the new initiation codon (ATG) of CamR was located 8 bp downstream of the Shine-Dalgarno sequence and the terminator (*trpA*) was located immediately downstream of the stop codon (TAG).

To prepare the DNA probe for DNase I footprinting, an *Sma*I site was prepared at 10 bases upstream from the native initiation codon (GTG) of CamR by site-directed mutagenesis with an oligonucleotide with the sequence 5′-TTGCCCG GGTCGATCCGA-3′ (the mismatch is underlined) (Fig. 2A). An *Sph*I (−326)-*Sma*I (−10) fragment containing the regulatory region of *camR* and the *camDCAB* operon was cloned into pUC19, yielding pHA37-1.

Site-directed mutagenesis. Mutagenesis was performed by the method of Künkel (17), with the Muta-Gene phargemid in vitro mutagenesis kit from Bio-Rad Laboratories. The sequences of the entire *camR* gene and all of the mutations were verified by DNA sequencing with Sequenase sequencing kits.

Assay of β-galactosidase. Cells were grown to the mid-log phase in L broth in the presence or absence of 5 mM D-camphor. The β-galactosidase activity of the *camR-lacZ* translational fusion was determined by the Miller assay (22). The basic promoter activities of the *camR* gene and the *camDCAB* operon were taken from the respective β-galactosidase activities in the *P. putida camR* strain (TN1126 which is CAM plasmid free). *P. putida camR*$^+$ strains were cultured in the presence or absence of D-camphor, and the repression ratios were estimated from the ratio of the induced/noninduced β-galactosidase activities.

Overproduction of CamR protein in *E. coli*. *E. coli* JM83 was transformed with CamR-overproducing plasmid pHAOV1. As a control, *E. coli* JM83 was transformed with expression vector pUC-p_L-cI. The transformants, JM83/pHAOV1 and JM83/pUC-p_L-cI, were cultured at 32°C in Terrific broth containing ampicillin (50 μg/ml) until the optical density at 600 nm reached 1.0. The cultures were then either supplemented with D-camphor to 5 mM or not supplemented and further cultured for 2 h at 42°C. The cells were harvested by centrifugation at 7,000 × *g* for 10 min at 4°C, suspended in TDEG buffer (50 mM Tris-HCl [pH 7.5]) containing 1 mM dithiothreitol, 0.1 mM EDTA, 10% [vol/vol] glycerol), and sonicated by six 30-s pulses with 30-s intervals under ice-cold conditions. After centrifugation at 100,000 × *g* for 60 min at 4°C, the supernatant was obtained and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (19) with a 13% acrylamide gel. The insoluble materials were suspended in a small amount of 10 mM sodium phosphate (pH 7.4) containing 1% SDS. A portion of the suspension was boiled with the sample buffer (19), and then the sample was also subjected to SDS-PAGE.

Repressor-operator binding. The cell lysate containing CamR was prepared from 400-ml cell cultures grown for 2 h at 42°C in the presence of D-camphor as described above. To prepare the probe for DNase I footprinting, plasmid pHA37-1 containing the *Sph*I (−236)-*Sma*I (−10) fragment (Table 1) was digested with *Eco*RI and *Hin*dIII and the 337-bp fragment was isolated after 5% acrylamide gel electrophoresis. The DNA fragment was end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. The probes labeled at one end were purified by acrylamide gel electrophoresis after secondary cleavage at the *Sph*I or *Sma*I site. Reaction mixtures containing 0.1 pmol of the ^{32}P-labeled DNA fragment and 0, 1.0, 2.5, 5.0, or 10 μg of a lysate of CamR-overproducing *E. coli* cells cultured in the presence of D-camphor were incubated for 20 min at 25°C for repressor-operator complex formation. As a control, 10 μg of a cell lysate from JM83/pUC-p_L-cI was used. The probe DNA was digested with 1 μg of DNase I per ml at 25°C for 1 min, and the digestion was stopped by adding EDTA to 4 mM. Samples were twice treated with phenol-chloroform (1:1), and then the DNA was recovered by ethanol precipitation with 0.3 M sodium acetate. Pellets were suspended in 10 μl of formamide dye (80% formamide, 1 mM EDTA, 10 mM NaOH, bromophenol blue and xylene cyanol, each at 0.1%), 3-μl aliquots were loaded onto a 6% acrylamide-urea sequencing gel, and electrophoresis was carried out for 2 h at 40 W.

Amino acid sequence. When the CamR-overproducing strain was cultured in the absence of D-camphor, the CamR protein was localized in the insoluble fraction. The insoluble materials were resuspended in 10 mM sodium phosphate (pH 7.4) containing 1% SDS, and CamR was isolated by Superose 12 gel filtration. Conversely, when the cells were cultured in the presence of D-camphor, CamR was localized in the soluble fraction. The soluble fraction was further fractionated by SDS-PAGE, and the gel was stained with Coomassie brilliant

TABLE 2. Expression of *camR-lacZ* translational fusions in *P. putida camR* and *camR*$^+$ strains

Plasmid	*camR* strain[a] β-Gal activity[b]		Ratio of +/− values	*camR*$^+$ strain[c] β-Gal activity[b]		Ratio of +/− values
	+	−		+	−	
pJP122	1.4	0.9	1.6	2.1	2.1	1.0
pJP311	2.2	1.7	1.3	4.7	3.7	1.3
pJP1013	3.0	2.8	1.1	2.4	2.4	1.0
pJP411	22	22	1.0	23	3.9	5.9
pJP1001	23	25	0.9	24	3.8	6.3
pJP913	21	21	1.0	23	3.9	5.9
pJP902	58	64	0.9	70	1.8	39

[a] *P. putida* TN1126 (*camR* mutant).
[b] β-Galactosidase (β-Gal) activities are expressed in Miller units. Cells were cultured as described in Materials and Methods. +, with D-camphor; −, without D-camphor.
[c] *P. putida* TN1126/pJP4 (*camR*$^+$).

blue R-250. The band for CamR was cut out, and the protein was extracted from the acrylamide gel. The N-terminal amino acid sequences of the two forms of CamR protein were determined on an Applied Biosystems 470-A Sequenator.

Other details. Sources were as follows: restriction enzymes and T4 DNA ligase, Nippon Gene; the large fragment of DNA polymerase I (Klenow fragment), Takara Shuzo Co.; T4 polynucleotide kinase and Sequenase sequencing kit, Toyobo; bovine serum albumin (fraction V), Boehringer Mannheim; Superose 12 and protein standards for SDS-PAGE, Pharmacia LKB Biotechnology Inc.; [α-^{32}P]dCTP and [γ-^{32}P]ATP, New England Nuclear. Isolation of plasmid DNA and plasmid construction and transformation were done as described by Sambrook et al. (29). Protein concentration was determined by the method of Bradford (3) with bovine serum albumin as the standard.

RESULTS

Autoregulation of the *camR* gene. We examined expression of the *camR* gene by monitoring the β-galactosidase activities of *camR-lacZ* translational fusions, since the repressor activity of CamR cannot directly be detected in vivo. *P. putida camR* (TN1126) and *camR*$^+$ (TN1126/pJP4) strains were transformed with the *camR-lacZ* translational fusion plasmids shown in Fig. 2A. pJP4 was constructed by inserting the *camR* gene into high-copy-number vector pTS1036 (20 to 30 copies per cell) to express CamR. β-Galactosidase activities were measured by using cell lysates of these transformants cultured in the presence or absence of D-camphor (Table 2). In the *P. putida camR* strain, the β-galactosidase activities from pJP122, pJP311, and pJP1013 were low (1 to 3 Miller units), both in the presence and in the absence of D-camphor. On the other hand, the β-galactosidase activities from pJP411, pJP1001, and pJP913 were 10-fold higher than those from the former plasmids, and this phenomenon was independent of the presence of D-camphor. Moreover, in the *P. putida camR*$^+$ strain, β-galactosidase activities from the latter plasmids were inducible by camphor and their induction ratios were approximately 6. These results indicate that the promoter-operator sites of the *camR* gene are located in a region between the *Bal*I (−218) and *Pst*I (−266) sites (Fig. 2A) and that expression of *camR* is negatively regulated by its own gene product, CamR.

Construction of a plasmid for CamR overproduction and expression of the CamR protein in *E. coli*. To determine the operator site of *camR* in vitro, a plasmid for CamR overproduction, pHAOV1, was constructed by placing the *camR* gene under control of the p_L promoter of expression vector pUC-p_L-cI as described in Materials and Methods. Since the GTG codon functions less efficiently as the initiator than does the ATG codon in *E. coli* (5, 23, 26), we changed the initiation codon of CamR from GTG to ATG by site-directed mutagenesis, accompanying the introduction of an *Nco*I site. Expression of the *camR* gene was induced by conventional heat treatment at 42°C. When JM83/pHAOV1 was cultured in the presence of D-camphor, a protein band with an apparent molecular mass of approximately 20 kDa, a value identical to that deduced from the nucleotide sequence of the *camR* gene (2), was abundant in the supernatant but was faint in the insoluble fraction (Fig. 3, lanes 7 and 8). In contrast, the band corresponding to CamR was abundant in the insoluble fraction but was faint in the soluble fraction from cells cultured in the absence of D-camphor (Fig. 3, lanes 5 and 6). In *E. coli* cells harboring pUC-p_L-cI, the band was not observed (Fig. 3, lanes 1 to 4). From the two fractions (Fig. 3, lanes 6 and 7), we isolated the protein from the major band (molecular mass, 20 kDa) and determined the N-terminal amino acid sequence. The sequence of the N-terminal 10 residues of both protein bands was Met-Asp-Ile-Lys-Gln-Ser-Leu-Leu-His-Ala, which is identical to that deduced from the nucleotide sequence of the *camR* gene (2). This result indicates that the CamR protein was overproduced as a soluble form in *E. coli* cells cultured in the presence of D-camphor and suggests that CamR can readily form an aggregate in the absence of D-camphor.

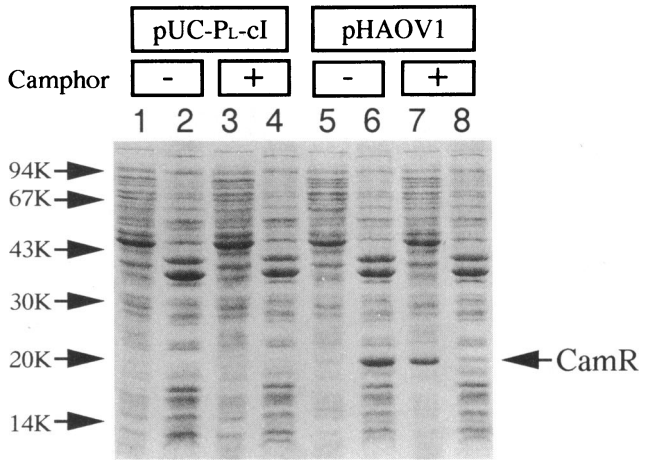

FIG. 3. Overproduction of CamR in *E. coli* JM83/pHAOV1. Details are described in Materials and Methods. Lanes: 1 to 4, proteins from JM83/pUC-p_L-cI; 5 to 8, proteins from JM83/pHAOV1; 1, 3, 5, and 7, proteins in the soluble fraction; 2, 4, 6, and 8, proteins in the insoluble fraction; 3, 4, 7, and 8, proteins from cells cultured in the presence of 5 mM D-camphor. The numbers to the left are molecular size standards (10^3).

Protection of operator DNA by CamR. By using the cell lysate containing the soluble form of CamR, the specific binding site of CamR was determined by DNase I footprinting assay. Figure 4A and B show the protection of the coding and noncoding strands of *camR*, respectively. When the amount of the cell lysate was 2.5 μg or more, protection was observed on both strands and the footprints disappeared on both strands when 5 mM D-camphor was added to the reaction mixture. The protected region covered 22 bp containing the 6-bp inverted repeat sequences shown in Fig. 4C. The binding site of CamR was consistent with the operator region of the *camR* gene

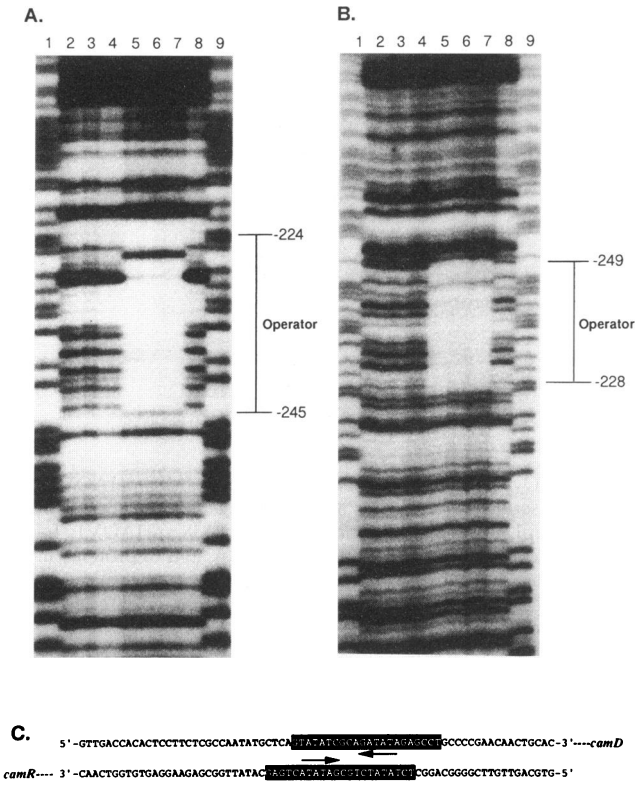

FIG. 4. DNase I footprinting analysis. In all of the experiments, cell lysates from CamR-overproducing cells cultured in the presence of D-camphor were used to protect DNA from DNase I. Lanes: 1 and 9, product of a G+A reaction prepared by the method of Maxam and Gilbert (20); 2, cell lysate from JM83/pUC-p_L-cI (10 μg); 3 to 7, cell lysate from JM83/pHAOV1 (0, 1.0, 2.5, 5.0, and 10 μg, respectively); 8, cell lysate from JM83/pHAOV1 (10 μg plus 5 mM D-camphor). Regions protected from DNase I are indicated on the right. (A) Protection pattern of the *camR* coding strand (*camDCAB* operon noncoding strand) of the *Sph*I-*Sma*I fragment labeled on the *Sph*I end. (B) Protection pattern of the *camR* noncoding strand (*camDCAB* operon coding strand) of the *Sph*I-*Sma*I fragment labeled on the *Sma*I end. (C) Nucleotide sequences of protected regions. Bases protected from DNase I digestion on each strand are highlighted by reverse type.

determined by monitoring the β-galactosidase activity of *camR-lacZ* translational fusions in vivo (Table 2; Fig. 2A). It is noteworthy that CamR bound only a single region between the *camR* gene and the *camDCAB* operon. Furthermore, the location of the promoter-operator regions of the *camDCAB* operon was downstream (the *camD* side) of the *Xho*I (−91) site (15). These results show that a single repressor-binding site serves as a regulatory locus, not only for the *camR* gene but also for the *camDCAB* operon. Camphor-induced expression of the *camR* gene and the *camDCAB* operon in *P. putida* is presumably caused by release of the CamR repressor from the operator region, as a result of direct interference by camphor with the binding. These results also suggest that the active CamR protein was present in the cell lysate prepared from JM83/pHAOV1 cultured in the presence of D-camphor. The number of active CamR proteins remains to be determined.

Promoter activities of the *camR* gene and the *camDCAB* operon. The promoter activity of the *camR* gene was compared with that of the *camDCAB* operon by monitoring β-galactosidase activity. As shown in Fig. 2A, the *Pst*I (−266)-*Pst*I (−118) fragment contains the promoter-operator regions of not only the *camR* gene but also the *camDCAB* operon. Plasmid pJP902 was constructed by ligating the *Pst*I (−266)-*Pst*I (−118) fragment to *lacZ* in the direction opposite to that of pJP913. Therefore, the expression of β-galactosidase in cells harboring pJP902 is controlled by the promoter-operator of the *camDCAB* operon. As shown in Table 2, in the *P. putida camR*$^+$ strain, the β-galactosidase activity from pJP902 was increased 40-fold in the presence of D-camphor. In the presence of D-camphor, the β-galactosidase activity from pJP913 was one-third of that from pJP902 in both the *P. putida camR* and *camR*$^+$ strains. This result suggests that the promoter activity of the *camR* gene is one-third of that of the *camDCAB* operon.

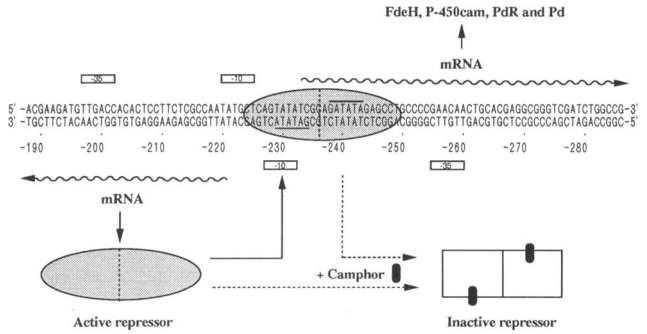

FIG. 5. Putative molecular mechanism of regulation of *cam* genes. The lower and upper lines show palindromic DNA sequences. FdeH, 5-*exo*-hydroxycamphor dehydrogenase; P450cam, cytochrome P-450 cam; PdR, putidaredoxin reductase; Pd, putidaredoxin.

DISCUSSION

We obtained evidence that *camR*, a repressor gene for the cytochrome P-450cam hydroxylase operon, is autogenously regulated by CamR, its own product. To determine the operator site of the *camR* gene, we constructed a plasmid for overproduction of CamR and overproduced it in *E. coli*. When the overproducing cells were cultured in the absence of D-camphor, the CamR protein was obtained in an insoluble form. On the other hand, in the presence of D-camphor, CamR was recovered in the soluble fraction. Even when the overproducing cells were cultured in the presence of an inducer (D-camphor), the washed cell lysate contained an active CamR protein which could bind to the operator DNA in vitro. In the DNase I footprinting assay, CamR protected only a single region between the *camR* gene and the *camDCAB* operon. Binding of CamR to the region was prevented in vitro by addition of D-camphor. This region contained the 6-bp (GATATA) inverted repeat sequences, satisfying the most important feature of a repressor-binding site (7). On the basis of the diameter of lysozyme (30 Å [0.3 nm]) as a global protein (11), the diameter of CamR with a molecular mass of approximately 20 kDa was calculated to be 33.6 Å. The length of operator DNA was calculated to be 74.8 Å. Also, by gel filtration, the purified CamR protein eluted as a single peak of protein with a molecular mass of 40 kDa (1). Thus, the active form of the CamR repressor would be a dimer, as is the case with the other repressors (24).

We propose that the molecular mechanism for regulation of the *camR* gene and the *camDCAB* operon in *P. putida* is as follows (Fig. 5). In the absence of camphor, the CamR protein binds to only a single region, which serves as a common operator of the *camR* gene and the *camDCAB* operon, and can repress

transcription of both the *camR* gene and the *camDCAB* operon; when camphor is added, repressors no longer bind and transcription of both genes can be induced. Camphor functions as an inducer by eliciting a conformational change in the CamR repressor through direct interaction with this protein.

By monitoring β-galactosidase activities with *camR-lacZ* translational fusions, we found that the promoter activity of the *camR* gene was one-third of that of the *camDCAB* operon in *P. putida*, a value compatible with the ratio of syntheses of *camR* mRNA and *camDCAB* mRNA in *P. putida* PpG1 (*camR*/*camDCAB* operon ratio, 1:4) determined by S1 nuclease mapping (6). It is likely that RNA polymerase bound to one promoter prevents occupation of the other promoter by another RNA polymerase molecule. We recently determined the transcriptional start sites of the *camR* gene and the *camDCAB* operon in *P. putida* PpG1 (6). As shown in Fig. 5, *camR* mRNA was initiated from the T at position −221 and was transcribed to the left side and *camDCAB* mRNA was initiated from the TAT at positions −231 to −233 and was transcribed to the right side. The promoters of *camR* and the *camDCAB* operon are TTGTTC-22 bp-TATACT and TTGACC-17 bp-TATGCT, respectively (Fig. 5). The promoter of the *camDCAB* operon exhibits good homology with both the −10 and the −35 regions of the promoter consensus sequence in *E. coli* (28), while the promoter of *camR* is poorly homologous since the spacing between the −10 and the −35 regions is 22 bp. In an in vitro transcription experiment (6), however, σ^{70}-like RNA polymerase ($P\sigma^{70}$) obtained from *P. putida* transcribed the *camR* promoter and the *tac* promoter, which has the typical consensus sequence for *E. coli* σ^{70} RNA polymerase, but not the *camDCAB* promoter. Studies on the mechanism for interaction among the *camR* promoter, the CamR protein, and RNA polymerase are ongoing.

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