JOURNAL OF BACTERIOLOGY, June 1986, p. 1089–1095
0021-9193/86/061089-07$02.00/0

Vol. 166, No. 3

camR, A Negative Regulator Locus of the Cytochrome P-450$_{cam}$ Hydroxylase Operon

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Received 27 December 1985/Accepted 12 March 1986

A 4.27-kilobase insert from a *Hin*dIII DNA library of *Pseudomonas putida* carrying the CAM plasmid allowed coordinate expression of genes *camD* and *camC* under control of *camR*, an upstream regulator. The *camC* gene specifies cytochrome P-450$_{cam}$, and *camD* specifies the 5-*exo*-alcohol dehydrogenase. A 1.38-kilobase deletion from the insert results in the constitutive expression of genes *camC* and *camD*; transformation in *trans* restores the substrate control, indicating that *camR* is a negative regulator.

The cloning of the gene *camC* encoding the heme sulfur protein P-450$_{cam}$ by complementation in *trans* (25) has opened to analysis the genetic organization and control of a multicomponent monoxygenase (35), the topic of this paper. The strain used, *Pseudomonas putida* PpG1, was originally isolated by enrichment culture on D-camphor as the carbon source (2). The reaction pathway was established (2, 7, 20), beginning with hydrocarbon oxygenation and proceeding through ring fission (Fig. 1), with the enzymes identified in cell extracts (7, 8, 21, 24). These studies provided entry to the enzymology, selectivity, and mechanism of alkane oxygenation; the hydroxylase, P-450$_{cam}$, became a primary prototype for P-450 monoxygenases (9, 17, 24). Genetic studies led to the discovery of chromosome mobilization (5, 6) and metabolic plasmids carrying self-fertility (34) and gave insight into genetic exchange among procaryote phenotypes (6, 34, 43). Phage isolation followed by transduction revealed gene clusters (16, 34), and transformation with cloned constructs demonstrated their operon control (12, 25, 43).

Observation of an unusual optical absorption band at 450 nm in the reduced CO complexes of hepatic tissue in the early 1960s led to an association of the "pigment 450" spectrum with heme, *b*-type cytochrome, and the multicomponent monoxygenase reactions (32). Their importance, multiple nature, and distribution have resulted in numerous studies (1, 3, 11, 28) until they are now well recognized and documented (18, 35, 41, 42).

In this paper we report the cloning of three genes, *camR*, *camD*, and *camC*, from the plasmid CAM and their restriction digest maps, location, and regulation. The *camD* promoter and nucleotide sequence for the N terminus is reported. The *camC* gene encodes the protein P-450$_{cam}$; *camD* encodes 5-*exo*-alcohol dehydrogenase (FdeH), which converts the hydroxyl of camphor 5-*exo*-alcohol to a ketone group. The negative regulator, *camR*, precedes the structural genes and responds to camphor as an inducer with coordinate expression of the FdeH and P-450 proteins. The finding of a negative regulator for CAM plasmid genes contrasts with the positive control of the TOL (13, 22) and NAH (43) metabolic plasmid systems.

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MATERIALS AND METHODS

Bacterial strains and plasmids. The *P. putida* and *Escherichia coli* strains and the vectors are listed in Table 1. The shuttle vectors pTS1036 (13.9 kilobases, Sm^r Su^r Km^r Ap^r) and pTS1210 (11.1 kilobases, Km^r Ap^r) are useful for cloning in gram-negative bacteria. pTS1036 carries double replicons, R1b679Tn*1* (Sm^r Su^r Ap^r) and pACYC177 (Ap^r Km^r), and pTS1210 carries pSa (Cm^r Sm^r Su^r Km^r) and pBR322 (Ap^r Tc^r) (T. Nakazawa, personal communication). The multicopy plasmid R1b679, of incompatibility group Inc P-4 (Q) (36), appears from the restriction map to be identical to RSF1010; pSa, of incompatibility group W, maintains a level of two to three copies per cell (37).

Media and antibiotics. L broth (1% polypeptone, 0.5% yeast extract, 0.5% NaCl, 0.1% glucose, pH 7.2) was used for liquid cultures; phosphate ammonium salts (PAS) served as minimal medium (7) for selection and growth of cells. The carbon source, D-camphor, was added as 2 M solution in dimethylformamide to a final concentration of 5 mM in liquid media; for agar plates 0.05 ml was added in the lid of the petri dish immediately before use. Cells for transformation were grown in LBS, which contains 0.5 M sucrose instead of glucose in LB. PY medium (1% polypeptone, 0.5% yeast extract, 0.5% NaCl, pH 7.2) was used to cultivate cells for plasmid DNA isolation. Antibiotics were added to agar plates at the following concentrations: streptomycin, 600 μg/ml; kanamycin, 40 μg/ml; and ampicillin, 1 mg/ml.

Chemicals and enzymes. Streptomycin and ampicillin were from the Meiji Co., Ltd., Tokyo, and kanamycin was from Sigma Chemical Co., St. Louis, Mo. *E. coli* C57 alkaline phosphatase and restriction enzymes were from Takara Shuzo Co., Ltd., Kyoto, and T4 DNA ligase was from New England BioLabs, Inc., Beverly, Mass.

***P. putida* PpG1 gene bank and clone preparation.** *Hin*dIII digests of vector pTS1036 and of PpG1 total cell DNA, prepared by the method of Miura (29), were jointed with T4 ligase and transformed into host cell MT301. Samples of transformed cells were plated on L agar containing 600 μg of streptomycin per ml. The remaining culture, amplified in 400 ml of PY medium with a similar streptomycin level, was used to isolate DNA. In a typical experiment, vector pTS1036 (18.4 μg) and cell DNA (7 μg) were each digested with 32 U

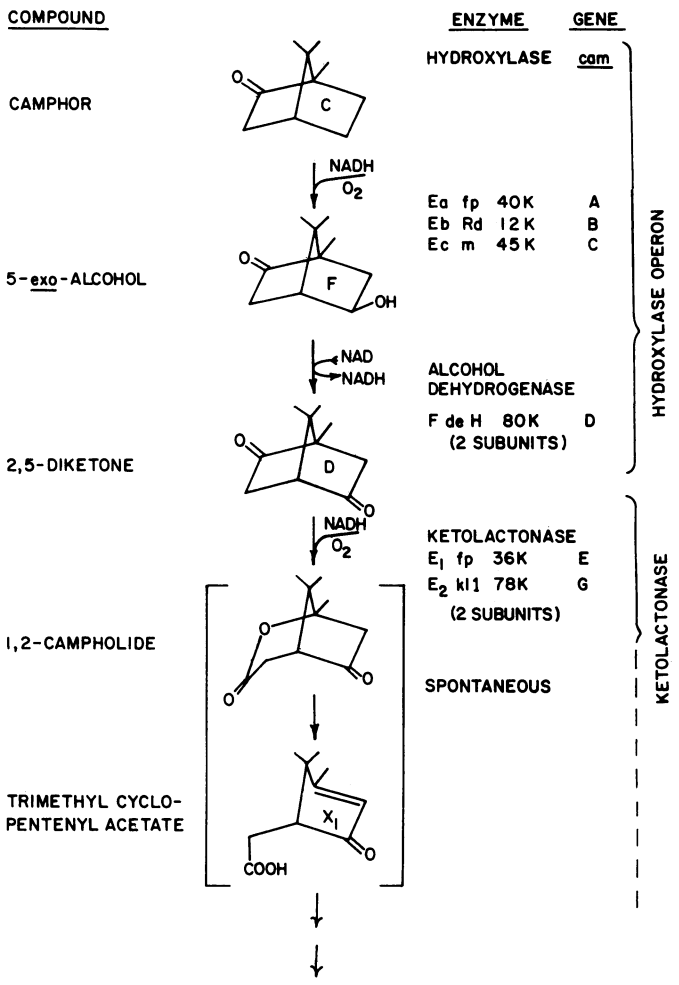

FIG. 1. D-Camphor oxidation pathway of *P. putida* PpG1. Enzymes: Ea fp, flavoprotein, NADH-redoxin oxidoreductase; Eb Rd, redoxin with ($Fe_2S_2Cys_4$) iron sulfur center; Ec m, monoxygenase P-450$_{cam}$. E_1 fp, NAD-FMN ketolactonase-1 reductase (39); E_2 kl1, D-camphor or 2,5-diketocamphane ketolactonase (8, 38).

of *Hin*dIII at 37°C in 10 mM Tris chloride–10 mM $MgSO_4$–50 mM NaCl (pH 7.4). The cell DNA was heated to 65°C for 20 min and used directly. The vector DNA was brought to 50 mM Tris by adding 1 M Tris chloride (pH 9.0), treated with 1 U of alkaline phosphatase, and then heated to 65°C for 2 h; the DNA was then phenol extracted and ethanol precipitated.

Transformation. For *P. putida*, a modified Molholt and Doskocil (30) procedure was used. Bacteria grown overnight at 30°C in L broth were inoculated at 1% into 40 ml of LBS and incubated until the cell density reached an optical density of about 0.6 at 600 nm. The cells were collected by centrifugation at 2°C and washed sequentially with 0.5 volume of cold transformation reagents I (10 mM NaCl, 0.5 mM sucrose) and II (10 mM Tris chloride [pH 7.6], 100 mM $CaCl_2$, 0.5 M sucrose, 1% polyethylene glycol 1000). The cells were incubated for 20 min in reagent II before sedimentation, and the pellet was suspended in 0.05 volume of transformation reagent III (10 mM Tris chloride [pH 7.6], 100 mM $CaCl_2$, 1% polyethylene glycol 1000). A 200-μl sample was mixed with 0.1 ml (about 0.1 μg) of transforming DNA and incubated in ice water for 1 h. The mixture was warmed to 42°C, held for 2 min, chilled, and added to 2.7 ml of L broth, and the cells were allowed to grow for 3 h at 28°C on a shaker. Samples were plated for appropriate selection.

Isolation and manipulation of plasmid DNA. The DNA was purified by a Triton X-100 cleared lysate procedure modified slightly from that of Kupersztoch-Portnow et al. (26). The DNA was digested as recommended by the suppliers, the enzymes were heat inactivated, and ligation was accomplished as indicated above.

Enzyme assays and spectral estimation of P-450$_{cam}$. Overnight cultures at 30°C in PAS medium with 20 mM monosodium glutamate and the appropriate antibiotic concentration were diluted 100-fold in the same medium and allowed to grow to an optical density of about 0.25 at 600 nm. A 40-ml sample was transferred to a Nalgene tube, harvested by centrifugation, and washed twice with 10 ml of T-O buffer (50 mM Tris chloride, 10 mM 2-mercaptoethanol, 5% glycerol, pH 7.5). The remaining culture was divided into two equal portions. Dimethylformamide was added to 1/400 volume for a noninduced sample, whereas 2 M camphor was added in the same ratio and solvent for the induced culture. The incubation was continued; 40-ml samples were removed at intervals, and the cells were pelleted, suspended in about 0.6 ml of T-O buffer, and broken by sonication. Extracts were prepared by centrifugation in a Beckman 50 Ti rotor for 40 min at 45,000 rpm and 4°C; this is sufficient to remove the ribosomes and minimize the NADH oxidation rate. The cell extracts were assayed for each enzyme by measuring the camphor-dependent rate of NADH oxidation at 340 nm in the presence of an excess of the other two hydroxylase proteins (21, 24). FdeH was measured at 340 nm by the F-alcohol-dependent reduction of NAD by the method of Hartline and Gunsalus (20) (Fig. 1). An enzyme unit is the amount required to oxidize 1 nmol of NADH or to reduce 1 nmol of NAD per s. In a 1.2-ml volume, 1-cm light path, the unit is 3.2 × the change in absorbance at 340 nm per min based on the 6.22 ε_{mM} extinction of NADH at 340 nm.

Ferrous-CO spectral estimation of P-450$_{cam}$. Cells were

TABLE 1. Bacteria and plasmids

Strain or plasmid	Phenotype or genotype	Reference or source
P. putida		
PpG1	Wild type/CAM	34
JPS11	CAM *camA514*	–[a]
PpG545	CAM *camB102*	34
PpG543	CAM *camC100*	34
PpG553	CAM *camD120*	34
MT301	*trpB615 recA801 fpa-514*[b]/ΔCAM	–[c]
MTC3011	Like MT301, CAM *camC100*	–[d]
PpG1343	Met$^-$, *met-616*/ΔCAM	25
JPS3	Met$^+$, revertant of PpG1343	25
E. coli	F$^-$ *hsdR514 supE44 supF58 ΔlacIZY6 galK2 galT22 metB1 trpR55*	31
Plasmids[e]		
pTS1036	Sm^r Km^r Ap^r Su^r	T. Nakazawa
pTS1210	Ap^r Km^r	T. Nakazawa
pBR322	Ap^r Tc^r	

[a] Isolated after ethylmethanesulfonate treatment of PpG1.
[b] *fpa* is fluorophenylalanine resistance (to 1 mg/ml), a spontaneous mutation.
[c] Isolated from PpG1400(*trpB615 rec-604*/ΔCAM) (43) by J. M. Ward; supplied by T. Nakazawa.
[d] Conjugal cross between donor strain PpG543 and recipient strain MT301.
[e] Antibiotic resistance: Sm, streptomycin; Km, kanamycin; Ap, ampicillin; Su, sulfonamide; Tc, tetracycline.

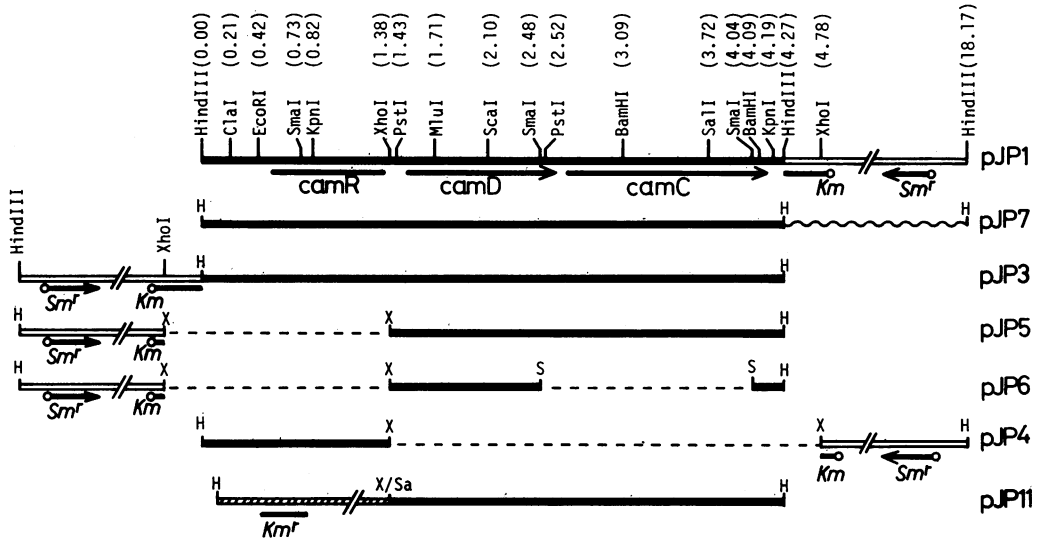

FIG. 2. Restriction nuclease map of plasmid pJP1 and the CAM insert. The gene *camC* encodes P-450$_{cam}$, *camD* encodes FdeH, and *camR* encodes regulator. Abbreviations: Sm, streptomycin; Km, kanamycin; Ap, ampicilin; Su, sulfonamide. Solid lines are CAM inserts. Vector segments (cut short): (▭), from pST1036 (Ap^r and Su^r loci deleted); (▨) from pTS1210; (∼∼∼), from pBR322. Restriction sites are given as kilobase distances from the left *Hin*dIII site at 0. Enzymes: H, *Hin*dIII; S, *Sma*I; Sa, *Sal*I; X, *Xho*I.

harvested from PAS. A 1.3-liter volume was inoculated with 1/100 volume of an overnight culture and incubated at 30°C until an optical density of about 0.25 was reached. The cells from 200 ml were collected by centrifugation and washed twice with 20-ml portions of T-O buffer. The remaining culture was divided into 400- and 600-ml portions; 1 ml of dimethylformamide was added to the former for noninduced cells, and 1.5 ml of the solvent containing 2 M camphor was added to the latter for induced cells. After continued incubation at 30°C, 200-ml induced samples were taken at 30, 60, and 90 min, and 200-ml noninduced samples were taken at 60 and 90 min. Cell pellets prepared as described above were suspended in about 1 ml of T-O buffer and disrupted by sonication. Extracts were prepared by centrifugation at 18,000 rpm for 10 min in a Hitachi RPR-20 rotor. The protein concentrations were adjusted to 4 mg/ml, and CO difference spectra were recorded (17). The P-450$_{cam}$ levels were estimated from the absorption difference at 446 and 490 nm, using an ϵ_{mM} of 93. Protein concentrations were estimated by the method of Lowry et al. (27) with a bovine serum albumin standard.

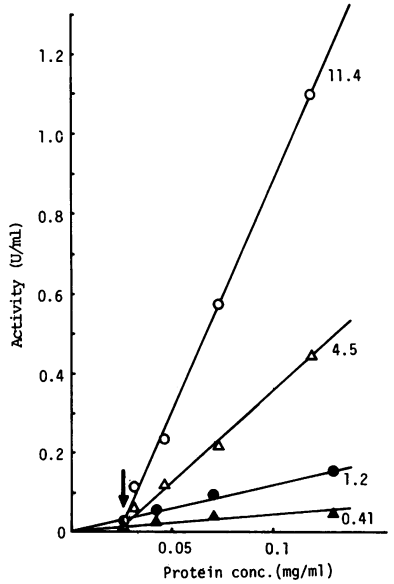

FIG. 3. Expression of the *cam* gene by strain JPS3(pJP1). Symbols: (○, ●), FdeH; (△, ▲), P-450$_{cam}$; (○, △), with camphor; (●, ▲), without camphor. PAS medium contains 20 mM monosodium glutamate; camphor was added where indicated by the arrow. The numbers indicate enzyme synthesis rates in units per milligram of cell protein (14, 20).

RESULTS

Recombinant plasmid isolation by mutant complementation. Due to the fragility to isolation of the large, 240-kilobase CAM plasmid (4, 10, 15, 19, 20, 23), an *Hin*dIII digest library was prepared from Cam$^+$ PpG1 total cell DNA. Transformation frequencies in *P. putida* were improved by replacing strain PpG277 with MT301.

The vector pTS1036 carrying Sm^r Km^r genes was cleaved with *Hin*dIII, treated with bacterial alkaline phosphatase, and ligated with the *Hin*dIII-digested PpG1 (CAM) DNA. The ligated mixture was transformed into MT301 cells, a portion of the culture was plated on streptomycin (600 μg/ml) agar, and the transformants were tested for insertional inactivation of the Km^r gene at the *Hin*dIII site—98 of the 100 colonies tested were Km^s. The remaining culture was used as inoculum for 400 ml of PY medium containing 600 μg of streptomycin per ml to prevent growth of nontransformed cells. The plasmid DNA was extracted and used to transform strain MTC3011 cells, which carry the *camC100* mutant locus on the CAM plasmid. Putative complementation was scored by plating the transformed cells on PAS agar with 600 μg of streptomycin per ml and camphor as the carbon source.

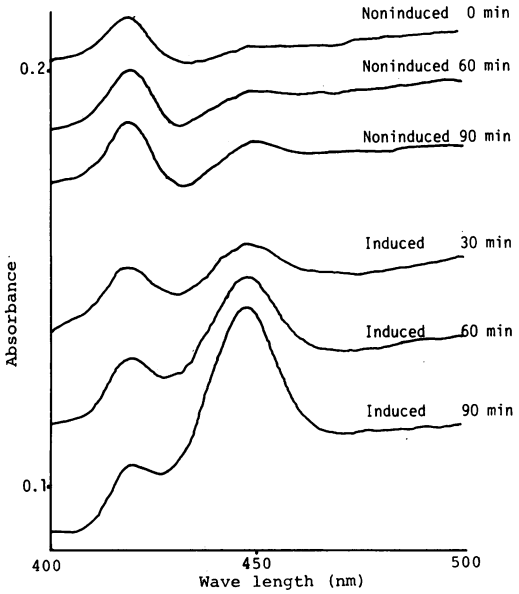

FIG. 4. P-450$_{cam}$ expression by strain JPS3(pJP1). The CO difference spectra are from cell extracts (17).

During 5 days of incubation at 30°C, 21 Cam$^+$ Sm^r colonies appeared. Plasmid DNA prepared from eight of the clones was found on *Hin*dIII digestion to provide two fragments, one the size of vector pTS1036, 13.9 kilobases, and the other a 4.27-kilobase insert (Fig. 2). One recombinant was selected and designated pJP1. The remaining transformants were not characterized.

***cam* gene expression by plasmid pJP1.** We have previously cloned the *camC* locus on a 2.3-kilobase *Pst*I insert (25); thus, a 4.27-kilobase *Hin*dIII insert suggested the possibility of additional *cam* genes. To examine this possibility, plasmid pJP1 was used to transform cells defective in the *camC*-linked genes *camA*, *camB*, and *camD*; complementation was found for the *camD* gene product, FdeH.

The synthesis of the *camD* and *camC* gene products, FdeH and P-450$_{cam}$, occurred on introduction of the plasmid pJP1 onto the CAM-free host JPS3 (Fig. 3). These JPS3(pJP1) cells were grown in the PAS-monosodium glutamate broth with and without camphor induction. Samples taken at 0, 15, 30, 60, and 90 min showed linear rates of induction with time, about 10-fold the noninduced rate (Fig. 4). The P-450$_{cam}$-induced levels were about 10^4 molecules per cell versus less than 10^3 per cell in the noninduced cells. Thus the insert carries, in addition to the structural genes, a regulator, which we tentatively term the *camR* locus.

***cam* gene locations in plasmid pJP1.** A restriction nuclease map of pJP1 was facilitated by subcloning the 4.27-kilobase *Hin*dIII insert into the vector pBR322 to transform *E. coli* strain LE392 (31). This plasmid, pJP7, isolated from an Ap^r Tc^s transformant, was digested with *Hin*dIII and shown to contain both pBR322 (4.32 kilobases) and the 4.27-kilobase CAM insert. The physical map, constructed by single and double digestions, is shown in Fig. 2. The CAM insert lacks restriction sites for *Aat*II, *Apa*I, *Bgl*II, *Dra*I, *Hpa*I, *Nco*I, *Nru*I, *Pvu*I, *Pvu*II, *Sac*II, and *Xba*I.

To locate the *cam* genes in pJP1, we prepared deletion and insertion derivatives. Complete digestion with *Hin*dIII and religation formed plasmid pJP3 with the CAM insert in the opposite orientation; both the *camC* and *camD* expressions were equivalent to those in the *P. putida* strain (Table 2).

On *Xho*I digestion and religation, pJP3 yielded pJP5 (Fig. 2), which lacks 1.38 kilobases of the 4.27-kilobase insert and 0.56 kilobase of the vector between the *Xho*I and *Hin*dIII sites. This structure was confirmed by the absence of the 1.94-kilobase *Xho*I fragment and the loss of the *Eco*RI and *Kpn*I sites. Cells transformed with pJP5 expressed the *camD* and *camC* genes constitutively.

To determine more precisely the location of the structural genes *camC* and *camD*, DNA from pJP5 digested with *Sma*I was religated and transformed into *camC100* mutant MTC3011 with Sm^r selection. The resultant plasmid, pJP6, was Sm^r *camC*$^-$ *camD*$^-$ (Table 2). The *camC* gene, known to lie between the 2.52- and 4.27-kilobase *Pst*I and *Hin*dIII sites, leaves about 1.3 kilobases upstream, near the 1.38-kilobase *Xho*I site, as a potential locus of *camD*. It may extend beyond 2.52 kilobases, because the *camC* start codon is 156 base pairs to the right (Fig. 2). Thus the *camD* location indicates two identical subunits, since they would contain about 365 amino acids and require coding by about 1,100 base pairs (Fig. 1).

Negative regulation of the *camD* and *camC* structural genes. Plasmid pJP5 expressed the *camD* and *camC* genes constitutively. To test the in *trans* effect of the 0- to 1.38-kilobase region of the *Hin*dIII insert on the expression of the 1.38- to 4.27-kilobase segment, they were separated and placed in compatible vectors. Plasmid pJP11 was constructed by insertion of the *Xho*I-*Hin*dIII fragment 1.38 and 4.27 kilobases from pJP5 between the *Sal*I and *Hin*dIII sites of vector pTS1210, which carries the replicon of plasmid pSa. The 0- to 1.38-kilobase region was subcloned from pJP3 DNA by digestion with *Xho*I and then religated and transformed into MTC3011 cells carrying the *camC100* mutation. A clone selected for the Sm^r phenotype was Cam$^-$ and shown to contain the CAM 0- to 1.38-kilobase insert. We prepared a recombinant host carrying the CAM plasmids pJP11 and pJP4 in *trans*. Gene expression (Table 2) showed camphor control of the structural genes to the level of plasmid pJP1. Thus the 0- to 1.38-kilobase portion of the CAM insert encodes a negative regulator, *camR*.

An operator-promoter region is implicated in the 1.38- to 4.27-kilobase segment of the pJP11 CAM insert. The DNA sequence is shown in Fig. 5, beginning with the *Xho*I site corresponding to the 1.38-kilobase site. The start codon for the *camD* gene begins with base pair 237 and is preceded by −35 consensus sequences, −10 transcriptional initiation sequences, and a translational start ribosome-binding site sequence of *E. coli*. The codes for the N-terminal amino acid residue of the gene product FdeH conform with the first 45

TABLE 2. *camC* and *camD* gene expression from recombinant plasmids[a]

Plasmid	Enzyme formation (U/mg of protein)			
	P-450$_{cam}$[b]		FdeH	
	Induced	Noninduced	Induced	Noninduced
pJP1	4.5	0.38	11.4	1.1
pJP3	3.9	0.27	12.5	0.77
pJP4	<0.01	<0.01	<0.01	<0.01
pJP6	<0.01	<0.01	<0.01	<0.01
pJP5	1.5	1.3	3.0	3.0
pJP11	2.3	2.3	9.3	9.6
pJP11 plus pJP4	2.3	0.1	8.4	0.14

[a] Structures of the plasmids are shown in Fig. 2.
[b] See references 17 and 24.

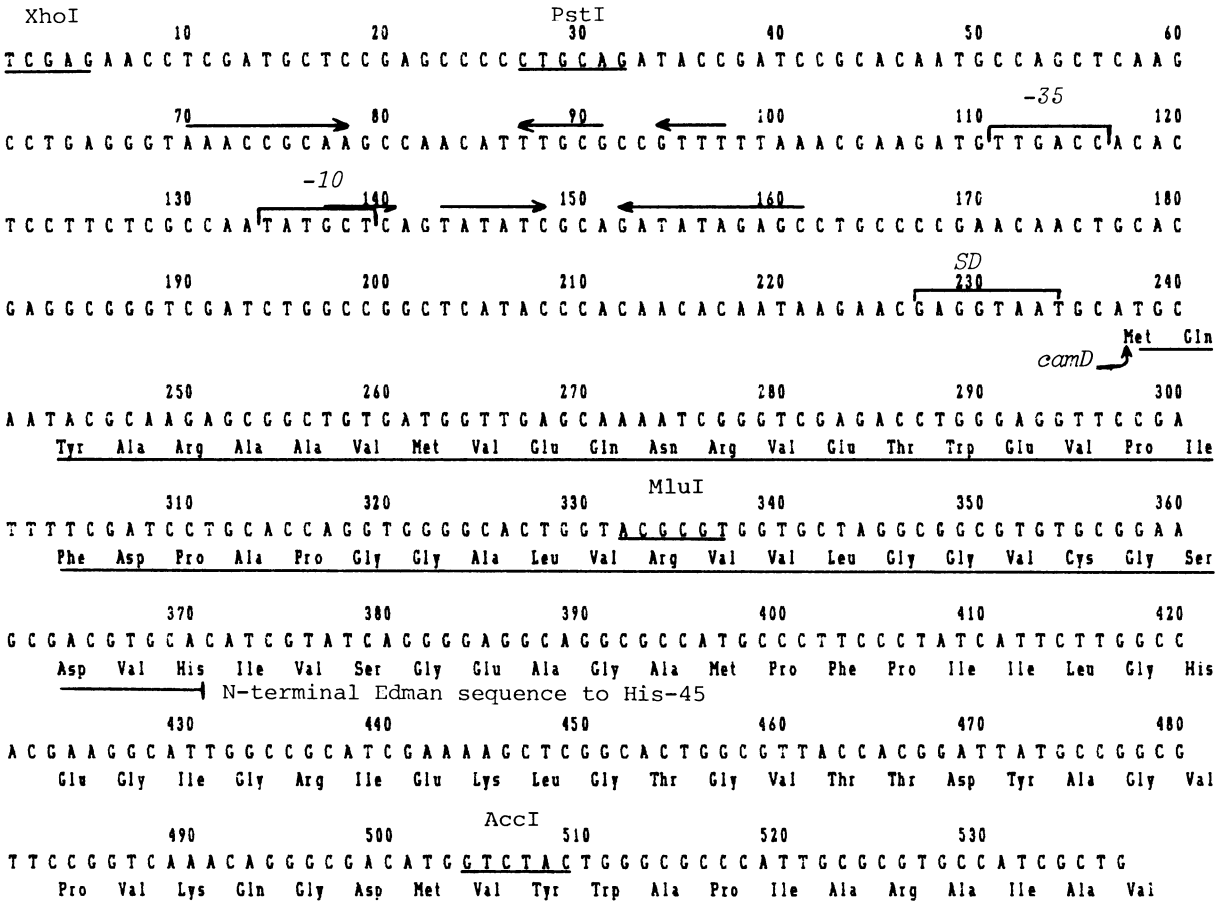

FIG. 5. Nucleotide sequence in *cam* promoter *camD* gene region. The nucleotides are numbered from the 5′ *Xho*I site at 1.38 kilobases of plasmid pJP1. The *camD* gene start signal is nucleotide 237; the first 45 codons conform to the Edman amino acid analyses. Restriction sites and −35, −10, and Shine and Delgano sequences are indicated. The opposed arrows at nucleotides 80 and 150 indicate possible secondary structures.

amino acids obtained by protein sequence analysis (N. Moriwaki and K. Yasunobu, unpublished data).

DISCUSSION

Cytochrome P-450$_{cam}$, the *camC* gene product from the *P. putida* CAM plasmid, is the best characterized of the heme thiolate monoxygenases. It has provided the model for physical and chemical studies of hydroxylase systems and, with the *camA* and *camB* gene products (putidaredoxin reductase and putidaredoxin), has given electron transport data on dioxygen reduction and its insertion into the product. The early transduction experiments showed a closely linked gene cluster, *camABCD*, encoding the hydroxylase subunits and the dehydrogenase for the alcohol product (34). Little was known of the organization and control, save that substrate induction by camphor leads to simultaneous expression of four enzymes.

We have previously (25) cloned *camC* on a 2.3-kilobase insert from a *Pst*I digest library of CAM plasmid DNA. A selection by *trans* complementation of CAM mutants from the Rheinwald et al. (34) collection has proven effective. Since the procedure of CAM plasmid isolation is not fully reliable, we applied selection from an *Hin*dIII library of total cell DNA and recovered a clone with the *camC* gene on a 4.27-kilobase DNA insert. This clone also carries the *camD* gene and a regulator gene, *camR*. This is the first example of negative control of metabolic plasmid genes. The deletion of the upstream 1.38 kilobases of the insert leads to constitutive expression of the *camC* and *camD* genes. Reintroduction of this fragment in *cis* or *trans* restores substrate control.

Members of *Pseudomonas* and related genera utilize an enormous variety of natural and synthetic hydrocarbon compounds as carbon sources (e.g., octane, toluene, naphthalene, camphor, nicotine, etc.); in certain instances this ability is specified by plasmid-borne genes (e.g., plasmids TOL, NAH, OCT, CAM, NIC, etc.). Positive regulation has been reported for *xyl* and *nah* operons by the *xylR* and *xylS* (12, 13, 22) and *nahR* (15, 43) genes. These are in marked contrast to the negative control of this *cam* operon.

The locations of the *camD* and *camC* genes and the direction of transcription were deduced from the amino acid and nucleotide sequences. In pJP1, the *Xho*I-*Pst*I fragment (1.38 to 2.52 kilobases) contains the N-terminal codons of the F-dehydrogenase at about 1.61 kilobase. The correspondence of the DNA sequence from base pair 237 of the isolated DNA with the first 45 amino acid residues taken from the peptide sequence data confirms the *camD* gene location.

The *camC* gene is located within the *Pst*I-*Hin*dIII 2.52- to 4.27-kilobase insert of plasmid pJP1, with the N and C termini, respectively, at about 2.67 and 4.14 kilobases (40). Based on the position of the *Pst*I site at 0 kilobase in the

pKG300 insert, this *Pst*I site is at 2.52 kilobases in pJP1 (25). The stop codon is at base pair 25 to the right of the *Bam*HI site at Asp_{407}, i.e., at 4.09 kilobases, to indicate the transcription of the structural genes from left to right on the pJP1 map (Fig. 2).

ACKNOWLEDGMENTS

We acknowledge many helpful discussions with T. Miki and the gift of vectors pTS1036 and pTS1210 from T. Nakazawa. We are indebted to Marcy Ludington for art and editorial work and to Elizabeth Brillhart for taking over the processing and editorial work in midstream with professional attention and skill.

The work was supported in part by National Science Foundation grant 83-07757 and by grants from the Ministry of Education, Science and Culture of Japan and from the Takeda Pharmaceutical Company in Japan. Terpene intermediates, proteins, and assay methods were provided by J. Ullah and D. Jollie, under the support of National Institutes of Health Grant AM00562.

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