The Journal of Microbiology, June 2003, p.102-108
Vol. 41, No. 2

Cloning and Sequence Analysis of Two Catechol-degrading Gene Clusters from a Phenol-utilizing Bacterium *Pseudomonas putida* SM25

Young-Hee Jung[1], Jong-Ok Ka[2], Choong-Ill Cheon[1], Myeong-Sok Lee[1], Eun-Sook Song[1], Soon-Young Choi[1], Daeho Cho[1], Sang-Ho Choi[3], Kwon-Soo Ha[4], Young Mok Park[5], Jong-Soon Choi[5], and Kyung-Hee Min[1]*

[1]Department of Biological Science, Sookmyung Women's University, Yongsan-ku, Seoul 140-742, Korea
[2]Department of Agricultural Biology, Seoul National University, Suwon 441-744, Korea
[3]Department of Food Science & Technology, Chunnam National University, Gwangju 500-757, Korea
[4]Department of Molecular Cellular Biochemistry, Kangwon National University School of Medicine, Kangwon-do, 200-701
[5]Korea Basic Science Institute, Daejeon 305-333, Korea

(Received February 10, 2003 / Accepted April 1, 2003)

A 6.1 kb *Sph* I fragment from the genomic DNA of *Pseudomonas putida* SM 25 was cloned into the vector pUC19. The open reading frame of *catB* was found to consist of 1,122 nucleotides. The sequence alignment of the *catB* gene products from different kinds of bacteria revealed an overall identity ranging from 40 to 98%. The *catC* gene contained an open reading frame of 96 codons, from which a protein with a molecular mass of about 10.6 kDa was predicted. The amino acids in the proposed active-site region of CatC were found to be almost conserved, including the charged residues. Since the *catBC* genes in *P. putida* SM25 were tightly linked, they could be regulated under coordinate transcription, and transcribed from a single promoter located upstream of the *catB* gene, as in *P. putida* RB1.

Key words: Catechol degradation, *catB* and *catC* genes, cloning and sequence analysis

Catechol ring cleavage reactions are catalyzed by two types of dioxygenases, intradiol and extradiol. β-ketoadipate pathway enzymes, including intradiol dioxygenase, are essential for the conversion of catechol to succinate and acetyl-coenzyme A via the intermediate β-ketoadipate. The structural and regulatory genes encoding these enzymes have been extensively studied in *Pseudomonas spp.* and *Acinetobacter calcoaceticus* (Wheelis and Stainer, 1970; Wheelis and Ornston, 1972; Wu *et al.*, 1972; Shanley *et al.*, 1986; Frantz and Chakrabarty, 1987; Frantz *et al.*, 1987; Aldrich and Chakrabarty, 1988).

It has also been reported that cloned *catB* and *catC* genes and the *catBC* operon encode *cis, cis*-muconate lactonizing enzyme and muconolactone isomerase, respectively (Shanley *et al.*, 1986; Aldrich *et al.*, 1987; Aldrich and Chakrabarty, 1988; Kukor *et al.*, 1988; Houghton *et al.*, 1995; Kim *et al.*, 1997a; Kim *et al.*, 1997b; Eulberg *et al.*, 1998; Murakami *et al.*, 1999; Ogawa and Miyashita, 1999).

The *catB* and *catC* genes are coordinately controlled and tightly linked in *P. putida* (Aldrich and Chakrabarty, 1988). In addition, these genes are clustered on the chromosome with the regulatory genes for catechol pathways, which give rise to catechol in *P. putida* (Wheelis and Ornston, 1972; Shanley *et al.*, 1986).

Pseudomonas putida catBC genes are expressed in response to *cis, cis*-muconate (Ornston, 1966). It has also been shown that CatR is a positive transcriptional activator of the *catBC* operon when *P. putida* is grown in the presence of benzoate (Holben *et al.*, 1992; Parsec *et al.*, 1992; Eulberg *et al.*, 1997). The nucleotide sequences of *catB* and *catC* from *P. putida* are of particular interest because they encode proteins with a crystal structure (Goldman *et al.*, 1985; Katti *et al.*, 1989; Houghton *et al.*, 1995). The CatB structure was previously shown to be similar to that of mandelate racemase with a comparable primary structure (Neidhart *et al.*, 1991; Hasson *et al.*, 1998). This finding is significant in metabolic evolution because CatB and mandelate racemase catalyze different reactions in the same metabolic pathway (Petsko *et al.*, 1993). In particular, the CatC protein is especially unusual in that it has five active sites between its protein subunits within a decameric array (Katz *et al.*, 1985; Katti *et al.*, 1989).

Recently, the current authors isolated the *P. putida* SM25 strain which utilizes phenol as a carbon source (Han *et al.*, 1996). Accordingly, the current report describes the cloning of the *catB* and *catC* genes from the chromosomal DNA of *P. putida* SM25 by colony hybridization with a probe. The pBC fragment carrying the genes was compared with restriction endonucleases and sequenced. The

* To whom correspondence should be addressed.
(Tel) 82-2-710-9415; (Fax) 82-2-706-3249
(E-mail) khm9415@sookmyung.ac.kr

amino acid sequences of the *catB* and *catC* gene products were then compared those of corresponding enzymes from other bacterial strains.

Materials and Methods

Bacterial strains and plasmids

The *Pseudomonas putida* SM25 strain was isolated from soils in Korea on a minimal medium containing 5 mM phenol (Han *et al.*, 1996). *Escherichia coli* NM522 was used as the host strain for the transformation with recombinant plasmids and for the expression of the *catB* genes. pUC19 was used as the cloning vector.

Media and growth conditions

M9 minimal medium and a complex medium were prepared as described previously (Kukor *et al.*, 1988; Seol *et al.*, 2001). Ampicillin was used as selective marker for *E. coli* NM522 at a concentration of 50 μg/ml. For the induction of *E. coli* NM522 harboring recombinant plasmid, 0.5 ml of overnight LB precultures were inoculated at 37°C. After induction with 0.4 mM IPTG (isopropylthio-β-D-galactoside) at an optical density of 1.0 at 600 nm, incubation was further continued for 3 h at 30°C.

Preparation of cell extracts

Cells were harvested by centrifugation at 10,000×g for 15 min. The cell pellets were washed twice with 20 mM Tris-HCl (pH 8.0), and then the cells were sonically disrupted using a sonic dismembrator, model 300 (Fisher, USA). Any cellular debris was removed by centrifugation at 100,000×g for 2 hr and the clear supernatant solution obtained was used immediately for the enzyme assay.

Enzyme assays and estimation of protein concentration

Catechol 1,2-dioxygenase activity was determined by the procedure of Hegeman (1966) and its specific activity was expressed as mmole of *cis, cis*-muconate formed per min per mg of protein. The *cis, cis*-muconate lactonizing enzyme was assayed by measuring the decrease in absorbance at 260 nm due to the disappearance of substrate as described previously (Ornston, 1966). The protein concentrations were determined by the method of Bradford (Bradford, 1976) with bovine serum albumin as the standard.

DNA manipulation

The plasmids used in this study were isolated using the alkaline lysis method. The DNA was resolved on a 0.8% agarose gel with a TAE buffer by electrophoresis and visualized by staining with ethidium bromide (Sambrook *et al.*, 1989). The DNA digestion with a restriction enzyme and ligation with T4 ligase were performed according to the supplier's directions (Boehringer Mannheim, Germany). The transformation of the *E. coli* strains with the recombinant plasmids was carried out according to the $CaCl_2$ procedure (Sambrook *et al.*, 1989). A QIAGEN kit (Qiagen, Germany) was used in the plasmid purification for the deletion and sequencing of the cloned DNA. For nucleotide sequencing, a minimal DNA fragment containing the *cat* genes subcloned in a pBluescript II KS(+) vector was deleted at 200 bp intervals using an Erase-a-base system (Promega, USA).

Construction of gene library

The chromosomal DNA of *P. putida* SM25 was purified as described previously (Sambrook *et al.*, 1989). The chromosomal DNA of *P. putida* SM25 (50 μg) was partially digested with *Sph*I. DNA fragments between 5 and 10 kb were then isolated by agarose gel electrophoresis and ligated to the *Sph*I site of pUC19 vector.

Synthesis of oligonucleotide probe and colony hybridization

The DNA primers were synthesized using the conserved region of the *catB* gene from the chromosomal DNA of *P. putida* PRS2000 (Houghton *et al.*, 1995). The hybridization probe was prepared by a PCR using a Takara Thermal Cycle MP. Colony hybridization was performed using an ECL kit (Amersham Biosciences, USA) according to supplier's instructions.

DNA sequencing

The DNA fragment containing the *cat* genes was sequenced by Sanger's method of dideoxynucleotide-mediated chain termination (Sambrook *et al.*, 1989), using a Sequenase Version 2.0, USB sequencing kit (Amersham Biosciences, USA). The specific primers for extension by DNA polymerase were T3 and T7 primers, plus M13 forward and M13 reverse primers. Multiple alignment of the amino acid sequence was carried out using Clustal X program.

Results and Discussion

Cloning and nucleotide sequences of cat genes

In the previous study, the current authors isolated *P. putida* SM25 from phenol-contaminated wastewater in Korea on a minimal medium containing phenol as the sole carbon source (Han *et al.*, 1996). The strain was found to utilize catechol, which was metabolized through the *ortho*-pathway. To further characterize the *cat* genes from *P. putida* SM25, in the present study we undertook to clone the genes on pUC19. Our initial effort involved the selection of a recombinant plasmid containing the *P. putida cat* genes in *E. coli* NM522 using a complementation test with the *cat* mutant of *catB* or *catC* of *P. putida* strains. However, this was not suitable for cloning the genes. Therefore, a *Sph*I digest of chromosomal SM25 DNA was ligated into the *Sph*I site of pUC19. A ligated fragment was introduced into *E. coli* NM522, and subjected to

Table 1. Bacterial strains and plasmids used in this study

Strains or plasmids	Relevant characteristics	Reference
Strains		
Pseudomonas putida SM25	Phenol-utilization, Ap^r	Han *et al.*(1996)
E. coli NM522	*supE thi* (*lac-pro*AB) *hsd*5(r^- m^-) F' *pro*AB *lacI*^{q} 2ΔM15	Promega
BL21(DE3)	F^- *ompT* *hsd*S_B($r_B^-m_B^-$)*gal dcm*(DE3)	Novagen
Plasmids		
pUC19	Ap^r and multicloning sites	Novagen
pBCA	pUC19 with 6.1 kb *Sph*I fragment encoding *catBC*, Ap^r	This study
pBC1	pUC19 with 4.0 kb *Sph*I-*Eco*RI fragment, Ap^r	This study
pBC2	pUC19 with 2.8 kb *Kpn*I-*Eco*RI fragment, Ap^r	This study
pBC3	pUC19 with 2.1 kb *Sph*I-*Pst*I fragment, Ap^r	This study

Ap^r is the resistance to ampicillin (50 μg/m*l*)

Table 2. Enzyme activities of *E. coli* carrying the recombinant plasmid

Enzyme	Specific activities (U/ protein)[a]	
	E. coli NM522	*E. coli* NM522 carrying pBC2
Catechol 1,2-dioxygenase	0.003	0.004
cis, cis-muconate lactonizing enzyme	0.002	0.460

[a]Details on induction and estimation of activities are given in Materials and Methods.

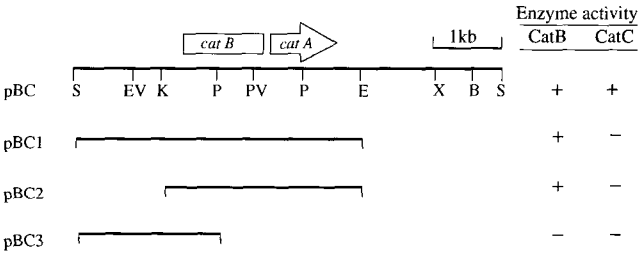

Fig. 1. Physical maps of pBC and its subclones carrying catB and catC. Abbreviations: S, *Sph* I; K, *Kpn* I; P, *Pst* I; E, *EcoR* I; EV, *Eco*RV; PV, *Pvu* I; B, *BamH* I; X, *Xba* I.

ampicillin selection. The selected plasmid, designated pBC, carried an insert of about 6.1 kbp and was screened by colony hybridization using a probe from the *catB* gene.

The resulting recombinant plasmid, pBC (8.8 kb), contained a 6.1 kb *Sph* I fragment carrying the *catBC* genes related to the degradation of catechol. A 4.5 kb fragment of pBC digested with *Sph* I and *Eco*R I was introduced into the polycloning site of the pUC19 vector to generate pBC1. The subcloning of pBC2 and pBC3 was achieved from pBC by digestion with various enzymes, as shown in Fig. 1.

The physical map and *cis, cis*-muconate lactonizing enzyme activities of these subclones are shown in Fig. 1. The *catBC* genes were found to be localized at the 2.1 kb *Kpn* I and *Eco*R I fragment found in pBC, and the precise locations of the *catB* and *catC* genes were determined by testing the activities of the *cis, cis*-muconate lactonizing enzyme and muconolactone isomerase. A more detailed mapping of pBC2 was constructed using restriction enzymes, and the nucleotide sequence of pBC2 was determined.

The nucleotide sequence of *catB* was found to be 1,119 bp, Gene Bank accession number (AY028997). The *catB* gene encoded a polypeptide chain with a molecular mass of 41.4 kDa consisting of 373 amino acid residues. A putative ribosome binding sequence, GGA, was identified 10 bp upstream from the initiation codon of the gene. However, the *catC* gene was initiated 20 bp downstream of the stop codon of the *catB* gene. The *catC* gene encoded a polypeptide with a molecular mass of 10.6 kDa consisting of 96 amino acid residues. A putative ribosome-binding sequence, AAGAAGA, was located about 5 bp upstream from the start codon of the *catC* gene. The GenBank accession number of the *catC* gene is AY028998. Since *catC* has been observed to follow *catB* in the *cat* gene clusters examined so far, the organization of the *catBC* gene was examined in the present study.

Enzyme Activities of cat gene products from pBC2 fragment

The *E. coli* NM522 stains harboring pBC2 expressed both *cis, cis*-muconate lactonizing enzyme activities, but no catechol 1,2-dioxygenase activities, as shown in Table 2. None of these enzymes was expressed in *E. coli* NM522 carrying pUC19 without the insert fragment.

Amino acid homology of cis, cis-muconate lactonizing enzyme and muconolactonone isomerase

The amino acid sequence of the *cis, cis*-muconate lacton-

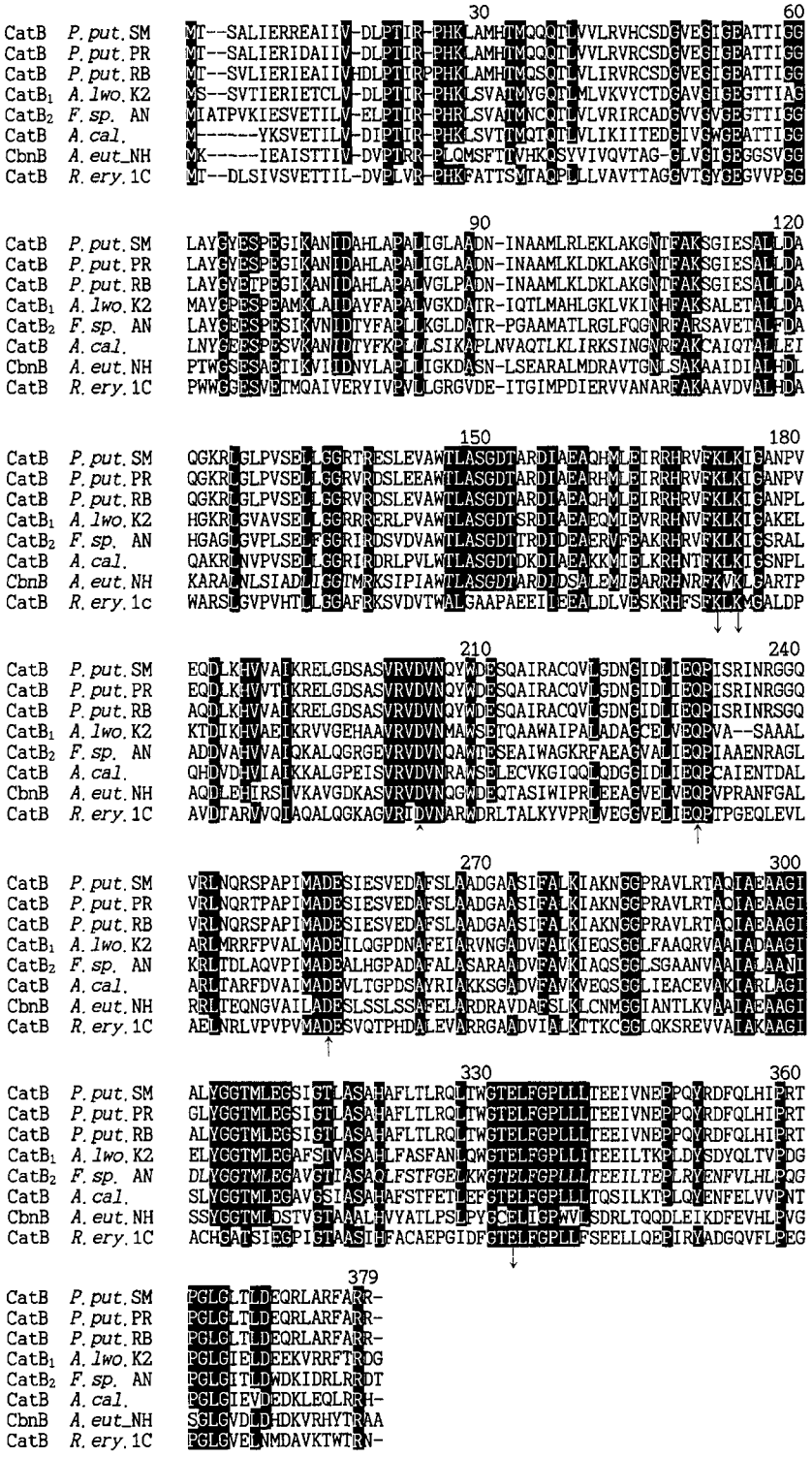

Fig. 2. Alignment of amino acid sequences of muconate lactonizing enzyme (CatB) as calculated by the Clustal X program. The numbers above the sequences refer to positions in the alignment, not single sequences. The amino acid residues in positions with more than seven identical sequences are highlighted. The aspartate and glutamate residues involved in manganese coordination are indicated by arrows pointing upward, whereas the lysine and glutamate residues directly involved in the enzyme mechanism are indicated by arrows pointing downward (Eulberg *et al.*, 1997; Helin *et al.*, 1995). The references and accession numbers in order of the aligned sequences are as follows: *P. put.* SM, *P. putida* SM25 (AY028997); *P. put.* PR, *P. putida* PRS2000 (Houghton *et al.*, 1995; U12557); *P. put.* RB, *P. putida* RB1 (Aldrich and Chakrabarty, 1998; M19460); A. *lwo.* K2, *Acinetobacter lwoffii* K24 (Kim *et al.*, 1997b; U77658); *F. sp.* AN, *Frateuria sp.* ANA18 (Murakami *et al.*, 1999; AB009373); *A. cal., A. calcoaceticus* (Neidle *et al.*, 1988; AF009224); *A. eut.* NH, *Alcaligenes eutrophus* NH9 (Ogawa and Miyashita, 1999; AB10932); *R. ery.* 1C, *Rhodococcus erythropolis* 1CP (Eulbrg *et al.*, 1997; X99622).

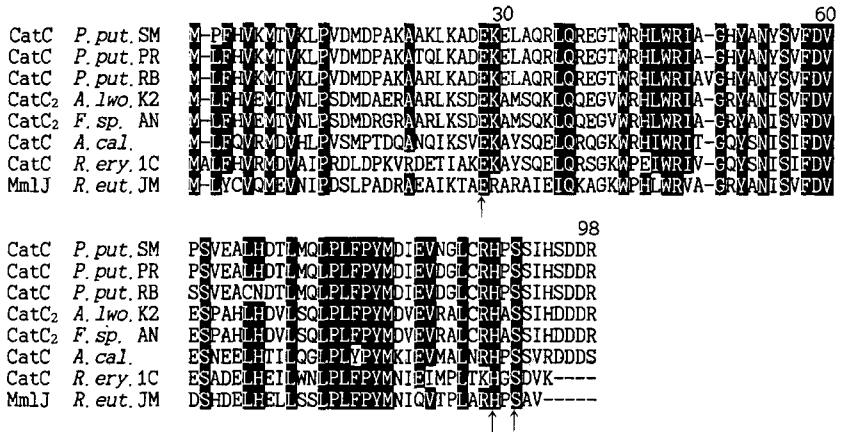

Fig. 3. Alignment of amino acid sequences of muconolactone isomerase (CatC) as calculated by Clustal X program. The numbering above the sequences refers to positions in the alignment, not single sequences. Those amino acids in more than seven identical positions in sequences are highlighted. Amino acid residues previously reported to contribute to the active site are indicated by arrows (Katti *et al.*, 1989; Eulberg *et al.*, 1997). The reference and accession numbers for the published sequences are as follows: *P. put.* SM, *P. putida* SM25 (AY028998); *P. put.* PR, *P. putida* PRS2000 (Houghton *et al.*, 1995; U12557); *P. put.* PB, *P. putida* RBI (Aldrich and Chakrabarty, 1988; M19460); *A. lwo.* K2, *Acinetobacter lwoffii* K24 (Kim *et al.*, 1997b; 033951); *F. sp.* AN, *Frateuria sp.* ANA18 (Murakami *et al.*, 1999; A009373); *A. cal.*, *A. calcoaceticus* (Neidle *et al.*, 1988 AF009224); *R. ery.* IC, *Rhodococcus erythropolis* ICP (Eulberg *et al.*, 1997; X99622); *R. eut.* JM, *R. eutropha* JMP134 (Erb *et al.*, 1988; CAA67959).

izing enzyme (MLE) deduced from the nucleotide sequence was aligned with seven corresponding enzymes reported in other bacterial strains, as shown in Fig. 2. The product of the *catB* gene showed high sequence similarities to known bacterial muconate lactonizing enzymes.

The deduced amino acid sequence of the *cis,cis*-muconate lactonizing enzyme (MLE) produced by *P. putida* SM25 exhibited a 96% identity with that of the enzyme produced by *P. putida* PRS2000 (Houghton *et al.*, 1995), 95% with that of the enzyme produced by *P. putida* RB1 (Aldrich and Chakrabarty, 1988), and 57% with that of the enzyme produced by *Acinetobacter lwoffii* K24 (Kim *et al*,. 1997b). However, its sequence identity was less than 45% when compared to the corresponding enzymes of *R. erythropolis* 1CP (Eulberg *et al.*, 1997), and *Acinetobacter eutrophus* NH9 (Ogawa and Miyashita, 1999).

The three-dimensional structure of MLE was originally solved from a twinned crystal form (Chang and Crawford, 1991) and proved to be similar to that of mandelate racemase. The structure of this crystal form was also successfully refined at high resolution (Helin *et al.*, 1995; Hasson *et al.*, 1998), and the active sites of MLE, madelate racemase, and enolase were compared (Hasson *et al.*, 1998). In the current study, the catabolic residues of MLE were found at conserved positions, Lys-172 and Lys-174, and the homologous residue in Glu-333 appear to play the catalytic role (Hasson *et al.*, 1998), as shown in Fig. 2. Based on a sequence comparison (Eulberg *et al.*, 1997; Eulberg *et al.*, 1998), the Asp-203, Asp-254 and Glu-229 residues, in particular, were found to be involved in manganese coordination.

The deduced amino acid sequence of the muconolactene isomerase produced by *P. putida* SM25 exhibited a 98% identity with that of the enzyme produced by *P. putida* RB1 (Aldrich and Chakrabarty, 1988), and 92% with that of the enzyme produced by *P. putida* PRS2000 (Houghton *et al.*, 1995). However, the homology was less than 55% when compared with the corresponding enzymes of *Rhodococcus erythropolis* 1CP (Eulberg *et al.*, 1997), *Ralstonia eutropha* JMP134 (Erb *et al.*, 1998), and *Acinetobacter calcoaceticus* (Neidle *et al.*, 1988).

CatC is unusual in that it only contains 97 amino acids in its primary structure, and the five active sites of the enzyme are formed between pairs of protein subunits within a decameric array (Katti *et al.*, 1989). In the current study, the putative active site was predicted to be situated at the interface of the subunits. In addition, Glu-29, His-89, and Ser-91 were identified as potential contributing catalytic groups. The amino acid residues in the proposed active-site region were found to be almost completely conserved, including the changed residues at the opening of the pocket. To determine the evolutionary relationship between the *cis, cis*-muconate lactonizing enzyme (MLE) of strain SM25 and corresponding enzymes from other strains, a phylogenetic analysis was carried out based on aligning the amino acid sequences of the enzymes using the CLUSTAL program. A dendrogram showing the homology among the MLE amino acid sequences is shown in Fig. 4. The amino acid sequences of MLEs of *P. putida* SM25, PRS2000, and RB1 more resembled to each other than those of *Alcaligenes eutrophus*, *Acinetobacter* spp., and *Rhodococcus erythropolis*. An earlier alignment of the amino acid sequences of CatB using a dendrogram was also investigated (Eulberg *et al.*, 1997). These findings suggest that MLEs are more closely

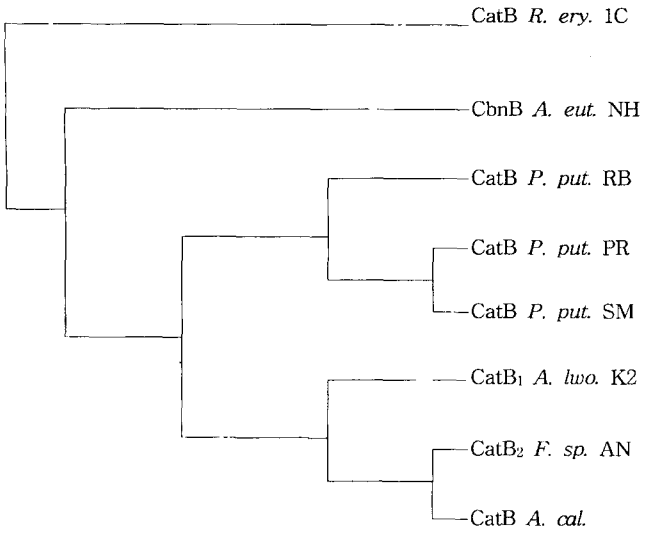

Fig. 4. Dendrograms showing relatedness of the amino acid sequences of cis, cis-muconate lactonizing enzymes. Clustering was performed by the Clustal X program. *R. ery.* IC, *Rhodococcus erythropolis* ICP (Eulberg *et al.*, 1997; X99622); *A. eut.* NH, *Alcaligenes eutrophus* NH9 (Ogawa and Miyashita, 1999; AB10932); *P. put.* RB, *P. putida* RBI (Aldrich and Chakrabarty, 1998; M19460); *P. put.* PR, *P. putida* PRS2000 (Houghton *et al.*, 1995; U12557); *P. put.* SM; *P. putida* SM25 (AY028997); *A. lwo.* K2, *Acinetobacter lwoffii* K24 (Kim *et al.*, 1997b; U77658); *F. sp.* AN; *Frateuria sp.* ANA-18 (Murakami *et al.*, 1999; AB009373); *A. cal, A. calcoaceticus* (Neidle *et al.*, 1988; AF009224).

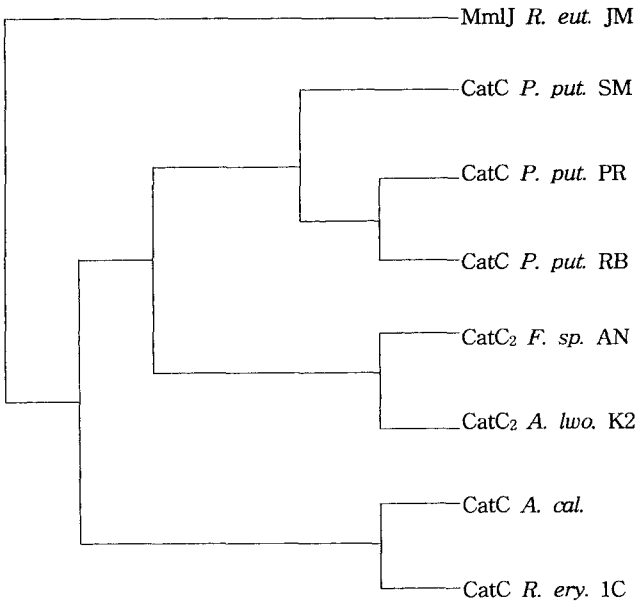

Fig. 5. Dendrograms showing relatedness of amino acid sequences of muconolactene isomerase. Clustering was performed by the Clustal X program. *R. eut.* JM, *Ralstonia eutropha* JMP134 (CAA67959); *P. put.* SM, *Pseudomonas putida* SM25 (AY028998); *P. put.* PR, *Psedomonas putida* PRS2000 (U12557); *P. put.* RB1, *Pseudomonas putida* RB1 (M19460); *F. sp.* AN, *Frateuria sp.* ANA-18 (AB009373); *A. lwo.* K2, *Acinetobacter lwoffii* K24 (O33951); *A. cal., Acinetobacter calcoaceticus* ADP1 (AF009224); *R. ery.* IC, *Rhodococcus erythropolis* ICP (X99622).

related to each other in *P. putida* strains than in other genus species.

The similarity patterns of muconolactone isomerase were found in the dendrograms based on clustering using the program CLUSTAL (Fig. 5). As such, their dendrograms would be expected to have similar branching patterns, thereby implying that one of denograms in Figs. 4 and 5 may not accurately reflect the evolutionary relationships, with the exception of the close relatedness of the cluster of *P. putida* strains.

Acknowledgments

This research was supported by a Sookmyung Women's University Research Grant 2001.

References

Aldrich, T.L. and A.M. Chakrabarty. 1988. Transcriptional regulation, nucleotide sequence, and localization of the promoter of the *catBC* operon in *Pseudomonas putida. J. Bacteriol.* 170, 1297-1304.

Aldrich, T.L., B.J. Frantz, F. Gill, J.J. Kilbane, and A.M. Chakrabarty, 1987. Cloning and complete nucleotide sequence determination of the *catB* gene encoding *cis, cis*-muconate lactonizing enzyme. *Gene* 52, 185-195.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248-254.

Chang, M. and I.P. Crawford. 1991. In vitro determination of the effect of indoleglycerol phosphate on the interaction of purified TrpI protein with its DNA-binding sites. *J. Bacteriol.* 173, 1590-1597.

Ehrt, S., F. Schirmer and W. Hillen. 1995. Genetic organization, nucleotide sequence and regulation of expression of genes encoding phenol hydroxylase and catechol 1,2-dioxygenase in *Acinetobacter calcoaceticus* NCIB8250. *Mol. Microbiol.* 18, 13-20.

Eulberg, D., L.A. Golovleva, and M. Schlomann. 1997. Characterization of catabolic genes from *Rhodococcus erythropolis. J. Bacteriol.* 179, 370-381.

Eulberg, D., E.M. Kourbatova, L.A. Golovleva, and M. Schlomann. 1998. Evolutionary relationship between chlorocatechol catabolic enzymes from *Rhodococcus opacus* 1CP and their counterparts in proteobacteria: sequence divergence and functional convergence. *J. Bacteriol.* 180, 1082-1094.

Erb, R.W., K.N. Timmis, and D.H. Pieper. 1998. Characterization of a gene cluster from *Ralstonia eutropha* JMP134 encoding metabolism of 4-methylmuconolactone. *Gene* 206, 53-62.

Frantz, B. and A.M. Chakrabarty. 1987. Organization and nucleotide sequence determination of a gene cluster involved in 3-chlorocatechol degradation. *Proc. Natl. Acad. Sci. USA* 84, 4460-4464.

Frantz, B., K.L. Ngai, D.K. Chatterjee, L.N. Ornston, and A.M. Chakrbarty. 1987. Nucleotide sequence and expression of *clcD,* a plasmid-born dienelactone hydrolase gene from *Pseudomonas* sp. strain B13. *J. Bacteriol.* 169, 704-709.

Goldman, A., D.L. Ollis, K. Ngai, and T.A. Seitz. 1985. Crystal

structure of muconate lactonizing enzyme at 6.5 resolution. *J. Mol. Biol.* 182, 353-355.

Han, E.M., Y.H. Jung, H.Y. On, M.S. Lee, Y.K. Yang, Y.S. Kim, C.K. Kim, K.S. Lee, and K.H. Min. 1997. Tn*5* tagging of the phenol-degrading gene on the chromosome of *Pseudomonas putida*. *Mol. Cells.* 7, 40-44.

Hasson, M.S., I. Schlichting, J. Moulai, K. Taylor, W. Barrett, G.L. Kenyon, P.C Babbitt, J.A. Gerlt, G.A. Petsko, and D. Ringe. 1998. Evolution of an enzyme active site: the structure of a new crystal form of muconate lactonizing enzyme compared with mandelate racemase and enolase. *Proc. Natl. Acad. Sci. USA.* 95, 10396-10401.

Hegeman, G.D. 1966. Synthesis of the enzymes of the mandelate pathway by *Pseudomonas putida*. I. Syntheis of enzymes by the wild type. *J. Bacteriol.* 91, 1140-1154.

Holben, W.E., B.M. Schroeter, V.G.M. Calabrese, R.H. Olsen, I.K. Kukor, V.O. Biederbeck, A.E. Smith, and J.M. Tiedje. 1992. Gene probe analysis of soil microbial populations selected by amendment with 2,4-dichlorophenoxyacetic acid. *Appl. Environ. Microbiol.* 58, 3941-3948.

Houghton, J.E., T.M. Brown, A.J. Appel, E.J. Hughe, and L.N. Ornston. 1995. Discontinuities in the evolution of *Pseudomonas putida cat* genes. *J. Bacteriol.* 177, 401-412.

Katz, B.A., O. David, and H.W. Wyckoff. 1985. Low resolution crystal structure of muconolactone isommerase a decamer with a 5-fold symmetry axis, *J. Mol. Biol.* 184, 311-318.

Katti, S.K., B. Katz, and H.W. Wyckoff. 1989. Crystal structure of muconolactone isomerase at 3.3 resolution. *J. Mol. Biol.* 205, 557-571.

Kim, S.I., S.H. Lee, J.S. Choi, and K.S. Ha. 1997a. Organization and transcriptional characterization of the cat_I gene cluster in *Acinetobacter lwoffii* K24. *Biochem. Biophys. Res. Comm.* 243, 289-294

Kim, S.I., S.H. Leem, J.S. Choi, Y.H. Chung, S.H. Kim, Y.M. Park, Y.K. Park, Y.N. Lee, and K.S. Ha. 1997b. Cloning and characterization of two *catA* genes in *Acinetobacter lwoffii* K24. *J. Bacteriol.* 179, 5226-5231.

Kukor, J.J., R.H. Olsen, and D.P. Ballon. 1988. Cloning and expression of the *catA* and *catBC* gene cluster from *Pseudomonas aeuroginosa* PAO. *J. Bacteriol.* 170, 4458-4465.

Murakami, S., A. Takashima, J. Takemoto, S. Takenaka, and R. Shinke. 1999. Cloning and sequence analysis of two catechol-degrading gene clusters from the aniline-assimilating bacterium *Frateuria* species ANA-18. *Gene*. 226, 189-198.

Neidhart, D.J., G.L. Kenyon, J.A. Gerlt, and G.A. Petsko. 1991. Mandelate racemase and muconate lactonizing enzyme are mechanistically distinct and structurally homologous. *Nature* 347, 692-694.

Neidle, E.L., C. Hartnett, S. Bonitz, and L.N. Ornston. 1988. DNA sequence of the *Acinetobacter calcoaceticus* catechol 1,2-dioxygenase structural gene *catA* : Evidence for evolutionary divergence of intradiol dioxygenases by acquisition of DNA sequence repetitions. *J. Bacteriol.* 170, 4874-4880.

Ogawa, N. and K. Miyashita. 1999. The chlorocatechol-catabolic transposon Tn*5707* of *Alcaligenes eutrophus* NH9, carrying a gene cluster highly homologous to that in the 1,2,4-trichlorobenzene-degrading bacterium *Pseudomonas* sp. strain P51, confers the ability to grow on 3-chlorobenzoate. *Appl. Environ. Microbiol.* 65, 724-731.

Ornston. L. N. 1966. The conservation of catechol and protocatechuate to β-ketoadipate by *Pseudomonas putida*. IV. Regulation. *J. Biol. Chem.* 241, 3800-3810.

Parsek, M.R., D.L. Shinabarger, R.K. Rothmel, and A.M. Chakrabarty. 1992. Roles of *catR* and *cis,cis*-muconate in activation of the *catBC* operon, which is involved in benzoate degradation in *Pseudomonas putida*. *J. Bacteriol.* 174, 7798-7806.

Petsko, G.A., G.L. Kenyon, J.A. Gerlt, D. Ringe, and J.W. Korzarich. 1993. On the origin of enzymatic species. *Trends Biochem. Sci.* 18, 372-376.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press.

Seol, J.Y., S.Y. Cho, and K.H. Min. 2001. Physical analysis of *nahQ* and *tnpA* genes from *Pseudomonas fluorescens*. *J. Microbiol.* 39, 338-342.

Shanley, M.S., E.L. Neidle, R.E. Parales, and L.N. Ornston. 1986. Cloning and expression of *Acinetobacter calcoaceticus catBCDE* gene in *Pseudomonas putida* and *Escherichia coli*, *J. Bacteriol.* 165, 557-563.

Wheelis, M.L., and L.N. Ornston. 1972. Gene control of enzyme induction in the β-ketoadipate pathway of *Pseudomonas putida*: deletion mapping of *cat* mutations. *J. Bacteriol.* 109, 790-795.

Wheelis, M.L. and R.Y. Stainer. 1970. The genetic control of dissimilartory pathways in *Pseudomonas putida*. *Genetics* 66, 245-266.

Wu, C.H., M.K. Ornston, and L.N. Ornston. 1972. Gene control of enzyme induction in the β-ketoadipate pathway of *Pseudomonas putida*: two-point cross with a regulartory mutant strain. *J. Bacteriol.* 109, 796-802.