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$P450_{cam}$ GENE CLONING AND EXPRESSION IN PSEUDOMONAS PUTIDA AND ESCHERICHIA COLI

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Summary: The gene camC, which encodes the cytochrome P450 monoxygenase protein, was cloned into the shuttle vector pKT240 and recovered as the recombinant pKG201 with a 2.3 kb insert from the CAM plasmid in the PstI site. The gene product is expressed constitutively in P. putida and in E. coli whereas the inverted insert clone lacks expression, indicating absence of an insert promoter.

Pseudomonas putida, strain PpG1/pRG1, uses D- or L-camphor as carbon and energy source, via a camphor inducible-plasmid encoded oxidative pathway, shown in Figure 1 (1,2). The initial reaction, 5-exo-hydroxylation, requires a three-component monoxygenase encoded by the genes camABC (3-5). The gene camA encodes a flavoprotein, camB an iron sulfide cluster redoxin, and camC the terminal hydroxylase catalyst (3).

Cytochrome P450 was first recognized optically in liver microsomes by the unique ferrous-CO Soret absorption band near 450 nm, and only later associated with monoxygenase reactions (6). The $P450_{cam}$ model has provided the structure (7,8) and to the reactions and mechanisms (9,10) for the microsomal and the mitochondrial/microbial systems. Thus we have taken the organization and control of the $P450_{cam}$ system as a genetic prototype (11,12) to understand also the mammalian enzyme systems.

In this paper we describe the molecular cloning of a 2.3 kb segment of the CAM plasmid encoding the camC gene, under constitutive control of a vector promoter.

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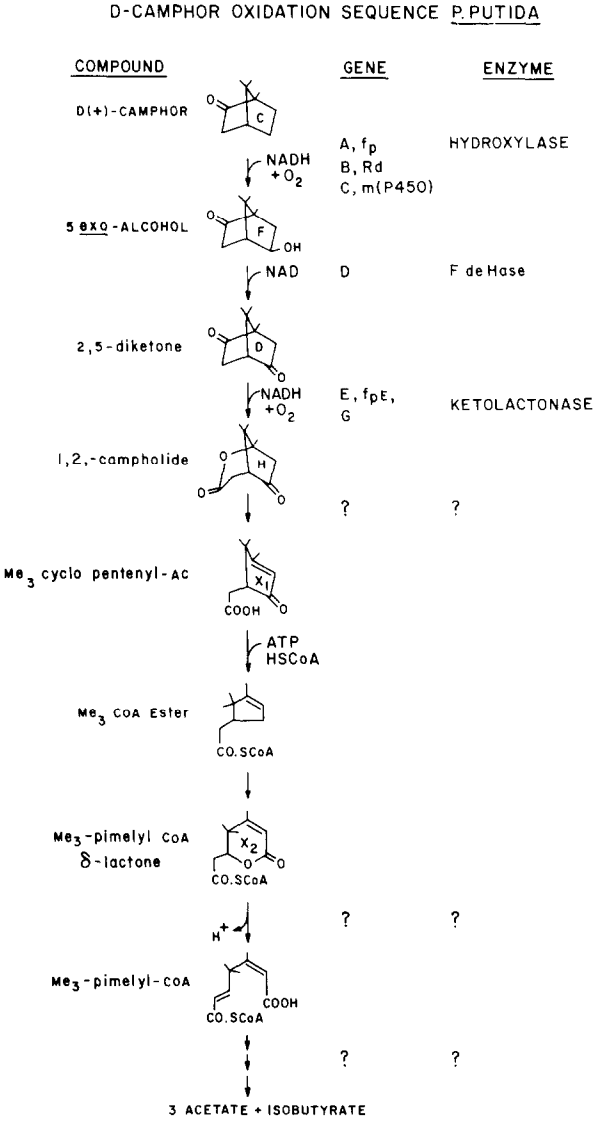

Fig. 1. D-camphor Oxidation Pathway: Plasmid born CAM, pRG1, hydroxylase genes.

MATERIALS and METHODS

Bacterial strains and plasmids. Table 1 lists the strains used.
Media. L broth was employed as complete medium in the isolation of plasmids. PAS (phosphate ammonium salts, 13) served as a selective medium, and to grow cells for the enzyme assays.
Plasmid isolation and analyses. For CAM, pRG1, alkaline denaturation was used (14), with slight modification according to Palchaudhuri, et al. (15). Vectors and recombinant plasmids were recovered according to Farrell (16). Restriction digests and ligation were as recommended by the suppliers.
Enzyme assays. Cells were grown to the midlog phase at 30°C in PAS broth containing for P. putida 20 mM MSG (monosodium glutamate), and for E. coli 10 mM glucose, plus the appropriate antibiotic and amino acid supplement. A 0 min sample was collected by centrifugation of a 40 ml aliquot and washed

Table 1. Bacterial Strains and Plasmids

Strain or Plasmid	Phenotype or Genotype	Ref.
P. putida		
PpG 1	wt/CAM	3
" 543	wt/CAM*camC100*	3
" 1343	Met^-,*met-616/CAMΔ*	14
JPS 3	Met^+,1343 revertant	This ms.
E. coli		
LE 392	*F⁻hsdR514 supE44 supF58Δ (lacIZY)6 galK2 galT22 metB1 trpR55*	18
Plasmid		
pRG 1*	camphor oxidation	3
pKT240	Ap^r Km^r	19,20
pBR322	Ap^r Tc^r	21

*CAM plasmid, pRG1, for Rheinwald, Gunsalus

twice with T-0 buffer (50 mM Tris, 10 mM 2-mercaptoethanol, 10% glycerol, pH 7.5). The remaining culture was divided. To one half camphor was added to 5 mM, and the incubations continued for 90 min, then the non-induced and induced 40 ml aliquots were pelleted and washed as with the 0 min sample. The cells were broken by sonication, clarified at 100 Kg, and the activity of the extracts measured for each of the hydroxylase components according to Katagiri, et al. (4).

Protein. Estimations were according to Lowry, et al. (17), with bovine serum albumin as standard.

RESULTS and DISCUSSION

Cloning of the gene camC. The DNA of CAM plasmid, pRG1, was digested with *PstI* and the fragments inserted by T4 DNA ligase into the *PstI* site of vector pKT240. The ligated mixture was used to transform the mutant strain PpG543 (this strain is unable to grow on a camphor plate, due to a mutation, locus *camC100*, on the pRG1 CAM plasmid). Putative complementation was determined by plating the transformed cells on PAS agar containing camphor as the carbon source and Km (Kanamycin) at 100 μg/ml. Cam^+Km^r clones appeared, were purified, the plasmid DNA isolated, and used to back transform PpG543. Of the 50 Km^r clones tested, all transformants grew on the PAS medium containing camphor. A fast growing clone was selected and the recombinant plasmid designated pKG201.

Restriction map of pKG201. A *PstI* digest of pKG201 yielded two fragments, 11.2 kb, corresponding to the vector pKT240, and the second 2.3 kb of CAM DNA.

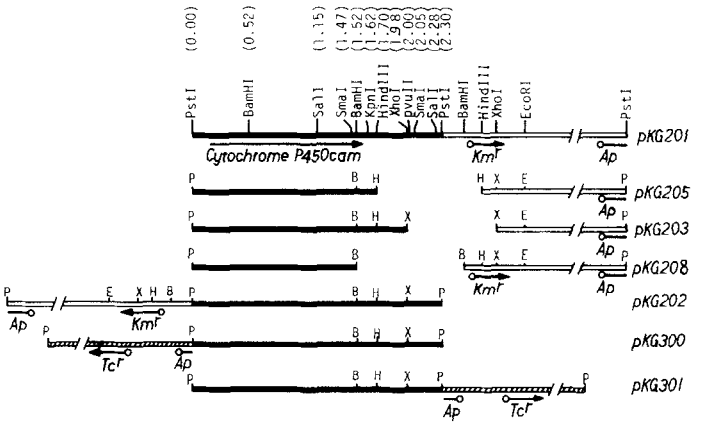

Fig. 2. Plasmid pKG201, and Subclones. Heavy solid line = CAM segment; double line = pKT240; hatched = pBR322 vectors. Circle = promotor; arrow = transcription direction. Numbered restriction loci are Kb from left end of CAM insert.

The restriction sites were determined from double digests, as shown in Figure 2. The insert is free of sites for BglII , ClaI, EcoRI, HpaI, and PvuI.

Expression of the camC gene. The plasmid pKG201 was transformed into the P. putida host JPS3, a Met^+ revertant of 1343, Table 1, and the rate of $P450_{cam}$ synthesis measured in the presence of camphor as inducer and in its absence. The rates were independent of the inducer, suggesting that the 2.3 kb insert lacks a regulatory gene. This expression could result from read through transcription from the Ap^r gene promoter of vector pKT240. The rate of $P450_{cam}$ synthesis in the JPS3/pKG201 recombinant is about twice that of the wild type PpG1/pRG1 and may result from the relative activities of the two promoters, or from the multiple copies of the pKG201 plasmid, relative to the very large pRG1 CAM plasmid of the parent. To further check the possibility that the insert lacks a native CAM control, we examined the effect of the insert orientation by PstI digestion and self ligation. A construct pKG202 was found by double digests to carry the 2.3 kb insert in the inverted orientation from pKG201, Figure 2.

The $P450_{cam}$ synthesis rate was found to be very much lower in cell extracts from JPS3/pKG202, Table 2, even in the induced condition, than in the same host carrying pKG201. The lack of camC expression by pKG202 would thus result from improper orientation to the Ap^r promoter.

Table 2. Cytochrome P450cam Synthesis Rates in P. putida and E. coli *

Host/Plasmid	P450cam Activity, nkat+ mg^{-1} Protein*	
P. putida	Non-Induced	Induced
JPS3/pKG201	4.1	4.0
" " 202	0.14	0.17
" " 208	<0.01	<0.01
" pKT240	--†	<0.01
PpG1/pRG 1	0.28	2.0
E. coli		
LE392/pKG300	4.8	4.8
" " 301	--†	0.22
" pBR322	--†	<0.01

*For procedures, see Materials and Methods, rate = enzyme units/ml culture/mg increase in cell protein, 0-90 min.
+Enzyme unit is nkat = n mole camphor dependent NADH oxidation sec^{-1}.
†Not determined.

In these studies, we were unable to detect activities in the extracts for the camA and B gene products, the putidaredoxin-reductase, and the redoxin.

camC gene locus in 2.3 kb PstI insert. Figure 2 illustrates a more precise map of the camC gene locus obtained by restriction digests of plasmid pKG201 with XhoI, HindIII and BamHI, followed by self ligation, transformation of the mutant PpG543, and selection for Cam^+ and/or Km^r clones. Restriction sites for XhoI and HindIII are found in the Km^r gene of the vector, thus their Cam^+ deletion clones are Km^s, whereas the Km^r phenotype is retained in the BamHI subclones, though no Cam^+ clones were recovered. Thus the camC gene lies between PstI (0.00 kb) and HindIII (1.7 kb) on the insert. The Cam^-Km^r subclone pKG208, obtained with BamHI, neither complemented the camC100 mutation nor produced $P450_{cam}$ in host JPS3, indicating that the BamHI site at 1.52 kb lies within the camC gene.

Recently, we have isolated another recombinant plasmid carrying a 7.17 kb HindIII segment of the CAM plasmid, which includes the 2.3 kb PstI fragment, and carries the cam hydroxylase operon under control of a regulatory gene camR, in the gene order camRDCAB. The camD gene encodes FdeH (5-exo-alcohol dehydrogenase); the details will be reported elsewhere.

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