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RESEARCH ARTICLE

Cloning and expression of the gene encoding (R)-specific carbonyl reductase from *Candida parapsilosis* CCTCC M203011

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Abstract The gene which encodes (*R*)-specific carbonyl reductase (rCR) from *Candida parapsilosis* CCTCC M203011 was cloned, sequenced and compared with genes from the GenBank. The results indicated that rCR gene was 1011 bp, encoding a protein of 336 amino acids with a molecular weight of 35.9 kDa, and its nucleotide sequence showed 99% similarity to those of other members of the alcohol dehydrogenase superfamily. The rCR gene could express in recombinant strain *Escherichia coli* JM109, and the expression plasmid could produce (*R*)-1-pheny-1,2-ethanediol (100% e.e., 80.14% yield) from *β*-hydroxyacetophenone without any additive to regenerate NAD^+ from NADH.

Keywords carbonyl reductase, expression, asymmetric reduction, (*R*)-1-phenyl-1, 2-ethanediol

1 Introduction

The production of optical pure chiral material has significant value in pharmacology and toxicology, etc., because two enantiomers of chiral compounds have different effects on these aspects. Optical pure 1-phenyl-1,2-ethanediol is an important intermediate with great demand for the synthesis of optical active pharmaceuticals, agrochemicals, pheromones and liquid crystals, etc. (Schmid et al., 2001; Bevinakatti and Banerji, 1991; Shinobu et al., 1992).

There is considerable interest in biocatalysts for the production of optical pure chiral alcohols. The enzymatic asymmetric reduction of prochiral carbonyl compounds is an effective way of obtaining chiral alcohols. Kataoka et al. (1999) coexpressed aldehyde reductase gene and glucose dehydrogenase (GDH) gene in *E. coli* to produce ethyl (*R*)-4-chloro-3-hydroxybutanoate [(*R*)-CHBE] with a molar yield of 94.1% and an optical purity of 91.7% enantiomeric excess. Glucose dehydrogenase was used as a catalyst to complete the regeneration cycle of the coenzyme in the reaction system. Weckbecker et al. (2004) coexpressed pyridine nucleotide transhydrogenase, $NADP^+$-dependent alcohol dehydrogenase and NAD^+-dependent formate dehydrogenase to obtain (*R*)-phenylethanol through the conversion of prochiral ketone, and 66% yield was achieved. In this case, PNT (pyridine nucleotide transferase) was used to catalyze the transfer of hydrogen from NADH to $NADP^+$, which resulted in the simultaneous regeneration of NAD^+ and NADPH. Kataoka et al. (2004) cloned and overexpressed the genes encoding the two NADPH-dependent conjugated polyketone reductases isozymes (CPRs) in *E. coli*. The recombinant *E. coli* could reduce ketopantoyl lactone to D-pantoyl lactone in the reaction system containing $NADP^+$ and glucose dehydrogenase with a >90% molar yield and optical purity.

In previous studies, we found that *C. parapsilosis* CCTCC M203011 could effectively catalyze the deracemization of racemic 1-phenyl-1,2-ethanediol (PED) to an (*S*)-enantiomer. Two enzymes which were related to the reaction were purified. One is (*R*)-specific alcoholdehydrogenase, which reduces 2-hydroxyacetophenone to (*R*)-PED, and the other is (*S*)-specific alcoholdehydrogenase, which reduces 2-hydroxyacetophenone to (*S*)-PED. Two purified proteins have been sequenced. Here we described the cloning and expression of *rcr* gene encoding (*R*)-specific alcoholdehydrogenase from *C. parapsilosis*. The recombinant *E. coli* reduced *β*-hydroxyacetophenone to optical pure (*R*)-PED without the additive of coenzymes or coexpression of other genes.

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2 Materials and methods

2.1 Strains and plasmids

C. parapsilosis CCTCC M203011, vector pTrc99A, *E. coli* XL-1 and *E. coli* JM109 were stored in our laboratory. Cloning vector pMD18-T was purchased from TaKaRa. Plasmid pT-rCR and pTrc-rCR were constructed by our laboratory.

2.2 Mediums

2 × YT medium: tryptone 20 g/L, yeast extract 10 g/L and NaCl 10 g/L (pH7.2). Screening medium: tryptone 10 g/L, yeast extract 5 g/L and NaCl 10 g/L, agar 15 g/L, X-gal 4 mg/mL and isopropylthiogalactoside (IPTG) 2.4 mg/mL (pH7.2). 50 mg/L Ampicillin (final concentration) was added before all of mediums were used.

2.3 Enzymes and chemicals

Snailase was purchased from Beijing Biodee Biotechnology Co., Ltd. Proteinase K was the product of Merck. DNTP was obtained from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. Restriction enzyme, T_4 DNA ligase, Taq DNA polymerase, DNA marker DL2000, X-gal and IPTG were purchased from TaKaRa. 3S spin agarose gel DNA purification kit and primers were provided by Shanghai Biocolor Bioscience & Technology Company. *β*-hydroxyacetophenone was synthesized in our lab. All other chemicals used in this experiment were of analytical grade and commercially available.

2.4 Extraction of genomic DNA

C. parapsilosis CCTCC M203011 cells of 0.1 grams were washed twice with TE buffer (pH8.0), resuspended in TE buffer containing *β*-mercaptoethanol and incubated at 100 r/min for 30 min. Then cells were collected, washed twice with SCE buffer (0.7 mol/L KCl, 10 mmol/L $MgCl_2$, 10 mmol/L Tris, pH8.0)) and resuspended in TE buffer. 1% sodium dodecyl sulfate (SDS) was added to lyse the cells, and proteinase K to a final concentration of 100 μg/mL. After the mixture was incubated at 37°C for 30 min, phenol-chloroform extraction and chloroform extraction were used to remove impurities. Total nucleic acids were precipitated from extracted aqueous layer with 2 volumes of 100% ethanol for 2 h at −20°C. After centrifugation at 15000 × g for 20 min at 4°C, the pelleted nucleic acids were washed with ice-cold 75% (v/v) ethanol and vacuum dried prior to dissolving in 50 μL of TE buffer.

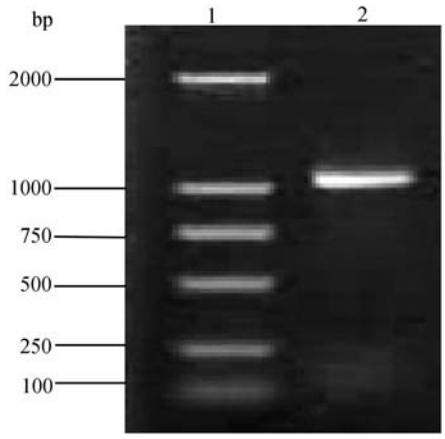

Fig. 1 Amplification of the DNA fragment of the *rcr* gene. Note: 1: DL2000 DNA markers; 2: PCR fragment

2.5 rCR protein sequencing

The purified rCR was sequenced using the LC/MS/MS system from Shanghai Genecore Biotechnologies Co., Ltd.

2.6 PCR

The amplification reactions were performed in 94°C for 3 min, 30 cycles of 94°C for 1 min, 45°C for 1 min, 72°C for 1 min, with a final extension step of 72°C for 10 min. PCR products were identified by electrophoresis on a 1.0% agarose gel.

2.7 Purification, ligation and transformation of PCR products

The amplified PCR products were purified by 3S spin agarose gel DNA purification kit and ligated into vector

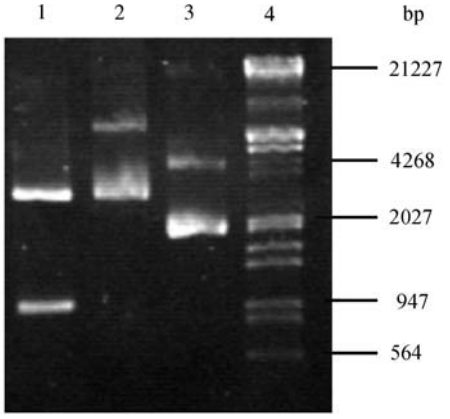

Fig. 2 Restriction enzyme analysis of the recombinant plasmid pT-rCR. Note: 1: pT-rCR/*Sal*I; 2: pT-rCR plasmid; 3: pMD18-T plasmid; 4: Marker (λDNA/*Eco*RI + *Hin*dIII)

Fig. 3 DNA sequence of the *rcr* gene

pMD-18T according to the manufacturer's instructions. Finally, the ligation products were transformed into *E. coli* XL-1 component cells. Positive clones were identified by blue-white spot screening.

2.8 Restriction enzyme analysis of positive clones

PT-rCR was extracted by using alkaline lysis method and stored at −20°C for restriction enzyme analysis.

2.9 Cloned gene sequencing

PCR products were sequenced at Shanghai Biocolor BioScience & Technolgy Co., Ltd using a pair of primers (pM13-R and pM13-F).

2.10 Construction of expression plasmid

The expression plasmid was constructed according to the laboratory manual (Sambrook et al., 1993)

2.11 SDS-PAGE analysis

The expression levels of rCR were analyzed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Wang and Fan (2004). The protein bands were visualized by staining with Coomassie brilliant blue.

2.12 Measurement of recombinant protein

A positive clone was selected for incubation in 2 × YT culture at 37°C for 8 h. After inducing with 1 mM IPTG for 16 h, the cells were collected by centrifugation and resuspended in buffer (20 mM Tris-HCl (pH8.0)). The induced cells were disrupted by sonication. Debris was removed by centrifugation (12000 r/min, 10 min, 4°C) to obtain the crude extract. The rCR activity was determined using the Vallee&Hoch method (Stellmach, 1992).

2.13 Asymmetric reaction

0.1 g cells (wet weight) were suspended in 1 mL solution containing 0.5‰ β-hydroxyacetophenone followed by incubation at 30°C for 48 h with shaking. After the reaction, cells were removed by centrifugation and (*R*)-PED was extracted with 2 mL ethyl acetate by vigorous mixing. The organic layer was analyzed by HPLC (HP 1100, Agilent, USA).

3 Results

3.1 Protein sequencing

The purified rCR was digested by trypsin, and the resulting peptides were analyzed by liquid chromatographyTandem mass spectrometry (LC-MS-MS). The sequences of three fragments were identified as NDLPVHKPKAGQLLLK, VACVGPNGCGGCKYCR and LKELPEYIEK. Three peptide sequences were blasted by PBD, and the result showed complete match to *CpSADH* (Yamaoto et al., 1999). Based on the amino acid sequence of *CpSADH*, a pair of primers was designed as follows: sense, 5′–aagtcgacatgtcaattccatcaa–3′; antisense, 5′–aactcgagctatg-gattaaaaacaac–3′ (underlined nucleotides indicate the *Sal*I and *Xho*I cleavage sites).

3.2 PCR amplification, purification, ligation and transformation

Agarose gel electrophoresis of PCR products are shown in Fig. 1. The length of the PCR fragment was estimated to be 1 kb. PCR products were purified and ligated into pMD18-T vector. After *E. coli* XL-1 was transformed with the ligation mixture, positive clones were obtained by blue-white spot screening.

3.3 Identification of recombinant plasmid

Because both sense primer and pMD18-T vector contain *Sal*I cleavage sites, *Sal*I was chosen to identify recombinant plasmid. Enzyme digestion indicated that the *rcr* gene had been successfully inserted into vector pMD18-T (Fig. 2).

3.4 DNA sequencing of *rcr* gene

The nucleotide acid sequences of the *rcr* gene are shown in Fig. 3. The underlined parts correspond to the sense and antisense primers. From ATG to TAG, the *rcr* gene consists of 1011 bp. The sequence showed 99% similarity to *CpSADH* by GenBank Blast. The *rCR* gene encodes a protein containing 336 amino acids and its molecular weight was calculated to be about 35.9 kDa. The sequence of the *rcr* gene had been submitted to GeneBank and the access number is DQ295067.

3.5 Analysis of the domains of *rcr* gene and phylogenesis

The DNA sequence of the *rcr* gene was submitted to NCBI (http://www.ncbi.nlm.nih.gov). Then the sequence was compared with the sequences in the GenBank's non-redundant Database using the BLASTX program (E $<10^{-27}$) with an e-value cut off of $<10^{-4}$, and 19 homologous genes were searched. Clustalw (http://www.ebi.ac.uk/clustalw/) software was used to align homologous genes. The ORF sequence of the *rcr* gene was subsequently used to identify the functional domains via InterProScan (http://www.ebi.ac.uk/InterProScan). The results showed that protein rCR had three functional domains: Adh_zinc binding domain, NAD^+_Bing domain and GroEs_like collapse domain (Fig. 4). The DNA sequence of the *rcr* gene was translated into amino acid sequence, and then neighbor-joining procedure from Phylip 3.57 software was conducted to construct the phylogenetic tree (Fig. 5). The shadowed part is *Candida parapsilosis* CCTCC M203011, which has the highest relation to *Candida orthopsilosis* and *Candida merapsilosis*, and lowest relation to *Zymamonas mobilis*.

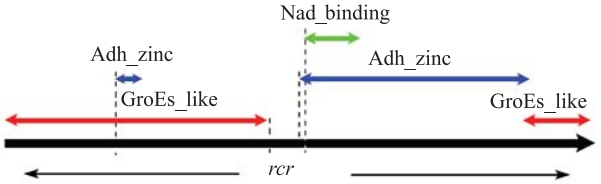

Fig. 4 Function domain of the *rcr* gene

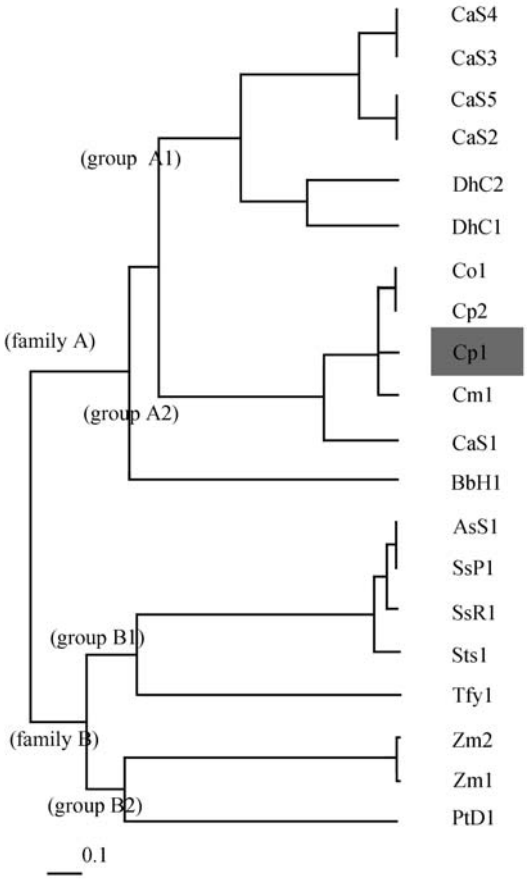

Fig. 5 Phylogenetic tree of the *rcr* gene

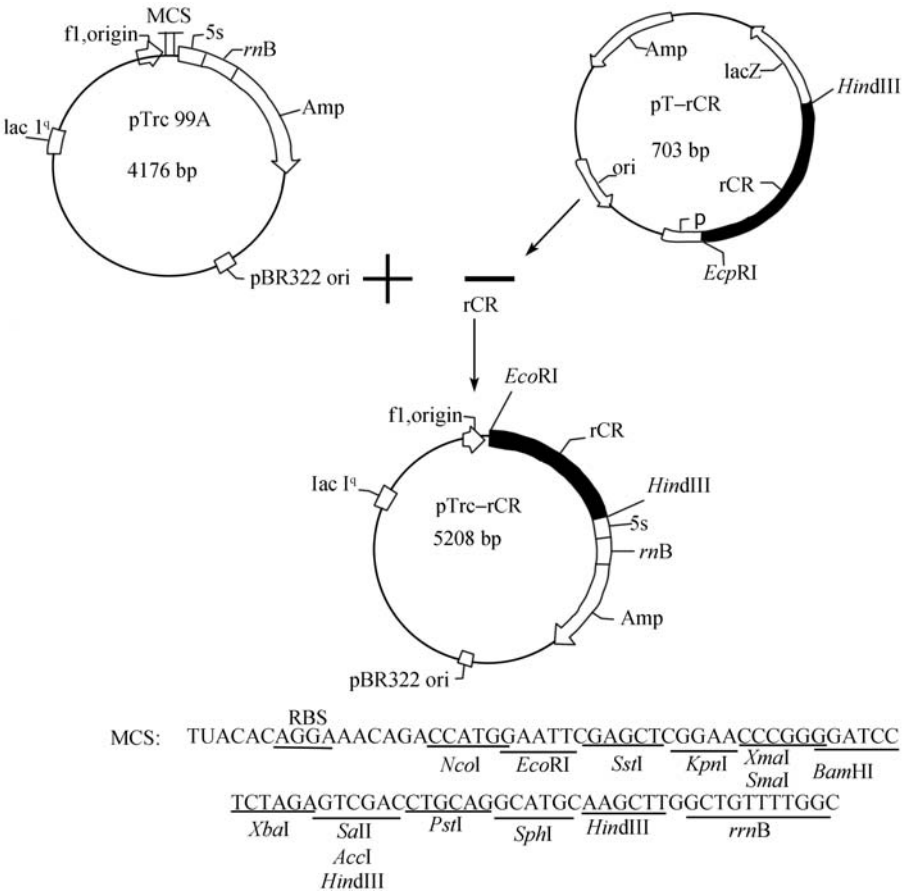

Fig. 6 Constructing process of expression plasmid

3.6 Construction of expression plasmid pTrc-rCR and restriction enzyme analysis

The procedure for the construction of expression plasmid pTrc-rCR is shown in Fig. 6. The recombinant plasmid was identified by *Sal*I restriction enzyme analysis because both sense primer and vector pTrc99A had the cleavage site. After digestion of *Sal*I, about 1 kb fragment was cut from the recombinant plasmid (Fig. 7). Then, pTrc-rCR was transformed into *E. coli* JM109 cell.

3.7 SDS-PAGE analysis

After inducing by IPTG, the cells that were harvested by centrifugation were disrupted by sonication and

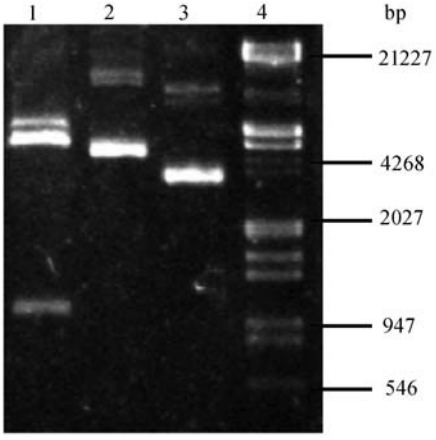

Fig. 7 Restriction enzyme analysis of expression plasmid pTrc-rCR. Note: 1: pTrc-rCR/Sal I; 2: pTrc-rCR plasmid; 3: pTrc99A plasmid; 4: Marker (λDNA/*Eco*R I + *Hin*dIII)

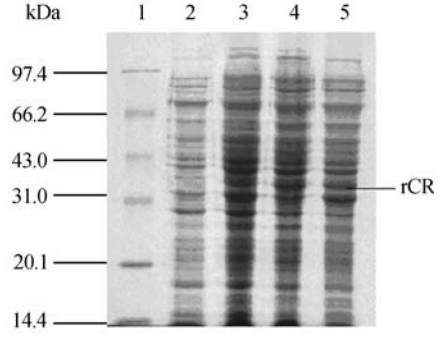

Fig. 8 SDS-PAGE analysis of the plasmid pTrc-rCR-inducted *E. coli* JM109. Note: 1: Protein molecular weight markers; 2: *E. coli* JM109/pTrc99A; 3–5: *E. coli* JM109/pTrc-rCR after induction

Table 1 Enzyme activity of the recombinant protein

substrate	*E. coli* JM109/pTrc99A/U·mg^{-1}	*E. coli* JM109/pTrc-rCR without induction/U·mg^{-1}	*E. coli* JM109/pTrc-rCR after induction/U·mg^{-1}
β-hydroxyacetophenone	0.04	0.129	0.223

suspended in the 1 × SDS buffer. The mixture was boiled at 100°C for 5 min, SDS-PAGE with 15 μL. SDS-PAGE showed that a new band appeared in the band 3–5 and confirmed that the *rcr* gene had expressed in *E. coli* JM109 (Fig. 8).

3.8 Assay of rCR activity

The enzyme activity of recombinant rCR was determined with β-hydroxyacetophenone as the substrate (Table 1). After induction by 1 mM IPTG, specific activity of rCR was much higher than that of the control. Even without induction, the rCR gene could express in the recombinant strain because the lac promoter in pTrc99A was a constitutive promoter.

3.9 Whole-cell biotransformations

Transformation of recombinant *E. coli* was determined by the reduction of β-hydroxyacetophenone to (*R*)-PED (Table 2). Although *E coli* containing pTrc99A could also convert the substrate, transformation effect was much lower than positive recombinant strain with a molar yield of 80.4% and an optical purity of 100% e.e. No corresponding PCR products were obtained using *E. coli* JM109 as the template with sense and antisense primers. Thus, *E. coli* containing pTrc99A could express some reductases whose functions were similar to rCR.

4 Discussion

According to the rCR sequence, the complete nucleotide acid sequence of the *rcr* gene was achieved. The analysis of NCBI showed that rCR protein had typical Adh_zinc domain and NAD^+_Binding domain. The purified rCR from *C. parapsilosis* CCTCC M203011 was Zn^{2+}-dependent and NAD^+-dependant, which is consistent with the domain analysis of gene cloning. The DNA sequence of the *rcr* gene provides a basis for further research such as enzymatic characteristics and catalysis mechanism of rCR, structure and function of *rcr* gene and directed evolution of cloning gene.

Table 2 Effect of whole-cell conversion using the recombinant strain

strain	yield/%	(*R*)-e.e./%
E. coli JM109 (pTrc99A)	18.5	91.5
E. coli JM109 (pTrc-rCR)	80.4	100

The gene which encodes (*R*)-specific carbonyl reductase (rCR) from *C. parapsilosis* CCTCC M203011 was cloned and expressed in *E. coli*. The recombinant strain could reduce asymmetrically β-hydroxyacetophenone to (*R*)-PED with a molar yield of 80.4% and an optical purification of 100% e.e. In some reported work, carbonyl reductase gene expressed and chiral compounds were obtained by asymmetric reduction of recombinant strains, and coenzymes were added or another gene was co-expressed to complete the regeneration cycle of coenzymes. These methods increase the complexity of operations or production cost. However, in our study, the recombinant *E. coli* containing pTrc-rCR could regulate regeneration cycle of coenzyme through its own redox. This effective and useful production system makes it possible to investigate the reaction process, produce industrial application of (*R*)-PED and create an enzyme with a desired stereospecificity by the approach of site-directed evolution.

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