Biochemical and Biophysical Research Communications 404 (2011) 511–516

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

Yoshikazu Fujii [a,d], Koji Norihisa [b], Tadashi Fujii [a], Yasuhide Aritoku [a], Yusuke Kagawa [d], Khalid Ibrahim Sallam [c,1], Osamu Johdo [a], Akira Arisawa [a,*], Tomohiro Tamura [c,d,*]

[a] Bioresource Laboratories, Mercian Corporation, 1808 Nakaizumi, Iwata, Shizuoka 438-0078, Japan
[b] Biotechnical Development Center, Mercian Corporation, 1808 Nakaizumi, Iwata, Shizuoka 438-0078, Japan
[c] Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), 2-17-2-1 Tsukisamu-Higashi, Toyohira-ku, Sapporo 062-8517, Japan
[d] Graduate School of Agriculture, Hokkaido University, Kita-9, Nishi-9, Kita-ku, Sapporo 060-8589, Japan

ARTICLE INFO

Article history:
Received 25 November 2010
Available online 6 December 2010

Keywords:
Pseudonocardia autotrophica
Pravastatin
Rolling-circle type replication
Acetone-inducible promoter
Biotransformation

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.

* Corresponding authors. Address: Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), 2-17-2-1 Tsukisamu-Higashi, Toyohira-ku, Sapporo 062-8517, Japan (T. Tamura). Fax: +81 538 21 1135 (A. Arisawa), fax: +81 11 857 8980 (T. Tamura).

E-mail addresses: arisawa-a@mercian.co.jp (A. Arisawa), t-tamura@aist.go.jp (T. Tamura).

[1] Present address: Department of Food Hygiene and Control, Faculty of Veterinary Medicine, Mansoura University, Mansoura, Egypt.

0006-291X/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2010.12.013

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

24 h induction, cells were harvested by centrifugation and resuspended in buffer A (50 mM potassium phosphate buffer pH 7.4, 10% glycerol). Cell-free extract was prepared as described in Supplementary Methods. The reduced CO difference spectra were measured with a cell-free extract by a UV–visible spectrophotometer U-3310 (Hitachi) as described previously [14].

2.8. Biotransformation of compactin (CP) to pravastatin (PV) by resting cells

Host strains *P. autotrophica* NBRC 12743, *P. autotrophica*/pTAOR, and *P. autotrophica*/pTAOR4-Rev-boxAB were cultured for 72 h at 28 °C in 100 ml LB medium containing 24 μg/ml apramycin. Protein expression was initiated by adding acetone to a final concentration of 1%. After 24 h induction, cells were harvested by centrifugation and resuspended in buffer B (50 mM potassium phosphate buffer pH 7.4 and 2% glycerol) to 16.3 mg of wet-cell weight/ml. Biotransformation was initiated by the addition of compactin (CP) to a final concentration of 3 mg/ml. Conversion was conducted for 24 h at 28 °C. The sample was mixed with a half volume of acetonitrile and methanol, followed by centrifugation at 15,000 rpm. The supernatant was analyzed by HPLC as described below.

2.9. Biotransformation of CP to PV in batch culture

P. autotrophica/pTAOR4-Rev-boxAB was cultured in 100 ml seed medium (1.5% D-glucose, 0.3% yeast extract, 1.5% polypeptone, 0.4% NaCl, 0.2% $CaCO_3$, pH 7.4) containing 24 μg/ml apramycin at 28 °C for 72 h. Then, 1 ml of the culture was inoculated into 100 ml of the main medium (2.0% D-glucose, 0.5% yeast extract, 1.0% polypeptone, 1.0% soy protein, 0.04% NaCl, 0.04% K_2HPO_4, 0.3% $CaCO_3$, pH 7.4) containing 24 μg/ml apramycin and was incubated at 28 °C for 48 h, followed by the addition of 1 ml acetone. After 24 h cultivation, biotransformation was carried out with CP (20 mg/ml in water) which was added to the culture at a final concentration of 4 mg/ml. Additional CP was added as it was consumed. Conversion samples (500 μl) were mixed with 250 μl acetonitrile and 250 μl methanol. After centrifugation at 15,000 rpm for 10 min, the supernatant was analyzed by HPLC.

2.10. HPLC analysis

PV and CP were analyzed by HPLC on a Chromolith Performance RP-18e (4.6 mm ID × 100 mm) column at 40 °C using a methanol gradient in aqueous solution (containing 0.1% triethylamine and 0.1% acetic acid) from 50% to 90% for 3 min, 90% for 0.5 min, and 50% for 2.5 min at a flow rate of 2.0 ml/min. The derivatives were detected by UV-monitoring at 238 nm. PV and CP had retention times of 1.8 min and 3.2 min, respectively.

2.11. Sequence information

The DNA sequences of pPA43082, pTAOR4-Rev-boxAB, and the *Cla*I-*Xho*I acetone-inducible fragment containing *aceA*-P*ace*-*orfA*-*orfB* are available in GenBank as accession numbers AB600171, AB600173, and AB600172, respectively.

3. Results and discussion

3.1. Plasmid isolation from P. autotrophica and identification of the replication origin

A *P. autotrophica*-specific host-vector system was required for our approach to efficient biotransformation with *P. autotrophica* strains in which improved *vdh* genes are expressed. However, such a system was unavailable, so we sought to construct an original expression vector for *P. autotrophica*.

The literature contains no prior report on the *Pseudonocardia* plasmid. We searched for the native endogenous plasmid in *P. autotrophica* strains. Ten of the 25 strains in the DSMZ culture collection were found to contain cryptic plasmids. Three plasmids were sequenced and found to be nearly identical. The plasmid referred to as pPA43082 was isolated from *P. autotrophica* DSM 43082; it is a circular plasmid of 8047 bp in size. The rolling-circle replication system of pPA43082 consists of the *rep* gene and double-strand origin (DSO)-like, and single-strand origin (SSO)-like sequences [15]. In addition to the replication origin, 3 open-reading frames were deduced; ORF1 showed significant similarity to partitioning protein ParA from *Clavibacter michiganensis* (34% identity); ORF2 showed significant similarity to transcriptional regulator TraB from *Streptomyces ghanaensis* (36% identity); ORF3 showed significant similarity to FtsK, which is involved in cell division in *Rhodococcus erythropolis* (31% identity) (Fig. 1A). The *rep* gene shared 40% identity with that of pAP1 from *Arcanobacterium pyogenes*.

The region essential for replication was determined using *P. autotrophica* NBRC 12743 as a host by assessing the transformation ability of plasmids carrying different lengths of *rep*-flanking regions. The DNA fragment (between nucleotides 4201 and 6300) was recognized as the essential region for replication of pPA43082. This fragment was inserted into pTNR-oriT to yield the *E. coli–P. autotrophica* shuttle vector pTNR-oriT-rep1.

3.2. Identification of the acetone-inducible promoter and construction of expression vector pTAOR4-Rev

To obtain an inducible promoter to regulate expression of cloned genes in *Pseudonocardia*, we searched for proteins whose expression is upregulated by solvents (acetone and DMSO) or chemicals (VD_2 and VD_3). After cultivation to mid-log phase, each solvent or chemical was added and the cultures were incubated for an additional 24 h. Cell-free extracts were prepared and analyzed by SDS–PAGE. The inducible protein was not observed in cultures to which VD_2, VD_3, and DMSO were added. In contrast, a protein with molecular weight of about 55 kDa was induced by acetone. Its expression increased with acetone in a dose-dependent manner and reached maximum expression at 1% acetone (Fig. 2A). 2D-PAGE was performed for more precise analysis and revealed that 3 proteins of ~55 kDa were induced significantly by acetone (Fig. 2B). One of the proteins was recovered from the 2D gel and subjected to amino acid sequence analysis. Two internal peptide sequences, GQYFENPTPITG and MLDHYQQTK, were determined and degenerate primers were designed to clone the gene encoding the protein. After sequencing the PCR products along with successive gene walking by inverse PCR, we obtained 5158 bp of sequence information and identified three open-reading frames, *aceA*-*orfA*-*orfB* (Fig. 2C). The deduced amino acid sequence of *aceA* (543 amino acids with a molecular weight of 59,003 Da), which runs divergently to *orfA*-*orfB*, contained the determined peptide sequences, indicating that this gene encoded the acetone-inducible protein. The deduced product of *aceA* shared strong similarity with aldehyde dehydrogenase from *Actinosynnema mirum* DSM 43827 (82% identity). Acetone, for its analogous structure to acetaldehyde, is assumed to act as an inducer in the putative acetaldehyde-inducible system. *orfA* is 341 bp upstream of *aceA* and its deduced product (475 amino acids with a molecular weight of 51,851 Da) shared significant similarity with a phytochrome sensor protein in *Kribbella flavida* DSM 17836 (54% identity). *orfB* encodes a protein comprising 221 amino acids with a molecular weight of 24,156 Da. It shares significant similarity with a putative fructose transport system kinase in *Streptomyces viridochromogenes*

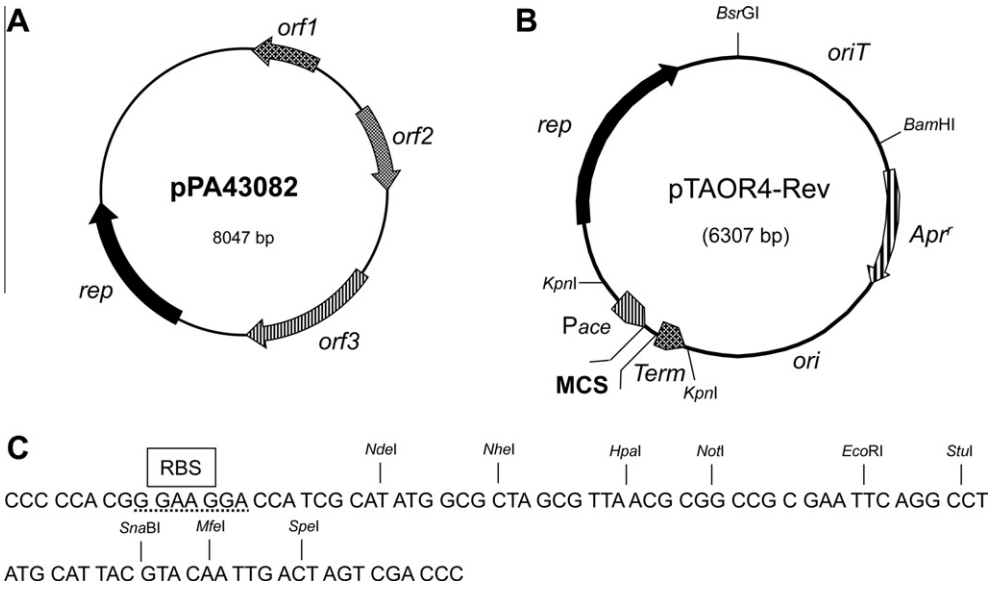

Fig. 1. Schematic maps of pPA43082 and pTAOR4-Rev. (A) Schematic map of cryptic plasmid pPA43082. Deduced amino acid sequences of ORF1, ORF2, and ORF3 showed significant similarity to PerA, TraB, and FtsK, respectively. The region including *rep* (nt 4201-6300 in pPA43082) was used for pTAOR4-Rev construction. (B) Schematic map of expression vector pTAOR4-Rev. *Apr*^{r}, apramycin resistance gene; *ori*, replication origin for *E. coli*; P*ace*, acetone-inducible promoter; MCS, multiple cloning site; *Term*, transcriptional terminator; fragment containing *rep*, minimum region derived from pPA43082 for autonomous replication of the plasmid in *P. autotrophica*. (C) Sequence of ribosome binding site (RBS) and multiple cloning site. The RBS sequence is dot-underlined. All restriction enzyme sites in the multiple cloning site are unique.

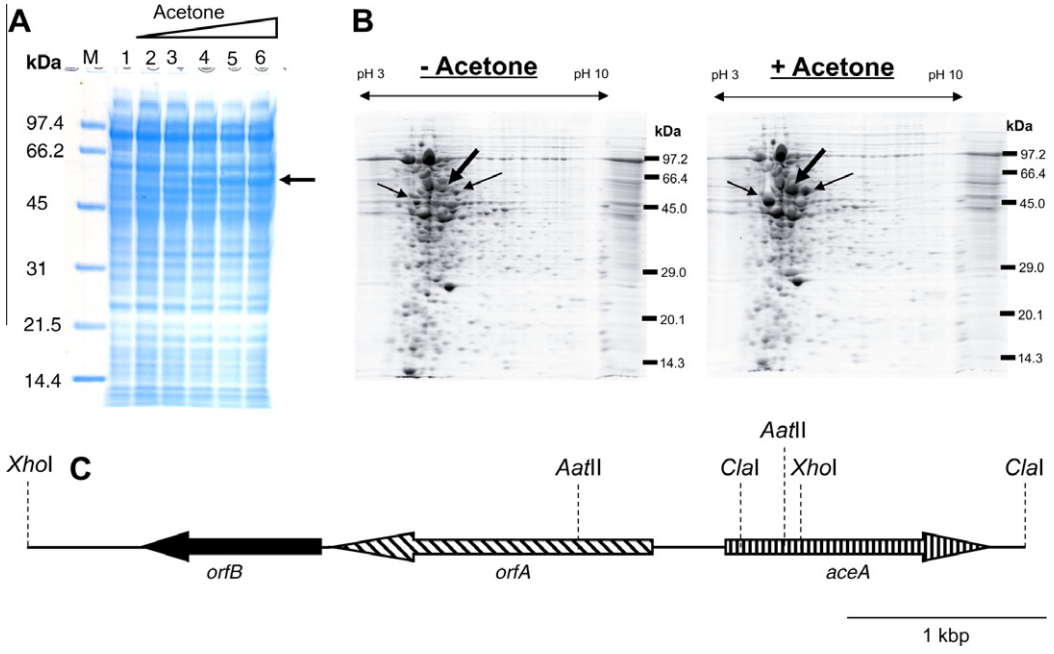

Fig. 2. Identification of acetone-inducible protein. (A) The cell-free extract samples prepared from *P. autotrophica* cultures induced with different concentrations of acetone: 0% (lane 1), 0.01% (lane 2), 0.05% (lane 3), 0.1% (lane 4), 0.5%, (lane 5), and 1.0% (lane 6). M, Molecular marker. The arrow represents acetone-inducible protein (AIP). Cell extracts (15 μg of protein) were resolved by SDS–PAGE with 12.5% polyacrylamide gel and proteins were stained with Coomassie Brilliant Blue G-250. (B) The cell-free extract samples shown in Fig. 2A, lane 1 and lane 6, were resolved by 2D-PAGE as described in Section 2. The arrows show AIPs. The bold arrow represents the most highly induced protein that was subjected to amino acid sequence analysis. (C) Restriction map of the *aceA* region. The arrows indicate ORFs.

DSM 40736 (65% identity). The functional correlation of *aceA* with *orfA* and *orfB* remains unclear. Putative promoter-35 and -10 regions were found 205 bp upstream [TTCACG] and 182 bp upstream [TATGGT] of the *ace*A start codon.

The expression vector pTAOR4-Rev was constructed with the replication origin of pPA43082 and *oriT*, along with the acetone-inducible promoter and a transcriptional terminator (Fig. 1B). In addition to plasmid transfer by conjugation between *E. coli* and *P. autotrophica*, plasmid transformation by electroporation was tested. The highest transformation efficiency was 5×10^2/μg of plasmid DNA when electroporation was performed in 0.1 cm gap Gene Pulser Cuvettes with 1.5 kV, 400 Ω resistance, and 25 μF. Although the transformation efficiency is not high, it is sufficient to obtain transformants for protein expression experiments. As

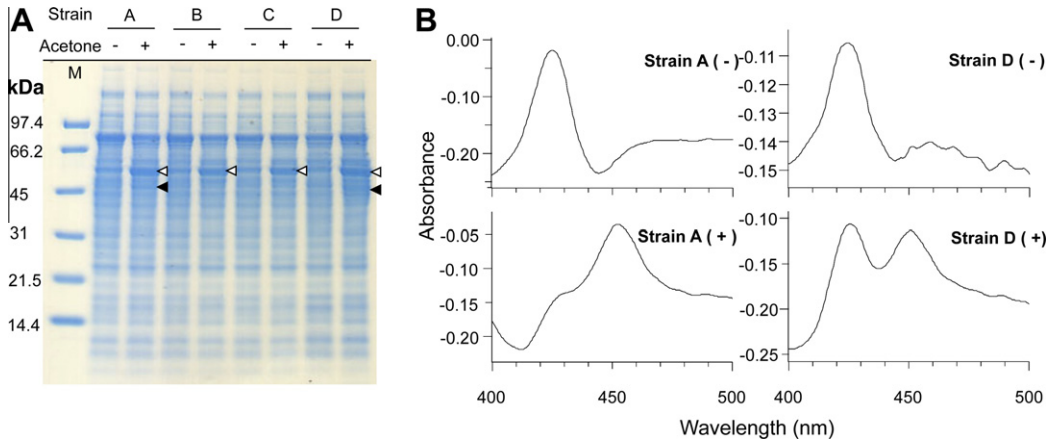

Fig. 3. Expression of recombinant P450s in *P. autotrophica*. (A) SDS–PAGE was performed on cell-free extracts. Strain A, *P. autotrophica*/pTAOR3-vdh; B, *P. autotrophica* NBRC 12743 (wild type); C, *P. autotrophica*/pTAOR; D, *P. autotrophica*/pTAOR4-Rev-boxAB. + and − represent presence and absence of acetone induction. Open and closed triangles represent acetone-inducible protein and expressed recombinant P450s, respectively. Cell extracts (15 μg of protein) were resolved by SDS–PAGE with 10–20% polyacrylamide gel and proteins were stained with Coomassie Brilliant Blue G-250. (B) Active P450 expression was detected by reduced CO difference spectral analysis (CDSA). Strain A (*P. autotrophica*/pTAOR3-vdh) and strain D (*P. autotrophica*/pTAOR4-Rev-boxAB) were cultivated in the presence and absence of acetone induction. Cell-free extracts were analyzed by CDSA. (+) and (−) represent presence and absence of acetone induction.

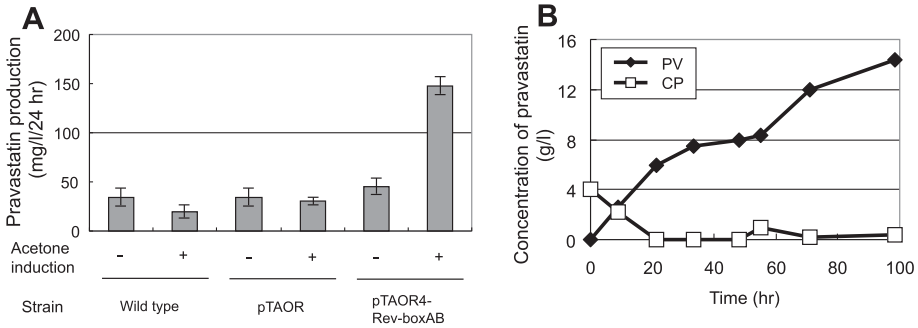

Fig. 4. Biotransformation of compactin (CP) to pravastatin (PV). (A) CP hydroxylation was performed with *P. autotrophica* NBRC 12743 (wild type), *P. autotrophica*/pTAOR, and *P. autotrophica*/pTAOR4-Rev-boxAB. The resting cells were prepared from the cultures in the presence or absence of acetone and biotransformation with the cells was performed for 24 h at 28 °C. PV production was measured in four independent tests. Gray bars show PV production activities (mg/l/24 h). (B) Time course of PV production in batch culture of *P. autotrophica*/pTAOR4-Rev-boxAB. The concentrations of PV and CP were monitored during CP biotransformation in batch culture. Filled diamonds and open squares represent the concentrations (g/l) of PV and CP, respectively. CP was supplied to culture as it was consumed.

P. autotrophica cells tend to aggregate during culture, cells were dispersed well before competent cell preparation.

3.3. Acetone-inducible expression of boxAB and vdh

The expression of *boxAB* and *vdh* was investigated with the inducible expression system in *P. autotrophica*. The fragment containing *boxAB* or *vdh* was inserted into pTAOR4-Rev to construct pTAOR4-Rev-boxAB and pTAOR3-vdh. Cell-free extracts prepared from *P. autotrophica* plasmid transformants were applied to SDS–PAGE and reduced CO difference spectral analysis (CDSA) to evaluate the yield of active P450. SDS–PAGE revealed acetone-dependent induction of BoxA and Vdh (Fig. 3A). The CDSA revealed that the spectral peak at 450 nm assigned to the active form of P450 was detected after acetone induction in both samples of BoxA and Vdh, whereas the cell-free extracts from the non-induced culture did not yield a noticeable peak at 450 nm (Fig. 3B). The *P. autotrophica* genome encodes more than 20 P450 genes (data not shown). Expression of these endogenous P450 genes appeared to be below detection limits under the conditions used here. These results suggest that active BoxA and Vdh were expressed under the control of the acetone-inducible promoter (P*ace*) in *P. autotrophica*. The expression yields of BoxA and Vdh were 121 nM and 179 nM in the culture base, respectively.

3.4. Biotransformation of compactin (CP) to pravastatin (PV)

The efficiency of CP to PV biotransformation was investigated in resting cells of *P. autotrophica*/pTAOR4-Rev-boxAB. The host strains *P. autotrophica*, *P. autotrophica*/pTAOR, and *P. autotrophica*/pTAOR4-Rev-boxAB were cultivated in the presence or absence of acetone. The PV yield was measured by HPLC. The host strain and *P. autotrophica*/pTAOR showed no enhancement of hydroxylation activity to CP by acetone induction while a low concentration of PV was observed, suggesting that *P. autotrophica* has weak endogenous CP 6β-hydroxylase activity (Fig. 4A). In contrast, *P. autotrophica*/pTAOR4-Rev-boxAB showed 3.3-fold increased activity after acetone induction. The PV productivities remained low without acetone induction and were similar to those observed in strains having no *boxA*, indicating that most CP was hydroxylated by BoxA during biotransformation with acetone induction and that the basal expression level from P*ace* is very low (Fig. 3, Fig. 4).

PV production was carried out in batch culture of *P. autotrophica*/pTAOR4-Rev-boxAB. *P. autotrophica*/pTAOR4-Rev-BoxAB was cultivated for 48 h in 100 ml of the main medium in a 500 ml flask, followed by addition of 1 ml acetone. After 24 h cultivation, CP was added to the culture to initiate PV production. CP was hydroxylated to PV almost stoichiometrically, implying highly efficient biotransformation. PV accumulated to 14.3 g/l after 100 h

biotransformation (Fig. 4B). The production profile was the same as that obtained in scale-up biotransformation in a 3 l jar fermentor (data not shown). PV production by biotransformation systems have been reported previously. Biotransformation with *Actinomadura* sp. ATCC 55678, *Streptomyces* sp. Y-110, and *Pseudonocardia autotrophica* BCRC 12444 yielded 0.8 g/l/7 days, 1.5 g/l/6 days, and 0.1 g/l/6 days PV production, respectively [7–9]. In comparison to these previously reported productivities, *P. autotrophica*/pTAOR4-Rev-boxAB yielded much higher PV accumulation.

PV accumulation with *Streptomyces sp*. TM-7, the origin strain of *boxAB*, and *boxAB*-expressing *E. coli* was evaluated. *Streptomyces* sp. TM-7 accumulated 4.6 g/l PV from 8.2 g/l CP after 160 h biotransformation in batch culture. The yield was about 50%. A *tolC/acrAB*-deficient strain of *E. coli* expressing *boxAB* showed high activity, converting 1.7 g/l CP completely to PV after 24 h biotransformation with resting cells [16]. Regardless of differences between growing and resting cell reactions, *E. coli* expressing *boxAB* achieved higher PV production (0.8 g/l/9 h) than did *Streptomyces* sp. TM-7 (0.3 g/l/9 h). However, *E. coli* could not accumulate more than 2 g/l, possibly due to its poor tolerance of CP and PV (data not shown). Judging strictly by PV accumulation rate, *Streptomyces* sp. TM-7 (4.6 g/l) was superior to *E. coli* expressing *boxAB*. *P. autotrophica*/pTAOR4-Rev-boxAB showed the best PV accumulation of all tested strains.

In this study, a novel expression vector in *P. autotrophica* was created and applied to a PV production system. The system may have utility in industrial PV production. Furthermore, the system may be used for biotransformation of other substrates to industrially important materials.

Acknowledgment

We thank T. Harada for providing technical assistance. We are also grateful to all our research group members for their kind help and valuable advice.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.12.013.

References

[1] T. Kieser, M.J. Bibb, M.J. Buttner, K.F. Chater, D.A. Hopwood, Practical Streptomyces Genetics, John Innes Foundation, Norwich, 2000.
[2] K. Takeda, T. Asou, A. Matsuda, K. Kimura, K. Okamura, R. Okamoto, et al., Application of cyclodextrin to microbial transformation of vitamin D_3 to 25-hydroxyvitamin D_3 and 1α, 25-dihydroxyvitamin D_3, J. Ferment. Bioeng. 78 (1994) 380–382.
[3] Y. Fujii, H. Kabumoto, K. Nishimura, T. Fujii, S. Yanai, K. Takeda, et al., Purification, characterization, and directed evolution study of a vitamin D_3 hydroxylase from *Pseudonocardia autotrophica*, Biochem. Biophys. Res. Commun. 385 (2009) 170–175.
[4] D.C. Lamb, T. Skaug, H.L. Song, C.J. Jackson, L.M. Podust, M.R. Waterman, et al., The cytochrome P450 complement (CYPome) of *Streptomyces coelicolor* A3(2), J. Biol. Chem. 277 (2002) 24000–24005.
[5] Y. Abe, S. Baba, T. Suzuki, C. Ono, K. Iwamoto, Molecular basis of ML-236B production in the high-producing mutant No. 41520 of *Penicillium citrinum*, J. Gen. Appl. Microbiol. 50 (2004) 169–176.
[6] I. Watanabe, F. Nara, N. Serizawa, Cloning, characterization and expression of the gene encoding cytochrome P-450sca-2 from *Streptomyces carbophilus* involved in production of pravastatin, a specific HMG-CoA reductase inhibitor, Gene 163 (1995) 81–85.
[7] Y. Peng, A.L. Demain, Bioconversion of compactin to pravastatin by *Actinomadura* sp. ATCC 55678, J. Mol. Catal. B Enzym. 10 (2000) 151–156.
[8] J.W. Park, J.K. Lee, T.J. Kwon, D.H. Yi, Y.J. Kim, S.H. Moon, et al., Bioconversion of compactin into pravastatin by *Streptomyces* sp, Biotechnol. Lett. 25 (2003) 1827–1831.
[9] C.H. Chen, H.Y. Hu, Y.C. Cho, W.H. Hsu, Screening of compactin-resistant microorganisms capable of converting compactin to pravastatin, Curr. Microbiol. 53 (2006) 108–112.
[10] T. Fujii, S. Hirosue, Y. Aritoku, Novel polypeptide, DNA encoding the polypeptide and use thereof, WO2002/099109.
[11] J. Sambrook, D.W. Russell, Molecular Cloning: A Laboratory Manual, 3rd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2001.
[12] K.I. Sallam, N. Tamura, T. Tamura, A multipurpose transposon-based vector system mediates protein expression in *Rhodococcus erythropolis*, Gene 386 (2007) 173–182.
[13] Z. Shao, W.A. Dick, R.M. Behki, An improved *Escherichia coli–Rhodococcus* shuttle vector and plasmid transformation in *Rhodococcus* spp. using electroporation, Lett. Appl. Microbiol. 21 (1995) 261–266.
[14] T. Omura, R. Sato, The carbon monoxide-binding pigment of liver microsomes, J. Biol. Chem. 239 (1964) 2370–2378.
[15] A.S. Khan, Plasmid rolling-circle replication: highlights of two decades of research, Plasmid 53 (2005) 126–136.
[16] T. Fujii, Y. Fujii, K. Machida, A. Ochiai, M. Ito, Efficient biotransformations using *Escherichia coli* with *tolC acrAB* mutations expressing cytochrome P450 genes, Biosci. Biotechnol. Biochem. 73 (2009) 805–810.