Research & Reviews: Journal of Microbiology and Biotechnology

e-ISSN:2320-3528
p-ISSN:2347-2286

Enhanced One-Step Fermentative Production of Epirubicin by Combination of Mutagenesis and Genetic Engineering in Doxorubicin-Producing *Streptomyces peucetius*

Xiaoru Wang, Xiaorong Tian, Xiaofang Shen, Yuanjie Wu, Songbai Yang and Shaoxin Chen*

Shanghai Institute of Pharmaceutical Industry, Shanghai, Gebaini Road 285, Shanghai 201203, P.R. China

Research Article

Received date: 08/03/2018
Accepted date: 28/03/2018
Published date: 31/03/2018

***For Correspondence**

Shaoxin Chen, Shanghai Institute of Pharmaceutical Industry, Shanghai, Gebaini Road 285, Shanghai 201203, P.R. China.

E-mail: sxzlb@263.net

Keywords: *Streptomyces peucetius*, epirubicin, metabolic engineering, rate-limiting genes

ABSTRACT

Epirubicin, a clinically important anthracycline-type antitumor drug, is industrially produced through a tedious chemical semisynthetic process. Here, we developed an engineered *Streptomyces peucetius* through a combinatorial strategy of strain mutation and metabolic engineering for efficient epirubicin biosynthesis. First, *S. peucetius* SIPI-DU-1557, which overproduces doxorubicin, was cultured through a doxorubicin-resistant screening method, and used as a host strain for genetic modification. Next, EvaE from Amycolatopsis orientalis, found through protein sequence comparisons of various exogenous TDP-4-ketoreductases, increased epirubicin production significantly. Subsequently, metabolic engineering strategies were used to enhance epirubicin production by co-expressing key biosynthesis pathway genes, dnrS/dnrQ and desIII/desIV, to strengthen metabolic flux toward epirubicin. The final epirubicin concentrations were 270 mg/L and 252 mg/L in the flask and 5 L fermenter, respectively. These are the highest levels reported, and show that the engineered S. *peucetius* has potential industrial application in green epirubicin production by direct fermentation with renewable resources.

INTRODUCTION

Epirubicin and doxorubicin are anthracycline-type antitumor antibiotics that are widely used as chemotherapeutics for the treatment of various cancers [1,2]. Epirubicin is more widely used in clinical practice due to its lower cardiotoxicity, resulting from a configurational difference at the C-4 hydroxyl group of daunosamine [2]. Currently, industrial epirubicin production involves chemical semisynthesis from naturally occurring daunorubicin as a starting material, through a tedious process with low yields and high production costs [3,4]. As a result, epirubicin is expensive (about $ 150000/kg). In contrast, microbial production processes that could use low-cost and renewable resources such as starch or glucose are a more sustainable alternative in meeting the

e-ISSN:2320-3528
p-ISSN:2347-2286

increasing market demand. Therefore, development of a direct biosynthetic process for the cost-effective and green production of epirubicin is commercially valuable.

The emerging synthetic biology technique offers unique opportunities to create natural product analogs by exploiting information about biosynthetic enzymes and pathways [5,6]. The biosynthesis pathways of doxorubicin, as well as its precursor daunorubicin in *Streptomyces peucetius* were characterized [7]. As shown in **Figure 1,** the first stage of the pathway was the biosynthesis of ε-rhodomycinone (RHO) by a type Ⅱ polyketide synthase (PKS) encoded by the dpsABCDGEFY genes [8]. Then the RHO connects with deoxysugar TDP-daunosamine synthesized by dnmLMUTJV genes to form rhodomycin D [9-11], and then undergoes post-modifications (methylation, decarboxylation, and hydroxylation) to form doxorubicin via daunorubicin [12,13].

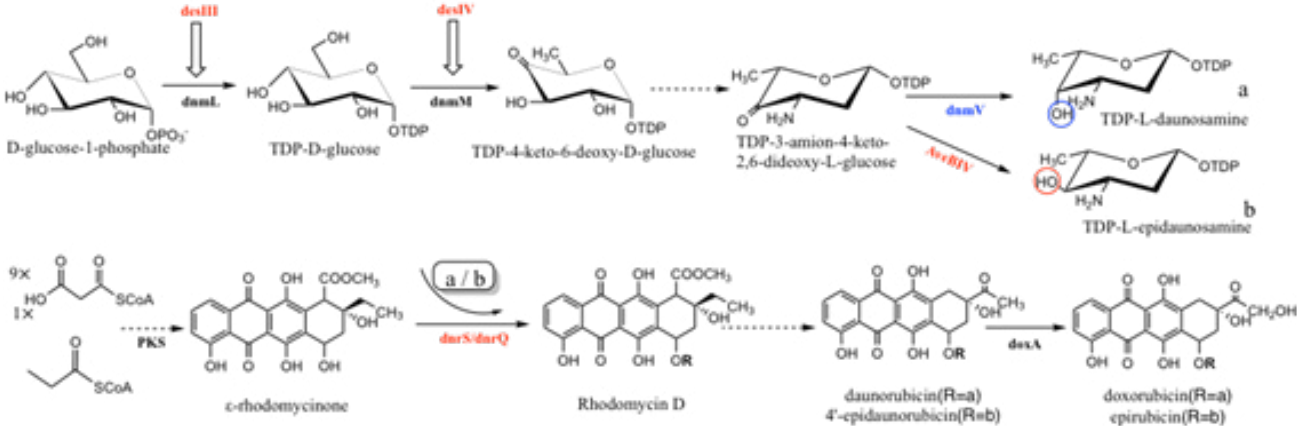

Figure 1: Doxorubicin biosynthesis pathway and genetic modification for epirubicin synthesis. TDP-daunosamine or TDP-epidaunosamine from glucose-1-phosphate and doxorubicin or epirubicin from propionyl-CoA and malonyl-CoA. Abbreviations: dnmL, glucose-1-phosphate thymidylyl transferase; desIII, glucose-1-phosphate thymidylyl transferase from Streptomyces venezuelae; dnmM, TDP-D-glucose 4,6-dehydratase; desIV, TDP-D-glucose 4,6-dehydratase from Streptomyces venezuelae; dnmU, TDP-4-keto-6-deoxyglucose-3(5)-epimerase; dnmV, TDP-4-ketodeoxyhexulose reductase. AveBIV, TDP-4-ketohexulose reductase; PKS, a type II polyketide synthase; dnrS, glycosyltransferase; dnrQ, an enzyme required for the glycosylation; doxA, cytochrome P-450 hydroxylase.

To obtain epirubicin and its precursor 4′-epidaunorubicin in *S. peucetius,* Hutchinson [14] constructed an engineered strain by introducing aveBIV into a dnmV-blocked mutant of the doxorubicin producer, *S. peucetius* ATCC 29050. The aveBIV gene, one of the avermectin biosynthesis genes in Streptomyces avermitilis, is related to oleandrose C-4 ketone reduction, which is opposite to the stereoselectivity of the ketoreduction governed by dnmV in TDP-daunosamine biosynthesis [15]. However, the genetically modified strains produced only 9.7 mg/L 4′-epidaunorubicin and only trace amounts of epirubicin, and therefore had no potential for industrial application. Recently, a 4′-epidaunorubicin-producing strain was obtained through the genetic engineering of daunorubicin-producing strains Streptomyces coeruleorubidus, but only a small amount of 4′-epidaunorubicin was detected in the fermentation broth [16]. To our knowledge, there were no further reports on epirubicin synthesis through metabolic engineering.

To effectively synthesize epirubicin via direct fermentation, the application of host strains with overproduction properties is an emerging trend [5,17]. Genetically modified industrial overproductive strains such as Saccharopolyspora erythraea and *S. avermitilis* can produce erythromycin and avermectin analogs in highly superior quantities than wild-type strains [18-20]. The *S. peucetius* ATCC 29050 strain produced only 9 mg/L doxorubicin [13], which explains why epirubicin yield was low after genetic modification. Therefore, a doxorubicin overproducing strain should be a more suitable host for constructing the epirubicin-producing strain. Second, to maximize epirubicin biosynthesis potential, metabolic engineering strategies were used to enhance expression of key enzymes in the biosynthesis pathway to push metabolic flux toward epirubicin. In this study, we attempted to construct a recombinant strain with a high epirubicin yield from a doxorubicin-overproducing *S. peucetius* strain by combining strain mutation and metabolic engineering.

MATERIALS AND METHODS

Strains, Plasmids, and Culture Conditions

The strains and plasmids used in this study are listed in **Table 1**. *S. peucetius* and its derivatives were cultured at 28°C in liquid Tryptic Soy Broth (TSB) medium at 28°C for mycelium preparations and total DNA isolation. Escherichia coli DH5α were used as a sub-cloning host for plasmid construction. *E. coli* ET12567/pUZ8002 was used for conjugal transfer from *E. coli* to *S.* peucetius. *E. coli* strains were cultured at 37°C in lysogeny broth (LB). Antibiotics (25 μg/mL chloramphenicol, 25 μg/mL kanamycin, 50 μg/mL apramycin, 50 μg/mL nalidixic acid) were added when necessary.

Strains or plasmids	Relevant characteristic	Reference or source

e-ISSN:2320-3528
p-ISSN:2347-2286

Strains		
E. coli		
DH5α	F^-, φ80dlacZΔM15, Δ (lacZYA-argF) U169, deoR, recA1, endA1, hsdR17 (rk^-, mk^+), phoA, supE44, λ^-, thi-1, gyrA96, relA1	CWBIO
ET12567/PUZ8002	Methylation defective, strain used in *E. coli*-streptomyces intergeneric conjugation	[21]
S. avermitilis	Avermectin producing strain, containing *aveBIV* gene	ATCC31267
A. orientalis	Chloroeremomycin producing strain, containing *evaE* gene	NRRL18099
S. peucetius		
Δ dnmV	SIPI-DU-1557 with disrupted *dnmV*	This study
EPI-1	Genome-based expression of *aveBIV* under the promoter P*erm*E* in *S. peucetius* ΔdnmV	This study
EPI-2	Genome-based expression of *evaE* under the promoter P*erm*E* in *S. peucetius* ΔdnmV	This study
EPI-3	Genome-based expression of *evaE*, *dnrQ* and *dnrS* under the promoter P*erm*E* in *S. peucetius* ΔdnmV	This study
EPI-4	Genome-based expression of *evaE*, *dnrQ*, *dnrS*, *desIII* and *desIV* under the promoter P*erm*E* in *S. peucetius* ΔdnmV	This study
Plasmids		
pSET152	E. coli replicon, Streptomyces ØC31 attachment site, Apr^R	[22]
pET-22b (+)	E. coli expressing and subcloning vector, Amp^R	This study
pΔdnmV	pSET152 with deletion of phiC31 integrase gene and two homologous arms containing the upstream and downstream DNA fragments of *dnmV*	This study
pAveB	pSET152 with the expression of *aveBIV* under the control of strong promoter P*erm*E*	This study
pEvaE	pSET152 with the expression of *evaE* under the control of strong promoter P*erm*E*	This study
pEQS	pSET152 with the expression of *evaE*, *dnrQ* and *dnrS*, under the control of strong promoter P*erm*E*	This study
pEQSd	pSET152 with the expression of *evaE*, *dnrS*, *dnrQ*, *desIII* and *desIV* under the control of strong promoter P*erm*E*	This study

Table 1: Strains and plasmids used in this study

Vector Construction

DNA isolation and manipulation in *Escherichia coli* and *Streptomyces peucetius* were performed using standard methods [23]. The primers used for construction of plasmids and strains are shown in **Table 2**. To knockdown dnmV (GenBank accession number AF006633) in S. peucetius, a 3.5kb DNA fragment containing mutated dnmV, in which the stop codon TAG is generated through a nucleotide substitution 252 bp downstream from the dnmV start codon, was amplified from S. *peucetius* SIPI-DU-1557 genomic DNA through overlap extension PCR using the following primers: Dau-HindIII-F, dnmV-252-R and dnmV-252-F, Dau-XbaI-R. A 3.1 kb DNA fragment was amplified from pSET152 plasmid using pSET152-XbaI-F and pSET152-HindIII-R primers. The 3.5 kb and 3.1 kb DNA fragments were digested with XbaI/HindIII and ligated to generate dnmV knock-out plasmid pΔdnmV.

Table 2: Primers used in this study.

Primers	Sequences (5′→3′)
Dau-*Hin*dIII-F	GGGAAGCTTGATCGCCCTCACGGAACT
dnmV-252-R	CGCAGATGCGACTACGTCATCTCC

e-ISSN:2320-3528
p-ISSN:2347-2286

dnmV-252-F	GGAGATGACGTAGTCGCATCTGCG
Dau-*Xba*I-R	GGGTCTAGAGCCGGCATGCGGATCG
pSET152-*Xba*I-F	GACTCTAGAGGATCCGCGGC
pSET152-*Hin*dIII-R	AAGCTTCTGCAGGTCGACGGA
ave-*Nde*I-F	CATATGATGGGGCGGTTTTCGGTGTG
ave-*Bam*HI-R	GGATCCTACACGTAAGCCGCCACC
eva-*Nde*I-F	CATATGATGAAGCTGATCACCGTGCTCGG
eva-*Bam*HI-R	GGATCCTCATGCGCGAGCCTTTCC
dnrS/dnrQ-EvaE-F	ATGGAAAGGCTCGCGCATGAGGATCATGCCCACACCCACGTCCGC
dnrS/dnrQ-*Bam*HI-R	GGATCCTCAGCGCAGCCAGACGGGCA
desIII/IV-dnrS/Q-F	GGCAGGGCGTCCGGCACTAGGGATCATGAAGGGAATAGTCCTGGCCG
desIII/IV-*Eco*RV-R	TCGATATCGCGCGCGGCCGCGGATCCTCACGCGGACACCTCCACG

To express aveBIV (GenBank accession number AB032523) in *S. peucetius*, a 1 kb DNA fragment that contained the complete aveBIV sequence was amplified from *S.* avermitilis genomic DNA using ave-NdeI-F and ave-BamHI-R primers, and then cloned into the pET-22b (+) vector, yielding pave-22b. A 0.4 kb synthesis DNA fragment containing a PermE* promoter and the ribosome binding site (RBS) was inserted into the XbaI/NdeI site of pave-22b, yielding pPerm-ave-22b. The 1.4 kb XbaI/BamHI fragment containing PermE*, RBS, and aveBIV were inserted into the corresponding sites of the pSET152 vector to obtain pAveB.

To express evaE (GenBank accession number AJ223998) in *S. peucetius*, a 1 kb DNA fragment containing the complete evaE gene sequence was amplified from the genomic DNA of Amycolatopsis orientalis NRRL 18099 by using EvaE-NdeI-F and EvaE-BamHI-R primers, and was inserted into the NdeI/BamHI of pAveB to obtain pEvaE.

To overexpress dnrS/dnrQ (GenBank accession number L47164) along with evaE in *S. peucetius*, a 1.2 kb DNA fragment containing the complete dnrS/dnrQ gene sequence was PCR amplified from *S. peucetius* genomic DNA using dnrS/dnrQ-EvaE-F and dnrS/dnrQ-BamHI-R primers, and cloned into the BamHI site of pEvaE using the seamless connection method (ClonExpress® II One Step Cloning kit) to obtain pEQS.

To express desIII/desIV (GenBank accession number: AF079762), dnrS/dnrQ along with evaE in *S. peucetius*, a 2 kb DNA fragment containing the complete sequence of desIII and desIV, was amplified from Streptomyces venezuelae genomic DNA using desIII/IV-dnrS/Q-F and desIII/IV-EcoRV-R primers, and then cloned into the EcoRV site of pEQS to obtain pEQSd.

Construction of Epirubicin-producing Strains

To construct the dnmV-disrupting strain, pΔdnmV was introduced into *S. peucetius* SIPI-DU-1557 through conjugation on MS medium at 28°C for 18-20 h [24]. The medium was covered with 1 mL 50 μg/mL apramycin and 50 μg/mL nalidixic acid, and incubated at 28°C for 7 days. The apramycin-resistance exconjugants were initially selected, then sub-cultured in TSB medium without apramycin to obtain the apramycin-sensitive strains.

To construct strains producing epirubicin, pAveB, pEvaE, pEQS, and pEQSd were separately introduced into dnmV-disrupted S. *peucetius* ΔdnmV through conjugation, and the apramycin-resistance strains were screened.

Fermentation of *S. peucetius*

S. peucetius and recombinant strains were cultured on solid medium (maltodextrin 20 g/L, yeast extract 15 g/L, enzymatic hydrolysis casein 5 g/L, glucose 4 g/L, soya pepton 2.5 g/L, KH_2PO_4 1.5 g/L, $CaCO_3$ 2 g/L, agar 20 g/L, pH 7.0) at 28°C for 7 days. The mycelium was then inoculated into a 250 mL flask containing 20 mL of the seed medium (corn starch 5 g/L glucose 5 g/L, soybean flour 30 g/L, yeast powder 1 g/L, NaCl 1 g/L, KH_2PO_4 1 g/L, $MgSO_4 \cdot 7H_2O$ 1 g/L, pH 7.2) at 28 °C, 220 rpm for 2 days. Next, 2.5 mL seed culture was inoculated into the same 250 mL flask containing 25 mL of the fermentation medium (maltodextrin 120 g/L, yeast powder 30 g/L, NaCl 2 g/L, $CaCl_2$ 3 g/L, $CaCO_3$ 3 g/L, pH 6.2) at 28°C, 220 rpm for 7 days. For epirubicin production in a 5 L fermenter, the strains were cultured in 100 mL of seed medium at 28°C in 750 mL flasks on a shaking incubator at 220 rpm. After 40 h, 100 mL of seed medium was transferred to a 5 L fermenter (BIOTECH-5BG-7000A, Baoxing Bioengineering Equipment Co., Shanghai, China) containing 2 L fermentation medium. The culture was grown at 28°C, and dissolved oxygen level was maintained at over 20% through aeration in 1 vvm (volume air per volume per minute) and varying stirring speeds.

Mutation and Screening of *S. peucetius*

Two milliliter mycelium suspension of *S. peucetius* SIPI-D-30 in sterile distilled water was transferred to an aseptic plate, and subjected to UV irradiation for 2 min at a distance of 30 cm from the UV lamp (254 nm wavelength, 30 W power). The mycelium suspension was then spread on the solid medium containing 40 to 275 mg/L doxorubicin, and the mutants were cultured in a shake flask to determine the doxorubicin yield.

HPLC Analysis Methods

e-ISSN:2320-3528
p-ISSN:2347-2286

To analyze doxorubicin and epirubicin production, the fermentation broth was extracted with absolute ethyl alcohol and centrifuged at 10000 g for 5 min after adjusting the pH to 1.5–1.8 using 6 mol/L HCl. The supernatant was analyzed by high-pressure liquid chromatography (HPLC) with an Agilent C18 column (4.6 × 150 mm; 3.5 µm). The analytic method was developed with mobile phase A (0.1% TFA in H2O) and B (0.1% TFA in acetonitrile) and a program of 0–5 min, 75% A/25% B to 70 A/30% B; 5–20 min, 70% A/30% B to 55% A/45% B; 20–23 min, 55% A/45% B to 10% A/90% B; 23–24 min, 10% A/90% B to 75% A/25% B; 24–30 min, 75% A/25% B at a flow rate of 0.8 mL/min and UV detection at 254 nm using an Agilent 1260 HPLC system. The identity of the compound was confirmed by liquid chromatography-mass spectrometry (LC-MS) analysis.

RESULTS AND DISCUSSION

Mutation and Screening of High-Doxorubicin–Production Strain

Doxorubicin is an epirubicin analog, and therefore we assumed that the doxorubicin overproducer would be an ideal host strain to metabolically engineer epirubicin production. Many studies have been performed to enhance doxorubicin production through genetic engineering to obtain a doxorubicin overproducer [25]. For example, expression of the global regulatory gene afsR and resistance genes in S. *peucetius* resulted in 4-fold and 5-fold overproduction of doxorubicin, respectively [26,27]. Similarly, over-expression of structural sugar and *glycosyltransferase* genes enhanced its production 5.6-fold [28]. However, the doxorubicin titers were still below 20 mg/L in these studies.

There are very little published data on strain improvement through traditional mutagenesis. Although its screening efficiency is low compared to genetic engineering, the opportunity to obtain high-yield strains can increase through appropriate screening methods. Considering that doxorubicin is a cytotoxic secondary metabolite and can inhibit the growth and metabolism of the producing strain, the doxorubicin-resistant screening method can be used to screen the mutated strain. **Figure 2** outlines the mutation and screening procedure.

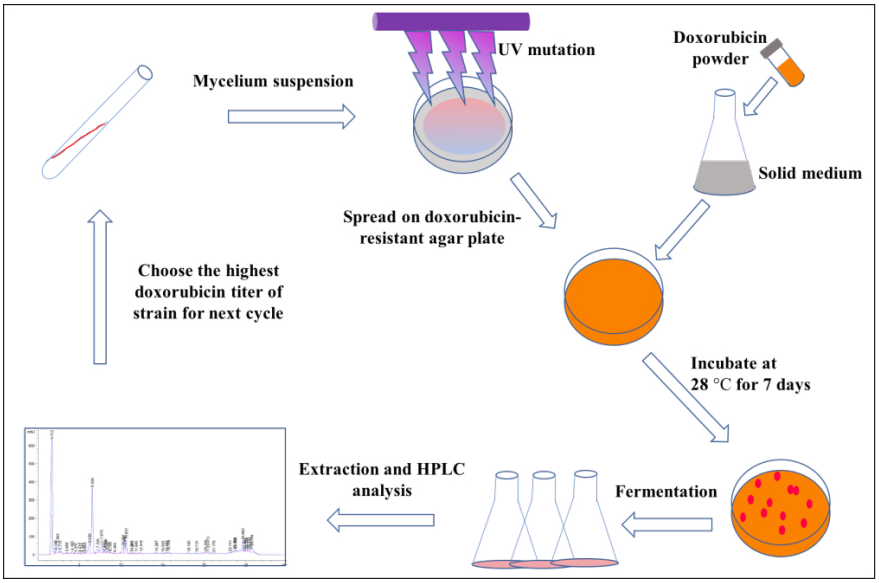

Figure 2: Flow chart outlining the doxorubicin-resistant screening method. The mycelium suspension of S. *peucetius* was subjected to UV irradiation and then spread on the solid medium containing different concentrations of doxorubicin to guarantee a 99% inhibitory rate. The mutants were cultured in shake flasks to screen the strain with the highest doxorubicin yield for the next cycle of mutation.

To determine the resistance of S. *peucetius* SIPI-D-30 to doxorubicin, the strain was plated on different concentrations of doxorubicin (10, 20, 30, 40, 50 mg/L). The results demonstrated that SIPI-D-30 could not tolerate concentrations of 40 mg/L doxorubicin or above. Therefore, plates with 40 mg/L doxorubicin were used to screen resistant mutants with UV treatment. Among the mutants, one strain, S. *peucetius* D-37, showed a 110% higher doxorubicin yield than that of the original strain (110mg/L). And the concentration of doxorubicin which the strain D-37 could not tolerate increased to 100 mg/L. Therefore, to screen S. *peucetius* with higher tolerances to doxorubicin, the concentration of doxorubicin was increased to ensure 100% lethality for the next UV mutagenic treatment. After five rounds of UV mutation, a mutant, SIPI-DU-1557 was obtained from a plate medium containing 275 mg/L doxorubicin. The SIPI-DU-1557 mutant strain produced 535 mg/L doxorubicin. Compared with the genetic engineering method, the traditional method of strain improvement by mutagenesis is still an important, reliable, and cost-effective procedure.

Genetic Engineering of Epirubicin-producing Strains in *S. peucetius* SIPI-D-U-1557

To construct an epirubicin-producing strain, dnmV was firstly inactivated in the S. *peucetius* SIPI-DU-1557 strain by inserting a stop codon 252 bp downstream from the dnmV start codon to obtain S. *peucetius* ΔdnmV **(Figure 3A)**. As shown in **Figure 3B**,

e-ISSN:2320-3528
p-ISSN:2347-2286

HPLC analysis of S. *peucetius* ΔdnmV fermentation broth revealed complete doxorubicin elimination and formation of the intermediate rhodomycinone as a main product, indicating dnmV gene disruption.

The aveBIV gene under the control of the strong promoter PermE* was then cloned into plasmid pSET152 to construct pAveB and introduced into S. *peucetius* ΔdnmV via site-specific integration in the S. *peucetius* ΔdnmV genome **(Figure 3A)**. Exconjugants were selected through apramycin resistance to generate S. *peucetius* EPI-1. As shown in **Figure 3C**, the S. *peucetius* EPI-1 produced 47 mg/L epirubicin, as analyzed by HPLC and LC-MS, which was a considerable improvement when compared with the yields reported in Hutchinson's study [28], indicating that a host strain with high doxorubicin productivity is the basis for high epirubicin production.

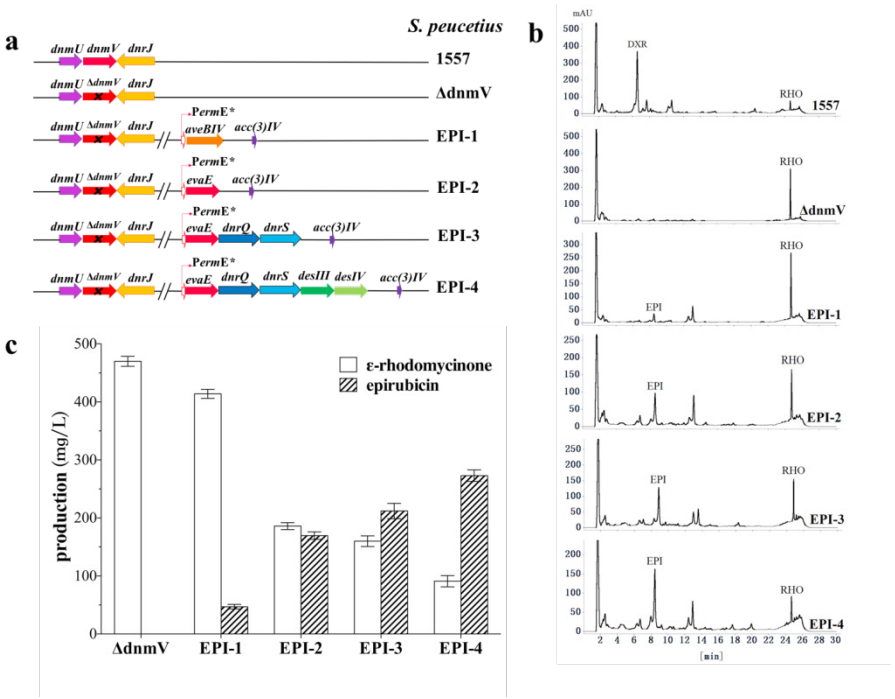

Figure 3: (A)- Genotypes of S. *peucetius* SIPI-DU-1557 and its recombinant strains. The dnmV gene was disrupted and the genes aveBIV, evaE, dnrQ, dnrS, desIII, desIV (under control of PermE*) were integrated into the S. *peucetius* ΔdnmV genome. (B- HPLC analysis of anthracycline antibiotics production in fermentation broths. The recombinant strains were fermented under the same condition used for S. *peucetius* SIPI-DU-1557, and the fermentation broth was taken on the 7th day. DXR (doxorubicin), EPI (epirubicin), RHO (ε-rhodomycinone). (C)- Comparison of the epirubicin and ε-rhodomycinone yield from S. *peucetius* recombinant strains. In all cases, data are shown as the mean ± standard deviation from at least three independent experiments.

Although the S. *peucetius* EPI-1 produced a considerable amount of epirubicin, a large quantity of the intermediate RHO (414 mg/L) was accumulated in the fermentation broth, while only 32 mg/L of RHO was found in parent strain SIPI-D-U-1557 **(Figure 3C)**. To convert more RHO to epirubicin, another copy of aveBIV was introduced into S. *peucetius* EPI-1, but the epirubicin productivity did not improve significantly (data not shown).

Expression of evaE in *S. peucetius* ΔdnmV to Increase Epirubicin Production

With the development of genome sequencing, abundant genetic information regarding secondary metabolite biosynthesis pathways has been discovered [29,30], providing a new avenue for obtaining a variety of TDP-4-ketohexulose reductases through bioinformatics analysis. Herein, we attempted to search new TDP-4-ketoreductases, aside from AveBIV, to increase the TDP-epidaunosamine pool, and enhance epirubicin production further. The AveBIV protein sequence was used as a reference to blast similar sequences in The Universal Protein Resource (UniProt) database. As a result, over 200 protein sequences were obtained. Five enzymes from diverse microorganisms with relatively high sequence similarities with AveBIV were selected for further investigation **(Figure 4)**. After analyzing the detailed biosynthesis pathway and enzyme-catalyzed products, EvaE from Amycolatopsis orientalis, which shares 51.1% amino acid identity with AveBIV was selected. EvaE was identified as an enzyme catalyzing the reduction of a TDP-6-deoxysugar C-4 ketone in the chloroeremomycin biosynthesis pathway [31]. The catalysis product, TDP-epivancosamine, is similar to TDP-epidaunosamine in terms of its chemical structure, except at C3′. TDP-epivancosamine contains a methyl group at C3′, whereas TDP-epidaunosamine possesses a hydrogen at the same position. Therefore, we hypothesized that EvaE could also produce TDP-epidaunosamine in dnmV-mutant strains.

The plasmid pEvaE was introduced into S. *peucetius* ΔdnmV via conjugation to produce S. *peucetius* EPI-2 (**Figure 3A**). The epirubicin yield of S. *peucetius* EPI-2 reached 170 mg/L, showing a 2.6-fold improvement over S. *peucetius* EPI-1. Simultaneously, the production of RHO decreased to 195 mg/L **(Figure 3C)**. This result indicated that EvaE had higher efficiency than AveBIV to

e-ISSN:2320-3528
p-ISSN:2347-2286

catalyze conversion of TDP-3-amino-4-keto-2,6-dideoxy-L-glucose to TDP-epidaunosamine, and that the deficiency of intercellular TDP-epidaunosamine was a critical factor in limiting epirubicin biosynthesis.

Co-expression of dnrS/dnrQ and desIII/desIV for Further Enhancement of Epirubicin Production

Although epirubicin production is greatly improved in *S. peucetius* EPI-2, its precursor RHO was still accumulated in the fermentation broth, indicating that subsequent genetic manipulations were essential to improve epirubicin production. Sohng's studies [27] showed that TDP-daunosamine formation and glycosylation were the rate-limiting steps in doxorubicin biosynthesis. In doxorubicin biosynthesis (**Figure 1**), DnrS catalyzes the glycosylation of TDP-daunosamine to RHO to form rhodomycin D, and dnrQ encodes an enzyme required for glycosylation [11]. Among the enzymes required for TDP-daunosamine biosynthesis in *S. peucetius*, two genes, dnmL and dnmM, were believed to be necessary for the first two steps [9]. DnmL and dnmM encode glucose-1-phosphate thymidylyl-transferase and TDP-D-glucose 4,6-dehydratase, respectively. However, overexpression of dnmL and dnmM has no effect on doxorubicin production. DesIII and desIV in Streptomyces venezuelae catalyze the same enzymatic reactions as dnmL and dnmM for the formation of the common intermediate TDP-4-keto-6-deoxy-D-glucose [32]. Overexpression of dnrS along with dnrQ, in the presence or absence of desIII/desIV, could increase doxorubicin production significantly. The biosynthesis pathways of doxorubicin and epirubicin were mostly similar, except for the last step of sugar moiety synthesis, which inspired us to further enhance epirubicin through the same strategy.

We sought to improve epirubicin titer by overexpressing dnrS/dnrQ in *S. peucetius* EPI-2. The *S. peucetius* EPI-3 strain was constructed by integrating dnrS/dnrQ in *S. peucetius* EPI-2 (**Figure 3A**). The yield of epirubicin was 212 mg/L (**Figure 3C**), a 3.5-fold increase over *S. peucetius* EPI-1. However, a slight decrease of RHO in EPI-3 fermentation may suggest an insufficient TDP-epidaunosamine supply. Therefore, the pEQSd containing evaE, dnrS/dnrQ, and desIII/desIV was introduced into *S. peucetius* ΔdnmV to construct the recombinant strain *S. peucetius* EPI-4, which produced 273 mg/L epirubicin, a 4.8-fold increase compared with *S. peucetius* EPI-1 (**Figure 3A** and **Figure 3C**). This result suggests that the epirubicin yield was further enhanced by co-overexpression of epirubicin biosynthesis pathway-related genes and that TDP-daunosamine formation and glycosylation were also the rate-limiting steps in epirubicin biosynthesis. However, when introducing an extra copy of dnrS/dnrQ and desIII/desIV into *S. peucetius* EPI-4, epirubicin and RHO production both dropped drastically due to poor growth of the engineered strain.

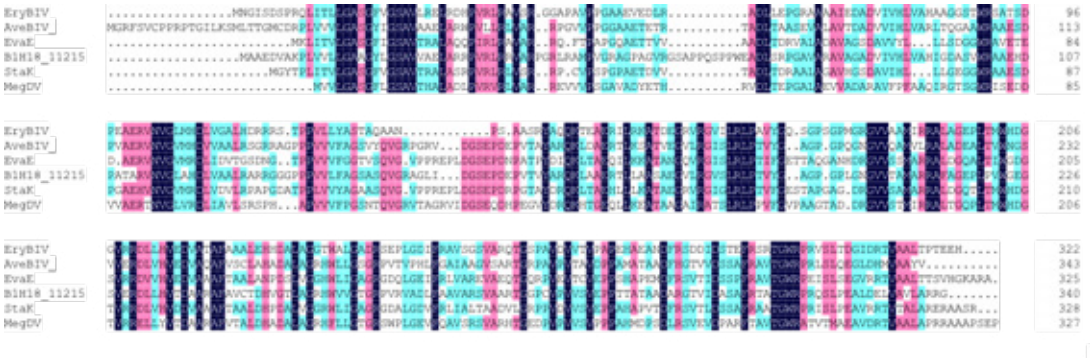

Figure 4: Amino acid alignments of TDP-4-ketoreductases using the DNAMAN Tool. Sequences are EryBIV from Saccharopolyspora erythraea (UniProtKB entry O33938), AveBIV from Streptomyces avermitilis (UniProtKB entry Q79ZM1), EvaE from Amycolatopsis orientalis (UniProtKB entry O52794), B1h18_11215 from Streptomyces tsukubensis (UniProtKB entry A0A1V4AAV6), StaK from Streptomyces sp. TP-A0274 (UniProtKB entry Q83WG0), MegDV from Micromonospora megalomicea subsp. nigra (UniProtKB entry Q9F834).

Production of Epirubicin in a 5 L Fermenter

The production of epirubicin by the *S. peucetius* EPI-4 strain was performed in a 5 L fermenter over a 192-h incubation period. As shown in **Figure 5**, total sugar continuously dropped during the incubation period, which indicated that sugar was the main requirement for cell growth and maintenance and epirubicin production. The stable pH across the entire period was a contributing factor for epirubicin production. Epirubicin yield was enhanced with an increase in the packed mycelial volume (PMV), which suggested that to increase PMV and obtain a higher level of epirubicin, optimization of fermentation conditions will be necessary in the future. The epirubicin production reached a maximum of 252 mg/L at 168 h, similar to the fermentation result obtained in a flask. These results showed that *S. peucetius* EPI-4 has potential industrial application in epirubicin production by direct fermentation.

e-ISSN:2320-3528
p-ISSN:2347-2286

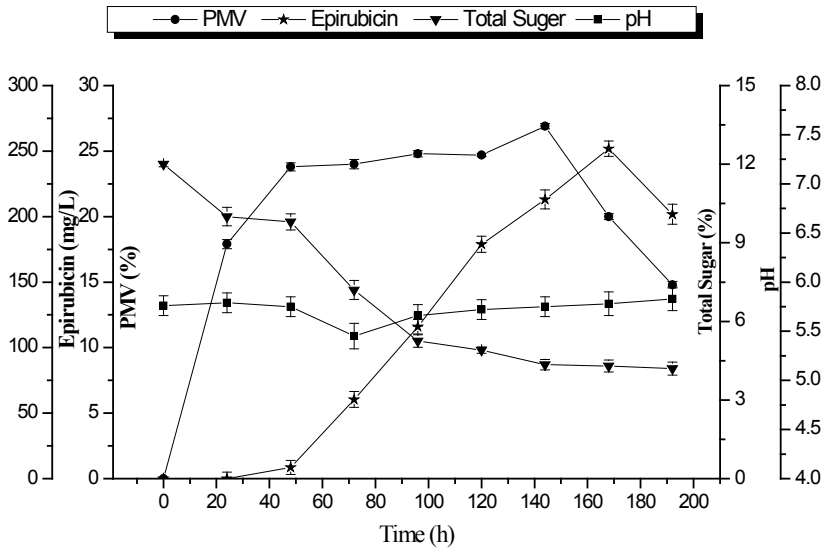

Figure 5: Time course analysis of epirubicin production, the packed mycelial volume (PMV) value, total sugar, and pH in a 5 L fermenter by S. *peucetius* EPI-4. The aeration rate was fixed at 1 vvm and the agitation rate was set to automatically respond to the dissolved oxygen (DO) to maintain it at ≥ 20%.

CONCLUSION

Here, a metabolically engineered S. *peucetius* strain was developed for potential cost-effective epirubicin production. High epirubicin production was achieved by combined screening of high-doxorubicin–producing strains for host suitability and metabolic engineering of the epirubicin biosynthesis pathway. EvaE, which catalyzes TDP-3-amino-4-keto-2,6-dideoxy-L-glucose to TDP-epidaunosamine more efficiently than aveBIV, was selected for epirubicin biosynthesis and could be eveloved by protein engineering to obtain higher activity. Co-expression of key epirubicin-biosynthesis genes improved epirubicin yield. The maximum epirubicin titer reached 270 mg/L and 252 mg/L in the flask and 5 L fermenter, respectively—the highest reported yields for genetically engineered S. *peucetius* to date.

ACKNOWLEDGEMENT

This work was supported by the National Major Scientific and Technological Special Project for “Significant New Drugs Development” (2014ZX09201001-005-001).

REFERENCES

1. Arcamone F, et al. New developments in antitumor anthracyclines. Pharmacology & Therapeutics 1997; 76: 117.
2. Minotti G, et al. Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. Pharmacological Reviews. 2004; 56: 185-229.
3. Arcamone F, et al. Synthesis and antitumor properties of new glycosides of daunomycinone and adriamycinone. Journal of Medicinal Chemistry. 1975; 6: 703-707.
4. Van Der RM, et al. Process for preparing epirubicin or acid addition salts thereof from daunorubicin, US 1999.
5. Kim E, et al. Reinvigorating natural product combinatorial biosynthesis with synthetic biology. Nature Chemical Biology. 2015; 11: 649-659.
6. Zhuo Y, et al. Synthetic biology of avermectin for production improvement and structure diversification. Biotechnology Journal. 2014; 9: 316–325
7. Hutchinson, C.R., 1997. Biosynthetic Studies of Daunorubicin and Tetracenomycin C. Chemical Reviews, 97: 2525-2535.
8. Grimm A, et al. Characterization of the Streptomyces peucetius ATCC 29050 genes encoding doxorubicin polyketide synthase. Gene. 1994; 151: 1-10.
9. Gallo MA, et al. The dnrM gene in Streptomyces peucetius contains a naturally occurring frameshift mutation that is suppressed by another locus outside of the daunorubicin-production gene cluster. Microbiology. 2006; 142: 269-275.

e-ISSN:2320-3528
p-ISSN:2347-2286

10. Otten SL, et al. Cloning and characterization of the Streptomyces peucetius dnmZUV genes encoding three enzymes required for biosynthesis of the daunorubicin precursor thymidine diphospho-L-daunosamine. Journal of Bacteriology. 1997; 179: 4446-4450.

11. Otten SL, et al. Cloning and characterization of the Streptomyces peucetius dnrQS genes encoding a daunosamine biosynthesis enzyme and a glycosyl transferase involved in daunorubicin biosynthesis. Journal of Bacteriology. 1995; 177: 6688-6692.

12. Dickens, M.L., Priestley, N.D., Strohl, W.R., 1997. In vivo and in vitro bioconversion of epsilon-rhodomycinone glycoside to doxorubicin: functions of DauP, DauK, and DoxA. Journal of Bacteriology, 179(8), 2641-2650.

13. Lomovskaya N, et al. Doxorubicin overproduction in Streptomyces peucetius: cloning and characterization of the dnrU ketoreductase and dnrV genes and the doxA cytochrome P-450 hydroxylase gene. Journal of Bacteriology. 1999; 181: 305-318.

14. Wohlert S, et al. Insights about the biosynthesis of the avermectin deoxysugar L-oleandrose through heterologous expression of Streptomyces avermitilis deoxysugar genes in Streptomyces lividans. Chemistry & Biology. 2001; 8: 681-700.

15. Shao L, et al. Overexpression of aveBIV leading to the improvement of 4'-epidaunorubicin production in Streptomyces coeruleorubidus strain SIPI-A0707. Applied Microbiology & Biotechnology. 2010; 87: 1057-1064.

16. Peano C, et al. Comparative genomics and transcriptional profiles of Saccharopolyspora erythraea NRRL 2338 and a classically improved erythromycin over-producing strain. Microbial Cell Factories. 2012; 11: 1-25.

17. Huang J, et al. Gene Replacement for the Generation of Designed Novel Avermectin Derivatives with Enhanced Acaricidal and Nematicidal Activities. Applied & Environmental Microbiology. 2015; 81: 5326-5334.

18. Kim MS, et al. Engineered biosynthesis of milbemycins in the avermectin high-producing strainStreptomyces avermitilis. Microbial Cell Factories. 2017 16: 9.

19. Rodriguez E, et al. Rapid engineering of polyketide overproduction by gene transfer to industrially optimized strains. Journal of Industrial Microbiology & Biotechnology 2003; 30: 480-488.

20. Macneil DJ, et al. Analysis of Streptomyces avermitilis genes required for avermectin biosynthesis utilizing a novel integration vector. Gene. 1992; 111: 61-68.

21. Bierman M, et al. Plasmid cloning vectors for the conjugal transfer of DNA from Escherichia coli to Streptomyces spp. Gene. 1992; 116: 43-49.

22. Sambrook J and Russell DW. Molecular Cloning: A Laboratory Manual, 2001 Third Edition.

23. Buttner M, et al.. Practical Streptomyces Genetics. 2000.

24. Hutchinson CR and ColomboAL,. Genetic engineering of doxorubicin production in Streptomyces peucetius : a review. Journal of Industrial Microbiology & Biotechnology. 1999; 23: 647-652.

25. Malla S, et al.. Self-resistance mechanism in Streptomyces peucetius: overexpression of drrA, drrB and drrC for doxorubicin enhancement. Microbiological Research. 2010; 165: 259-267.

26. Parajuli N, et al. Identification and characterization of the afsR homologue regulatory gene from Streptomyces peucetius ATCC 27952. Research in Microbiology. 2005; 156: 07-712

27. Malla S, et al. Enhancement of doxorubicin production by expression of structural sugar biosynthesis and glycosyltransferase genes in Streptomyces peucetius. Journal of Bioscience & Bioengineering. 2009; 108: 92-98.

28. Madduri K, et al. Production of the antitumor drug epirubicin (4'-epidoxorubicin) and its precursor by a genetically engineered strain of Streptomyces peucetius. Nature Biotechnology. 1998; 16: 69-74.

29. Doroghazi JR and Metcalf WW. Comparative genomics of actinomycetes with a focus on natural product biosynthetic genes. Bmc Genomics. 2013; 14: 611.

30. Harrison J and Studholme DJ. Recently published Streptomyces genome sequences. Microbial Biotechnology. 2014; 7: 373-380.

31. Chen H, et al. Deoxysugars in glycopeptide antibiotics: enzymatic synthesis of TDP-L-epivancosamine in chloroeremomycin biosynthesis. Proceedings of the National Academy of Sciences of the United States of America. 2000; 97: 11942-11947.

32. Xue Y, et al. A Gene Cluster for Macrolide Antibiotic Biosynthesis in Streptomyces venezuelae: Architecture of Metabolic Diversity. Proceedings of the National Academy of Sciences of the United States of America. 1998; 95: 12111-12116.