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Matsumura, Eitaro ; Sakai, Masashi ; Hayashi, Katsuaki ; Murakami, Shuichiro ; Takenaka, Shinji ; Aoki, Kenji

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Constitutive expression of *catABC* genes in the aniline-assimilating bacterium *Rhodococcus* species AN-22: production, purification, characterization and gene analysis of CatA, B and C

Eitaro MATSUMURA*, Masashi SAKAI†, Katsuaki HAYASHI†, Shuichiro MURAKAMI†, Shinji TAKENAKA† and Kenji AOKI†[1]

*Division of Life Science, Graduate School of Science and Technology and †the Laboratory of Applied Microbiology, Department of Biofunctional Chemistry, Faculty of Agriculture, Kobe University, Rokko, Kobe 657-8501, Japan

(SHORT TITLE)

Constitutive CatA, B, C in aniline-assimilating bacterium

Key words: aniline-assimilating bacterium, *Rhodococcus*, constitutive expression, *cis*,*cis*-muconate cycloisomerase, muconolactone isomerase, primer extension analysis

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Abbreviations used: CatA, catechol 1,2-dioxygenase; CatB, *cis,cis*-muconate cycloisomerase; CatC, muconolactone isomerase; CatD, β-ketoadipate enol-lactone hydrolase; CatIJ, β-ketoadipate: succinyl CoA transferase; CatR, regulatory protein of catechol degrading pathway; buffer A, 20 mM Tris-HCl (pH 8.0); and RT-PCR, reverse transcriptase-PCR.

[1]To whom correspondence should be addressed (email: kaoki@kobe-u.ac.jp).

The nucleotide sequences reported in this paper have been submitted to the DDBJ, EMBL and GenBank nucleotide sequence databases with accession number AB167712.

(SYNOPSIS)

The aniline-assimilating bacterium *Rhodococcus* sp. AN-22 was found to constitutively synthesize *cis*,*cis*-muconate cycloisomerase (CatB) and muconolactone isomerase (CatC) in its cells growing on non-aromatic substrates, in addition to the previously reported catechol 1,2-dioxygenase (CatA). The bacterium maintained the specific activity of the three enzymes at an almost equal level during cultivation on succinate. CatB and C were purified to homogeneity and characterized. CatB was a monomer with the molecular mass of 44 kDa. The enzyme was activated by Mn^{2+}, Co^{2+} and Mg^{2+}. Native CatC was a homooctamer with the molecular mass of 100 kDa. The enzyme was stable between pHs 7.0 and 10.5 and resistant to heating up to 90°C. Genes coding for CatA, B and C were cloned and named *catA*, *B* and *C*, respectively. The *catABC* genes were transcribed as one operon. The deduced amino acid sequences of CatA, B and C showed high identities with those from other gram-positive microorganisms. A regulator gene such as *catR* encoding a regulatory protein was not observed around the *cat* gene cluster of *Rhodococcus* sp. AN-22, but a possible relic of *catR* was found in the upstream region of *catA*. Reverse transcriptase-PCR and primer extension analyses showed that the transcriptional start site of the *cat* gene cluster was located 891 bp upstream of the *catA* initiation codon in the AN-22 strain growing on both aniline and succinate. Based on these data, we concluded that the bacterium constitutively transcribed the *catABC* genes and translated its mRNA into CatA, B and C.

INTRODUCTION

Aniline is a major industrial raw material and an intermediate used for the production of dyes, plastics, herbicides, etc. Studies of the biodegradation of aniline as an environmental pollutant were made by Lyons *et al*. [1]. We have isolated many bacteria [2] from soil that grew on aniline as the sole carbon, energy and nitrogen source, and have investigated their metabolic

pathways [3], enzyme and gene systems [4–7]. It was revealed that all the isolated bacteria degrade aniline via catechol, which is then metabolized by the enzymes encoded by the *cat* gene cluster [8].

Through these investigations, we found that the aniline-assimilating bacterium *Rhodococcus* sp. AN-22 constitutively synthesized catechol 1,2-dioxygenase (CatA, EC 1.13.11.1) in cells grown on a medium without aniline [9], as well as on aniline medium, although other aniline-assimilating bacteria metabolized aniline with inducible enzymes including CatA [10]. CatA is a key enzyme in the *ortho*-cleavage pathway of aromatic compounds by microorganisms. This enzyme catalyzes the ring fission of catechol with the consumption of one mol of O_2 per mol of substrate and produces *cis*,*cis*-muconate. A variety of CatAs are distributed to a large extent in microorganisms metabolizing aromatic compounds through the *ortho*-cleavage pathway. Almost all the previously reported enzymes are inducible.

Some reports have suggested the existence of the constitutive CatAs in the 3-aminophenol-assimilating bacterium *Arthrobacter* sp. mA3 [11], a *p*-nitrophenol-assimilating phototrophic bacterium [12] and a phenol-assimilating bacterium *Acinetobacter radioresistens* S13 [13]. However, the level of constitutive synthesis of the CatAs was markedly low and the constitutive enzyme production was not characterized. On the other hand, aniline-assimilating *Rhodococcus* sp. AN-22 produced more CatA on 11 non-aromatic substrate media, such as sugars and amino and organic acids, than that on the aniline medium. The constitutive enzyme CatA was then purified and characterized [9].

In this study, our objective was to examine whether or not other enzymes responsible for the catechol catabolism in the AN-22 strain are constitutive. For this purpose, we prepared the cell extracts from cells grown on succinate and aniline media, and estimated the *cis*,*cis*-muconate cycloisomerase (CatB, EC 5.5.1.1), muconolactone isomerase (CatC, EC

5.3.3.4), β-ketoadipate enol-lactone hydrolase (CatD, EC 3.1.1.24) and β-ketoadipate: succinyl CoA transferase (CatIJ, EC 2.8.3.6). In addition, CatB and C were purified and characterized. Furthermore, we attempted the cloning and analysis of genes coding for the catabolic enzymes and regulating their expression, which will provide more definitive information for the constitutive synthesis of these enzymes.

The production, purification, characterization and gene analysis of catabolic enzymes for catechol in aniline-assimilating *Rhodococcus* sp. AN-22 are described in this article.

EXPERIMENTAL

Chemicals

Aniline hydrochloride, catechol and 4-methylcatechol were purchased from Wako Pure Chemical, Osaka, Japan; 3-methylcatechol, 3-chlorocatechol, 4-chlorocatechol and 3-fluorocatechol were from Tokyo Kasei, Tokyo, Japan; DE52 (DEAE) cellulose was from Whatman Chemical Separation, Brentford, Middlesex, U.K.; DEAE-Toyopearl 650S, Phenyl-Toyopearl 650S and Toyopearl HW-55S were from Tosoh Corporation, Tokyo, Japan; and restriction endonucleases were from Takara Bio Incorporation, Otsu, Japan.

Bacterial strains, plasmids and growth conditions

Rhodococcus sp. AN-22 was used throughout this study as a producer of enzymes for the catechol catabolism and a source of their genes. *Escherichia coli* XL1-Blue (Stratagene, La Jolla, CA, U.S.A.) was used as a host for constructing a gene library. A pBluescript II KS+ (Stratagene) was used for the construction of a gene library and subcloning of DNA fragments.

Rhodococcus sp. AN-22 was cultured at 30°C with shaking on the aniline medium as previously described [14] or on a succinate medium containing 0.9% (w/v) sodium succinate

and 0.15% (w/v) $(NH_4)_2SO_4$, instead of aniline. For the preparation of DNA and RNA, the aniline and succinate media supplemented with 0.6% (w/v) glycine were used. *E. coli* XL1-Blue was cultured at 37°C with shaking on Luria-Bertani (LB) broth, if necessary, supplemented with 100 μg/ml of ampicillin, 12.5 μg/ml of tetracycline, 1 mM isopropyl-β-D-thiogalactopyranoside and 0.04% (w/v) X-Gal. *E. coli* XL1-Blue carrying the plasmid pED14 [8] and pUC9A [15] were used as the producers of CatD and chlorocatechol 1,2-dioxygenase, respectively.

Enzyme assays

The activities of CatA and CatB were spectrophotometrically measured by the methods of Aoki *et al*. [4] and Murakami *et al*. [16], respectively. The activity of CatC was measured essentially by the method of Ornston [17] in the presence of an excess of partially purified CatD, which was prepared from the cell extracts of recombinant *E. coli* XL1-Blue carrying the *catD* gene derived from another aniline-assimilating bacterium *Frateuria* sp. ANA-18 [8]. The molar extinction coefficient of 1.43×10^3 at 230 nm for muconolactone was used [18]. The activities of CatD and CatIJ were measured by the methods of Ornston [19] and Yeh and Ornston [20], respectively.

One unit of activity for CatB and C was defined as the amount of each enzyme that consumed 1 μmol of substrate per min. The specific activity was defined as units per mg of protein. Protein concentrations were measured by the method of Lowry *et al*. [21].

Production and estimation of CatA, B, C, D and IJ

Rhodococcus sp. AN-22 was cultured with shaking on 67 ml of aniline or succinate medium in a 500-ml flask. At appropriate intervals, the turbidity of the culture was monitored at 660 nm. Cells were harvested by centrifugation at 8000 × *g* for 10 min and washed twice with

0.8% (w/v) NaCl. The washed cells were suspended in 20 mM Tris-HCl (pH 8.0) (buffer A) and disrupted with a Kubota 201M ultrasonic oscillator (Kubota Shoji, Tokyo, Japan) at 180 W for 5 min. After centrifugation at 20 000 × *g* for 20 min, the supernatant was used as the cell extracts. Streptomycin sulfate (20%, w/v) was added to the cell extracts with stirring to a final concentration of 1% (w/v). The solution was stirred for 30 min and centrifuged. The supernatant was recovered and used for the enzyme assays of CatA, B, C, D and IJ.

Purification of CatB

Cells used for the enzyme purification were cultured on the succinate medium. All operations for the purification were done at 0–4°C. All centrifugations were at 20 000 × *g* and 4°C for 20 min.

Preparation of cell extracts

The obtained frozen cells (30 g, wet weight) were thawed and suspended in 300 ml of buffer A. The cells suspended in 30-ml batches were disrupted with the ultrasonic oscillator as mentioned above. After removing the intact cells and cell debris by centrifugation, the supernatant, designated as the cell extracts, was collected (fraction 1; 310 ml).

Streptomycin sulfate treatment

Streptomycin sulfate (20%, w/v) was added to fraction 1 with stirring to a final concentration of 1% (w/v). The solution was then stirred for 30 min and centrifuged. The supernatant (320 ml) was recovered (fraction 2) and the precipitate was discarded.

$(NH_4)_2SO_4$ fractionation

Fraction 2 was brought to 30% 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 60% saturation. After stirring for 30 min, the precipitate was collected by centrifugation and dissolved in buffer A. The solution was dialyzed against buffer A with two changes of buffer. The final volume of the dialyzed solution (fraction 3) was 30 ml.

Chromatography on DE52 cellulose

Fraction 3 was applied to a column (2 × 22 cm) of DE52 cellulose equilibrated with buffer A. Proteins were eluted with a linear gradient (0 to 0.4 M) of NaCl in 1.4 l of buffer A; the protein concentration and enzyme activity of the fractions were then assayed. Fractions with a high specific activity were pooled to yield fraction 4 (210 ml).

Chromatography on DEAE-Toyopearl 650S

Fraction 4 was dialyzed against buffer A and the dialyzed solution was applied to a column (2 × 12 cm) of DEAE-Toyopearl 650S equilibrated with buffer A. Proteins were eluted with a linear gradient (0 to 0.35 M) of NaCl in 800 ml of buffer A; the protein concentration and enzyme activity of the fractions were assayed. Fractions with a high specific activity were pooled to yield fraction 5 (40 ml).

Chromatography on Toyopearl HW-55S

Fraction 5 was concentrated to 0.5 ml with a collodion bag (Sartorius, Goettingen, Germany). The concentrated sample was placed on top of a column (3 × 60 cm) of Toyopearl HW-55S equilibrated with buffer A containing 0.2 M NaCl. Proteins were eluted with buffer A

containing 0.2 M NaCl. After estimating the protein concentration and enzyme activity, fractions with a high specific activity were pooled to yield fraction 6 (8 ml).

Chromatography on Phenyl-Toyopearl 650S

To fraction 6, $(NH_4)_2SO_4$ was added to make a 30% (w/w) solution. The enzyme solution was applied to a column (1.7 × 12 cm) of Phenyl-Toyopearl 650S equilibrated with buffer A containing 30% (w/w) $(NH_4)_2SO_4$. Proteins were eluted with a linear gradient (30 to 0%, w/w) of $(NH_4)_2SO_4$ in 400 ml of buffer A; the protein concentration and enzyme activity of the fractions were assayed. The purity of the enzyme in each fraction was determined by PAGE. Fractions showing a single protein band on the gel were pooled (fraction 14 ml).

Purification of CatC

Frozen cells (9.6 g, wet weight) were thawed and suspended in 96 ml of buffer A. The preparations of the cell extracts (fraction 1, 100 ml), streptomycin sulfate treatment (fraction 2, 103 ml), $(NH_4)_2SO_4$ fractionation (fraction 3, 30 ml), chromatographies on DE52 cellulose (fraction 4, 18 ml), DEAE-Toyopearl 650S (fraction 5, 20 ml) and Phenyl-Toyopearl 650S (fraction 6, 3 ml) were carried out essentially by the same methods as used for the purification of CatB.

Determination of molecular masses

The molecular masses of the native enzymes were measured by gel filtration, and those of the enzyme subunits were measured by SDS-PAGE [22]. Size markers used for gel filtration were those in the calibration proteins gel chromatography kit from Boehringer Mannheim, Mannheim, Germany. The electrophoresis calibration kit LMW (Pharmacia Fine Chemicals,

Uppsala, Sweden) and the LMW calibration kit for SDS electrophoresis (Amersham Biosciences, Piscataway, NJ, U.S.A) were used as size markers for SDS-PAGE.

Determination of NH_2-terminal and inner amino acid sequences

Purified CatB and C were electroblotted using the method of Matsudaira [23] and then the NH_2-terminal amino acid sequences were determined by automated Edman degradation with a Shimadzu PPSQ-10 protein sequencer (Shimadzu, Kyoto, Japan). The inner amino acid sequences of CatB were also determined with the sequencer after fractionation of BrCN-digested peptides on an SDS-polyacrylamide gel.

Substrate specificity

Methyl, chloro and fluoro derivatives of *cis*,*cis*-muconate were enzymatically synthesized with chlorocatechol 1,2-dioxygenase from *Ralstonia eutropha* NH9 as previously described [24]. 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 [25]. K_m and V_{max} values of the purified enzyme were calculated by non-linear regression with the Enzfitter program (Biosoft, Cambridge, U.K.).

Gene manipulation and construction of gene library

Standard methods were used for the plasmid DNA purifications, restriction enzyme digestions and *E. coli* transformations [26]. The total DNA of *Rhodococcus* sp. AN-22 was prepared according to the protocol described by DiLella and Woo [27]. The purified DNA (11 μg) was partially digested with *Sau*3A I, and the DNA fragments were fractionated by ultracentrifugation through sucrose gradients. A gene library was constructed by ligating 5- to

10-kb fragments to a pBluescript II KS- vector digested with *Bam*H I. *E. coli* XL1-Blue was transformed with the ligated plasmids. Subcloning experiments were performed using conventional techniques [26].

PCR

On the basis of NH_2-terminal and inner amino acid sequences of CatB, the two degenerate primers catB-N (5‘-ACIACIATHATHGAYGT-3‘) and catB-I (5‘-CCCCAIGCRTCRTGCAT-3‘) were synthesized. The cycling of primers was performed at 95°C (1 min), 43°C (1 min) and 72°C (1 min) for 30 cycles and additionally at 72°C (2 min) for one cycle to complete the amplifying reaction.

Preparation of [α-^{32}P]dCTP-labeled probe and colony hybridization

Amplified DNA fragments were ligated to a pGEM-T vector (Promega, Madison, WI, U.S.A.) and completely sequenced to ensure that the ligated fragments constituted a portion of the *catB* gene. After sequencing, the recombinant plasmid constituting *catB* gene was digested with *Eco*R I. The inserted DNA was purified and labeled with a random primer DNA labeling kit version 2 (Takara) and [α-^{32}P]dCTP (ICN, Costa Mesa, CA, U.S.A.). Recombinant plasmids of transformants from a gene library of *Rhodococcus* sp. AN-22 were fixed on a Hybond-N+ membrane (Amersham) according to the manufacturer’s instructions. Colony hybridization was done under standard conditions [26].

DNA sequencing

A FlexiPrep kit (Amersham) was used for preparing the double-stranded DNA for sequencing. Sequencing reactions were performed using a Thermo Sequenase primer cycle sequencing kit

with 7-deaza dGTP (Amersham). Reaction mixtures were run on a Shimadzu DSQ-2000L sequencer (Shimadzu).

Preparation of total RNA, reverse transcriptase (RT)-PCR and primer extension analysis

Total RNA was prepared from cells of *Rhodococcus* sp. AN-22 grown on the aniline and succinate media using an RNeasy mini kit (Qiagen, Hilden, Germany). On the basis of the nucleotide sequences of the *catABC* genes, the two primers OF (5'-CACCTGATGGTGAAGGCTCC-3') and OR (5'-GCGCTGCAGATCCTGACTGT-3') were synthesized (Figure 1). cDNA was synthesized with a RevertAid M-MuLV reverse transcriptase (MBI Fermentas, Vilnius, Lithuania) using an OR primer and total RNA of *Rhodococcus* sp. AN-22. The cycling of the primers was performed at 94°C (1 min), 58°C (1 min) and 72°C (1 min) for 30 cycles and additionally at 72°C (2 min) for one cycle to complete the amplifying reaction. For the primer extension analysis, the FITC-labeled primer PE (5'-CCCGAATGGGTCGGGATGTG-3') was designed. Reverse transcriptional reactions were performed as mentioned above. After incubation for 1 h, the synthesized cDNA was precipitated with ethanol and prepared for electrophoresis and sequencing, according to the method reported previously [28].

RESULTS

Constitutive synthesis of CatB and C

Table 1 shows that *Rhodococcus* sp. AN-22 synthesized CatB and C in its cells grown on all the non-aromatic substrates examined, as seen in the case of CatA [9]. The cells grown on D-mannose, L-malate and L-leucine produced higher specific activities of CatB and C than those grown on aniline. When the bacterium was incubated with acetate, which is a metabolic

member of glyoxylate cycle, the two enzyme activities markedly decreased, although satisfactory cell growth was observed. The strain maintained the specific activities of CatA, B and C at an almost equal level during cultivation on the succinate medium (Figure 2).

On the other hand, the specific activities of CatD and IJ in cells grown on succinate were less than one-eighth and one-25th, respectively, than those in cells obtained from the aniline medium, indicating that the two enzymes were synthesized essentially in the presence of aniline or its metabolites.

Purification and characterization of CatB

Table 2 is a summary of a typical enzyme purification for CatB from cells of *Rhodococcus* sp. AN-22 grown on succinate. The specific activity of the final preparation of CatB was 52 units per mg with an overall recovery of 18%. The final enzyme preparation had a 370-fold increase in the specific activity and showed a single protein band on both native and denaturing polyacrylamide gels (Figure 3A).

The apparent molecular masses of CatB were determined to be 43 kDa by gel filtration (Figure 4) and 44 kDa by SDS-PAGE (Figure 3B). These findings indicated that the enzyme is a monomer. The purified enzyme showed a maximal activity at pH 8.0. It was stable between pHs 6.0 and 9.0. The enzyme maintained 90% activity up to 55°C after 10 min of incubation at pH 8.0, and the enzyme activity was lost at 60°C.

Table 3 shows that the activity of CatB for *cis*,*cis*-muconate increased in the presence of Mn^{2+}, Co^{2+}, Mg^{2+} and Ni^{2+}. Of the metals tested, Co^{2+} produced the highest CatB activity.

The effects of metal salts and sulfhydryl and chelating agents on the activity of CatB were tested (Table 4). $HgCl_2$, $CuSO_4$ and CH_2ICOOH inhibited the enzyme. Other sulfhydryl and chelating agents tested did not evidently affect the activity.

The CatB of *Rhodococcus* sp. AN-22 showed substantial activities for the cyclization of

2-methyl-, 3-methyl- and 2-chloro-*cis*,*cis*-muconate, in addition to the original substrate *cis*,*cis*-muconate (Table 5). The turnover number (k_{cat}) of CatB for *cis*,*cis*-muconate was higher than those for methyl and 2-chloro-derivetives, while the affinity for *cis*,*cis*-muconate was lower than the others on the basis of K_m values.

Purification and characterization of CatC

Table 6 shows a summary of a typical enzyme purification for CatC from succinate-grown cells. The specific activity of the final preparation was 860 units per mg with an overall recovery of 2.7%. The final enzyme preparation had a 930-fold increase in the specific activity and showed a single protein band on both native and denaturing polyacrylamide gels (Figure 3B).

The apparent molecular masses of CatC were 100 kDa by gel filtration and 12 kDa by SDS-PAGE (Figure 3B). These findings indicated that the enzyme is a homooctamer.

The optimal activity of the purified CatC was not determined, because the reaction mixture for the enzyme assay contained another enzyme affecting the optimal pH for CatC. It was stable between pHs 7.0 and 10.5. The enzyme maintained more than 80% activity up to 90°C after 10 min of incubation at pH 8.0. In addition, it maintained 64% activity at 95°C. The kinetic parameters of CatC for muconolactone were as follows: K_m, 640 ± 70 μM; V_{max}, 330 ± 20 U mg^{-1}; and k_{cat}, 4000 ± 300 min^{-1}.

Cloning and sequence analysis of *cat* genes from *Rhodococcus* sp. AN-22

The NH_2-terminal amino acid sequences of purified CatB and CatC were SDPDLKIASVTTTIIDVPLIRPHKFAT and ALFHVRMDVD, respectively. The inner amino acid sequences of a certain peptide of CatB were HDAWGALGVPVAXLLGGA. On the basis of these amino acid sequences, catB-N and catB-I primers were synthesized. When the

catB-N and catB-I primers and total DNA purified from *Rhodococcus* sp. AN-22 as a template were incubated, a 320-bp DNA fragment (probe, Figure 1) was amplified. The sequencing of both strands of the amplified fragment showed that this fragment encoded a portion of CatB. The sequenced fragment was labeled with [α-^{32}P]dCTP. The labeled fragment was used as a probe for colony hybridization. Among the 4600 transformants of a gene library from the AN-22 strain, two transformants harboring plasmids p42E6 and p44E8 were positive (Figure 1). The DNA fragments of the two plasmids were subcloned and sequenced.

The DNA fragments inserted into p42E6 and p44E8 covered 7187 bp. Three complete ORFs (ORF1-3) were found in the determined nucleotide sequences. ORF1 (positions 1067 to 1915) consisted of 849 bp encoding 283 amino acid residues. The deduced amino acid sequences were identical with the inner amino acid sequences of the purified CatA from *Rhodococcus* sp. AN-22 [9]. The molecular mass of a polypeptide encoded by ORF1 was calculated to be 31 kDa, which corresponded to the value of 30 kDa estimated by SDS-PAGE. The deduced amino acid sequences of ORF1 showed high identities with those of CatAs from *Rhodococcus rhodochrous* NCIMB 13259 (86%, AF043741) [29], *Rhodococcus erythropolis* AN-13 (74%, D83237) [6] and *Rhodococcus opacus* 1CP (69%, X99622) [30]. These results show that ORF1 was a gene encoding the CatA from *Rhodococcus* sp. AN-22. We named the gene *catA*.

The deduced amino acid sequences of ORF2 (positions 2068 to 3192) were identical with the NH_2-terminal and inner amino acid sequences of CatB. The deduced molecular mass of an ORF2 product was 40 kDa, which was almost the same as that of the purified CatB. The deduced amino acid sequences of ORF2 showed high identities with those of the CatBs from *R. erythropolis* AN-13 (81%, D83237) [6] and *R. erythropolis* 1CP (76%, X99622) [30], but low identities to those of the CatBs from gram-negative bacteria. ORF2 was named *catB*.

ORF3 (positions 3233 to 3514) consisted of 282 bp. The deduced NH_2-terminal amino acid sequences corresponded to that of CatC. The deduced molecular mass of an ORF3 product was 12 kDa, which corresponded to the subunit value (12 kDa) of CatC. The deduced amino acid sequences of ORF3 showed high identities with those of CatCs from *R. opacus* 1CP (76%, X99622) [30], from *Streptomyces setonii* ATCC 39116 (68%, AF277051) [31] and $CatC_1$ from *Frateuria* sp. ANA-18 (62%, AB009343) [8]. These data showed that ORF3 encoded CatC. ORF3 was named *catC*.

In the region upstream of *catA* of *Rhodococcus* sp. AN-22, a possible relic of *catR* was found as shown in Supplementary Figure S1. The reduced amino acid sequences of the relic of *catR* were high identity with those of *catR* from *R. opacus* 1CP [32] and *R. erythropolis* AN-13 [6]. The relic of *catR* neighbored to a homologous sequence of IS*204* (positions 800 to 1), an insertion sequence from *Nocardia asteroides* YP21 [33]. The IS*204*-like sequence of the AN-22 strain contained a predicted inverted repeat, but not ORFs encoding significant proteins such as transposase.

In the 3672 bp region downstream of *catC*, no ORFs encoding significant proteins were found.

Transcriptional start site and operon structure of *cat* genes

RT-PCR analyses carried out using three primer pairs on the basis of the sequences upstream of *catA* showed that a transcriptional start site of the *cat* genes existed between 531 and 159 bp upstream of a *catA* initiation codon (Supplementary Figure S2). Primer extension analyses were then employed to determine the amount of single primer extension products and the exact transcriptional start site of the *cat* genes. Almost the same amount of them was synthesized in the presence of the PE primer and total RNA obtained from aniline- and succinate-grown cells of *Rhodococcus* sp. AN-22 (Figure 5). Both transcription started at the

same position of 891-bp (thiamine residue on the coding strand) upstream of the *catA* initiation codon.

When RT-PCR analysis of total RNA from succinate-grown cells was carried out using a primer pair that spanned across *catABC*, a DNA fragment was amplified, indicating that readthough transcription occurred in the region containing these three genes (Figure 6). Since no PCR product was observed in the negative control sample without a reverse transcriptase, possible DNA contamination was excluded.

DISCUSSION

In this study, we found that aniline-assimilating *Rhodococcus* sp. AN-22 constitutively synthesized CatB and C in its cells, which were then purified and characterized, in addition to the previously reported CatA [9]. On the other hand, CatD and IJ were inducibly produced. This is the first report describing the finding, purification and characterization of constitutive CatB and C from microorganisms degrading aromatic compounds. The CatB of *Rhodococcus* sp. AN-22 exhibited the activity as a monomer, although all the inducible CatBs reported are activated by the polymerization of a subunit to a polymer with the molecular mass of 200-400 kDa. The CatB of the AN-22 strain was resistant to inhibitors such as sulfhydryl and chelating agents, compared with the enzymes inducibly produced from other microorganisms [34]. Although the specific constant (k_{cat}/K_m) for *cis*,*cis*-muconate of the CatB from the AN-22 strain was similar to those of CatBs from *Arthrobacter* sp. BA-5-17 [24], *Acinetobacter calcoaceticus* ADP1 [35] and *R. opacus* 1CP [36], the k_{cat} value of the former was higher than those from other bacterial strains. In addition, the CatB showed the activity in the presence of Co^{2+} and Mg^{2+}, besides Mn^{2+}. For these reasons, constitutive CatB of *Rhodococcus* sp. AN-22 seems to be peculiar in terms of its enzyme chemistry.

The CatC of the AN-22 strain was quite stable to heating and maintained more than an

80% activity at 90°C, although that of *Pseudomonas putida* rapidly lost the activity at 65°C [17]. In an SDS-polyacrylamide gel, the CatC band was extensively diffused, probably because of its small molecular mass (Figure 3B). Since the reaction mixture for the CatC assay contained CatD, CatCs from various microorganisms including *Rhodococcus* sp. AN-22 have not been fully characterized [36].

We cloned *catA*, *B* and *C* genes encoding the constitutive CatA, B and C, respectively, from *Rhodococcus* sp. AN-22. The three genes formed an operon and showed high identity with other corresponding genes from gram-positive microorganisms (see the results section), although the CatB of the AN-22 strain had several characteristics mentioned above. The location of the *catA*, *B* and *C* genes of *Rhodococcus* sp. AN-22 was identical with those of two other *Rhodococcus* stains, which are inducibly expressed [6,18], and apparently different from those of a *Streptomyces* strain and gram-negative strains (Supplementary Figure S3). The *catDIJ* genes of *Rhodococcus* sp. AN-22 were not observed in the cloned DNA fragments in this study, as these genes were not found in the *catABC* locus in most bacteria reported [8,30,31,37]. In addition, CatD and CatIJ were synthesized inducibly in *Rhodococcus* sp. AN-22. These facts suggest that the *catDIJ* genes of the AN-22 strain were distant from *catABC* and expressed inducibly by another regulatory system. Since CatA, B and C are synthesized constitutively and CatD and IJ inducibly in the cells, β-ketoadipate enol-lactone converted from catechol by CatA, B and C could be accumulated readily at the early phase of catechol metabolism. This intermediate metabolite is valuable as a substrate used for the assay of CatD

Although the regulatory gene *catR*, which is commonly contained in the gene cluster for catechol catabolism [10,32], was absent around the *catABC* genes of *Rhodococcus* sp. AN-22, a possible relic of *catR* was adjacent to an IS*204*-like element in the region upstream of *catA*. These findings indicate that the *catR* encoding a regulator of *catABC* was disrupted by the

transposition of IS*204*-like element into *Rhodococcus* sp. AN-22. It was reported that some sequences of IS-like element are present in the region upstream of a constitutive 2,3-dihydroxybiphenyl 1,2-dioxygenase gene from dibenzofuran-degrading *Rhodococcus* sp. YK2 [38].

When *Rhodococcus* sp. AN-22 grew on aniline and succinate, almost the equal amount of two single primer extension products was synthesized (Figure 5). The transcription started at the same site in both cases, and putative σ^{70}-type -10 (TTCCCC) and -35 (TCCCAT) promoter sequences were identified 8 bp upstream of the transcriptional start site. Since this site exists in the IS-like element, the promoter is not regulated by the original CatR. It seems likely that the promoter found in the element acted for the constitutive expression of *catABC* genes.

These data led us to the assumption that the *catABC* genes of this bacterium are always expressed with any growth substrate or its metabolite. The assumption was then supported by the data in the present and previous reports [9] that the CatA, B and C are synthesized on all the growth substrates examined. However, the cells cultured with acetate expressed much lower activities of CatA, B and C than those in the cells cultured with organic acids belonging to the TCA cycle. These results suggest that possible regulation to repress the expression of *catABC* genes may function in the glyoxylate cycle, in competition with the TCA cycle.

The actinomycete genus *Rhodococcus* is of interest for the metabolic abilities of substituted hydrocarbons and other chemicals and the solvent tolerance because of the aliphatic chains of mycolic acid present in their cell walls [39,40]. For these reasons, a *Rhodococcus*-based host-vector system will be useful for many applications to bioremediation, biodegradation and bioconversion. Since *Rhodococcus* sp. AN-22 has the constitutive expression system, the constructed host-vector based on this system will be able to synthesize a target protein steadily without an inducer, such as isopropyl-β-D-thiogalactopyranoside.

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REFERENCES

1 Lyons, C. D., Katz, S. E. and Bartha, R. (1984) Mechanisms and pathways of aniline elimination from aquatic environments. Appl. Environ. Microbiol. **48**, 491-496

2 Aoki, K., Shinke, R. and Nishira, H. (1982) Identification of aniline-assimilating bacteria. Agric. Biol. Chem. **46**, 2563-2570

3 Aoki, K., Shinke, R. and Nishira, H. (1983) Metabolism of aniline by *Rhodococcus erythropolis* AN-13. Agric. Biol. Chem. **48**, 1611-1616

4 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

5 Aoki, K., Konohana, T., Shinke, R. and Nishira, H. (1984) Two catechol 1,2-dioxygenases from an aniline-assimilating bacterium, *Frateuria* sp. ANA-18. Agric. Biol. Chem. **48**, 2097-2104

6 Murakami, S., Kodama, N., Shinke, R. and Aoki, K. (1997) Classification of catechol 1,2-dioxygenase family: 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

7 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

8 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* species ANA-18. Gene **226**, 189-198

9 Matsumura, E., Ooi, S., Murakami, S., Takenaka, S. and Aoki, K. (2004) Constitutive synthesis, purification, and characterization of catechol 1,2-dioxygenase from the aniline-assimilating bacterium *Rhodococcus* sp. AN-22. J. Biosci. Bioeng. **98**, 71-76

10 Takashima, A., Murakami, S., Takenaka, S. and Aoki, K. (2001) Regulation by two CatR proteins that differ in binding affinity to *catB* promoters expressing two *cat* gene clusters. Biosci. Biotechnol. Biochem. **65**, 2146-2153

11 Lechner, U. and Straube, G. (1988) Degradation of 3-aminophenol by *Arthrobacter* spec. mA3. J. Basic Microbiol. **28**, 629-637

12 Roldan, M. D., Blasco, R., Caballero, F. J. and Castillo, F. (1998) Degradation of *p*-nitrophenol by the phototrophic bacterium *Rhodobacter capsulatus*. Arch. Microbiol. **169**, 36-42

13 Caposio, P., Pessione, E., Giuffrida, G., Conti, A., Landolfo, S., Giunta, C. and Gribaudo, G. (2002) Cloning and characterization of two catechol 1,2-dioxygenase genes from *Acinetobacter radioresistens* S13. Res. Microbiol. **153**, 69-74

14 Aoki, K., Ohtsuka, K., Shinke, R. and Nishira, H. (1983) Isolation of aniline-assimilating bacteria and physiological characterization of aniline biodegradation in *Rhodococcus erythropolis* AN-13. Agric. Biol. Chem. **47**, 2569-2575

15 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

16 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

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

18 Moiseeva, O. V., Solyanikova, I. P., Kaschabek, S. R., Groning, 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

19 Ornston, L. N. (1966) The conversion of catechol and protocatechuate to β-ketoadipate by *Pseudomonas putida*. II. Enzymes of the protocatechuate pathway. J. Biol. Chem. **241**, 3787-3794

20 Yeh, W.-K. and Ornston, L. N. (1981) Evolutionarily homologous $\alpha_2\beta_2$ oligomeric structures in β-ketoadipate succinyl-CoA transferases from *Acinetobacter calcoaceticus* and *Pseudomonas putida*. J. Biol. Chem. **256**, 1565-1569

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

22 Weber, K. and Osborn, M. (1969) The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. **244**, 4406-4412

23 Matsudaira, P. (1987) Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J. Biol. Chem. **262**, 10035-10038

24 Murakami, S., Kohsaka, C., Okuno, T., Takenaka, S. and Aoki, K. (2004) Purification, characterization, and gene cloning of *cis*,*cis*-muconate cycloisomerase from

benzamide-assimilating *Arthrobacter* sp. BA-5-17. FEMS Microbiol. Lett. **231**, 119-124

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

26 Sambook, J., Frisch, E. F. and Maniatis, T. (1989) Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, NY

27 DiLella, A. G. and Woo, S. L. C. (1987) Cloning large segments of genomic DNA using cosmid vectors. Methods in Enzymology **152**, 199-212

28 Murakami, S., Sawami, Y., Takenaka, S. and Aoki, K. (2004) Cloning of a gene encoding 4-amino-3-hydroxybenzoate 2,3-dioxygenase from *Bordetella* sp. 10d. Biochem. Biophys. Res. Commun. **314**, 489-494

29 Strachan, P. D., Freer, A. A. and Fewson, C. A. (1998) Purification and characterization of catechol 1,2-dioxygenase from *Rhodococcus rhodochrous* NCIMB 13259 and cloning and sequencing of its *catA* gene. Biochem. J. **333**, 741-747

30 Eulberg, D., Golovleva, L. A. and Schlömann, M. (1997) Characterization of catechol catabolic genes from *Rhodococcus erythropolis* 1CP. J. Bacteriol. **179**, 370-381

31 Park, H.-J. and Kim, E.-S. (2003) An inducible *Streptomyces* gene cluster involved in aromatic compound metabolism. FEMS Microbiol. Lett. **226**, 151-157

32 Eulberg, D. and Schlömann, M. (1998) The putative regulator of catechol catabolism in *Rhodococcus opacus* 1CP–an IclR-type, not a LysR-type transcriptional regulator. Antonie Van Leeuwenhoek **74**, 71-82

33 Yao, W., Yang, Y. and Chiao, J. (1994) IS*204*: an insertion sequence from *Nocardia asteroides* (*mexicana*) YP21. Plasmid **32**, 262-269

34 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

35 Vollmer, M. D., Hoier, H., Hecht, H. J., Schell, U., Gröning, J., Goldman, A. and Schlömann, M. (1998) Substrate specificity of and product formation by muconate cycloisomerases: an analysis of wild-type enzymes and engineered variants. Appl. Environ. Microbiol. **64**, 3290-3299

36 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

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

38 Iida, T., Mukouzaka, Y., Nakamura, K., Yamaguchi, I. and Kudo, T. (2002) Isolation and characterization of dibenzofuran-degrading actinomycetes: analysis of multiple extradiol dioxygenase genes in dibenzofuran-degrading *Rhodococcus* species. Biosci. Biotechnol. Biochem. **66**, 1462-1472

39 Bell, K. S., Philp, J. C., Aw, D. W. J. and Christofi, N. (1998) The genus *Rhodococcus*. J. Appl. Microbiol. **85**, 195-210

40 O'Brien, X. M., Parker, J. A., Lessard, P. A. and Sinskey, A. J. (2002) Engineering an indene bioconversion process for the production of cis-aminoindanol: a model system for the production of chiral synthons. Appl. Microbiol. Biotechnol. **59**, 389-399

Table 1 Constitutive synthesis of CatB and C

All values are indicated as means ± S.D. for five separate experiments.

Substrate	OD_{660}	CatB activity (U/mg)	CatC activity (U/mg)
Aniline	1.2	0.052 ± 0.010	0.76 ± 0.36
D-Mannose	1.4	0.087 ± 0.015	0.96 ± 0.05
D-Glucose	1.9	0.080 ± 0.005	0.67 ± 0.04
Sucrose	1.5	0.019 ± 0.003	0.47 ± 0.01
L-Malate	2.0	0.20 ± 0.01	0.82 ± 0.03
Succinate	1.9	0.12 ± 0.02	0.91 ± 0.07
Acetate	2.2	0.016 ± 0.002	0.22 ± 0.01
L-Isoleucine	1.2	0.051 ± 0.007	0.77 ± 0.05
L-Leucine	1.5	0.10 ± 0.01	1.1 ± 0.1

Table 2 Purification of CatB from succinate-grown cells of *Rhodococcus* sp. AN-22

Fraction	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)
Cell extract	240	1800	0.14	100
Streptomycin sulfate	200	2600	0.078	82
Ammonium sulfate	220	470	0.48	92
DE52	140	51	2.7	57
DEAE-Toyopearl 650S	140	6.4	22	58
HW-55S	80	1.6	50	33
Phenyl-Toyopearl 650S	42	0.81	52	18

Table 3 Effects of bivalent metal ions on the activity of CatB

The enzyme reaction was performed by the standard assay methods in the presence of various bivalent ions instead of manganese ion.

Metal ions	Relative activity (%)
None	43
Mn^{2+}	100
Co^{2+}	170
Mg^{2+}	75
Ba^{2+}	40
Ca^{2+}	34
Ni^{2+}	55
Zn^{2+}	30

Table 4 Effects of various compounds on the activity of CatB

The enzyme (0.7 units) was incubated with various compounds in 1.0 ml of buffer A at 24°C for 10 min. An aliquot (0.1 ml) of the incubation mixture was subjected by the enzyme assay using *cis*,*cis*-muconate as a substrate.

Compound	Concentration (mM)	Remaining activity (%)
None	-	100
$HgCl_2$	1.0	20
$CuSO_4 \cdot 5H_2O$	1.0	65
CH_2ICOOH	0.2	58
PCMB*	0.1	92
α, α'-Dipyridyl	1.0	93
N-Ethylmaleimide	1.0	90
Tiron	1.0	100
EDTA	1.0	101
o-Penanthroline	0.05	101
NaN_3	1.0	96

**p*-Chloromercuribenzoic acid.

Table 5 Substrate specificity of CatB

Substrate	K_m* (μM)	V_{max}* (U mg^{-1})	k_{cat}† (min^{-1})	k_{cat}/K_m (min^{-1} μM^{-1})
cis,*cis*-Muconate	660 ± 70	390 ± 30	17000 ± 1000	26
2-Methyl-*cis*,*cis*-muconate	380 ± 70	8.5 ± 1.4	330 ± 60	1.1
3-Methyl-*cis*,*cis*-muconate	310 ± 20	24 ± 2	1100 ± 100	3.1
2-Chloro-*cis*,*cis*-muconate	300 ± 60	6.0 ± 2.5	260 ± 110	1.4
3-Chloro-*cis*,*cis*-muconate		(< 0.01)‡		
2-Fluoro-*cis*,*cis*-muconate		(< 0.01)‡		

*K_m and V_{max} are indicated as means ± S.D. for five determinations.

†k_{cat} values were calculated on the basis of the molecular mass of 44 kDa.

‡Detection limit for specific activity under the experimental conditions.

Table 6 Purification of CatC from succinate-grown cells of *Rhodococcus* sp. AN-22

Fraction	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)
Cell extract	440	480	0.92	100
Streptomycin sulfate	500	480	1.0	110
Ammonium sulfate	320	71	4.5	73
DE52	100	7.2	14	23
DEAE-Toyopearl 650S	75	1.8	42	17
Phenyl-Toyopearl 650S	12	0.014	860	2.7

(FIGURE LEGENDS)

Figure 1 Location of cloned DNA fragments and *cat* genes

Open arrows indicate ORFs and directions of their transcripts. The probe synthesized by PCR using catB-N and catB-I primers was used for colony hybridization. The primers used for RT-PCR and primer extension analyses are indicated by horizontal arrows. A solid triangle indicates a transcriptional start site of *cat* gene cluster. Abbreviations used: A, *Apa* I; C, *Cla* I; E, *Eco*N I; N, *Nco* I; and S, *Sac* I.

Figure 2 Production of CatA, B and C from *Rhodococcus* sp. AN-22 on succinate medium

The bacterium was incubated at 30°C with shaking. At appropriate intervals, cell growth (closed diamond), CatA (closed triangle), CatB (closed square) and CatC (closed circle) were messured. Results are indicated as means ± S.D. for three determinations.

Figure 3 Native PAGE (lane 1) and SDS-PAGE (lane 2) of CatB (A) and C (B) from *Rhodococcus* sp. AN-22

Lane 1, Native PAGE. The purified enzymes (5 μg each) were run on 7.5% (w/v) gels of pH 8.9 at 2 mA/tube for 2.5 h in a running buffer (pH 8.3) of Tris-glycin. Lane 2, SDS-PAGE. The purified enzymes (4 μg each) denatured with SDS were on 7.5% (w/v) gels containing 0.1% (w/v) SDS at 6 mA/tube for 3 h (A) or 2 h (B) in a running buffer (pH 7.2) of 0.1% (w/v) SDS-0.1 M sodium phosphate. Size markers used for SDS-PAGE were α-lactalbumin (molecular mass, 14 kDa), trypsin inhibitor (20 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 and 45 kDa), bovine serum albumin (67 and 66 kDa) and phosphorylase *b* (94 and 97 kDa). The gels used for the native PAGE and SDS-PAGE were stained with 0.25% (w/v) Coomassie Brilliant Blue R-250 in a solvent of ethanol-acetic acid-H_2O (9 : 2 : 9, v/v).

The molecular masses of three markers were different in (A) and (B), because the marker products used for (A) and (B) were purchased from Pharmacia and Amersham, respectively. See the text for details.

Figure 4 Determination of molecular mass of CatB by gel filtration

The enzyme (24 units) and markers (2 mg each) were placed on the top of a column (3.0 × 60 cm) of Toyopearl HW-55S, and then eluted with buffer A containing 0.2 M NaCl. Symbols: 1, cytochrome *c* (12.5 kDa); 2, chymotrypsinogen A (25 kDa); 3, ovalbumin (45 kDa); 4, bovine serum albumin (68 kDa); and closed circle, CatB.

Figure 5 Primer extension analysis of *cat* gene transcripts

Total RNA prepared from aniline- and succinate-grown cells of *Rhodococcus* sp. AN-22 was used for the reverse transcription reaction with the PE primer. This primer was also used for a dideoxy sequencing reaction using plasmid p42E6 as a template. The reverse transcription products obtained (lanes 1 and 2) corresponded in size to the thymine residue (T) marked by * in the coding strand.

Figure 6 Agarose gel electrophoresis of RT-PCR product

Total RNA prepared from succinate-grown cells of *Rhodococcus* sp. AN-22 was used for the synthesis of cDNA as a templete. PCR was performed with the synthesized cDNA using OF and OR primers as amplification primers (lane 3). Control experiments were as follows: PCR was carried out using total RNA as a template without a reverse transcription step (lane 2) and using total DNA (lane 4) as a template. A 1-kb DNA ladder (New England Biolabs, Beverly, MA, U.S.A.) was used as markers (lane 1).

Figure 1

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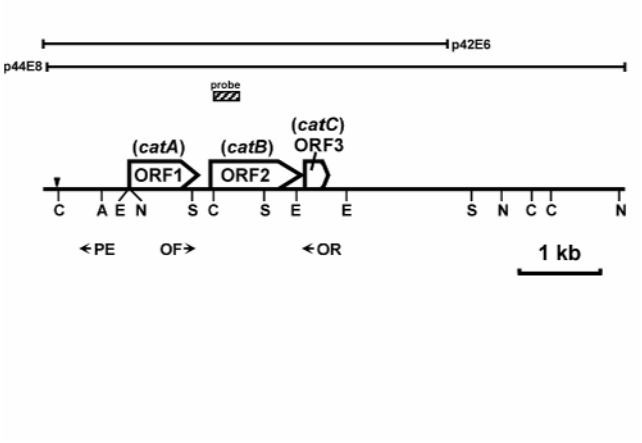

Figure 2

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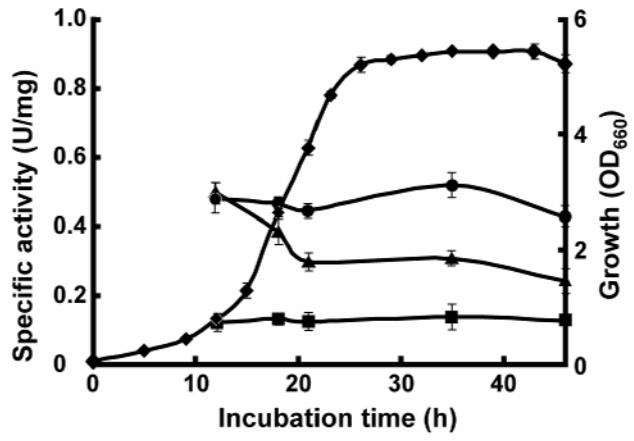

Figure 3

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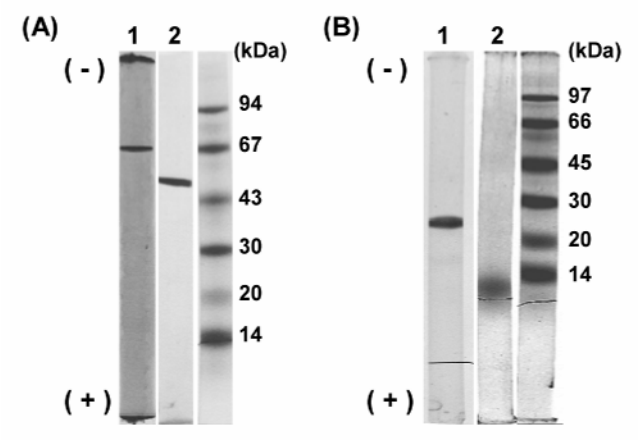

Figure 4

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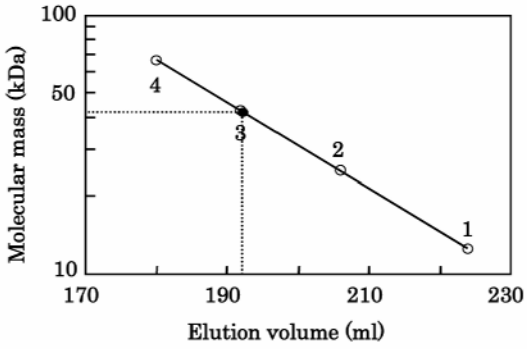

Figure 5

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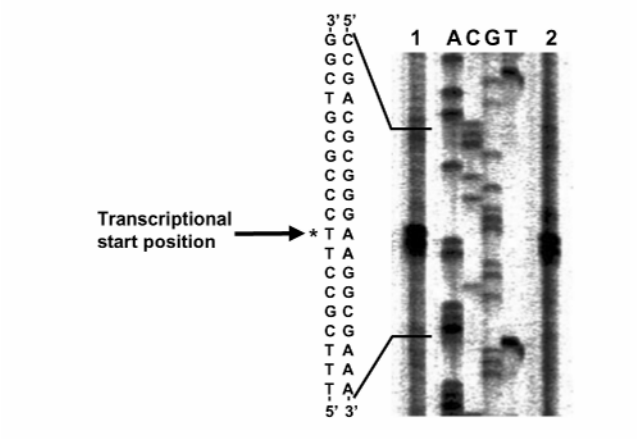

Figure 6

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