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

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-keto reductases, 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.
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 dpsABCDGFY 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.

<table>
<thead>
<tr>
<th>Strains or plasmids</th>
<th>Relevant characteristic</th>
<th>Reference or source</th>
</tr>
</thead>
</table>

RR: J Microbiol Biotechnol | Volume 7 | Issue 1 | March, 2018
### Strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli DH5α</td>
<td>F, p80dlacZΔM15, Δ (lacZYA-argF) U169, deoR, recA1, endA1, hsdR17 (rk, mk*), phoA, supE44, λ, thi-1, gyrA96, relA1</td>
<td>CWBIO</td>
</tr>
<tr>
<td>ET12567/PUZ8002</td>
<td>Methylation defective, strain used in E. coli-streptomyces intergeneric conjugation</td>
<td>[21]</td>
</tr>
<tr>
<td>S. avermitilis</td>
<td>Avermectin producing strain, containing aveBIV gene</td>
<td>ATCC31267</td>
</tr>
<tr>
<td>A. orientalis</td>
<td>Chloroeremomycin producing strain, containing evaE gene</td>
<td>NRRL18099</td>
</tr>
<tr>
<td>S. peucetius ΔdnmV</td>
<td>SIPI-DU-1557 with disrupted dnmV</td>
<td>This study</td>
</tr>
<tr>
<td>EPI-1</td>
<td>Genome-based expression of aveBIV under the promoter PermE* in S. peucetius ΔdnmV</td>
<td>This study</td>
</tr>
<tr>
<td>EPI-2</td>
<td>Genome-based expression of evaE under the promoter PermE* in S. peucetius ΔdnmV</td>
<td>This study</td>
</tr>
<tr>
<td>EPI-3</td>
<td>Genome-based expression of evaE, dnrQ and dnrS under the promoter PermE* in S. peucetius ΔdnmV</td>
<td>This study</td>
</tr>
<tr>
<td>EPI-4</td>
<td>Genome-based expression of evaE, dnrQ, dnrS, desIII and desIV under the promoter PermE* in S. peucetius ΔdnmV</td>
<td>This study</td>
</tr>
</tbody>
</table>

### Plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSET152</td>
<td>E. coli replicon, Streptomyces ØC31 attachment site, AprR</td>
<td>[22]</td>
</tr>
<tr>
<td>pET-22b (+)</td>
<td>E. coli expressing and subcloning vector, AmpR</td>
<td>This study</td>
</tr>
<tr>
<td>pΔdnmV</td>
<td>pSET152 with deletion of phiC31 integrase gene and two homologous arms containing the upstream and downstream DNA fragments of dnmV</td>
<td>This study</td>
</tr>
<tr>
<td>pAveB</td>
<td>pSET152 with the expression of aveBIV under the control of strong promoter PermE*</td>
<td>This study</td>
</tr>
<tr>
<td>pEvaE</td>
<td>pSET152 with the expression of evaE under the control of strong promoter PermE*</td>
<td>This study</td>
</tr>
<tr>
<td>pEQS</td>
<td>pSET152 with the expression of evaE, dnrQ and dnrS, under the control of strong promoter PermE*</td>
<td>This study</td>
</tr>
<tr>
<td>pEQSd</td>
<td>pSET152 with the expression of evaE, dnrQ, desIII and desIV under the control of strong promoter PermE*</td>
<td>This study</td>
</tr>
</tbody>
</table>

**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.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dau-HindIII-F</td>
<td>GGGAAGCTTGATGCCCCCCTACGGAACCT</td>
</tr>
<tr>
<td>dnmV-252-R</td>
<td>CGCAGATGCAGCTACGTCATCTCC</td>
</tr>
</tbody>
</table>
Research & Reviews: Journal of Microbiology and Biotechnology

HPLC Analysis Methods

RR: J Microbiol Biotechnol | Volume 7 | Issue 1 | March, 2018
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](image)

**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-D-U-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,
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-epidau nosamine 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
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.

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.
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 evolved 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


