# Purification, characterization, and gene cloning of cis,cis-muconate cycloisomerase from benzamide-assimilating Arthrobacter sp. BA-5-17

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**Title:** Purification, characterization, and gene cloning of *cis*,*cis*-muconate cycloisomerase from benzamide-assimilating *Arthrobacter* sp. BA-5-17

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## Abbreviations

CD, catechol 1,2-dioxygenase; CMC, chloro-*cis,cis*-muconate cycloisomerase; MC, *cis,cis*-muconate cycloisomerase; ORF, open reading frame


## Abstract

*cis,cis*-Muconate cycloisomerase (MC) was purified to homogeneity from benzamide-assimilating *Arthrobacter* sp. BA-5-17. The purified enzyme showed high activities for *cis,cis*-muconate and 3-methyl-*cis,cis*-muconate, and preferred the 3-substituted derivatives over the derivatives with the same substituent at the 2 position as a substrate. A gene encoding MC of strain BA-5-17 was cloned and named *catB*. The *catB* gene was clustered with *catR* encoding a putative LysR-type regulator, *catC* encoding a putative muconolactone isomerase, and *catA-II* encoding the catechol 1,2-dioxygenase isozymes CD-III-1 and III-2. These genes showed the same orientations in transcriptional directions and the organization of cloned genes was *catRBCA-II*. In the phylogenetic analysis of MCs and chloro-*cis,cis*-muconate cycloisomerases, the BA-5-17 and *Streptomyces setonii* MCs formed a subfamily, clearly distinguished from those of other MCs.


## 1. Introduction

Catechol is well-known as one of the central intermediates in the metabolisms of aromatic compounds by microorganisms, and metabolized through the *ortho*- or *meta*-cleavage pathway. In the first step of the *ortho*-cleavage pathway of catechol,

catechol is converted to *cis,cis*-muconate through dioxygenation and cleavage of a benzene ring by catechol 1,2-dioxygenase (CD). *cis,cis*-Muconate cycloisomerase (MC) catalyzes the cycloisomerization of *cis,cis*-muconate in the second step of the pathway, and has been purified from many bacteria and characterized [1-4].

*Arthrobacter* sp. BA-5-17 metabolizes benzamide through the *ortho*-cleavage pathway of catechol [5]. We purified four CD isozymes, CD-I, II, III-1, and III-2 from benzamide-induced cells of the bacterium, and characterized them [6]. The purified CD isozymes showed differences in the thermostability, effects of inhibitors on enzyme activity, and absorbance spectra of the enzymes. In particular, the $NH_2$-terminal amino acid sequence analysis indicated that CD-I and II, and III-1 and III-2 were encoded by the same genes. Bacteria-producing CD isozymes have been reported in gram-negative strains [7,8], and two MC genes were found in them [8,9]. Although CDs from *Arthrobacter* sp. BA-5-17 is reported as the first characterized CD isozymes produced by a gram-positive bacterium, nothing is known about MC catalyzing the next step in the bacterium. We, therefore, purified MC from *Arthrobacter* sp. BA-5-17 and cloned a gene encoding the enzyme to clarify the catechol-degrading pathway of this strain.

## 2. Materials and methods

### *2.1. Chemicals*

3-Methylcatechol, 4-methylcatecol, 3-chlorocatecol, 4-chlorocatechol, and 3-fluorocatechol were purchased from Tokyo Kasei Kogyo (Tokyo, Japan), and 4-ethylcatechol was from Avocado (Lancashire, England). Meat extract (Extract

Ehlrich) was from Wako Pure Chemical (Osaka, Japan), and polypepton and dried yeast extract S were from Nihon Seiyaku (Tokyo, Japan). DE52 cellulose was from Whatman (Madison, WI., USA). DEAE-Toyopearl 650S, Phenyl-Toyopearl 650M, and Toyopearl HW-55S were from Toyo Soda MFG (Tokyo, Japan).

*2.2. Bacteria and growth conditions*

*Arthrobacter* sp. BA-5-17 was cultured in benzamide medium [5] at 30°C with shaking. *Escherichia coli* XL1-Blue was cultured in Luria-Bertani medium [10] at 37°C, if necessary, supplemented with ampicillin (100 μg $ml^{-1}$), tetracycline (12.5 μg $ml^{-1}$), isopropyl-β-D(-)-thiogalacto-pyranoside (1 mM), and X-Gal (0.04%). *E. coli* XL1-Blue carrying the plasmid pUC9A with a *cbnA* gene encoding chlorocatechol 1,2-dioxygenase from *Ralstonia eutropha* NH9 [11] was used for the enzymatic conversion of methyl, ethyl, chloro-, and fluoroderivatives of catechol to the corresponding derivatives of *cis,cis*-muconate.

*2.3. Enzyme assay*

*cis,cis*-Muconate was converted from catechol enzymatically and purified as described previously [12]. MC activity was measured spectrophotometrically at 260 nm and 24°C with a reaction mixture which contained 33 mM Tris-HCl (pH 8.0), 0.67 mM $MnCl_2 \cdot 4H_2O$, 0.1 mM *cis,cis*-muconate. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the cycloisomerization of 1 μmol of *cis,cis*-muconate per min. The molar extinction coefficient of 16 800 $M^{-1}$ $cm^{-1}$ was used for *cis,cis*-muconate [13]. Protein concentrations were measured by the method of

Lowry et al [14]. Specific activity was defined as units $mg^{-1}$ protein.

*2.4. Enzyme purification*

All steps of the enzyme purification were carried out at 0-4°C. All centrifugations were 20 000×*g* and 4°C for 10 min.

A wet weight of 17.84 g of *Arthrobacter* sp. BA-5-17 cells was obtained from a 1 200 ml culture in benzamide medium containing 1.0% (w/v) polypepton, 1% (w/v) meat extract, and 1% (w/v) dried yeast extract S. The preparation of the cell extract (step 1, fraction 1) and the streptomycin sulfate treatment to remove nucleic acid from the cell extract solution (step 2, fraction 2) essentially followed previously described methods [15].

Step 3: $(NH_4)_2SO_4$ fractionation. Fraction 2 was brought to 35% saturation with $(NH_4)_2SO_4$. The mixture was stirred for 30 min and centrifuged; the supernatant was collected, and the precipitate was discarded. $(NH_4)_2SO_4$ was added to the supernatant to 50% saturation. After stirring for 30 min, the precipitate was collected by centrifugation and dissolved in 20 mM Tris-HCl (pH 8.0) buffer (buffer A). The solution was dialyzed against 1 000 ml of buffer A with two changes of buffer (fraction 3).

Step 4: DE52 cellulose column chromatography. Fraction 3 was applied to a column (2.3×26 cm) of DE52 cellulose equilibrated with buffer A. Proteins were eluted with a linear gradient (0.1-0.45 M) of NaCl in 1 600 ml of buffer A (flow rate, 60 ml $h^{-1}$). Fractions with the enzyme activity of greater than 0.58 U $ml^{-1}$ were pooled to yield fraction 4.

Step 5: DEAE-Toyopearl 650S column chromatography. Fraction 5 was dialyzed against 1 000 ml of buffer A and applied to a column (1.5×14 cm) of

DEAE-Toyopearl 650S equilibrated with buffer A. Proteins were eluted with a linear gradient (0.15-0.4 M) of NaCl in 600 ml of buffer A (flow rate, 80 ml $h^{-1}$). Fractions with the enzyme activity of greater than 1.0 U $ml^{-1}$ were pooled to yield fraction 5.

Step 6: Phenyl-Toyopearl 650M column chromatography. Fraction 6 was brought to 35% saturation with $(NH_4)_2SO_4$ and applied to a column (1.5×10 cm) of Phenyl-Toyopearl 650M equilibrated with buffer A containing 35% saturation of $(NH_4)_2SO_4$. Proteins were eluted with a linear gradient (35-0% saturation) of $(NH_4)_2SO_4$ in 500 ml of buffer A (flow rate, 80 ml $h^{-1}$). The enzyme purity in each fraction with the enzyme activity of greater than 2.7 U $ml^{-1}$ was verified by PAGE [16]. Fractions showing a single protein band on the gel were pooled (fraction 6).

### *2.5. Determination of molecular masses*

The molecular mass of the native enzyme was determined by gel filtration on Toyopearl HW-55S, and that of the enzyme subunit was measured using SDS-PAGE [17]. Size markers used for the gel filtration were those in a calibration protein gel chromatography kit from Boehringer Mannheim (Mannheim, Germany). The electrophoresis calibration kit LMW (Amersham Bioscience) was used as markers for the SDS-PAGE.

### *2.6. Substrate specificity*

Methyl, ethyl, chloro-, and fluoroderivatives of *cis,cis*-muconate were enzymatically synthesized with chlorocatechol 1,2-dioxygenase (0.02 U for

catechol as a substrate) from *R. eutropha* NH9 in reaction mixtures containing 33 mM Tris-HCl (pH 8.0) and 0.103 mM catechol derivatives at 24°C. After conversion of catechol derivatives, reaction mixtures were diluted, and 20 mM $MnCl_2 \cdot 4H_2O$ was added. The final preparations of reaction mixtures, containing 0.67 mM $MnCl_2 \cdot 4H_2O$ and authentic *cis,cis*-muconate or *cis,cis*-muconate derivatives at a range of 0.025 to 0.1 mM, were used for the determination of kinetic parameters of the purified enzyme. The following molar extinction coefficients were used: 17 100 $M^{-1}$ $cm^{-1}$ for 2-methyl-*cis*-*cis*-muconate, 12 400 $M^{-1}$ $cm^{-1}$ for 3-methyl-*cis*-*cis*-muconate, 18 000 $M^{-1}$ $cm^{-1}$ for 2-chloro-*cis*-*cis*-muconate, 13 900 $M^{-1}$ $cm^{-1}$ for 3-chloro-*cis*-*cis*-muconate, and 14 900 $M^{-1}$ $cm^{-1}$ for 2-fluoro-*cis*-*cis*-muconate [13]. The molar extinction coefficient of 3-ethyl-*cis,cis*-muconate was established as 12 100 $M^{-1}$ $cm^{-1}$. $K_m$ and $V_{max}$ values of the purified enzyme were calculated by nonlinear regression with the Enzfitter program (Biosoft, Cambridge, United Kingdom).

### *2.7. Determination of $NH_2$-terminal amino acid sequence*

The $NH_2$-terminal amino acid sequence of the purified MC was determined as previously described [3].

### *2.8. Gene manipulation, gene cloning, and nucleotide sequence analysis*

Standard methods were used for the plasmid DNA purifications, restriction enzyme digestions, and *E. coli* transformations [10]. Subcloning experiments were performed in pBluescript vectors (Stratagene). The purification of the total DNA and construction of a gene library of *Arthrobacter* sp. BA-5-17 were performed as

previously described [18]. Cell materials from each *E. coli* consisting of the gene library were fixed on a Hybond-N+ membrane (Amersham Biosciences) according to the manufacturer's instructions. A synthesized nucleotide, 5'-ATGAA(AG)AT(TCA)GA(AG)(CA)GIAT-3', corresponding to the determined $NH_2$-terminal amino acid sequence of MKIERI, was radiolabeled as previously described [9]. A transformant showing a positive signal was selected by colony hybridization using the radiolabeled probe under standard conditions [10], and a recombinant plasmid, named p29D10, was isolated from the transformant. A 4.7-kb region in the insert DNA (9.0 kb) of p29D10 (Fig. 1) was sequenced using subcloned fragments as described previously [9]. The computer analyses of cloned genes and deduced amino acid sequences were accomplished through the use of the FASTA and BLAST database searching programs, respectively, at the DNA Data Bank of Japan. A multiple sequence alignment was performed by CLUSTALW 1.7 at the DNA Data Bank of Japan. A phylogenetic tree was obtained from CLUSTALW 1.7 and the software TreeView 1.6.6 supplied on the Internet. The DDBJ/EMBL/GenBank accession number for the reported sequence in this paper is AB109791.

## 3. Results and discussion

### *3.1 Purification and properties of the purified enzyme*

MC from benzamide-assimilating *Arthrobacter* sp. BA-5-17 was purified 471-fold from 17.84 g (wet weight) of cells with an overall yield of 19% (Table 1). The purified enzyme exhibited a single protein band on both the native and

denaturing polyacrylamide gels (data not shown). The molecular mass was determined to be 280 kDa by gel filtration and 43 kDa by SDS-PAGE (data not shown).

The purified enzyme showed optimal pH at pH 7.5, and retained more than 70 % activity after incubation of the enzyme (0.085 mg/ml) for 24 h at 4°C in 20 mM phosphate buffer (pH 7.0-7.5), 20 mM Tris-HCl (pH 7.5-9.5), and 20 mM carbonate buffer (pH 9.5-11.0). The enzyme (0.085 mg/ml) retained more than 90% activity against incubating at 65°C for 10 min in 20 mM Tris-HCl (pH 8.0), but lost the activity by incubation at 70°C for 10 min. The purified enzyme showed 25% relative activity in a reaction mixture without $Mn^{2+}$ compared with that in the standard reaction mixture containing $Mn^{2+}$. When various bivalent metal ions in place of $Mn^{2+}$ were added into the reaction mixture as a cofactor at a concentration of 0.67 mM, the enzyme showed 56% relative activity in a reaction mixture containing $Mg^{2+}$ for that under the standard conditions. No activities were observed in a reaction mixture containing $Co^{2+}$, which is reported as a metal cofactor that is available for the catalysis of MC from Gram-positive *Rhodococcus erythropolis* AN-13 [3]. The $NH_2$-terminal amino acid sequence of the enzyme was determined to be MKIERIEAIPYSIPYAKPLKFA.

Table 2 shows substrate specificity of the purified enzyme. Turnover numbers ($k_{cat}$) for methyl derivatives were lower than that for *cis,cis*-muconate. However, a relative specific constant ($k_{cat}/K_m$) for 3-methyl-*cis,cis*-muconate was the similar values for *cis,cis*-muconate because $K_m$ value for 3-methyl-*cis,cis*-muconate was lower than that for *cis,cis*-muconate. On the other hand, turnover number for 2-methyl-*cis,cis*-muconate was so low that a relative specific constant for 2-methyl-*cis,cis*-muconate was extremely low in contrast to that for 3-methyl-*cis,cis*-muconate. This, therefore, is the first report describing the

purification of MC showing high activities for both 3-methyl-*cis,cis*-muconate and *cis,cis*-muconate. The enzyme preferred 3-chloro-*cis,cis*-muconate to 2-chloro-*cis,cis*-muconate as a substrate as well as methyl derivatives although relative specific constants for the chlorinated derivatives were lower than those for methyl derivatives. The enzyme also catalyzed the cycloisomerization of 3-ethyl-*cis,cis*-muconate.

### *3.2. Cloning of genes encoding MC and other enzymes involved in catechol degradation*

A positive clone was selected by colony hybridization using a nucleotide probe designed from the $NH_2$-terminal amino acid sequence of the purified MC, and the isolated plasmid was named p29D10. We found 4 open reading frames (ORF) with the same orientations in a 4 744 bp sequence, which was determined in the 9.0 kb insert DNA of the plasmid p29D10 (Fig. 1).

The second ORF encoded 380 amino acid residues, and an $NH_2$-terminal sequence deduced from the ORF corresponded with that of the purified MC. The molecular mass of the deduced amino acid sequence was 40 926 Da, and was similar to that of the subunit size of 43 kDa determined by SDS-PAGE. These results show that the second ORF was a gene encoding MC from *Arthrobacter* sp. BA-5-17, and the ORF was named *catB*. The deduced amino acid sequence of MC encoded by *catB* shared 54% identical positions with that of putative MC from *Streptomyces setonii* (Accession No. AF435013), and less than 35% identical positions with those of the previously characterized MCs and CMCs. However, the amino acid residues, which are involved in manganese coordination or in the enzymatic mechanism of cycloisomerization [19,20], are conserved in aligned

sequences containing the BA-5-17 MC (Fig. 2).

The fourth ORF consisted of 849 bp, and the deduced amino acid sequence of the ORF contained the $NH_2$-terminal sequences of CD isozymes, CDIII-1 and III-2, purified in the previous study [6]. The molecular mass of the deduced amino acid sequence of the fourth ORF was calculated to be 31 137 Da, and was similar to those of the subunit sizes of 33 kDa [6]. *Arthrobacter* sp. BA-5-17 produced CD-I and II with identical $NH_2$-terminal sequences, which probably transcribed from a gene [6]. In consideration of the gene encoding CD-I and CD-II, we named the fourth ORF *catA-II* encoding CD-III-1 and III-2. The amino acid sequence deduced from *catA-II* showed 79 and 61% identical positions with those of the CDs from gram-positive bacteria, *Arthrobacter* sp. mA3 (AJ000187) and *Rhodococcus opacus* 1CP (X99622), respectively, and 66% identical positions with that of putative CD from *Streptomyces setonii* (Accession No. AF435013).

The first and third ORFs consisted of 792 and 279 bp, respectively. The deduced amino acid sequence of the first ORF showed 41 and 26 % identical positions with those of putative LysR-type regulators, CatR from *S. setonii* (AF435013) and *Ralstonia eutropha* (AF042281), and 36% identical positions with that of the LysR-type regulator CbeR from *Burkholderia* sp. NK8 (AB024746). The product of the third ORF showed 69-71% identical positions with those of putative muconolactone isomerases encoded by *catC* from *Burkholderia* sp. NK8 (AB024746), *catC1* from *Burkholderia* sp. TH2 (AB035483), and *catC1* from *Ralstonia eutropha* 335 (AF042281).

### *3.3. Phylogenetic analysis of MSs and CMCs*

Fig. 3 shows a phylogenetic tree of the MCs and CMCs. The MCs and CMCs

from gram-negative bacteria except TfdDII from *Ralstonia eutropha* JMP134 are classified into each subfamily as described by Moiseeva et al [21]. The gram-positive *Arthrobacter* sp. BA-5-17 and *S. setonii* MCs were localized in the same branch, clearly distinguished from other branches containing MCs from gram-positive bacteria, *Rhodococcus opacus* 1CP and *R. erythropolis* AN-13. The *Rhodococcus* MCs show lower activity for 3-methyl-*cis,cis*-muconate compared with that for *cis,cis*-muconate [2,3]. Furthermore, MC from *R. erythropolis* AN-13 showed higher activity in a reaction mixture containing $Co^{2+}$ ion in place of $Mn^{2+}$ ion than that in the standard reaction mixture containing $Mn^{2+}$ [3]; the BA-5-17 MC, on the contrary, didn't catalyzed the cycloisomerization of catechol in the reaction mixture containing $Co^{2+}$ ion. Thus, the BA-5-17 MC differed in substrate specificity and catalytic property from reported MCs of gram-positive bacteria in addition to the difference in the subfamily.

*Arthrobacter* sp. BA-5-17 produces four CD isozymes, encoded by two different genes under growth conditions used for the MC purification [6]. However, A gene encoding CD isozymes, CD-I and CD-II, was not found in the cloned fragment. Further genetic studies are needed to clarify catechol-degrading gene cluster of this bacterium.

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## References


[1] Ornston, N.L. (1966) The conversion of catechol and protocatechuate ro β-ketoadipate by Pseudomonas putida. III. Enzymes of the catechol pathway. J. Biol. Chem. 241, 3795-3799.

[2] Solyanikova, I.P., Maltseva, O.V., Vollmer, M.D., Golovleva, L.A. and Schlömann, M. (1995) Characterization of muconate and chloromuconate cycloisomerase from *Rhodococcus erythropolis* 1CP: indications for functionally convergent evolution among bacterial cycloisomerases. J. Bacteriol. 177, 2821-2826.

[3] Murakami, S., Takemoto, J., Takenaka, S., Shinke, R. and Aoki, K. (1998) Purification and characterization of muconate cycloisomerase from aniline-assimilating *Rhodococcus erythropolis* AN-13. J. Ferment. Bioeng. 85, 521-524.

[4] Murakami, S., Takemoto, J., Takashima, A., Shinke, R. and Aoki, K. (1998) Purification and characterization of two muconate cycloisomerase isozymes from aniline-assimilating *Frateuria* species ANA-18. Biosci. Biotechnol. Biochem. 62, 1129-1133.

[5] Murakami, S., Nakanishi, Y., Shinke, R. and Aoki, K. (1991) Catechol 1,2-dioxygenase isozymes in soil bacteria metabolizing aromatic compounds. Soil Biol. Biochem. 23, 815-819.

[6] Murakami, S., Wang, C.L., Naito, A., Shinke, R. and Aoki, K. (1998) Purification and characterization of four catechol 1,2-dioxygenase isozymes from the benzamide-assimilating bacterium *Arthrobacter* species BA-5-17. Microbiol. Res. 153, 163-171.

[7] Aoki, K., Konohana, T., Shinke, R. and Nishira, H. (1984) Two catechol

1,2-dioxygenase from an aniline-assimilating bacterium, *Frateuria* sp. ANA-18. Agric. Biol. Chem. 48, 2097-2104.

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

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

[10] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

[11] Liu, S., Ogawa, N. and Miyashita, K. (2001) The chlorocatechol degradative genes, *tfdT-CDEF*, of *Burkholderia* sp. strain NK8 are involved in chlorobenzoate degradation and induced by chlorobenzoates and chlorocatechols. Gene 268, 207-214.

[12] Murakami, S., Hayashi, T., Maeda, T., Takenaka, S. and Aoki, K. (2003) Cloning and functional analysis of aniline dioxygenase gene cluster, from *Frateuria* species ANA-18, that metabolizes aniline via an *ortho*-cleavage pathway of catechol. Biosci. Biotechnol. Biochem. 67, 2351-2358.

[13] Dorn, E. and Knackmuss, H.-J. (1978) Chemical structure and biodegradability of halogenated aromatic compounds. Substitution effects on 1,2-dioxygenation of catechol. Biochem. J. 174, 85-94.

[14] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.

[15] Aoki, K., Konohana, T., Shinke, R. and Nishira, H. (1984) Purification and

characterization of catechol 1,2-dioxygenase from aniline-assimilating *Rhodococcus erythropolis* AN-13. Agric. Biol. Chem. 48, 2087-2095.

[16] Davis, B.J. (1964) Disc electrophoresis. II. Method and application to human serum proteins. Ann. N. Y. Acad. Sci. 121, 404-427.

[17] Weber, K. and Osborn, M. (1969) The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 247, 2358-2370.

[18] Murakami, S., Kodama, N., Shinke, R. and Aoki, K. (1997) Classification of catechol 1,2-dioxygenase family: sequence analysis of a gene for the catechol 1,2-dioxygenase showing high specificity for methylcatechols from gram+ aniline-assimilating *Rhodococcus erythropolis* AN-13. Gene 185, 49-54.

[19] Helin, S., Kahn, P.C., Guha, B.L., Mallows, D.G. and Goldman, A. (1995) The refined X-ray structure of muconate lactonizing enzyme from *Pseudomonas putida* PRS2000 at 1.85 Å resolution. J. Mol. Biol. 254, 918-941.

[20] Schell, U., Helin, S., Kajander, T., Schlömann, M. and Goldman, A. (1999) Structural Basis for the activity of two muconate cycloisomerase variants toward substituted muconate. Proteins 34, 125-136.

[21] Moiseeva, O.V., Solyanikova, I.P., Kaschabek, S.R., Gröning, J., Thiel, M., Golovleva, L.A. and Schlömann, M. (2002) A new modified *ortho* cleavage pathway of 3-chlorocatechol degradation by *Rhodococcus opacus* 1CP: genetic and biochemical evidence. J. Bacteriol. 184, 5282-5292.

Table 1

Purification of *cis,cis*-muconate cycloisomerase from *Arthrobacter* sp. BA-5-17

| Fraction[a] | Total activity (U) | Total protein (mg) | Specific activity (U/mg) | Recovery (%) |
|---|---|---|---|---|
| 1: Cell extract | 260 | 3000 | 0.087 | 100 |
| 2: Streptomycin sulfate | 260 | 3000 | 0.087 | 100 |
| 3: Ammonium sulfate | 170 | 840 | 0.20 | 65 |
| 4: DE52 | 140 | 40 | 3.5 | 54 |
| 5: DEAE-Toyopearl 650S | 110 | 4.5 | 24 | 42 |
| 6: Phenyl-Toyopearl 650S | 49 | 1.2 | 41 | 19 |

[a] Fractions 1-6 refer to the fractions obtained as the end of steps 1-6 of the purification procedure.

Table 2

Substrate specificity of the *cis,cis*-muconate cycloisomerase from *Arthrobacter* sp. BA-5-17

| Substrate | $K_m$ ($\mu$M)[a] | $V_{max}$ (U·mg$^{-1}$)[a] | $k_{cat}$ (min$^{-1}$)[b] | $k_{cat}/K_m$ (min$^{-1}$·$\mu$M$^{-1}$) |
|---|---|---|---|---|
| *cis,cis*-Muconate | 150±19 | 77±6.6 | 3,300 | 22 |
| 2-Methyl-*cis,cis*-muconate | 29±5.2 | 1.0±0.086 | 43 | 1.5 |
| 3-Methyl-*cis,cis*-muconate | 75±8.9 | 41±5.8 | 1,800 | 24 |
| 3-Ethyl-*cis,cis*-muconate | 670±32 | 220±21 | 9,500 | 14 |
| 2-Chloro-*cis,cis*-muconate | | (<0.02)[c] | | |
| 3-Chloro-*cis,cis*-muconate | 46±5.5 | 3.9±0.10 | 170 | 3.7 |
| 2-Fluoro-*cis,cis*-muconate | | (<0.02)[c] | | |

[a] $K_m$ and $V_{max}$ values were calculated by nonlinear regression with the program Enzfitter, and are indicated as the mean of five determinations ± SD.

[b] The $K_{cat}$ values were calculated on the basis of a subunit molecular mass of 43 kDa.

[c] Detection limit for specific activity under the experimental conditions (e.g., at a substrate concentration of 0.1 mM)

**Figure legends**

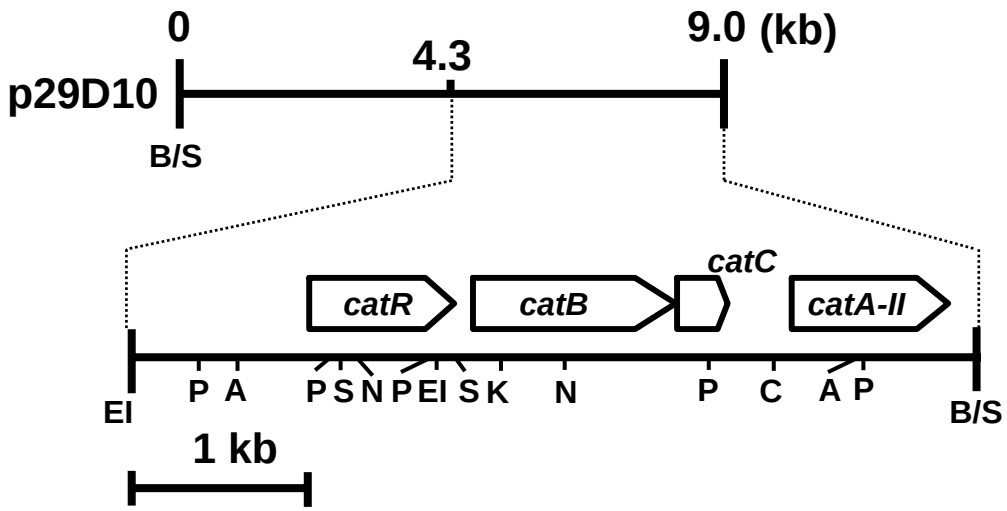
Fig.1. Restriction map of a sequenced region in the insert of p29D10 and cloned genes. Open arrows show ORFs and directions of their translation. Abbreviations; A, *Apa*I site; B/S, a site where *Bam*HI and *Sau*3AI sites are ligated; C, *Cla*I site; EI, *Eco*RI site; K, *Kpn*I site; N, *Not*I site; P, *Pst*I site; S, *Sac*I site.

Fig. 2. Sequence alignment of CatB from *Arthrobacter* sp. BA-5-17 (A) with MC from *S. setonii* (B) and MC from *Rhodococcus opacus* 1CP (C). Asterisks indicate positions where all amino acid residues are identical. The conserved amino acid residues, which are involved in manganese coordination or in the enzymatic mechanism of cycloisomerization, are indicated in squares.

Fig. 3. A phylogenetic tree of MCs and CMCs. Accession numbers for the published sequences are as follows: ClcBI from *R. opacus* 1CP, AF003948; ClcBII from *R. opacus* 1CP, AJ439407; TcbD from *Pseudomonas* sp. P51, M57629; TfdD from *R.eutropha* JMP134, M31458; TfdD from *Burkholderia* sp. NK8, AB050198; $CatB_1$ from *Frateuria* sp. ANA-18, AB009343; CatB from *Acinetobacter* sp. ADP1, AF009224; CatB2 from *Burkholderia* sp. TH2, AB035325; $CatB_2$ from *Frateuria* sp. ANA-18, AB009373; CatB from *Burkholderia* sp. NK8, AB024746; CatB from *R. eutropha* 335T, AF042281; CatB from *Pseudomonas* sp. CA10, AB047272; CatB from *P. putida* PRS2000, U12557; TfdDII from *R. eutropha* JMP134, U16782; CatB from *Arthrobacter* sp. BA-5-17, AB109791; CatB from *S. setonii*,

AF435013; CatB from *R. opacus* 1CP, X99622; CatB from *R. erythropolis* ANA-18, D83237.

Fig. 1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B/S
catC
catR
catB
catA-II
EI
P A
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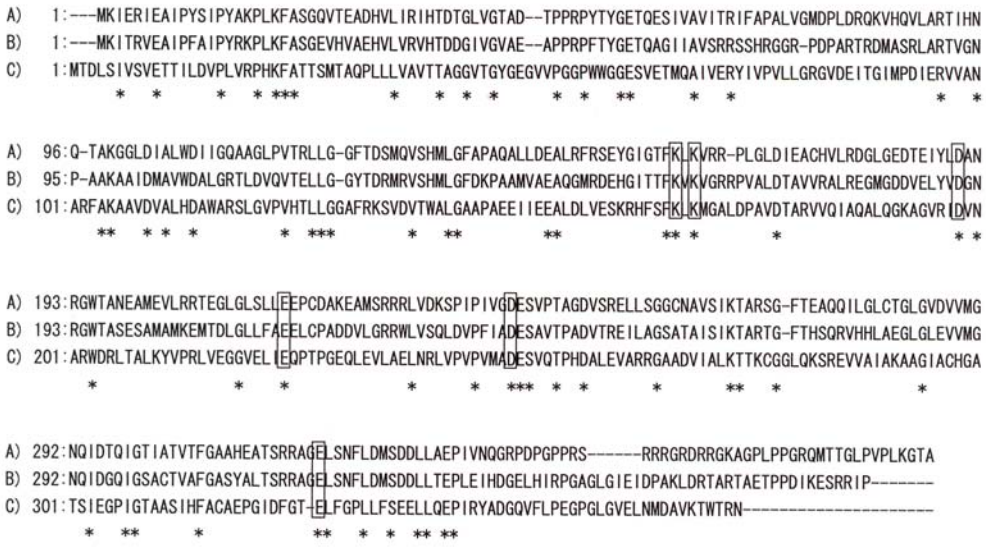
N
P
C
A P
B/S
1 kb

Fig. 2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B) 1:---MKITRVEAIPFAIPYRKPLKFASGEVHVAEHVLVRVHTDDGIVGVAE--APPRPFTYGETQAGIIAVSRRSSHRGGR-PDPARTRDMASRLARTVGN
C) 1:MTDLSIVSVETTILDVPLVRPHKFATTSMTAQPLLLVAVTTAGGVTGYGEGVVPGGPWWGGESVETMQAIVERYIVPVLLGRGVDEITGIMPDIERVVAN
A) 96:Q-TAKGGLDIALWDIIGQAAGLPVTRLLG-GFTDSMQVSHMLGFAPAQALLDEALRFRSEYGIGTFKLKVRR-PLGLDIEACHVLRDGLGEDTEIYLDAN
B) 95:P-AAKAAIDMAVWDALGRTLDVQVTELLG-GYTDRMRVSHMLGFDKPAAMVAEAQGMRDEHGITTFKWKVGRRPVALDTAVVRALREGMGDDVELYVDGN
C) 101:ARFAKAAVDVALHDAWARSLGVPVHTLLGGAFRKSVDVTWALGAAPAEEIIEEALDLVESKRHFSFKLKMGALDPAVDTARVVQIAQALQGKAGVRIDVN
A) 193:RGWTANEAMEVLRRTEGLGLSLLEEPCDAKEAMSRRRLVDKSPIPIVGDESVPTAGDVSRELLSGGCNAVSIKTARSG-FTEAQQILGLCTGLGVDVVMG
B) 193:RGWTASESAMAMKEMTDLGLLFAEELCPADDVLGRRWLVSQLDVPFIADESAVTPADVTREILAGSATAISIKTARTG-FTHSQRVHHLAEGLGLEVVMG
C) 201:ARWDRLTALKYVPRLVEGGVELEQPTPGEQLEVLAELNRLVPVPVMADESVQTPHDALEVARRGAADVIALKTTKCGGLQKSREVVAIAKAAGIACHGA
A) 292:NQIDTQIGTIATVTFGAAHEATSRRAGELSNFLDMSDDLLAEPIVNQGRPDPGPPRS------RRRGRDRRGKAGPLPPGRQMTTGLPVPLKGTA
B) 292:NQIDGQIGSACTVAFGASYALTSRRAGELSNFLDMSDDLLTEPLEIHDGELHIRPGAGLGIEIDPAKLDRTARTAETPPDIKESRRIP-------
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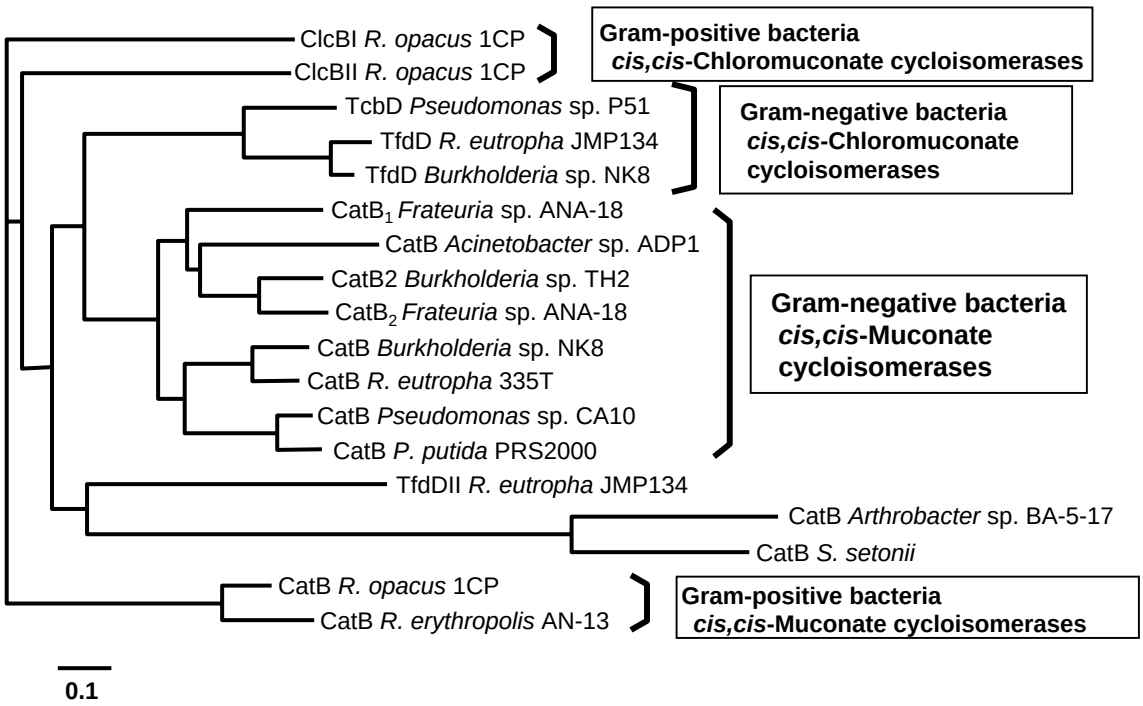
# Fig. 3


ClcBI R. opacus 1CP
ClcBII R. opacus 1CP
Gram-positive bacteria
cis,cis-Chloromuconate cycloisomerases
TcbD Pseudomonas sp. P51
TfdD R. eutropha JMP134
TfdD Burkholderia sp. NK8
Gram-negative bacteria
cis,cis-Chloromuconate
cycloisomerases
CatB1 Frateuria sp. ANA-18
CatB Acinetobacter sp. ADP1
CatB2 Burkholderia sp. TH2
CatB2 Frateuria sp. ANA-18
CatB Burkholderia sp. NK8
CatB R. eutropha 335T
CatB Pseudomonas sp. CA10
CatB P. putida PRS2000
Gram-negative bacteria
cis,cis-Muconate
cycloisomerases
TfdDII R. eutropha JMP134
CatB Arthrobacter sp. BA-5-17
CatB S. setonii
CatB R. opacus 1CP
CatB R. erythropolis AN-13
Gram-positive bacteria
cis,cis-Muconate cycloisomerases
0.1