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Cloning and Expression of *Pseudomonas cepacia catB* Gene in *Pseudomonas putida*

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The enzyme, *cis,cis*-muconate lactonizing enzyme has been proposed to play a key role in the β-ketoadipate pathway of benzoate degradation. A 3.2-kb *Eco*RI fragment termed as pRSU2, isolated from a *Pseudomonas cepacia* genomic library was able to complement the *catB* defective mutant. Several relevant restriction enzyme sites were determined within the cloned fragment. In *Pseudomonas putida* SUC2 carrying pRSU2, the enzyme activity was relatively higher than those of the induced or partially induced state of wild type *P. putida* PRS2000. It was probably due to higher expression of *P. cepacia catB* in *P. putida*. One possible interpretation of these results is that the *catB* promoter in *P. cepacia* is recognized within *P. putida*, resulting in the almost same expression level.

Key words: Cloning of *catB* gene, *cis,cis*-muconate lactonizing enzyme, restriction mapping, expression of *cat* gene

Aromatic hydrocarbons are degraded by *Pseudomonas* spp. and other soil bacteria via an intermediate catechol. Catechol ring cleavage reactions are catalyzed by two types of dioxygenases, intradiol and extradiol. Catechol 1, 2-dioxygenase, intradiol type, plays a central role in catabolic pathway of many aromatic growth substrates, converts catechol to *cis,cis*-muconate, and further metabolizes to tricarboxylic acid cycle intermediates via β-ketoadipate pathway.

Considerable research has been carried out on the regulation of enzyme induction of the β-ketoadipate pathway in *Pseudomonas putida* and *P. aeruginosa* (10, 17, 18). Three enzymes involved in this pathway, catechol 1,2-dioxygenase (*catA*), *cis,cis*-muconate lactonizing enzyme (*catB*), and muconolactone isomerase (*catC*) share a common inducer, *cis,cis*-muconate (10, 17, 19). In *P. putida*, *catB* and *catC* genes are coordinately controlled and have been found to be closely linked on the chromosomes (16, 26). In *P. aeruginosa*, transductional analysis has shown that *catA*, *catB*, and *catC* are linked on the chromosome and that this cluster is grouped with several independently regulated genes that code for enzymes with related catabolic functions (7, 22).

Shanley *et al.* (25) reported the isolation and preliminary characterization of a 5.0 kb *Eco*RI DNA restriction fragment carrying the *catBCDE* gene from *Acinetobacter calcoaceticus*. The properties of the cloned fragment demonstrate the physical linkage of the *catBCDE* gene and suggest that they are coordinately transcribed in *P. putida* and *E. coli*. The complete nucleotide sequence of a DNA segment cloned from the chromosome of *P. putida* encompassing the structural gene for *cis,cis*-muconate lactonizing enzyme has been reported (1). The *catB* and *catC* gene from *P. putida* share a single inducible promoter which is located at the upstream of *catB* gene. Expression of the gene was found to be regulated at the transcriptional level and the transcription initiation site was identified in *P. putida* (2). The nucleotide sequence of the promoter region was obtained and compared with other positively regulated procaryotic promoters (2). The *catA*, and *catBC* gene clusters from *P. aeruginosa* have been cloned and expressed in blocked mutants of *P. aeruginosa* at elevated levels, but in mutants of *P. putida* at low level of expression and the absence of an inducible response, suggesting an incompatibility between the regulation and expression of the *cat* genes in two species (6, 11).

In *P. putida*, the *catB* and *catC* genes are coordinately

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regulated and tightly linked (27). The *catBC* operon was positively regulated, and the promoter was located 64-bp upstream of the *catB* translational start site. Transcription of mRNA from mutant promoters was determined by primer extension. Comparision of the initiation start site of mutant promoters with that of the wild-type promoter identified a single functional promoter (3). In addition, a regulatory gene, designated *catR*, is believed to be located upstream of *catBC*. The product of this gene is required for expression of the *catBC* operon and the operon is positively regulated by the catR protein (1, 2, 23, 28).

In this paper, we report the molecular cloning of the *catB* gene from *P. cepacia*, which is able to utilize phenol or benzoate as a sole energy source. The 3.2-kb fragment containing the gene was isolated from *P. cepacia* genomic DNA library. The cloned *catB* gene complemented *P. putida* PRS2015 (*catB*) and showed higher level of expression in *P. putida*.

Material and Methods

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study, together with their relevant characteristics, are listed in Table 1.

Media and growth conditions

Pseudomonas cepacia, *P. putida*, and *E. coli* were grown on LB broth medium. Minimal medium supplemented with 20 mM glucose or 5 mM benzoate, was prepared as described previously (1). Supplements were added as needed at the following final concentrations: ampicillin, 50 μg/ml; tetracycline, 100 μg/ml; 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), 0.006%; isopropyl-β-D-thiogalactopyranoside (IPTG), 1 mM.

Table 1. Bacterial strains and plasmids

Strains	Genotype or Phenotype	Reference or Source
E. coli		
C600	F⁻ *thi*-1 *thr*-1 *leu*B6 *lac*Y *ton*A21 *sup*E44 λ⁻	Appleyard(4)
DH5α	*sup*E44 Δ*lac*U169 (φ 80*lacZ*Δ M15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*- *rel*A	
P. cepacia		
SM16	Prototroph (*catB*⁺)	This study
P. putida		
PRS2000	*per*-1103, Wild type (*catB*⁺)	Wheelis and Ornston(26)
PRS2015	*per*-1103, *catB* 1123	Wheelis and Ornston(26)
SUC2	*per*-1103, *catB*⁺, pRK415	This study
Plasmid		
pBluescript	SK⁺, Ap⁺	
pRK2013	Km^R, *mob*⁺	Figurski and Helinski(8)
pRK415	T$_c^R$,	Keen *et al.*(9)
pRSU2	T$_c^R$, *catB*⁺	This study
pCO2	Ap^R, *catB*	This study

Preparation of genomic DNA

Genomic DNA was isolated from *Pseudomonas cepacia* by a modification of the Marmur's method (14). Other common media and buffers used here is described as in Sambrook's manual (24). A single colony was grown in 100 ml of LB broth and were cultured in 1 l of LB broth at 37°C for 6 hrs. Cells were centrifuged at 4,000 rpm for 10 min at 4°C. Pellets were suspended in 2 ml of saline-EDTA with lysozyme (10 mg/ml), incubated for 30 min at 37°C, and frozen at -70°C for 30 min. The solution was treated with 20 ml of Tris-SDS and incubated in a water bath at 60°C. The lysate was extracted with 10 ml of buffered phenol (pH9.0) and centrifuged at 8000 rpm for 10 min. The upper supernatant was transferred into chilled sterile glass beaker on ice. DNA was precipitated with 2 volume of ethanol and harvested the supernatant with chilled glass rod. A bundle of DNA was rinsed with 70%, 80% and 90% ethanol for 5 min, respectively. DNA was resuspended in 10 ml of TE buffer and stored at 4°C. The solution was treated with RNase A (20 μg/ml) at 37°C for 1 hr. The ethanol-precipitated DNAs were dried, dissolved in 5 ml TE buffer, and stored at 4°C.

Construction of genomic library

The chromosomal DNA of *P. cepacia* was partially digested with *Eco*RI. Genomic DNA fragments between 2~8-kb in size were isolated by agarose gel electrophoresis. The recombinant plasmid library was constructed by ligating the genomic DNA fragments into *Eco*RI site of a vector pRK415. The resulting plasmids were introduced into *E. coli* DH5α competent cells using electroporator. The transformed bacterial cells were spread on LB plates containing ampicillin, IPTG, and X-gal. The preparation of recombinant plasmid DNA from bacterial cells was carried out as described by Sambrook *et al.* (24).

Mating experiment

The donor strain was *E. coli* DH5α carrying recombinant plasmids of genomic library. Conjugations were carried out by filter mating experiments. *E. coli* donor and *P. putida* PRS2015 (catB) as a recipient were mated using helper plasmid, pRK2013 in *E. coli* C600 (8). Donor, recipient, and helper strains were grown in 2 ml LB broth medium overnight, respectively. Each of these cells were washed with 0.85% NaCl solution two times. Cell pellets were resuspended in 1 ml of the saline. Each 100 μl of

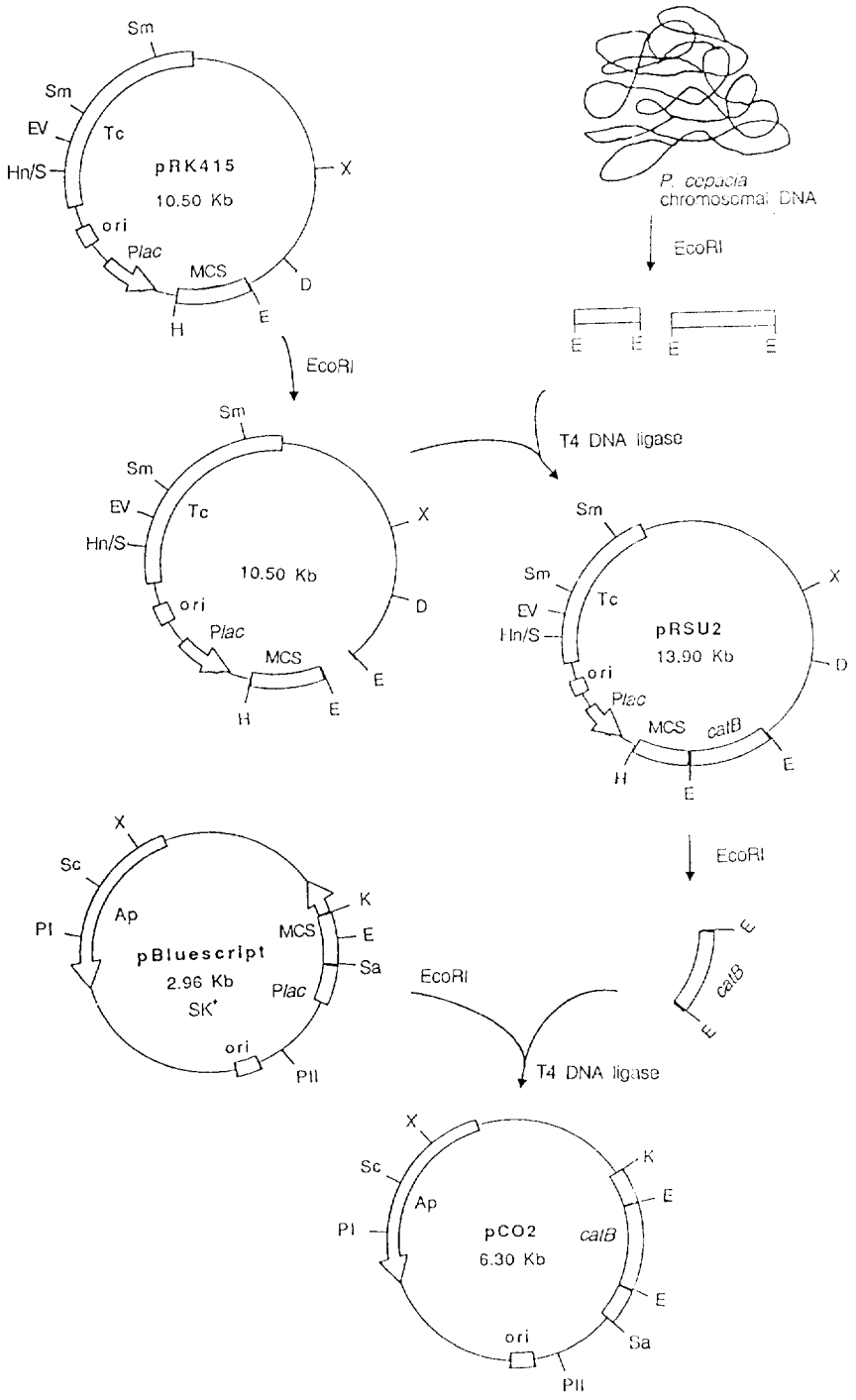

Fig. 1. Construction of pRSU2 and pCO2. *P. cepacia* DNA was digested with *Eco*RI and ligated into pRK415 to give the plasmid pRSU2. The 3.2-kb *Eco*RI insert was subcloned into multicloning sites on pBluescript to yield pCO2 Multicloning sites, Sm; *Sma*I, EV; *Eco*RV, Hn; *Hinc*II, X; *Xmn*I, D; *Dra* PI; *Pvu*I, PII; *Pvu* II, Sc; *Sca*I, K; *Kpn*I, and Sc; *Sac*I.

donors, recipients and helpers was mixed in 10 ml of saline, filtered in nitrocellulose membrane, placed onto an LB plate, and incubated for 10 min at 36°C. The solution was appropriately diluted and spread on the minimal plates containing 5 mM benzoate as the carbon source and 100 μg/ml of kanamycin to select transconjugants.

Measurement of enzyme activity

Cells were grown on minimal broth supplemented with 20 mM glucose or 5 mM benzoate overnight and were harvested by centrifugation at 10,000 rpm for 15 min. The pelleted cells were washed twice in 50 mM Tris-HCl (pH8.0), and were disrupted sonically with ul-

trasonic dismembrator, Model 300 (Fisher Co., USA). Cellular debris was removed by centrifugation at 10,000 rpm for 2 hrs. and the clear supernatant solution was used for immediately for enzyme assay. Activities for *cis, cis*-muconate lactonizing enzyme encoded by the *catB* gene were determined by established procedure (17). Protein was determined by the method of Lowry *et al.* (12) with bovine serum albumin as a standard. Specific activities are represented as micromoles of product formed per minute per milligram of protein.

Chemicals and reagents

All the chemicals, enzymes, and reagents used in this study were of the highest purity commercially available. Enzymes and reagents used for DNA manipulation were purchased from Promega Co., Boerhinger-Mannheim (BM, Germany), and Sigma Chemical Co. (St. Louis, Mo, USA). cis,cis-Muconate was supplied from Dr. Ornston, Yale University.

Results and Discussion

Molecular cloning of *catB* gene by complementing a *catB* mutant

Chromosomal DNA from *P. cepacia* SM16 was used to construct a DNA library in plasmid pRK415 as shown in Fig 1. All clones were introduced into *E. coli* DH5α to make gene library in *E. coli*, using tetracycline, X-gal and IPTG for selection. Their recombinant plasmids in *E. coli* transformants, donor strain, was conjugated into *P. putida* PRS2015 (*catB*) using helper plasmid, pRK2013 by the method of mating experiment. The clones were selected on the agar plate with tetracycline and reselected on the minimal plates containing 5 mM benzoate (Fig 2). One of the clones, pRSU2, was able to complement the *catB* mutation of *P. putida* PRS2015, thus allowing it to grow on benzoate minimal plate. The clone contained approximately 3.2 kb of chromosomal DNA fragment.

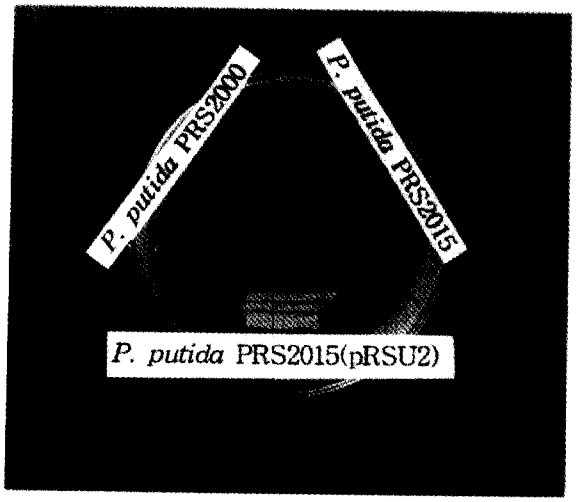

Fig. 2. Complementation test of *Pseudomonas putida* PRS 2015(*catB*) by recombinant plasmid pRSU2 on minimal medium containing benzoate(5 mM).

From the previous studies by others, there were numerous genes related to the *catB* gene reported. For example, *catBCDE* gene clusters 3.0-kb was isolated from *Acinetobacter calcoaceticus* and a cloned *Eco*RI fragment contained *catBCDE* DNA fragment (25). Aldrich *et al.* (1) cloned a 10.2-kb *Eco*RI fragment in pKT240 isolated from a *P. putida* genomic library which complemented a mutant deficient in *catB* gene. The *catB* gene was localized to a 1.6-kb fragment and the enzyme activity was determined from crude extracts of benzoate-grown *P. putida* harboring the recombinant plasmid pTA 11. The essential size of the DNA for *catB* was 5.7-kb *Xho*I-*Hind*III, in which has *Pst*I and *Cla* Isites. A 9.9-kb *Bam*HI restriction endonuclease fragment encoding the *catA* and *catBC* gene clusters was also selected from a gene bank of the *Pseudomonas aeruginosa* PAO1c chromosome (11). The *catBC* gene in pRO1783 are tightly linked and are transcribed from a single promoter. The *catB* gene can be positioned in the smaller *Eco*RI-*Sal*I fragment (3.5-kb) of pRO1783 and proceeds through the *EcoR*I site at map coordinately 3.5-kb in pRO1875 (11). The portion of the *catB* clone was analyzed by DNA sequence analysis.

In this study, we isolated 3.2-kb DNA fragment carrying *catB* from *P. cepacia*. From these results, the *catC* gene may be positioned in the *Eco*RI fragment (3.2-kb) of pRSU2 even if the enezyme activity could not be assayed because its substrate is not available. However, on the basis of DNA fragment size and restriction analysis, it was found that our *catB* fragment is considerably dif-

Table 2. Specific activities of *cis,cis*-muconate lactonizing enzyme of the cell-free extracts from *P. putida*

Strain		Growth medium[a]	Specific activities (u/mg protein)[b]
P. putida			
PRS2000	wild type	G	0.75
		B	2.70
PRS2015	catB	G	0.36
		B	NG
SUC2	transformant	G	1.15
		B	3.02

[a]Cultures were grown in minimal medium supplemented with 20 mM glucose (G), or 5 mM benzoate (B).
[b]One unit is defined as the amount of enzyme necessary to cause the disappearance of 1.0 μmol of substrate per min at 25℃.
NG, no growth.

ferent from others in fragment size and restriction map.

Expression of *catB* gene in *P. putida*

In this study, further evidence that the *catB* structural gene had been cloned was obtained by assaying clones for *cis,cis*-muconate lactonizing enzyme. *P. putida* PRS 2000 showed 0.75 units of specific activity of *cis,cis*-muconate lactonizing enzyme when induced partially with glucose, but this activity was 3.6 fold lower than that found in the induced cultur with benzoate as shown in Table 2. On the other hand, *P. putida* PRS2015, *catB* defective mutant, showed 0.36 units of specific activity of the enzyme when induced partially, but no detectable enzyme activity when induced with benzoate because this mutant can not grow on minimal benzoate medium. This result suggests that the mutated enzyme is probably leaky that it has a low enzyme activity. Thus, this benzoate medium allows induction of the *catB* gene product as shown by activities of *cis,cis*-muconate lactonizing enzyme of *P. putida* PRS2015 harvoring pRSU2.

However, *P. putida* SUC2 carrying pRSU2 had induced the enzyme activities at higher level than the induced or partially induced state of PRS2000 as shown in Table 2. It was probably due to higher expression of *P. cepacia catB* in pRK415 in *P. putida*. Thus, this clone which apparently has a *catB* gene, grows abundantly on benzoate plate overnight.

Other reports also showed that the *catB* gene product, *cis,cis*-muconate lactonizing enzyme, was induced by growth on benzoate (14, 28). *CatB* mutant was unable to grow on benzoate as a sole carbon source but grew if the medium was also supplemented with glucose. These findings are consistent with the results in our study.

When strain *P. putida* pRS2241 carrying pAN1 was grown in the absence of inducer, the *catBCDE* gene products from *A. calcoaceticus* were produced at elavated levels, comparable to those found in induced cultures (20, 25). In principle, the constitutive expression of the *catBCD* genes in *P. putida* pRS2241 (pAN1) could be due to transcription of chromosomal *P. putida* genes. In addition, each *Acinetobacter calcoaceticus cat* gene is expressed constitutively in *P. putida* (25). Expression of the the *catBCDE* gene from pKT230 is substantially higher in *P. putida* than in *E. coli*, further demonstrating that there are no barriers to the expression of foreign genes in *Pseudomonas* species (5, 15).

In this work, when carried in mutant of *P. putida*, the cloned *catB* gene from *P. cepacia* was expressed at the almost same level as that found in fully induced cells. In addition, the cloned *P. cepacia catB* gene in *P. putida* showed significant elevation of enzyme activity under the induced condition. Thus, one possible interpretation of our results is that the *P. cepacia* promoter for *catB* are recognized in *P. putida*, expressing almost the same level of enzyme activity.

However, the *catA*, *catB*, and *catC* gene of *P. aeruginosa* were expressed at very low levels in *P. putida*, levels which were similar to those found in uninduced cells of *P. putida* (11). The level of activity for each of the enzymes in catabolic pathway was 1/10th to 1/50th of that found in fully induced cells of the wild-type *P. aeruginosa* PAO1c. These results seem unlikely in the light of previous successful expression of cloned catabolic genes in *P. putida*.

Restriction mapping of *Eco*RI insert of *catB* gene

In order to determine nucleotide sequences of *catB* gene, the *Eco*RI insert fragment of 3.2-kb in *P. putida* PRS2015 was subcloned into pBluescript SK (+) vector, named pCO2. The isolated plasmid pCO2 was digested with several restriction enzymes and analyzed by agarose gel-electrophoresis. Restriction pattern of pCO2 is shown in Fig. 3. A restriction map of plasmid pCO2 carrying *catB* structural gene is shown in Fig 4. No

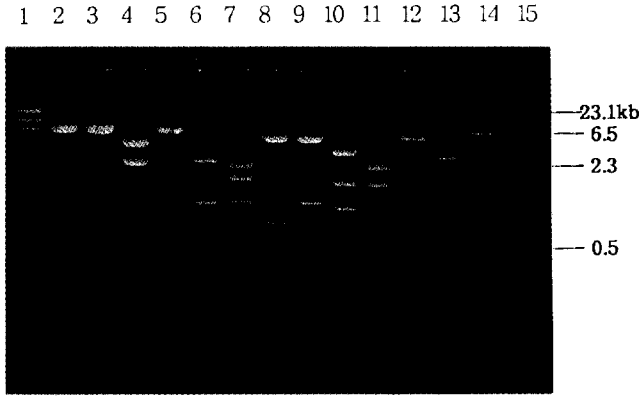

Fig. 3. Digestion profiles of recombinant plasmid pCO2 by various endonucleases. Lamda DNA was digested with *Hind*III(lane 1, 15), and pCO2 with *Bam*HI(lane 2), *Xho*I(lane 3), *Pvu*II(lane 4), *Kpn*I (lane 5), *Hind*III/*Sca*I(lane 6), *Eco*RI/*Sca*I(lane 7), *Hind*III/*Xho*I (lane 8), *Hind*III(lane 9), *Eco*RI/*Pvu*II(lane 10), *Sca*I(lane 11), *Pvu*II/*Xho* I (lane 12), *Eco*RI(lane 13), and *Cla*I(lane 14), respectively.

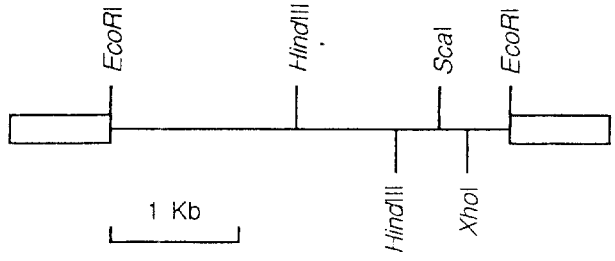

Fig. 4. Physical map of the recombinant plasmid pCO2.

*Bam*HI, *Kpn*I, and *Cla*I sites were detected inside the insert fragment. On the contrary, two *Eco*RI and *Hind*III sites were found in this fragment, whereas *Xho*I and *Sca*I have a single restriction site, respectively.

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