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ORIGINAL PAPER

Cloning and expression of a cytochrome P450 hydroxylase gene from *Amycolatopsis orientalis*: hydroxylation of epothilone B for the production of epothilone F

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Abstract Degenerate PCR primers were used to amplify cytochrome P450 gene fragments from the high-GC gram-negative bacteria *Amycolatopsis orientalis*, which catalyzes the hydroxylation of epothilone B to produce epothilone F. The amplified fragments were used as hybridization probes to identify and clone two intact cytochrome P450 genes. The expression of one of the cloned genes in a *Streptomyces lividans* transformant resulted in the biotransformation of epothilone B to epothilone F. The conversion of epothilone B to epothilone F by the *S. lividans* transformant was confirmed by mass spectrometry and nuclear magnetic resonance spectroscopy.

Keywords Epothilone · Cytochrome P450 · Hydroxylation · *Amycolatopsis orientalis* · Biotransformation

Introduction

Epothilones are a new class of hybrid peptide-polyketide compounds that were first identified as secondary metabolites produced by the myxobacterium *Sorangium cellulosum* [10]. These compounds were initially investigated as plant protective agents due to their antifungal properties [7]. It is now known that epothilones exert microtubule-stabilizing effects and cytotoxic activity against rapidly proliferating cells such as tumor cells or other hyperproliferative cellular diseases [4]. Epothilones A and B are the main products of the fermentation of *S. cellulosum*, although 37 natural epothilone related compounds have been isolated from the same fermentation broth (Fig. 1) [9]. One of these products, epothilone F is produced by the hydroxylation of epothilone B at the 21 position. Epothilone F can also be produced by the addition of epothilone B to the cultures of *S. cellulosum* [8], *Amycolatopsis orientalis*, and *Amycolata autotrophica* [15]. Epothilone F is used to produce the investigational anti-tumor compound BMS 310705 by substituting the 21-hydroxyl group with an amine. BMS 310705 is more water soluble than epothilone B and has demonstrated anti-tumor activity [14].

A high-GC gram-negative bacterium, identified as a soil isolate of *A. orientalis* (BMS strain SC15847), was found to produce a hydroxylase enzyme, which converts epothilone B to epothilone F. The yield of epothilone F from this bioconversion was low at ~30%, with loss of the remaining epothilone B to a cell-associated activity. Efforts to improve the process yield through process and strain development did not result in significant improvements. In order to improve the yield of this process, the gene encoding the enzyme catalyzing this hydroxylation reaction was cloned and expressed in *Streptomyces lividans*.

Materials and methods

Chemicals and enzymes

Epothilone B was produced by fermentation of *S. cellulosum* at Bristol-Myers Squibb Company, Syracuse.

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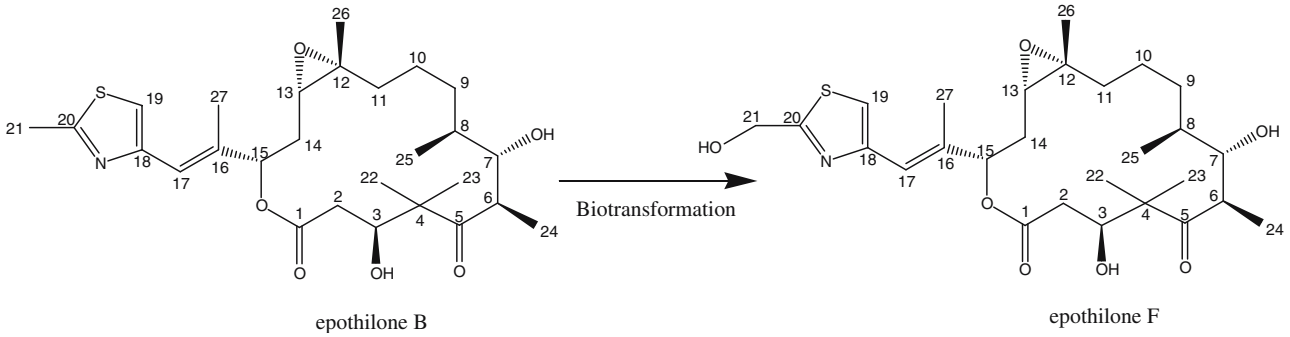

Fig. 1 Structures of epothilones B and F. Epothilone F was first isolated as a minor component from *Sorangium cellulosum* epothilone B fermentation broths. Epothilone F is produced by the biotransformation of epothilone B by *Amycolatopsis orientalis* and *Amycolata autotrophica* [15]

Unless otherwise noted, chemicals used were purchased from Sigma Chemical Co. (St Louis, MO, USA). Reagent salts and organic solvents were obtained from Fisher Scientific (Pittsburgh, PA, USA). DNaseI, RNAseH, Superscript II enzyme, random hexamers, DNAzol, and Trizol were obtained from Invitrogen (Carlsbad, CA, USA). Restriction enzymes were purchased from New England Biolabs (Beverly, MA, USA). Big Dye Sequencing kits were purchased from Applied Biosystems (Foster City, CA, USA).

Media and buffers

The *Amycolatopsis orientalis* cultures were grown in F7 media (Glucose 2.2%, Yeast extract 1.0%, malt extract 1.0%, peptone 0.1%, pH 7.0). *Escherichia coli* cultures were grown in Luria broth (LB, 1% tryptone, 0.5% yeast extract, 0.5% NaCl) or LB agar (Luria broth supplemented with 1.5% agar). *S. lividans* was grown in R2YE or YEME media prepared as described in the Practical Streptomyces Genetics Manual [12].

Microbial strains

Escherichia coli DH10B (F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Δ80*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*K rpsL(Str^R) *end*A1 *nup*G) was obtained from Invitrogen. *A. orientalis* strains ATCC 43491, ATCC 14930, ATCC 53630, ATCC 53550, ATCC 39444, ATCC 43333 and ATCC 35165 were obtained from American Tissue Culture Collection (ATCC, Manassas VA, USA). *Amycolatopsis orientalis* SC15847 was obtained by a BMS soil screening program and identified by 16S rDNA sequence analysis. The *S. lividans* strain TK24 was obtained from the John Innes Centre (Norwich, UK). The plasmid pANT849 was obtained from Dr. William Strohl at Ohio State University.

Isolation of genomic DNA from *A. orientalis*

Total DNA was isolated from *A. orientalis* cultures using the DNAzol reagent. Twenty ml of culture was grown 24 h at 28°C, spun down 10 min and resuspended in 20 ml of DNAzol. The procedure followed the manufacturer's protocol.

Preparation of cDNA

Total RNA was isolated from *A. orientalis* cultures using the Trizol reagent. Ten milliliter of culture was incubated with 5 mg/ml lysozyme for 5 min at room temperature. The mycelia were collected by centrifugation 5 min and the pellet resuspended in 10 ml of Trizol. The procedure was followed according to the manufacture's protocol. The RNA was treated with DNaseI, incubated 37°C, 25 min and inactivated. For the cDNA synthesis, 5 μg of total RNA was used as the template with random hexamers and the Superscript II enzyme. The reaction was incubated 25°C 10 min, 42°C 50 min and 70°C 15 min and 1 μl of RNase H added incubated 37°C 20 min and 70°C 15 min.

DNA sequencing

DNA Sequencing was performed using the Big-Dye Dye terminator system (Applied Biosystems). The reactions were purified using DyeEx 2.0 spin kit (Qiagen, Santa Clarita, CA, USA) and analyzed using an ABI310 or Avant3100 Sequence Detection System (Applied Biosystems).

PCR using degenerate primers to conserved regions within cytochrome P450 enzymes

Three highly conserved regions were identified in sequence alignments of cytochrome P450 enzymes

from bacteria. Degenerate PCR primers were designed to the three conserved regions: Primers P450-1$^+$ (5′-TG CTGCTSDTCGCCGGBCABGASAC-3′), P450-1a$^+$ (5′-TGMTSSYSNTCGSCGSBCAGASAC-3′), P450-2$^+$ (5′-CGGVGCSVTSGAGGARMTGCTGCG-3′) and P450-3$^-$(5′-GCCCAGGCASAHCACSYVVGGCDY BGGCTT-3′) (IUB Codes are used for mixed base positions).

Polymerase chain reactions (PCR) using the primer pairs (P450-1$^+$, P450-3$^-$), (P450-1a$^+$, P450-3$^-$) and (P450-2$^+$, P450-3$^-$) were performed using both genomic and cDNA templates. After PCR amplification, the reaction products were separated by gel electrophoresis and fragments of the expected size were excised. The DNA was extracted from the agarose gel slices using the Qiaquick gel extraction procedure (Qiagen). The fragments were then cloned into the PCRscript vector (Stratagene, La Jolla, CA, USA) using the PCRscript Amp cloning kit (Stratagene). Colonies containing inserts were picked to 1–2 ml of LB broth with 100 μg/ml ampicillin, 30–37°C, 16–24 h, 230–300 rpm. Plasmid isolation was performed using the Mo Bio miniplasmid prep kit (Mo Bio, Solano Beach, CA, USA). The cloned PCR products were sequenced. The sequence of the inserts was used to perform a TblastX search, using the protocol of Altschul et al. of the non-redundant protein database [2].

Genomic cloning of cytochrome P450 genes

Two PCR amplified cytochrome P450 fragments, NPB10 and NPB29 were labeled with digoxigenin (Roche, Indianapolis, IN, USA) using PCR. The probes were hybridized to genomic blots of *A. orientalis*. In a *Bgl*II genomic digest, a ~10-kb band was identified which hybridized to probe NPB10 and a ~14-kb fragment was identified which hybridized to the probe NPB29. A 9–12-kb fraction and a 12–16-kb fraction of *Bgl*II digested DNA was ligated to vector pWB19N digested with *Bam*HI and transformed to *E. coli*. DH10B [3]. Colony blot hybridization was performed using the labeled PCR fragments and detected using the Dig detection system (Roche) [3]. The hybridizing fragments were then sequenced using the primers designed to the gene fragments amplified using the degenerate oligonucleotides.

Expression in *Streptomyces lividans*

PCR primers were designed to amplify the coding region of the cytochrome P450 and ferredoxin and with *Bgl*II and *Hin*dIII restriction sites for insertion into the expression plasmid pANT849. The ligation reactions were transformed into *S. lividans* TK24 protoplasts and selected on R2YE media with 10 μg/ml thiostrepton [12]. Transformants were picked to R2YE agar slants with 50 μg/ml thiostrepton and grown 7 days at 28°C. The slants were resuspended in 2 ml of dH_2O mixing with an equal volume of 15% sucrose, 15% glycerol and stored at −20°C. For initial activity screening, 500 μl of the frozen spore prep was inoculated to 20 ml of R2YE or YEME media grown 48 h at 30°C, 1 ml transferred to 15 ml sterile tubes and 1 μl of a 25 mg/ml epothilone B solution added. The cultures were incubated 72 h at 28°C. The samples were extracted with an equal volume of 25% methanol: 75% butanol, vortexed, and allowed to settle 5 min. Five microliter was injected to an Agilent HP1100 system using a Whatman partisil 5 ODS-3 column 250 × 4.6 mm, 5.0 μM, 1.0 ml/min flow rate using a 60% ACN mobile phase.

NMR analysis of biotransformation broth of *S. lividans* TK24 (pANT849-NPB29)$^+$

NMR spectra were acquired on samples dissolved in $CDCl_3$ (sample concentrations were ~0.5–2 mg/ml) on a Bruker DRX-360 NMR spectrometer system operating at a proton resonance frequency of 360.132 MHz. Chemical shifts were referenced internally to the residual chloroform resonance 7.26 ppm for proton and 77 ppm for carbon. Spectral assignments were supported by the appropriate supplementary NMR data (COSY, HMQC and HMBC).

LC/MS analysis of biotransformation broth of *S. lividans* TK24 (pANT849-NPB29)$^+$

One milliliter samples of a 20 ml bioconversion in YEME media at 28°C were mixed with 1 ml of acetonitrile (ACN), vortexed 15 s and the upper layer transferred to second tube. The extraction was repeated twice and the total volume evaporated. The dry sample was reconstituted with 200 μl of ACN and brought to 1 ml with water. Five microliter was injected to an Agilent HP1100 system using a Waters Symmetry Shield RP8 column 150 × 4.6 mm, 3.5 μM, 1.0 ml/min flow rate using a 12–28% ACN gradient to 6 min, 28% for 4 min and 28–100% ACN in 20 min. The masses were determined using a Finnegan Matt TSQ 7000.

PCR amplification of corresponding P450 enzyme from other isolates of *A. orientalis*

Genomic DNA was isolated from *A. orientalis* strains. A primer set, NPB29-16F (5′-AGGAAACCACCGC GACCTTGCCACT-3′) and NPB29-17R (5′-ACCGA

ATCCGAAGGCGACGTGATGC-3′) was designed to the NPB29 sequence and used to amplify related sequences from the other *A. orientalis* strains. The resulting PCR fragments were separated on an agarose gel, excised, and purified using a Qiagen gel extraction procedure. The purified fragments were then sequenced using primers NPB29-16F and NPB29-17R.

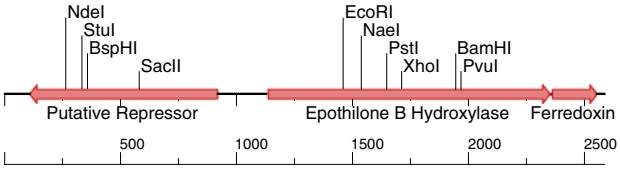

Fig. 2 Genetic organization of the epothilone B hydroxylase, ferredoxin and putative repressor

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  1  MTDVEETTAT LPLARKCPFS PPPEYERLRR ESPVSRVGLP SGQTAWALTR
 51  LEDIREMLSS PHFSSDRQSP SFPLMVARQI RREDKPFRPS LIAMDPPEHG
101  KARRDVVGEF TVKRMKALQP RIQQIVDEHI DALLAGPKPA DLVQALSLPV
151  PSLVICELLG VPYSDHEFFQ SCSSRMLSRE VTAEERMTAF ESLENYLDEL
201  VTKKEANATE DDLLGRQILK QRESGEADHG ELVGLAFLLL IAGHETTANM
251  ISLGTVTLLE NPDQLAKIKA DPGKTLAAIE ELLRIFTIAE TATSRFATAD
301  VEIGGTLIRA GEGVVGLSNA GNHDPDGFEN PDTFDIERGA RHHVAFGFGV
351  HQCLGQNLAR LELQIVFDTL FRRVPGIRIA VPVDELPFKH DSTIYGLHAL
401  PVTW
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Fig. 3 Amino acid sequence of the EBH enzyme of *Amycolatopsis orientalis*. Residues in *bold* indicate the amino acid positions at which polymorphisms were identified in EBH homologues isolated from other strains of *A. orientalis*

Results

Identification of the *ebh* gene

Degenerate oligonucleotide primers designed to conserved sequences found in bacterial cytochrome P450 enzymes were used to amplify P450 sequence fragments from genomic DNA and cDNA templates [11]. The PCR amplified DNA fragments were cloned into the PCRscript vector and sequenced. Using this approach, a total of nine unique cytochrome P450 sequences were identified from SC15847. Of the 50 cDNA clones analyzed, two sequences were predominant, NPB10 and NPB29 with twenty clones each, suggesting a higher expression level of these two mRNAs. The genes corresponding to these two sequences were then cloned from the genomic DNA.

The DNA sequence of the *Bgl*II genomic clone hybridizing to the NPB29 probe contains an open reading frame coding for a hypothetical cytochrome P450 protein of 404 amino acids (Fig. 2) and a predicted molecular weight of 44.7 kDa (Fig. 3). The amino acid sequence of this polypeptide was found to share 51% identity with the NikF protein of *S. tendae* [6] and 48% identity with the Sca-2 protein of *S. carbophilus* [20]. Both of these enzymes belong to the cytochrome P450 family 105 (CYP105) [16]. The invariable cysteine found in the heme-binding domain of all cytochrome P450 enzymes is found at residue 356 (Fig. 3). When the coding sequence of this gene was expressed in *S. lividans*, it conferred the ability to hydroxylate epothilone B to epothilone F. The gene for epothilone B hydroxylase was named *ebh* and the gene sequence was submitted to GenBank (accession no. DQ641255). The ATG start codon of a putative ferredoxin gene of 64 amino acids is found 9 bp downstream from the stop codon of *ebh*. This enzyme was found to share 50% identity with ferredoxin genes of *S. griseoulus* [18] and *S. noursei* [5]. A 269 amino acid ORF was found upstream of the *ebh* gene with similarity to the tetR-family of transcriptional regulatory elements [1].

The DNA sequence of the genomic clone hybridizing to the NPB10 fragment probe was found to contain a hypothetical cytochrome P450 protein of 396 amino acids and a downstream ferredoxin of 71 amino acids. This P450 was found to share 58% identity with the translated product of the *lnmZ* ORF of *Streptomyces atroolivaceus* and 52% identity with the product of *mycG* from *Micromonospora griseorubida*. The MycG protein has both hydroxylation and epoxidation functions in the mycinamicin biosynthetic pathway [13].

Expression of the *ebh* gene in *S. lividans*

The coding region of the NPB10 and NPB29 cytochrome P450 genes and downstream ferredoxins were amplified by PCR and inserted into the *Streptomyces* expression vector pANT849 and transformed to *S. lividans* TK24 protoplasts. The transformants were picked to R2YE slants and inoculated into 20 ml of YEME media. The cultures were incubated with epothilone B in 1 ml culture tubes for 72 h and assayed by HPLC for the production of epothilone F. The pANT849-NPB29 transformants were found to hydroxylate epothilone B to epothilone F (Fig. 4), while incubation epothilone B with pANT849-NPB10 and the *S. lividans* TK24 host did not produce epothilone F and little change in the epothilone B area percent was observed over 72 h. (Data not shown).

Bioconversion broth from *S. lividans* TK24 $(pANT849\text{-}NPB29)^{+}$ was extracted with ethyl acetate

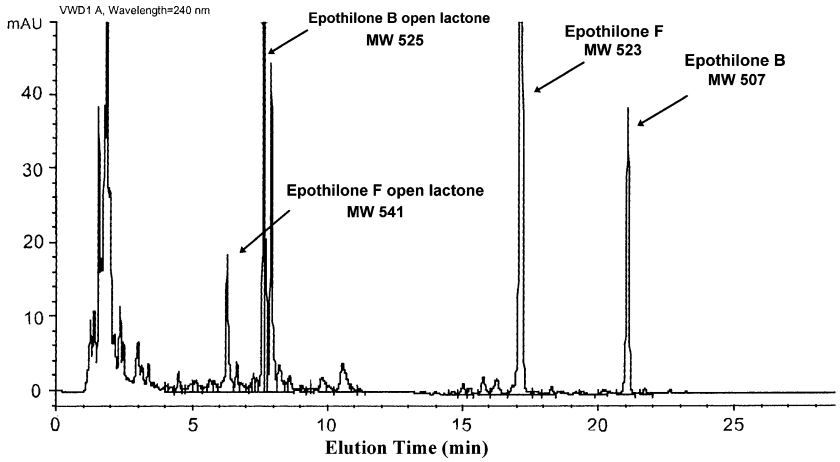

Fig. 4 LC/UV Analysis of 72 h Bioconversion with *S. lividans* (pANT849-NPB29)

and analyzed by NMR. Proton shifts at H-19, 7.14 ppm and H-21, 4.95 ppm conform to an epothilone F reference standard and differentiate epothilone F from epothilone B (H-21, 6.96 ppm and H-19, 2.69 ppm).

LC-MS analysis of the bioconversion broths demonstrated that in addition to the presence of epothilone B and epothilone F, several more polar peaks were observed. The masses of two of these peaks (525 and 541) at retention times of 7.631 and 6.278 min are consistent with open lactone ring products produced by the hydrolysis of epothilone B and epothilone F, respectively.

Cloning of ebh homologues from other *A. orientalis* strains

The *ebh* gene sequence was used to isolate homologues from other strains of *A. orientalis*. Using primers NPB29-16F and NPB29-17R, PCR fragments were amplified from the ATCC 43491, ATCC 14930, ATCC 53630, ATCC 53550, ATCC 39444, ATCC 43333 and ATCC 35165 strains. The translated PCR fragments from ATCC 35165 and ATCC 53550 were found to have the same amino acid sequence as the original EBH. Amino acid substitutions were found at 13 positions within the other five epothilone B hydroxylase (EBH) enzymes (Table 1).

Table 1 Amino acid substitutions of *A. orientalis ebh* homologues

EBH variant	Substitutions
ATCC 39444	His 398 Arg
ATCC 14930	Gly 100 Ser, Lys 101 Arg, Ile 130 Leu, Ser 192 Gln, Ser 224 Thr, Ile 285 Val
ATCC 43333	Ser 69 Asn, Gly 100 Ser, Ser 224 Thr, Val 256 Ala, Ile 285 Val
ATCC 43491	Gly 100 Ser, Leu 133 Met, Ser 224 Thr, Val 256 Ala, Ile 285 Val, Asp 326 Glu, Thr 333 Ala
ATCC 53550	Ala 93 Ser, Gly 100 Ser, Ser 224 Thr, Val 256 Ala, Ile 285 Val, Asp 326 Glu, Thr 333 Ala

Discussion

Using degenerate primers designed to conserved sequences within bacterial cytochrome P450 enzymes, an epothilone B hydroxylase was cloned from *A. orientalis* and expressed in *S. lividans*. This cytochrome P450 enzyme was found to be a member of the CYP105 family, which includes the Cyt$P450_{sca\text{-}2}$ enzyme from *S. carbophilus* and NikF from *S. tendae*. An iron-sulfur ferredoxin protein is found immediately downstream of the coding region of the cytochrome P450, but an NADH oxidoreductase was not found associated with the P450 gene. The clustering of a cytochrome P450 and ferredoxin is observed with one CYP from *S. coelicolor*, five CYPs from *S. avermitilis*, but no CYPs in *S. peucetius* [19]. This arrangement is more common in the CYP105 family, which contains the bacterial enzymes with the broadest substrate specificities. Upstream of the cytochrome P450, divergently expressed, is an ORF with similarity to the tetR-family of transcriptional regulatory elements [1]. This suggests a regulated expression of the cytochrome P450 and ferredoxin genes. Transformation of *S. lividans* with a plasmid containing a 14 kb *Bgl*II genomic fragment including the ebh gene, ferredoxin and its endogenous promoter and with the upstream putative regulatory sequence did not result in the hydroxylation of epothilone B to epothilone F. It is hypothesized that the presence of this putative repressor on the *Bgl*II genomic fragment prevented expression of the *ebh* gene in *S. lividans*.

The natural occurring variation in the enzyme from different *A. orientalis* strains suggests this enzyme may

serve as a defense system for detoxification, rather than act on a defined substrate. Degradation of macrolide antibiotics by hydroxylation and hydrolysis are resistance mechanisms utilized by both antibiotic producers and target organisms [16]. Differences in epothilone B to epothilone F bioconversion yields were observed with these isolates, but these differences cannot be definitively attributed to the sequence variations as these enzymes were not expressed in the *S. lividans* host.

The expression of the enzyme in a heterologous host with little epothilone B degrading activity did not result in improved yield of epothilone F, indicating that this degradation activity is also catalyzed by the EBH enzyme. Two of the major bioconversion products identified by LC-MS have masses consistent with hydrolysis of the of epothilone lactone. Oxidative hydrolysis of lactones catalyzed by a cytochrome P450 enzyme has been described [17].

While the heterologous expression of this enzyme did not result in an improvement in the yield of epothilone F from the biotransformation process, it did identify the epothilone B degrading activity as a function of the EBH enzyme. The cloning and expression of the *ebh* gene provides the opportunity for improving this biotransformation process through mutagenesis of this cytochrome P450 and screening for improved yield of epothilone F.

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