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Construction of a novel expression vector in *Pseudonocardia autotrophica* and its application to efficient biotransformation of compactin to pravastatin, a specific HMG-CoA reductase inhibitor

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ABSTRACT

The novel plasmid vector (pTAOR4-Rev) suitable for gene expression in actinomycete strains of *Pseudonocardia autotrophica* was constructed from 2 *P. autotrophica* genetic elements, the novel replication origin and the acetone-inducible promoter. The replication origin was isolated from the endogenous plasmid of strain DSM 43082 and the acetone-inducible promoter was determined by analysis of the upstream region of an acetaldehyde dehydrogenase gene homologue in strain NBRC 12743. *P. autotrophica* strains transformed with pTAOR4-P450, carrying a gene for cytochrome P450 monooxygenase, expressed P450 from the acetone-inducible promoter, as verified by SDS–PAGE and spectral analysis. The biotransformation test of acetone-induced resting cells prepared from a strain of *P. autotrophica* carrying pTAOR4 that harbors a compactin (CP)-hydroxylating P450 gene revealed 3.3-fold increased production of pravastatin (PV), a drug for hypercholesterolemia. Biotransformation of CP by the same strain in batch culture yielded PV accumulation of 14.3 g/l after 100 h. The expression vector pTAOR4-Rev and its function-enhancing derivatives provide a versatile approach to industrial biotransformation by *Pseudonocardia* strains, which can be good hosts for P450 monooxygenase expression.

1. Introduction

Host-vector systems are indispensable for the expression of recombinant proteins for research or industrial purposes. In the industrial application of heterologous gene expression, bacterial strains are used preferentially as hosts because they are easy to handle and their bioprocesses are easily manipulated. In addition to the general *Ecsherichia coli* system, streptomycete host-vector systems are noteworthy because the actinomycete strains have the potential to produce industrially valuable bioactive materials such as antibiotics, antitumor agents, and statins via fermentation or biotransformation [1]. The streptomycete vectors are generally incompatible and of limited utility in non-streptomycete hosts such as *Pseudonocardia autotrophica*, which is utilized for biotransformation of vitamin D_3 (VD_3) to calcitriol (CT) [2]. *P. autotrophica* catalyze hydroxylation of VD_3 at C-25, and then C-1α to CT. In our previous study of efficient biocatalytic processes of VD_3 hydroxylation, we identified cytochrome P450 monooxygenase (P450) as the hydroxylase responsible for VD_3 transformation. This P450, designated as Vdh, catalyzed the 25- and 1α-hydroxylations. The Vdh gene (*vdh*) was cloned and expressed in *E. coli*, in which the enzymatic properties of the recombinant protein were determined. *E. coli* expression and biotransformation systems were established and enabled to increase the hydroxylation activity of Vdh by directed evolution [3]. Improved biotransformation was obtained with *P. autotrophica* than with Vdh-expressing *E. coli* (data not shown). A *Pseudonocardia*-specific expression system was required for expressing improved *vdh* genes to enhance VD_3 hydroxylation activity.

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl-Coenzyme A; Vdh, vitamin D_3 hydroxylase; Fdx, ferredoxin; Fdr, ferredoxin-NADP$^+$ reductase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; HPLC, high-performance liquid chromatography.

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Actinomycete strains have many P450 genes in their genome [4]. These enzymes are involved in the production of secondary metabolites or degradation of xenobiotics. Hydroxylations catalyzed by actinomycete P450s are used in industrial applications, such as the production of pravastatin (PV) and CT. PV is a highly potent and specific inhibitor of HMG-CoA reductase, a key enzyme in cholesterol biosynthesis [5]. Because of its pharmaceutical value as a cholesterol-reducing agent, there are many reports on PV production by compactin (CP) hydroxylation in actinomycete strains. The industrial manufacturing of PV was established in *Streptomyces carbophilus*, in which P450sca-2 was shown to catalyze CP hydroxylation [6]. Afterwards, several reports on PV production described biotransformation with *Actinomadura* [7], *Streptomyces* [8], and *Pseudonocardia* [9]. CT production by biotransformation with *P. autotrophica* is used for industrial production of CT for osteoporosis and psoriasis [2]. Actinomycete strains provide a useful P450 source and have a suitable redox background with electron transport proteins required for P450 reactions. Therefore, actinomycete strains should be good candidates as host strains for P450 expression.

In our study, *Streptomyces* sp. TM-7 was isolated as a PV-producing strain. This strain yields PV accumulation of 4.6 g/l from 8.2 g/l CP after 6 days [10]. The genes for CP 6β-hydroxylating P450 (*boxA*) and ferredoxin (*boxB*) were cloned from *Streptomyces* sp. TM-7. We attempted to express BoxA and BoxB in *P. autotrophica*, known as a CP-resistant [9] and VD_3-hydroxylating strain. Utilization of hosts that are resistant to substrates and products is an appropriate strategy to improve biotransformation productivity. In this report, we describe the construction of a novel acetone-inducible expression vector (pTAOR4-Rev) for *P. autotrophica* and its application in the efficient biotransformation of CP to PV in *boxAB*-expressing *P. autotrophica*.

2. Materials and methods

2.1. Bacterial strains and culture conditions

P. autotrophica NBRC 12743, *P. autotrophica* DSM 43082, *P. autotrophica* DSM 535, *E. coli* DH5α, *E. coli* S17-1, and *E. coli* BL21(DE3) were cultured in LB medium (1% polypeptone, 0.5% yeast extract, and 1.0% NaCl) with 200 μg/ml kanamycin or 24 μg/ml apramycin for *P. autotrophica* transformants and 25 μg/ml kanamycin, 50 μg/ml ampicillin, or 50 μg/ml apramycin for *E. coli* transformants.

2.2. Recombinant DNA techniques

Restriction enzymes and DNA ligation reagents were purchased from TaKaRa Bio. Inc. (Shiga, Japan). DNA manipulation was conducted by standard methods [11] or as instructed by specific kit suppliers. Plasmid DNA was prepared with a miniprep purification kit (QIAGEN, Hilden, Germany), and polymerase chain reactions (PCR) were carried out in an automated thermal cycler (Applied Biosystems Inc., CA, USA) using KOD plus DNA polymerase (Toyobo, Osaka, Japan). Total DNA from *Pseudonocardia* strains was isolated with ISOPLANT II (Nippongene, Tokyo, Japan).

2.3. Identification of replication origin in P. autotrophica

Endogenous plasmid pPA43082 was identified in *P. autotrophica* DSM 43082 and the plasmid was sequenced as described in Supplementary Methods. Based on the sequence of pPA43082, we predicted that the replication system functions by the rolling-circle mechanism. Different fragment lengths containing the pPA43082 replication origin were amplified and ligated into the *Bsr*GI and *Bgl*II sites of pTNR-oriT, replacing *istAB* [12]. Plasmid constructs were tested for transformation ability in *P. autotrophica* NBRC 12743. Transformation was conducted by conjugation with *E. coli* S17-1 as described in Supplementary Methods.

The shortest DNA region containing essential elements for replication was amplified from pPA43082 with primers rep-1F (5′-GCC GGATCCCTCCCGCCGCCCCGCACGGCA-3′; *Bam*HI site is underlined) and rep-7R (5′-GCCTGTACATGACCCGCACCCGCCAGGCGT-3′; *Bsr*GI site is underlined). The amplified 2.1-kb DNA fragment was ligated into the *Bgl*II and *Bsr*GI sites of pTNR-oriT to yield pTNR-oriT-rep1. The plasmid in which the fragment was inserted in the opposite orientation did not yield transformants.

2.4. Sequence analysis of the region encoding acetone-inducible protein

A protein induced by acetone addition in the culture was detected by SDS–PAGE in *P. autotrophica* NBRC 12743. The acetone-inducible protein (AIP) was isolated by 2D-PAGE and the internal amino acid sequence was determined. Based on the internal amino acid sequence, the gene encoding AIP and its upstream region was sequenced to identify the acetone-inducible promoter (P*ace*). The detailed experimental procedure is described in Supplementary Methods.

2.5. Expression vector construction

The DNA fragment encoding *oriT* was amplified from pTNR-oriT and ligated into the *Bsr*GI and *Bam*HI sites of pTNR-AA to construct pTNR-AA-oriT. After removal of the IS2 and ampicillin-resistance gene of pTNR-AA-oriT, the DNA fragment encoding the replication origin for *P. autotrophica* was ligated into the *Kpn*I and *Bsr*GI sites to create pTAOR. The DNA fragments encoding P*ace*, *vdh* (GenBank Accession No. AB 456955), and a transcriptional terminator were inserted into pTAOR to produce pTAOR3-vdh. The DNA fragment encoding *boxAB* (GenBank Accession No. AB180845) was inserted into pTAOR3-vdh to replace *vdh* and create pTAOR3-boxAB. pTAOR3-boxAB was digested with *Hin*dIII and *Afl*II, blunted by T4 DNA polymerase, and self-ligated to yield pTAOR4. The DNA fragment encoding P*ace*-*boxAB*-terminator was ligated into the *Kpn*I site of pTAOR4 to create pTAOR4-For-boxAB and pTAOR4-Rev-boxAB. The detailed experimental procedure is described in Supplementary Methods.

2.6. Transformation by electroporation

P. autotrophica DSM 535 was cultured in LB medium with glass beads (5 mm in diameter) and competent *Pseudonocardia* cells were prepared according to the procedure for *Rhodococcus* strains [13]. A 100 μl aliquot of chilled competent *Pseudonocardia* cells was gently mixed with 3 μl (0.6 μg) pTAOR in a microcentrifuge tube and placed on ice for 30 min. The cell-DNA mixture was transferred to a prechilled 0.1 cm electrode gap Gene Pulser Cuvette (Bio-Rad Laboratories, CA, USA) for electroporation (Bio-Rad Gene Pulser). The electroporation mixture was resuspended in 1 ml LB and incubated at 30 °C for 3 h. The mixture was plated on LB agar plates containing 30 μg/ml apramycin and incubated at 30 °C for 6 days to select apramycin-resistant *Pseudonocardia* colonies.

2.7. Reduced CO difference spectral analysis

To measure the concentration of P450s in the *P. autotrophica* cells, reduced CO difference spectral analyses were performed. *P. autotrophica*/pTAOR3-vdh and *P. autotrophica*/pTAOR4-Rev-boxAB were cultured at 28 °C in 50 ml LB medium containing 24 μg/ml apramycin to an OD_{600} between 0.4 and 0.7. Protein expression was initiated by adding acetone to a final concentration of 1%. After