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Purification and Characterization of a *cam* Repressor (CamR) for the Cytochrome P-450cam Hydroxylase Operon on the *Pseudomonas putida* CAM Plasmid

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The cytochrome P-450cam hydroxylase operon of *Pseudomonas putida* PpG1 (ATCC 17543) encodes proteins responsible for early steps of the degradation of D-camphor. Transcription of this operon is negatively controlled by the *cam* repressor (CamR), and the expression of *camR* is autoregulated. CamR was purified from *Escherichia coli* harboring an overproducing plasmid. The repressor forms a homodimer with a molecular mass of 40 kDa, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis and gel filtration. CamR protected a specific DNA region from attack by DNase I. This region contains a palindromic operator of the cytochrome P-450cam hydroxylase operon and of the *camR* gene. Protection was inhibited by the addition of 60 μM D-camphor and also by certain camphor analogs and degradation products, including D-3-bromocamphor, adamantane, 2-adamantanone, 5-*exo*-hydroxycamphor, and 2,5-diketocamphane. These analogs and degradation products induced cytochrome P-450cam hydroxylase operon expression in vivo.

The cytochrome P-450cam hydroxylase operon (*camDCAB*) on the CAM plasmid of *Pseudomonas putida* PpG1 (ATCC 17453) is responsible for early steps of the D-camphor degradation pathway for catabolism of camphor to isobutyrate (17, 18). D-Camphor degradation initiates oxidation to 5-*exo*-hydroxycamphor by a monooxygenase system which consists of three 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) (7, 10). The second step is the conversion of alcohol to 2,5-diketocamphane, which is dehydrogenated by 5-*exo*-hydroxycamphor dehydrogenase (FdeH, 80 kDa; encoded by the *camD* gene) (14). Expression of the *camDCAB* operon and the *camR* gene is negatively regulated through interaction of the CamR protein with the single operator located in the overlapping promoter region between the *camDCAB* operon and the *camR* gene. In the presence of D-camphor, these genes are divergently transcribed from the overlapping promoters (3, 12). When examining structural and functional properties of the CamR repressor, we constructed a heterologous expression system for overproduction of the CamR protein, with *Escherichia coli* harboring a plasmid for expression of CamR (3).

We report here the purification and characterization of the CamR protein from an *E. coli* culture, in the presence of D-camphor. Both in vitro and in vivo, various camphor analogs inhibited binding of the CamR protein to the operator DNA.

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MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used in this study were *E. coli* JM83 [F′ *ara* Δ(*lac-proAB*) *rpsL*(ϕ80*lacZ*ΔM15)] (21) and *P. putida* PpG277 (16). A CamR-overproducing plasmid, pHAOV1, was constructed as follows. The translational initiation codon of the *camR* gene was changed from GTG to ATG to increase translational efficiency, and then the *camR* gene was placed under the control of a bacteriophage λ P_L promoter of the expression vector pUC-P_L-cI (3, 19). Plasmid pHAOV1 was introduced into *E. coli* JM83, and then the *E. coli* cells harboring the expression plasmid (JM83/pHAOV1) were used to overproduce the CamR protein. Plasmids pHA3 and pHA37-1 (3) were constructed by cloning *Pst*I-*Sph*I and *Sma*I-*Sph*I DNA fragments containing promoter/operator regions of the *camDCAB* operon and the *camR* gene into *Pst*I-*Sph*I and *Sma*I-*Sph*I sites of pUC19 (28), respectively (Fig. 1). Plasmid pJP39 was constructed by cloning the *Eco*RI-*Hin*dIII DNA fragment containing the *camDCAB* operon and the *camR* gene of the CAM plasmid into *Eco*RI-*Hin*dIII sites of pKT240 (6), a shuttle vector between *E. coli* and *P. putida* (2).

Purification of CamR. *E. coli* JM83/pHAOV1 was cultured in LB medium (23) containing ampicillin (50 μg/ml) at 32°C. An overnight culture was inoculated at a 1/50 volume into Terrific broth (23) containing ampicillin (50 μg/ml). The cells were cultured at 32°C with continuous shaking to an A_{595} of 1.0 before D-camphor was added to the culture to a final concentration of 1 mM and the preparation was further incubated for 2 h at 42°C. All the operations described below were carried out at 0 to 4°C. The cells (about 20 g [wet weight]) were harvested from a 4-liter culture by centrifugation at 7,000 × *g* for 10 min, suspended in 100 ml of buffer A (50 mM Tris-HCl [pH 7.5] containing 1 mM dithiothreitol, 0.1 mM EDTA, and 10% [vol/vol] glycerol), and then sonicated by six 30-s pulses at 30-s intervals with a Branson sonifier 250. Cell lysate was obtained by centrifugation at 100,000 × *g* for 1 h with a Beckman type 55.2 Ti rotor. Powdered ammonium sulfate (0.25 g/ml of cell lysate) was added to the cell lysate, and the preparation was continuously stirred for 1 h. The precipitate was collected by centrifugation at 31,000 × *g* for 90 min with a Kubota RA-6F rotor. The precipitate was suspended in buffer A, and the suspension was dialyzed against 100 volumes of buffer A containing 50 mM NaCl, with two changes. The column operations were carried out by fast protein liquid chromatography (FPLC; Pharmacia LKB Biotechnology Inc.). The dialyzed enzyme solution was applied to a DEAE-trisacryl column (22 by 95 mm, type M; IBF Biotechnics) equilibrated with buffer A containing 50 mM NaCl. The column was washed with 10 column volumes of the equilibration buffer, and CamR was eluted with a linear gradient of 50 to 500 mM NaCl in 10 column volumes of buffer A. The peak fractions of DNA binding activity were combined and filtered through an FPLC-HiLoad 16/60 Superdex 75pg column (121 ml; Pharmacia LKB Biotechnology Inc.) equilibrated with buffer A containing 200 mM NaCl at a flow rate of 0.5 ml/min. Fractions with DNA binding activity were combined and diluted to

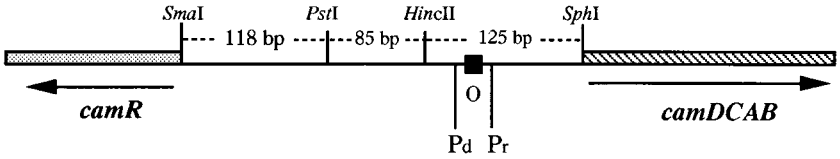

FIG. 1. Configuration of flanking region between the *camR* gene and *camDCAB* operon. The operator (O) common to the *camR* gene and the *camDCAB* operon is indicated by the solid box. The promoters for the *camR* gene (Pr) and the *camDCAB* operon (Pd) are indicated. The locations of *camR* and *camDCAB* and their directions of transcription are indicated by hatched boxes and arrows, respectively.

a 1/3 ratio with buffer A. The diluted enzyme solution was again subjected to chromatography with a DEAE-trisacryl column (10 by 50 mm) with a linear gradient of 50 to 500 mM NaCl in 10 column volumes of buffer A.

Gel mobility shift assay. A gel mobility shift assay was done for the in vitro assay of the DNA binding activity of CamR (3), and the amount of the protein-DNA complex formed was measured. A 125-bp fragment (Fig. 1, *Hin*cII-*Sph*I) containing the overlapping promoter/operator region of the *camDCAB* operon and the *camR* gene was purified from plasmid pHA3 by extracting the DNA fragment from the polyacrylamide gel (23). The isolated DNA fragment was labeled at one end of each strand with [γ-^{32}P]ATP and T4 polynucleotide kinase for use as a probe in the gel mobility shift assay. The standard assay system (20 μl) contained 0.1 ng of the labeled DNA probe and an appropriate amount of the enzyme fraction in 10 mM Tris-HCl (pH 7.5) containing 10% (vol/vol) glycerol, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 1 μg of poly(dI-dC) · poly (dI-dC). The components, except for the enzyme fraction, were combined in a total volume of 17 μl (premixture), before the enzyme fraction (3 μl) was added to the premixture. The mixture was incubated for 20 min at 25°C to allow formation of the CamR-DNA complex. Loading dye solution for polyacrylamide gel electrophoresis (PAGE) was added to give a final concentration of 2.5 mM EDTA, 0.25% glycerol, 0.025% bromophenol blue, and 0.025% xylene cyanol. The mixture was immediately applied to a nondenaturing 5% polyacrylamide gel. Electrophoresis was carried out in 67 mM Tris-HCl (pH 7.5) containing 33 mM sodium acetate and 10 mM EDTA for 30 min at 7.2 V/cm and further for 2 h at 14.3 V/cm. After being dried, the gel was subjected to autoradiography. The intensities of the bands of the autoradiogram were measured with a Bioimaging Analyzer BAS2000 (Fuji Photo Film Co., Ltd.). One unit of the DNA binding activity of CamR protein was defined as the activity converting 5% of the probe DNA to the protein-DNA complex in the standard assay. As required, enzyme fractions were diluted with 50 mM Tris-HCl (pH 7.5) containing 10% (vol/vol) glycerol, 50 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 200-μg/ml bovine serum albumin.

Sedimentation assay for DNA binding. The operator-carrying pHA37-1 plasmid (3-kbp) was linearized at the unique *Sca*I site to eliminate the topoisomer. The CamR repressor dimer (1.3 pmol) was incubated with or without the operator-carrying DNA (20 pmol) in 20 μl of a standard buffer consisting of 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 10% (vol/vol) glycerol. After reaction for 40 min at 25°C, the incubated mixtures were layered onto 1.7 ml of 15 to 60% (vol/vol) glycerol-standard buffer gradients and centrifuged at 150,000 × *g* for 4 h at 4°C in a model TL-100 with a TLS-55 rotor (Beckman). The samples were removed in 12 fractions, and 12 μl of each fraction was subjected to 0.7% agarose gel electrophoresis. To estimate the amount of DNA, the gel was stained with 0.5 μg of ethidium bromide per ml and photographed and the negative was scanned with a laser densitometer 2222-010 (LKB). The other portions of fractions were analyzed by enzyme-linked immunosorbent assay (ELISA) to quantify the CamR protein, as described previously (9). Antiserum against CamR was prepared by immunizing rabbits with purified CamR (4). Anti-CamR immunoglobulin G (polyclonal antibody) was purified from the antiserum by chromatography on a protein G-Sepharose affinity column (Pharmacia LKB Biotechnology Inc.).

Fluorometric assay for DNA binding. Emission spectra were recorded on a Shimadzu RF-540 spectrofluorometer at 22°C. Fluorescence was excited at 287 or 295 nm, and the emission intensity was measured with a bandwidth of 2 nm. CamR repressor dimer was diluted to 0.5 μM in 0.6 ml of 10 mM Tris-HCl (pH 7.5) containing 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 10% (vol/vol) glycerol. In a 10-mm quartz cuvette, the repressor solution was successively replaced by a mixture of 0.5 μM repressor and 2 μM operator DNA. The protein concentration was kept constant (0.5 μM). The DNA was the 32-bp hairpin duplex operator (discussed below). The total loss of fluorescent intensity from mixtures by pipetting was confirmed to be less than 5%. All measurements were duplicated.

Deoxyoligonucleotide 5′-GCAGGCTCTATATCTGCGATATACTGAGCA TATCCCCCATATGCTCAGTATATCGCAGATATAGAGCCTG-3′ was obtained from Toa Gousei Co. and was used as the *cam* operator. The synthetic 70-mer sequence creates an enlarged 5-C hairpin (boldface type), the stem of which forms a stable 32-bp duplex, irrespective of concentration, as follows:

5′-GCAGGCTCTATATCTGCGATATACTGAGCATAT-**CC**

C

3′-GTCCGAGATAGACACGCTATATGACTCGTATA-**CC**

Such a hairpin construction prevents strand separation of a short double-stranded oligomer (13). (The CamR binding site is underlined.)

DNase I footprinting analysis. DNase I footprinting assays were carried out by the method of Aiba (1). To prepare the probe for DNase I footprinting, plasmid pHA3 was digested with *Eco*RI and *Hin*dIII at the polylinker sites of pUC19. The *Eco*RI-*Hin*dIII fragment harbors the 210-bp *Pst*I-*Sph*I fragment containing the *cam* operator (Fig. 1). 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 *Pst*I site. One nanogram of the ^{32}P-labeled DNA fragment was incubated with various amounts of the CamR protein for 20 min at 25°C in 100 μl of 20 mM Tris-HCl (pH 7.9) containing 3 mM $MgCl_2$, 5 mM $CaCl_2$, 100 mM NaCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 50-μg/ml bovine serum albumin. DNase I (Takara Shuzo Co., Ltd.) was added to the mixture to a final concentration of 1 μg/ml, and the preparation was incubated for 1 min at 25°C. Digestion was stopped by adding 4 mM EDTA, and the DNA was treated twice with phenol-chloroform (1:1). The DNA was precipitated with ethanol before the pellet was solubilized in 5 μl of loading dye solution for urea PAGE (80% formamide, 1 mM EDTA, 10 mM NaOH, 0.1% bromophenol blue, and 0.1% xylene cyanol). A 3-μl aliquot was loaded onto a 6% acrylamide-urea sequencing gel for electrophoretic resolution, and electrophoresis was carried out for 2 h at 40 W.

For the reaction in the presence of D-camphor, products of D-camphor degradation, or camphor analogs, 0.5 μg of the CamR protein was incubated with 1 ng of 5′ end-labeled DNA probe for 20 min at 25°C. D-Camphor or the compound was added to the reaction mixture to a final concentration of 1 mM before the mixture was incubated for another 20 min and subjected to DNase I digestion.

Amino acid analysis and N-terminal sequencing. Amino acid analysis of the CamR protein was carried out with a Hitachi L-8500 amino acid analyzer after hydrolysis of the samples in 6 N HCl in a vacuum for 22 h at 110°C. The N-terminal amino acid sequence of the CamR protein was determined with a gas phase Sequenator (Applied Biosystems model 470-A).

Assay of 5-*exo*-hydroxycamphor dehydrogenase (FdeH). The in vivo assay of repressor activity was carried out by monitoring the activity of the *camD* gene product, FdeH. *P. putida* PpG277/pJP39 was cultured at 30°C in LB medium containing ampicillin (1 mg/ml) until the optical density at 660 nm reached 0.35. For the induced culture, a 100 mM solution of D-camphor, products of D-camphor degradation, or camphor analogs in dimethylformamide were added to the culture to yield a final concentration of 1 mM; for noninduced cultures, an equal volume of solvent was added to the culture. Cultures were further incubated for 2 h at 30°C. Cells were harvested by centrifugation at 7,000 × *g* for 10 min at 4°C, suspended in T-0 buffer (50 mM Tris-HCl [pH 7.5] containing 10 mM 2-mercaptoethanol and 5% [vol/vol] glycerol) and sonicated with six 30-s pulses at 30-s intervals, under ice-cold conditions, with a Branson sonifier 250. The cell lysate was obtained by centrifugation at 100,000 × *g* for 1 h at 4°C with a Beckman type 40.2 rotor and then was used for the FdeH assay, as described elsewhere (17). One unit of FdeH activity was defined as the amount required to reduce 1 nmol of NAD per s.

Other procedures. Isolation of plasmid DNA, plasmid construction, transformation, and general DNA techniques were done as described by Sambrook et al. (23). Sodium dodecyl sulfate (SDS)-PAGE (20) was carried out with a 13% separating gel and a 4% stacking gel. Proteins were stained with Coomassie Brilliant Blue R-250. Standards for molecular mass or pI measurements were purchased from Pharmacia LKB Biotechnology Inc. Protein concentrations were determined with a Bio-Rad protein determination kit, with bovine serum albumin as a standard.

RESULTS

Establishment of the assay system for DNA binding activity of CamR protein. We first examined the DNA binding activity of the CamR protein in vitro by using a gel mobility shift assay. A 210-bp fragment (Fig. 1, *Pst*I-*Sph*I) containing the overlapping promoter/operator region of the *camDCAB* operon and the *camR* gene was purified from plasmid pHA3 by extracting the DNA fragment from the polyacrylamide gel (23). The

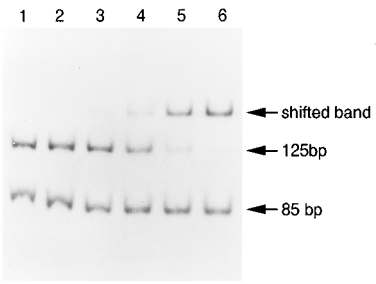

FIG. 2. Gel mobility shift assay with cell lysate of *E. coli* overproducing CamR. A mixture of the 125-bp fragment containing the promoter/operator region of the *camDCAB* operon and the *camR* gene and 85-bp fragment of non-promoter/operator region was used as a DNA probe. The appropriate amount of cell lysate of *E. coli* overproducing CamR was incubated with 0.1 ng of the DNA probe for 20 min at 25°C. The reaction mixture was analyzed by nondenaturing PAGE on a 5% gel plate followed by autoradiography. Lane 1, DNA probe alone; lanes 2 to 6, DNA probe plus 0.1, 0.2, 0.4, 0.8, and 1.6 μg of the cell lysate, respectively.

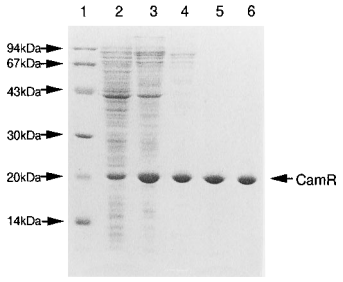

FIG. 3. SDS-PAGE analysis of preparation at each step of purification. Electrophoresis was carried out on a 13% polyacrylamide gel in the presence of 0.1% SDS with Mini-PROTEAN II (Bio-Rad). Three micrograms of the protein preparation at each step was treated with SDS and applied to the gel. Lane 1, molecular mass standards (94 kDa, phosphorylase *b*; 67 kDa, bovine serum albumin; 43 kDa, ovalbumin; 30 kDa, carbonic anhydrase; 20 kDa, soybean trypsin inhibitor; and 14 kDa, α-lactalbumin); lane 2, cell lysate; lane 3, fraction obtained by ammonium sulfate precipitation; lane 4, fraction obtained by chromatography on DEAE-trisacryl; lane 5, fraction obtained by gel filtration through an FPLC-Superdex 75pg column; lane 6, fraction obtained by second-round chromatography on DEAE-trisacryl.

isolated DNA was labeled at one end of each strand with [γ-^{32}P]ATP and then was cut with *Hin*cII to produce a 125-bp piece (*Hin*cII-*Sph*I) containing the promoter/operator sequence and an 85-bp non-promoter/operator piece (*Pst*I-*Hin*cII). The latter piece was used as an internal control. A mixture of these two DNA fragments was used as a probe in the pilot test. The mixed probes were incubated with the cell lysate of JM83/pHAOV1 cultured in the presence of D-camphor. When the amount of cell lysate protein was increased, the 125-bp DNA fragment but not the 85-bp DNA fragment quantitatively formed the DNA-protein complex (Fig. 2). When a cell lysate of JM83/pUC-P$_L$-cI, the same host harboring only the vector, was used as the control, a DNA-protein complex did not form (data not shown). These results indicate that the CamR protein specifically binds to the promoter/operator region of the *camDCAB* operon and the *camR* gene. Thus, the 125-bp DNA fragment was used as a probe for the gel mobility shift assay and the DNA binding activity of CamR was determined, as described in Materials and Methods.

Purification of CamR protein. We purified the CamR protein from the cell lysate of a CamR-overproducing strain, JM83/pHAOV1, as described in Materials and Methods. Table 1 summarizes the steps of a typical purification. The yield of CamR protein was 13 mg from 20 g (wet weight) of cells cultured in the presence of D-camphor. Figure 3 shows results of SDS-PAGE analysis of the preparations at each step of purification. The N-terminal amino acid sequence of the purified CamR protein was determined by gas phase Edman degradation through 33 steps. Each cycle, especially that with the N-terminal 10 residues, showed a clear major peak (data not shown). The estimated sequence was identical to that deduced from the nucleotide sequence of the *camR* gene (4). The amino acid composition, with the exception of cysteine and tryptophan, also showed excellent agreement with that deduced from the nucleotide sequence (4) (Table 2). These results indicate that the CamR protein synthesized in this heterologous system had a native primary structure and was not degraded by heat treatment (42°C, 2 h) or by the addition of an inducer (D-camphor).

TABLE 1. Purification of CamR

Step	Total protein (mg)	Total activity (U)	Sp act (U/mg of protein)	Purification (fold)	Yield (%)
Cell lysate	751	5.5×10^7	7.3×10^4	1.0	100
Ammonium sulfate	173	3.5×10^7	2.0×10^5	2.7	63.6
1st DEAE-trisacryl	34.0	2.1×10^7	6.0×10^5	8.3	38.2
Superdex 75pg	15.2	1.2×10^7	7.8×10^5	10.7	21.8
2nd DEAE-trisacryl	13.0	1.0×10^7	7.7×10^5	10.5	18.2

As shown in Fig. 3, the final preparation of CamR protein showed a single band with a molecular mass of 20 kDa. Analysis of purified CamR by isoelectric focusing showed a single band corresponding to a pI value of 5.8 (data not shown). These values are identical to those calculated from the nucleotide sequence of the *camR* gene (4), i.e., 20.4 kDa and a pI of 5.84, respectively. On the other hand, the purified CamR showed a single peak with a molecular mass of 40 kDa by gel filtration analysis (Fig. 4). These results suggest that the CamR protein forms a homodimer with a molecular mass of 40 kDa.

DNA binding activity of purified CamR protein. To determine to what extent the purified CamR protein retained DNA

TABLE 2. Amino acid composition of CamR expressed in *E. coli*

Amino acid	No. of residues	
	Analysis[a]	Predicted value[b]
Gly	17.0	17
Leu	22.0	23
Arg	10.4	11
Val	7.8	8
Ala	24.8	26
Ile	6.7	7
Asx	12.3	12
Glx	22.5	22
Thr	9.9	11
Pro	8.0	8
Tyr	6.6	7
Ser	4.9	5
Lys	5.7	6
Phe	5.0	5
His	6.3	7
Met	6.1	7
Cys	ND[c]	2
Trp	ND	2

[a] CamR purified from the culture with D-camphor.
[b] These values were calculated from the nucleotide sequence data (4).
[c] ND, not determined.

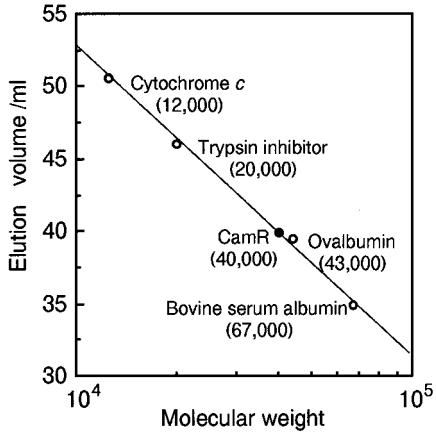

FIG. 4. Determination of molecular mass of CamR protein by gel filtration through an FPLC-HiLoad 16/60 Superdex 75pg column. A mixture of CamR and the standard proteins (500 μg each) was filtered through a column of Superdex 75pg at a flow rate of 0.5 ml/min. The filtrate was traced by a UV monitor and was also analyzed by SDS-PAGE. The solid and open circles show CamR and standard proteins, respectively.

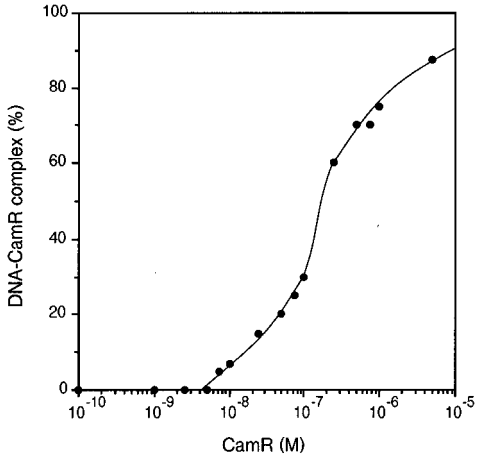

FIG. 6. K_d of CamR protein for binding to operator DNA. A labeled *Hin*cII-*Sph*I fragment (125 bp) containing the promoter/operator region of the *camD-CAB* operon and the *camR* gene was used as a DNA probe. Various amounts of the CamR protein were incubated with a 1×10^{-9} M DNA probe for 20 min at 25°C. The reaction mixture was subjected to nondenaturing PAGE on a 5% gel plate. The intensities of the bands of DNA-CamR complex were measured with a Fuji Bioimaging Analyzer BAS2000. A 100% conversion of CamR-DNA complex meant that all of the probe DNA converted to the CamR-DNA complex. In each assay, the total of the intensities of the bands of CamR-DNA complex and the remaining free probe was constant.

binding activity, a sedimentation assay was done. CamR was incubated with a 15-fold molar excess of a 3-kbp DNA fragment containing the *cam* operator, and fractions that formed complexes with DNA were separated from free species by glycerol gradient centrifugation. Quantification was performed by ELISA for CamR and by densitometry after agarose gel electrophoresis for DNA. The sedimentation profiles are shown in Fig. 5. CamR dimer repressor appeared at the top of the glycerol gradient (fractions 11 and 12) in the absence of operator DNA, and free 3-kbp-long operator DNA itself was detected in fractions 6 and 7 (Fig. 5A). A part of the protein was also detected in fraction 9, suggesting that purified CamR contained a small amount of aggregates. Next, CamR and operator DNA were allowed to equilibrate and were then centrifuged (Fig. 5B). The repressor-DNA complex was observed as a major peak at fraction 8, while CamR not in a complex remained in fraction 12. The peak of operator DNA shifted from fraction 7 to 8 upon formation of the complex. These observations indicate that the molar volume of operator DNA increased by 3%, perhaps because of a conformational change by the binding of CamR.

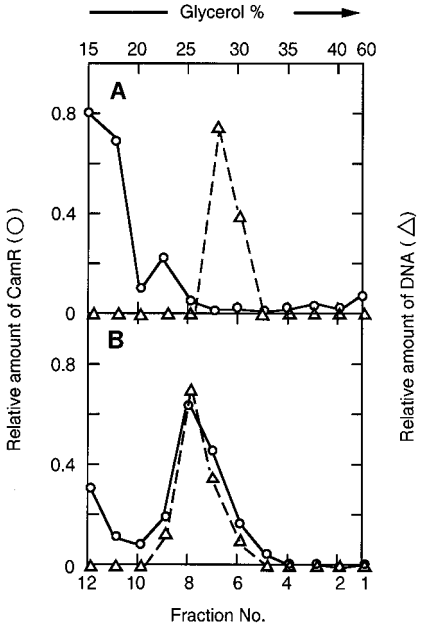

FIG. 5. Sedimentation assay for DNA binding of purified CamR repressor. CamR protein (0.065 μM) was preincubated with or without 3-kbp operator-carrying DNA (1 μM) and was then subjected to glycerol gradient centrifugation. Sedimentation separation was carried out as described in Materials and Methods. The scale of ordinates was normalized to the particular maximal values. (A) Sedimentation profiles of free CamR and operator-carrying DNA and (B) those of CamR and the DNA after the incubation. The direction of sedimentation is indicated by the arrow.

The active fraction of purified CamR can be calculated as the percentage of CamR cosedimented with the DNA. As described below, the concentration of DNA used in the sedimentation assay was well beyond the apparent dissociation constant between CamR and the operator. From peak areas of the distribution of CamR in panel B, we determined that 86% of the purified protein was in an active form.

Binding affinity to operator DNA. The apparent dissociation constant [K_d (app)] for binding of the CamR protein to the operator DNA fragment was estimated from the gel mobility shift assay and by using the following formula: for CamR + DNA → CamR-DNA, K_d (app) = [CamRfree] × [DNAfree]/[CamR-DNA complex]; where [CamRfree], [DNAfree], and [CamR-DNA complex] denote concentrations of free CamR, free DNA, and CamR-DNA complex, respectively. In the reaction mixture, the concentration of 125-bp operator DNA was $<5 \times 10^{-10}$ M and that of the CamR protein was $>5 \times 10^{-9}$ M. When the concentration of CamR protein is in excess of that of the operator DNA, [DNAfree] is considered to be equal to [CamR-DNA complex] at the half-maximal binding point. Thus, K_d (app) is equal to [CamRfree] and the value is close to the total CamR concentration. As shown in Fig. 6, the K_d (app) for binding of the CamR protein to the operator DNA was 1.5×10^{-7} M, under the conditions used in this study.

Fluorescent alteration of CamR after operator binding. CamR protein is predicted to contain two tryptophan residues

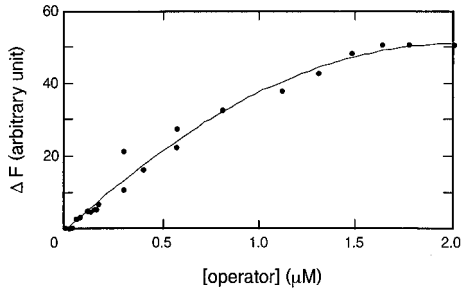

FIG. 7. Fluorometric titration of CamR repressor with *cam* operator. The fluorescent intensities at 348 nm (ΔF) were plotted as a function of the concentration of the added operator. The excitation wavelength was 287 nm. Other assay conditions are described in Materials and Methods. The curve was obtained by least-square fitting to the following equation: $\Delta F/[CamR] = [operator]/([operator] + K_d)$.

(Table 2). The fluorescent properties of tryptophan often provide clues about quantitative values for interactions between proteins and DNA. Similarly, for CamR, such fluorescence measurements of the protein facilitated direct monitoring of events of repressor-operator binding.

The emission spectrum of CamR repressor excited at 287 nm exhibited a typical tryptophan fluorescence pattern (data not shown), indicating that one or more residues of tryptophan exist in the primary sequence of CamR, as expected. The emission spectra were not affected when the excitation wavelength was 295 nm (data not shown). The spectrum of the free *cam* operator (32-bp long) was at a trace level on this scale. When the operator was added to the repressor, the emission intensity of CamR changed, reflecting the binding of CamR to the *cam* operator. Figure 7 shows data from a titration of the CamR repressor with the operator. Emissions were measured at 348 nm. The shape of the curve indicates that the binding was successively quantitative. According to the Michaelis-Menten type analysis, the apparent equilibrium dissociation constant of repressor-operator binding was 5.7×10^{-7} M.

Interestingly, after binding, a more-than-twofold increase of protein intensity, instead of the quenching observed with other proteins, was observed. The tryptophan residue(s) buried in the interior of CamR may not have been stacked with DNA but rather exposed to bulk solution by operator binding.

Binding of CamR to the operator DNA region. Using the purified CamR protein, we determined the specific DNA region bound with the CamR protein by DNase I footprinting assay. Figure 8A and B shows protection patterns of the coding strand and noncoding strand of the *camR* gene, respectively. These strands correspond to the noncoding strand and coding strand of the *camDCAB* operon, respectively. When the amount of CamR protein was 0.11 μg or more, the effect of protection was clearly observed on both strands and the protected region contained a 22-bp sequence containing 6-bp inverted repeat sequences (Fig. 8C). This finding is consistent with that of our previous DNase I footprinting assay which used crude cell lysates from CamR-overproducing *E. coli* cultured in the presence of D-camphor (3).

Next, we carried out a DNase I footprinting assay to examine the effect of D-camphor on the interaction between the CamR protein and the operator DNA. The CamR protein was preincubated for 20 min at 25°C with the DNA fragment containing the promoter/operator region of the *camDCAB* operon and the *camR* gene. After addition of D-camphor, incubation was continued for an additional 20 min before the reaction mixture was treated with DNase I. As shown in Fig. 9, the CamR

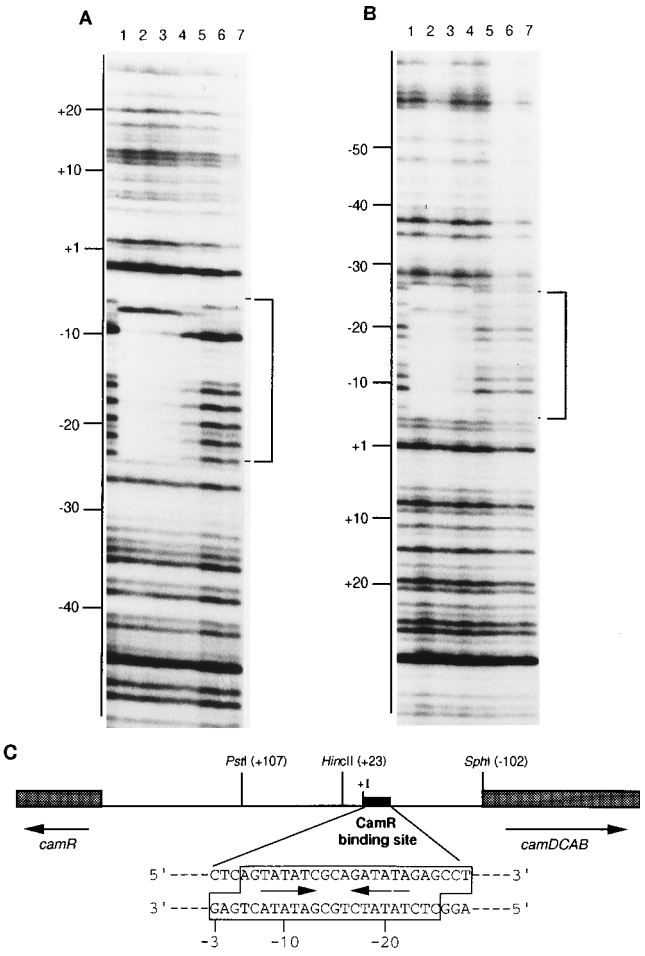

FIG. 8. Protection of operator region of *camR* gene and *camDCAB* operon from DNase I by CamR protein. The conditions for DNase I digestion are described in Materials and Methods. The protected region is indicated by a solid bracket in the right margin. Numbers in the left margin indicate the position of each base relative to the transcription start site of the *camR* gene. Lane 1, no CamR protein; lanes 2 to 6, 1.00, 0.33, 0.11, 0.037, 0.012, and 0.004 μg of CamR protein, respectively. (A) Protection pattern of *camR* coding strand (*camDCAB* operon noncoding strand) of DNA fragment labeled on the *Sph*I side. (B) Protection pattern of *camR* noncoding strand (*camDCAB* operon coding strand) of DNA fragment labeled on the *Pst*I side. (C) Nucleotide sequences of the protected regions. Bases protected from DNase I digestion on each strand are boxed. The 6-bp inverted repeat sequences are indicated by the arrows in the box.

protein did not protect the operator region from DNase I when the concentration of D-camphor was 60 μM or higher. This result means that D-camphor separated the CamR protein from the operator DNA. Our proposal that the camphor-induced expression of the *camDCAB* operon and the *camR* gene in *P. putida* is caused by a release of the CamR protein from the operator site, as a result of direct interference by D-camphor on the binding of the CamR protein, is given support (3).

Effects of camphor-related compounds on CamR-DNA complex. To study the interaction between CamR protein and the inducer, we asked whether binding of the CamR protein to the operator DNA would be inhibited in the presence of products of D-camphor degradation and camphor analogs. As shown in Fig. 10, among the D-camphor degradation products, 5-*exo*-hydroxycamphor (lane 4) and 2,5-diketocamphane (lane 5) inhibited the binding of CamR to the same extent as seen with D-camphor (lane 3) but 3,4,4-trimethyl-5-carboxylmethyl-2-

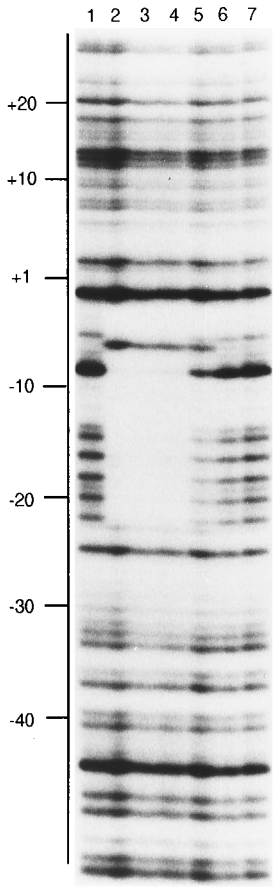

FIG. 9. Effects of camphor on the interaction between CamR protein and operator DNA. The probe used was the same as that used in the experiment for Fig. 8A. The assay conditions are described in Materials and Methods. Lane 1, no CamR protein; lanes 2 to 7, 0.5 μg of CamR protein plus 0, 0.004, 0.015, 0.06, 0.25, and 1 mM D-camphor, respectively. Numbers in the left margin indicate the position of each base relative to the *camR* transcription start site.

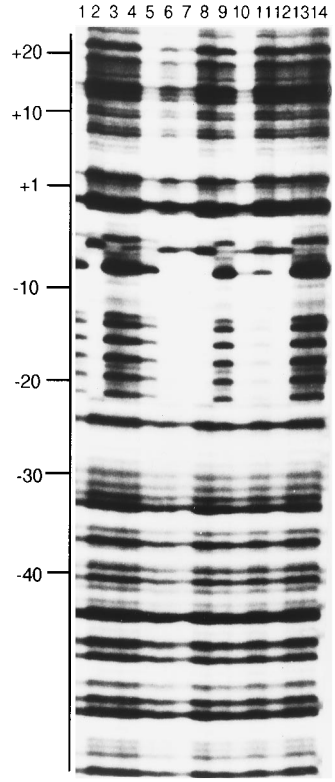

FIG. 10. Effects of camphor-related compounds on binding of CamR protein to operator DNA. The assay conditions are described in Materials and Methods. Lane 1, no CamR protein or compound; lane 2, 0.5 μg of CamR protein and no compound; lanes 3 to 14, 0.5 μg of CamR protein with 1 mM concentrations of each compound: D-camphor (lane 3), 5-*exo*-hydroxycamphor (lane 4), 2,5-diketocamphane (lane 5), 3,4,4-trimethyl-5-carboxymethyl-2-cyclopentenone (lane 6), acetic acid (lane 7), D-camphor-10-sulfonic acid (lane 8), D-3-bromocamphor (lane 9), norcamphor (lane 10), norbornane (lane 11), norbornadiene (lane 12), adamantane (lane 13), and 2-adamantanone (lane 14).

pentane (lane 6) and acetic acid (lane 7) did not. Camphor analogs D-3-bromocamphor (lane 9), norcamphor (lane 10), norbornane (lane 11), adamantane (lane 13), and 2-adamantanone (lane 14) inhibited the binding of CamR protein, but D-camphor-10-sulfonic acid (lane 8) and norbornadiene (lane 12) did not.

To determine whether these compounds which inhibited the binding of CamR protein would function as inducers of the *camDCAB* operon in vivo, expression of the operon was monitored via the enzyme activity of FdeH, the product of *camD*. As shown in Table 3, FdeH was significantly induced by the addition of D-camphor, 5-*exo*-hydroxycamphor, 2,5-diketocamphane, D-3-bromocamphor, norcamphor, adamantane, and 2-adamantanone. Thus, all compounds but norbornane that inhibited the binding of CamR protein to the operator DNA in vitro induced the activity of FdeH in vivo. The exception of norbornane may be attributable to (i) its high dissociation constant or (ii) its reduced permeability across cell membranes.

DISCUSSION

The regulatory gene *camR* autoregulates its expression (3), with the translational initiation codon of CamR at GTG (4). Thus, the number of CamR protein molecules in *P. putida* cells is likely to be small. To purify the CamR protein, a plasmid for the overproduction of CamR was constructed by placing the *camR* gene under the control of the PL promoter. The translational initiation codon of CamR was changed by site-directed

TABLE 3. Effects of D-camphor-related compounds on the expression of *camD* gene from recombinant plasmid pJP39 in *P. putida* PpG277

Compound	Sp act of FdeH (U/mg)	Ratio of +/− values[a]
None	0.10	
D-Camphor	2.26	22.6
5-*exo*-Hydroxycamphor	0.88	8.80
2,5-Diketocamphane	0.74	7.40
3,4,4-Trimethyl-5-carboxymethyl-2-cyclopentenone	0.08	0.80
Acetic acid	0.11	1.10
D-Camphor-10-sulfonic acid	0.11	1.10
D-3-Bromocamphor	0.52	5.20
Norcamphor	0.46	4.60
Norbornane	0.09	0.90
Norbornadiene	0.08	0.80
Adamantane	1.06	10.6
2-Adamantanone	2.62	26.2

[a] +, with compound; −, without compound.

mutagenesis from GTG to ATG to improve translational efficiency (3). Thus, we purified the CamR protein from transformed *E. coli*. As shown in Table 1 and Fig. 3, we purified the CamR protein to homogeneity using four steps: ammonium sulfate fractionation, DEAE-trisacryl chromatography, gel filtration through a Superdex 75pg column, and a second round of DEAE-trisacryl chromatography. The yield of the CamR protein was high, about 13 mg from a 4-liter culture. When the cells were cultured in the presence of D-camphor, most of the CamR protein was recovered in the soluble fraction and it was present in about 10% of the total soluble proteins (Fig. 3, lane 1). In contrast, when cells were cultured in the absence of D-camphor, most of the CamR protein was recovered in the insoluble fraction. We also purified CamR protein from the soluble fraction of cells cultured in the absence of D-camphor. The yield was about 0.47 mg from a 4-liter culture (data not shown). Judging from the gel mobility shift assay, the DNA binding activities of the two CamR proteins purified from cells cultured in the presence or absence of D-camphor were equal (data not shown). These results suggest that D-camphor contributes to proper folding of the CamR protein but could easily be separated from the repressor by gel filtration, since D-camphor inhibits binding of the repressor to the operator DNA (as described below).

Judging from SDS-PAGE analysis (Fig. 3), the elution profile of gel filtration (Fig. 4), and N-terminal amino acid sequence analysis, the CamR protein synthesized in this heterologous system seems to be a homodimer form consisting of two identical subunits, each with a molecular mass of 20 kDa. In the DNase I footprinting assay, the CamR protein protected only a single region between the *camDCAB* operon and the *camR* gene (Fig. 8). This region contains 6-bp (GATATA) inverted repeat sequences. These results suggest that the active binding form of CamR is a dimer of an identical polypeptide and both subunits of the repressor bind simultaneously to the operator, as in the case of LacI and TrpR repressors (15, 22, 26). The binding of the CamR protein to the DNA fragment containing the operator region showed dissociation constants of 1.5×10^{-7} M in the gel mobility shift assay (Fig. 6) and 5.7×10^{-7} M in the fluorometric assay (Fig. 7), respectively, under the conditions used. In sedimentation assays, we determined that 86% of the purified CamR protein was capable of binding DNA (Fig. 5). Thus, the dissociation constant of CamR is probably 10^{-7} M and is larger than those of the *lac* repressor and *trp* repressor (8, 11).

We observed that several camphor degradation products and camphor analogs inhibited the binding of CamR to the operator DNA in vitro and that these compounds induced FdeH (that is, expression of the *camDCAB* operon) in vivo. The magnitude of the inhibition of the CamR binding and that of the induction of FdeH were parallel among most of the compounds examined (Fig. 10; Table 3). These results strongly suggest that a conformational change on the CamR protein was caused by binding of the inducer and that this change led to a release of the repressor from the operator DNA. It is noteworthy that norcamphor, adamantane, and 2-adamantanone are included in the above-mentioned "effective" compounds. These analogs are substrates of cytochrome P-450cam (5, 27). The binding site(s) to the inducer of the CamR protein is presumed to have a structure analogous to that of the substrate-binding site of cytochrome P-450cam.

On the other hand, a putative helix-turn-helix region of CamR showed some homology to Bm3R1, the gene repressor protein of *Bacillus megaterium*, which controls both expression of its own gene (*bm3R1*) and expression of the cytochrome P-450_{BM} gene in this bacterium at the transcriptional level; and binding of Bm3R1 to the operator site on the cytochrome P-450_{BM} gene is inhibited by barbiturate (24, 25). Since the mechanism of regulation of the cytochrome P-450cam operon and the *camR* gene resembles that of the cytochrome P-450_{BM} gene and the *bm3R1* gene, CamR and Bm3R1 may be derived from a common ancestor protein that resembled TetR (12a). To clarify the ternary structure of the CamR repressor, crystallization of the CamR protein and analysis of its binding site(s) to the inducer and/or to the operator DNA are under way.

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REFERENCES

1. **Aiba, H.** 1985. Transcription of the *Escherichia coli* adenylate cyclase gene is negatively regulated by cAMP-cAMP receptor protein. J. Biol. Chem. **260:** 3063–3070.
2. **Aramaki, H., M. Fujita, Y. Sagara, A. Amemura, and T. Horiuchi.** 1994. Heterologous expression of *camR* and *camDCAB* of *Pseudomonas putida* in *Escherichia coli*. FEMS Microbiol. Lett. **123:**49–54.
3. **Aramaki, H., Y. Sagara, M. Hosoi, and T. Horiuchi.** 1993. Evidence for autoregulation of *camR*, which encodes a repressor for the cytochrome P-450cam hydroxylase operon on the *Pseudomonas putida* CAM plasmid. J. Bacteriol. **175:**7828–7833.
4. **Aramaki, H., Y. Sagara, K. Takeuchi, H. Koga, and T. Horiuchi.** 1994. Nucleotide sequence of the gene encoding a repressor for the cytochrome P-450cam hydroxylase operon on the *Pseudomonas putida* CAM plasmid. Biochimie **76:**63–70.
5. **Atkin, W. M., and S. G. Sligar.** 1987. Metabolic switching in cytochrome P-450cam: deuterium isotope effects on regiospecificity and the monooxygenase/oxidase ratio. J. Am. Chem. Soc. **109:**3754–3760.
6. **Bagdasarian, M. M., E. Aman, R. Lurz, B. Ruckert, and M. Bagdasarian.** 1983. Activity of the hybrid trp-lac(tac) promoter of *Escherichia coli* in *Pseudomonas putida*. Construction of broad-host-range, controlled-expression vectors. Gene **26:**273–282.
7. **Bradshaw, W. H., H. E. Conrad, E. J. Corey, I. C. Gunsalus, and D. Lednicer.** 1959. Microbiological degradation of (+)-camphor. J. Am. Chem. Soc. **81:**5507.
8. **Carey, J.** 1988. Gel retardation at low pH resolves *trp* repressor-DNA complexes for quantitative study. Proc. Natl. Acad. Sci. USA **85:**975–979.
9. **Coligan, J. E., A. M. Kruisbeek, D. H. Marglies, E. M. Shevach, and W. Strober.** 1992. Current protocol in immunology, p. 2.0.3–2.8.10. Wiley, New York.
10. **Conrad, H. E., R. DuBus, and I. C. Gunsalus.** 1961. An enzyme system for cyclic ketone lactonization. Biochem. Biophys. Res. Commun. **6:**293–297.
11. **Fried, M., and D. M. Crothers.** 1981. Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis. Nucleic Acids Res. **9:**6505–6525.
12. **Fujita, M., H. Aramaki, T. Horiuchi, and A. Amemura.** 1993. Transcription of the *cam* operon and *camR* genes in *Pseudomonas putida* PpG1. J. Bacteriol. **175:**6953–6958.

12a. **Fulco, A.** Personal communication.

13. **Haran, T. E., A. Joachimiak, and P. B. Sigler.** 1992. The DNA target of the *trp* repressor. EMBO J. **11:**3021–3030.
14. **Hartline, R. A., and I. C. Gunsalus.** 1971. Induction specificity and catabolite repression of the early enzymes in camphor degradation by *Pseudomonas putida*. J. Bacteriol. **106:**468–478.
15. **Hecht, M., H. Nelson, and R. Sauer.** 1983. Mutations in λ repressor's amino-terminal domain: implications for protein stability and DNA binding. Proc. Natl. Acad. Sci. USA **80:**2676–2680.
16. **Hermann, M., G. K. Garg, and I. C. Gunsalus.** 1979. Fertility factors in *Pseudomonas putida*: selection and properties of high-frequency transfer and chromosomal donors. J. Bacteriol. **137:**28–34.
17. **Koga, H., H. Aramaki, E. Yamaguchi, K. Takeuchi, T. Horiuchi, and I. C. Gunsalus.** 1986. *camR*, a negative regulator locus of the cytochrome P-450_{cam} hydroxylase operon. J. Bacteriol. **166:**1089–1095.
18. **Koga, H., E. Yamaguchi, K. Matsunaga, H. Aramaki, and T. Horiuchi.** 1989. Cloning and nucleotide sequences of NADH-putidaredoxin reductase gene (*camA*) and putidaredoxin gene (*camB*) involved in cytochrome P-450cam hydroxylase of *Pseudomonas putida*. J. Biochem. **106:**831–836.
19. **Kuroki, M., M. Murakami, M. Wakisaka, S. Ikeda, S. Oikawa, T. Oshima,**

H. Nakazato, G. Kosaki, and Y. Matsuoka. 1992. Immunoreactivity of recombinant carcinoembryonic antigen proteins expressed in *Escherichia coli*. Immunol. Invest. **21:**241–257.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
21. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101:**20–78.
22. **Pabo, C., and M. Lewis.** 1982. The operator-binding domain of λ repressor: structure and DNA recognition. Nature (London) **298:**443–447.
23. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Shaw, G. C., and A. J. Fulco.** 1992. Barbiturate-mediated regulation of expression of the cytochrome $P450_{BM-3}$ gene of *Bacillus megaterium* by Bm3R1 protein. J. Biol. Chem. **267:**5515–5526.
25. **Shaw, G. C., and A. J. Fulco.** 1993. Inhibition by barbiturate of the binding repressor to its operator site on the barbiturate-inducible cytochrome $P450_{BM-3}$ gene of *Bacillus megaterium*. J. Biol. Chem. **268:**2997–3004.
26. **Steitz, T. A., D. H. Ohlendorf, D. B. Mckay, W. F. Anderson, and B. W. Mattews.** 1982. Structural similarity in the DNA-binding domains of catabolite gene activator and *cro* repressor proteins. Proc. Natl. Acad. Sci. USA **79:**3097–3100.
27. **White, R. E., M.-B. McCarthy, K. D. Egeberg, and S. G. Sligar.** 1984. Regioselectivity in the cytochromes P-450: control by protein constraints and by chemical reactivities. Arch. Biochem. Biophys. **228:**493–502.
28. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.