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Cloning and Nucleotide Sequences of NADH-Putidaredoxin Reductase Gene (camA) and Putidaredoxin Gene (camB) Involved in Cytochrome
P-450cam Hydroxylase of Pseudomonas putida1

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Pseudomonas putida PpGl, which carries the CAM plasmid encoding enzymes involved in the degradation pathway of D-camphor, can utilize D-camphor as a sole carbon source. Cytochrome P-450cam and related enzymes participate in the early oxidation steps of D-camphor degradation metabolism. We cloned from a HindIII DNA library of PpGl a 2.9 kbp CAM segment which carries the major part of camA gene encoding NADH-putidaredoxin reductase and the entire camB gene encoding putidaredoxin. The 2.9kbp CAM segment was adjacent to the 4.27kbp HindIII CAM segment which has been previous-ly cloned (Koga et al.(1986) J. Bacterial. 166, 1089-1095). Thus, the total 7.17kbp HindIII CAM directed all the genes responsible for early steps of D-camphor degradation, i.e. 5-exo-hydroxycamphor dehydrogenase (camD gene), cytochrome P-450cam (camC), NADH-putidaredoxin reductase (camA), and putidaredoxin (camB). These cam genes form an operon, camDCAB, and are under negative control by the gene camR located immediate-ly upstream from the camD gene. The total number of amino acids deduced from the nucleotide sequence is 422 for putidaredoxin reductase, and 106 for putidaredoxin.

Pseudomonas putida strain PpGl, ATCC17453, was iso-lated as a microorganism able to grow on media containing D(+)-camphor as a sole carbon source, and shown to carry a plasmid termed CAM which encodes a pathway for catabolism of D- or L-camphor by oxidative enzymes, leading to isobutyrate (Fig. 1).
The oxidation of camphor is initiated to produce com-pound F (5-exo-hydroxycamphor) by a monooxygenase system with coupled redox components. This system for 5-exo-hydroxylation of camphor is composed of three enzymes:(a) NADH-putidaredoxin reductase, an FAD-protein of 45kDa encoded by gene camA,(b) putidaredox-in, an iron-sulfide redox protein of 12kDa encoded by gene camB,(c) cytochrome P-450cam, a terminal hydroxylase component of 47kDa encoded by gene camC (1, 2). The second step is the dehydrogenation of compound F to form compound D (2,5-diketocamphane) by F-dehydrogenase encoded by gene camD (3). The enzymic constitution of the first camphor hydroxylation system resembles the mito-chondrial P-450(scc) system in the bovine adrenal cortex involved in side chain cleavage of cholesterol; except that NADH is used in the former, and NADPH in the latter as an electron donor. Amino acid sequence homologies have been observed between P-450cam and P-450(scc), particularly in a presumed heme-binding region (4), and in an assumed iron-sulfide binding region around cysteine residues be-tween putidaredoxin and adrenodoxin (5).
We have previously reported the cloning of a 2.19kbp PstI CAM plasmid fragment which carries the structural gene camC, encoding cytochrome P-450cam (6). This clone

pKG201 or pKG300, cloned onto the shuttle vector pKT240 or pBR322, respectively, lacked its own promoter and expressed P-450cam protein by read-through tran-scription from the vector promoter. More recently, we constructed another recombinant plasmid, pJP1, which carries the 4.27kbp HindIII CAM fragment containing camD, camC, and their promoter (7). Expression of the genes camD, and camC was cooperatively induced by adding camphor to the P. putida cells carrying pJP1, thereby indicating the presence of the regulatory gene, camR, in the 4.27kbp HindIII CAM segment. The regula-tory function of the camR gene was shown to be negative control for the camDC gene expressions. The cam gene alignment was determined to be camR-camD-camC (7). Unger et al. sequenced the entire camC gene and found the presence of the NH2 terminal portion of the gene camA downstream of the gene camC, in the 2.19kbp PstI CAM segment (8). Since the 5-exo-hydroxylase of camphor is composed of a multicomponent hydroxylase system, the gene products of camA, camB, and camC, it is important to understand the protein-protein interaction mechanism in electron transfer from NADH to the FAD protein-FeS protein-cytochrome P-450cam. For this, knowledge of the amino acid and the nucleotide sequences of the putida-redoxin reductase and putidaredoxin are necessary for investigation of active site residues in the electron transfer step of monooxygenation. In this paper, we report the cloning of the entire camA and camB genes and the nucleotide sequences of these genes. The constitution of the cam operon (camDCAB), negatively regulated by camR, is also outlined.
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MATERIALS AND METHODS

Bacterial Strains and Plasmids-The Pseudomonas

putida and Escherichia coli strains and the plasmids are
listed in Table I.

Media-L-broth (12) served for cell growth, and phos-

Fig. 1. D-Camphor oxidation pathway of P. putida PpG1. The oxidation pathway downstream from FdeH is omitted.

phate-ammonium salts (PAS) medium (13) was used to
grow cells supplemented with D-camphor or its metabolites
as a carbon source. PASG containing 20mM monosodium
glutamate in PAS medium was used to grow cells for
enzyme assays.

Transformation-The transformation procedure was
essentially the same as described previously (7), except for
changing LBS (L-broth-sucrose, instead of glucose) to
original L-broth to prepare log phase cells.
Isolation and Manipulation of Plasmid DNA-Isolation
of plasmid DNA, digestion with restriction enzymes, and
ligation of restriction fragments were as described (7).
Enzyme Assays-Ultracentrifuged supernatant of the
sonicated cells was obtained, as described earlier (7).
Cultures were grown in PASG at 30•Ž to an A600 of 0.25,
then divided into two equal portions. One served as the
control, and the other was supplemented with D-camphor to
5mM. Samples of 40ml were removed at intervals, and
the cells were pelleted, suspended in 1ml of T-O buffer (50
mM Tris-chloride, 10mM 2-mercaptoethanol, 5% glycerol,
pH 7.5), and sonicated. Extracts were prepared by centrif-
ugation in a Beckman 50 Ti rotor for 40min at 45,000rpm
and 4•Ž. The cell extracts were assayed for each cam
enzyme by measuring the camphor-dependent rate of
NADH oxidation at 340nm in the presence of an excess of
the other two hydroxylase proteins. FdeH (camD activity)
was measured at 340nm by compound F-dependent reduc-
tion of NAD, according to Hartline and Gunsalus (3). One
unit of enzymic activity is the amount required to oxidize 1
nmol of NADH or to reduce 1nmol of NAD per s; the unit
is 3.2•~the change in absorbance at 340nm per min based
on an E=6.22 for NADH and a final reaction volume of 1.2
ml.
DNA Sequencing—Nucleotide sequences were deter-
mined by the dideoxy chain termination method (14) using
several custom-synthesized oligonucleotides as primers.

RESULTS

1. Cloning of the camB Gene-To clone the camB gene,
we used the camB mutant strain JPS11 (7) which carries
the camB514 mutant locus on the CAM plasmid, using the

TABLE I. Bacteria and plasmids. Abbreviations for antibiotic resistance: Ap, ampicillin; Km, kanamycin; Sm, streptomycin; Su

,

sulfonamide; Tc, tetracycline.

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Fig. 2. Physical and functional map of the camDCAB operon and structure of the recombinant plasmids carrying different lengths of CAM insert.

mutant complementation technique. This mutant (Cam-
Sms) cannot grow on a PAS plate containing camphor as a
sole carbon source but can grow on the plate containing
compound F (first metabolite of camphor, see Fig. 1), and
fails to produce putidaredoxin (the camB gene product). A gene library had been constructed as a collection of HindIII restriction enzyme fragments of the total cellular DNA of PpGI into vector pTS1036 (7). This gene bank DNA was used to transform JPS11 and was selected for Cam+Smr transformants. One of the resulting transformants was purified and used to prepare plasmid DNA (designated as pJP32, see Fig. 2). Digestion of plasmid pJP32 with HindIII yielded two fragments: an insert of 2.9kbp, and the 13.9kbp linearized vector pTS1036.
2. Restriction Map of cam Genes-A restriction map of pJP32 was facilitated by subcloning the 2.9kbp HindIII insert into the vector pBR322 to transform E. coli strain LE392. This plasmid, pJP48, isolated from an AprTcs transformant was used to construct the physical map of the 2.9kbp HindIII CAM insert (Fig. 2). We previously iso-lated a recombinant plasmid, pKG201 or pKG300, which carries the 2.19kbp PstI CAM fragment (2.70-4.89kbp sites, see Fig. 2) (6). Therefore, we compared the restric-tion map of the 2.9kbp HindIII CAM with that of 2.19kbp PstI CAM fragment, and found a local resemblance be-tween the left part of the former (pJP48) and the right part of the latter (pKG300, see Fig. 2). This result, together with evidence of a local resemblance between the left part of the 2.19kbp PstI CAM and the right part of the 4.27kbp HindIII CAM segments of pJP1 (7), strongly suggested that the two 4.27 and 2.9kbp HindIII CAM fragments were contiguous. For confirmation, we carried out double diges-

tions of pKG300 and pJP48, respectively, with HindIII, in combination with XhoI, PvuII, SmaI, and SalI. The diges-tion products yielded the same size fragments in the region between the 4.27-kbp site and the 4.89-kbp site (data not shown, see Fig. 2), thereby indicating that the two HindIII CAM fragments are contiguous. This conclusion was again confirmed by regenerating active camA-putidaredoxin reductase in the cell carrying the recombined plasmid (pJP45) (see Fig. 3; details will be described in the next section), whereas cells carrying pJP1 or pJP32 did not produce active putidaredoxin reductase at all (data not shown), because of truncation of the gene camA in them. The whole cam restriction map, 0.00-7.17kbp, is shown in Fig. 2.
3. Cam Gene Expression by Plasmid pJP45-Two CAM fragments, EcoRI (0.31kbp site)- HindIII (4.27kbp site) CAM and HindIII (4.27kbp site)-HindIII (7.17kbp site) CAM were ligated and inserted into the EcoRI and HindIII sites of the vector pTS1210. The resultant plasmid was named pJP45, and was expected to carry the genes camR, camD, camC, camA, and camB in that order. We measured the rates of synthesis of cam enzymes in JPS3 cells carrying pJP45 grown in PASG medium, with or without comphor. As shown in Fig. 3, the camA, camB, camC, and camD enzymes were all cooperatively induced by camphor in the pJP45-carrying cells. Thus, pJP45 contained all the cam genes involved in the NADH-electron transport sys-tem of camphor monooxygenation and camphor-5-exo-alcohol dehydrogenase, including gene camR. The induced synthesis rates of cam enzymes were around 6-12 times the noninduced rates. Also these experiments strongly suggest that the camB gene is located downstream from the camA

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Fig. 3. Expression of the cam genes produced by strain
JPS3(pJP45).

gene.
4. Nucleotide Sequences of the camA and camB Genes
-Nucleotide sequences of the 2.0kbp SmaI fragment

(4.01kbp site-6.01kbp site) which is expected to carry at least genes camA and camB were determined on both strands from overlapping DNA sequences. We found the camA and camB genes on the SmaI fragment, as was expected. The entire nucleotide sequences of these cam genes and their predicted amino acid sequences are pre-sented in Fig. 4. The nucleotide sequence of the 3' end of the gene camC, determined by Unger et al. (8) and that of the 5' part (154nt), corresponding to about one-tenth, of the camA gene determined by Unger et al. (8) and Romeo et al.
(15) were in complete agreement with our sequencing. The camB gene was found 55 nucleotides downstream of the camA gene. The putidaredoxin reductase, the camA gene product, consisted of 422 amino acids with amolecular weight of 45,578. The amino acid sequence deduced from the nucleotide sequence of putidaredoxin, the camB pro-tein, agreed well with that reported for the protein by Tanaka et al. (5) except for Glu-14 substituted for Gln. Methionine corresponding to the initiation codon must be

TABLE II. Synthesis rates of cam enzymes in P.putida carrying recombinant plasmids containing CAM fragment.

processed to expose Ser, because the N-terminus of native putidaredoxin is Ser. There were 106 amino acids with a molecular weight of 11,419. Thus, it is clear that the direction of transcription of the genes, camD, C, A, B, is from left to right as shown in Fig. 2.
5. Negative Regulation of Genes camD, C, A, B-In a previous paper, we reported that introduction of plasmid pJP4 carrying 0.00-1.43kbp CAM into the cells carrying plasmid pJP11, which contains 1.43-4.27kbp CAM, re-presses the expression of the camD and camC genes on pJP11, while cells carrying pJP11 alone show constitutive expression of these cam genes (7). From these experi-ments, the negative regulator gene camR was found in the 0.00-1.43kbp region of CAM. Thus, it was of interest to determine whether the camR gene product also controls the expression of the camA and camB genes. In order to test this, XhoI (1.43kbp site)-HindIII (7.17kbp site) CAM fragment was then inserted into the region between the SalI and HindIII sites of the vector pTS1210, to produce pJP43. Rates of synthesis of the camA, camB, camC, and camD enzymes were then measured in the cells of JPS3 carrying pJP43. Synthesis rates of all four cam enzymes were high, irrespecitve of the presence of camphor in the cell culture. The ratios of the induced/noninduced synthe-sis rates were about one for all four cam enzymes, indicat-ing that the CAM segment of 1.43-7.17kbp sites abolishes regulation of the expression of the cam gens (Table II). Next, we introduced plasmid pJP4 into the cells carrying pJP43 and incubated the double transformant, with or without camphor. As shown in the same table, the synthesis rates of all the cam enzymes were remarkably low in the absence of camphor, whereas they were enhanced in the presence of camphor. These results demonstrate that gene camR negatively controls the expression of not only camD and camC genes, but also camA and camB genes.

DISCUSSION

The cytochrome P-450cam monooxygenase system in the PpGl substrain of P.putida carries out the two-electron reduction of dioxygen with concomitant hydroxylation of the camphor molecular at the 5-exo position. Hydroxycam-phor (compound F) is then oxidized to the diketone by a dehydrogenase encoded by gene camD. The first monoox-ygenase system, for 5-exo-hydroxylation of D-camphor, contains three protein components, NADH-specific FAD-containing putidaredoxin reductase encoded by gene camA, the iron-sulfur protein putidaredoxin encoded by gene

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Fig. 4. The complete nucleotide sequences of NADH-putidaredoxin reductase gene (camA), putidaredoxin gene (camB), and their predicted amino acid sequences.

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camB, and P-450cam encoded by gene camC. These four
cam enzymes were all found to be induced by camphor (3).
In this work, we found that the genetic organization of the
cam genes consists of camDCAB genes in cluster and the
camR, negative regulator gene, precedes the camD gene.
The camR gene product is responsive to camphor, i. e., provides coordinate induction of the camDCAB genes. The orientation of transcription of each cam gene was deter-mined to be from left to right (Fig. 2), but that of the gene camR is in the opposite direction (unpublished results). With respect to camR, data will be reported elsewhere. The nucleotide sequence of the regulatory region, 296bp, beginning with the 1.43kbp site of XhoI, was searched for a possible E. coli promoter consensus sequence, and we found the canonical structure at the -10 region (TATGCT) separated by 17bp from the -35 region (TTGACC)(7). Possible promoter structure could not be found in the camC, camA, and camB genes and their boundary regions. Together with the fact of the same orientation of transcrip-
tion of the camDCAB genes, the results suggest that these cam genes form an operon array. We already knew of a putative operator sequence (GCTCAGTATATCG-CAGATATAGAGC) carrying the palindrome structure,

upstream from the camD gene, overlapped in an as-sumed -10 region (7). The palindrome sequences which resemble the putative operator were not found in the 5' flanking regions of, or in, the camC, camA, and camB genes. Presumably only one operator, to which the camR protein binds, is present upstream from the camD gene, and it regulates the expression of the genes camDCAB. The ratios, induced/noninduced, of the camDCAB gene expression in the double transformants of pJP4 and pJP43 were always higher (20-91 times, see Table II) than those in cells carrying pJP45 alone (6-12 times, see Fig. 5). For example, the induction ratio of F-dehydrogenase (camD gene product) was 18.3/0.2 in the former cells, whereas it was 3.6/0.4 in the latter ones. The marked difference in the

recombinant plasmids among the two strains is that pJP4 plays the role of camR protein donor trans in the double transformants, and pJP45 carries gene camR cis. It seems unlikely that the amount of the camR protein in the

pJP45-carrying cells is higher than in the double trans-formants, since in a noninduced condition the rate of syntheis of F-dehydrogenase in cells carrying pJP45 is about twice that in the double transformants. Both the cam

fragments of pJP43 and pJP45 are in the low copy vector

pTS1210 (2-3 copies/P. putida cell)(16), and that of pJP4 is in the multicopy vector pTS1036 (40 copies/chromo-some)(17). If there is a gene dosage effect, pJP4-pJP43-carrying cells would be expected to produce much more of the camR repressor than the cells carrying pJP43 alone, leading to less induction by camphor in the double trans-formant than the pJP45-carrying cells. This is inconsistent with our observations. In comparing the DNA structure difference among the two plasmids, the 0.00-0.31kbp CAM segment region is deleted in pJP45, but not in pJP4. We presume that the region is involved in other aspects of the camDCAB gene induction process, such as camphor binding or receptor, or enhancement of inactivation of the camR repressor. There is another possibility, that the camR protein might be converted to the regulatory acti-vator by binding with camphor or its metabolite. These

problems remain to be clarified.

The putative ribosome-binding site, Shine-Dalgarno sequence (18), was similar in each cam gene; ACGAG for camD (7), AGGAG for camC (8), GGGAG for camA, GTGAG for camB.

Among an abundance of cytochromes P-450 present in organisms (mammalia, yeast, and some bacteria), P-450cam seems to provide an excellent model system for understanding the structure-function relationship. In par-ticular, X-ray crystallographic studies of the cytochrome P-450cam elucidated by Poulos et al.(19-21) have opened the way to study the reaction mechanism (for example, substrate and oxygen pocket structures, oxygen activation and its cleavage, etc.) of the P-450cam by changing amino acid residues through the use of the site-directed muta-genesis (22, 23). Utilizing the information on the cam gene nucleotide sequences, we have started studies on the structure-function relationship of the putidaredoxin re-ductase and putidaredoxin with use of their mutated genes as a tool.

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