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## Ribosome engineering and fermentation optimization leads to overproduction of tiancimycin A, a new enediyne natural product from *Streptomyces* sp. CB03234

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### Abstract

Tiancimycin (TNM) A, a recently discovered enediyne natural product from *Streptomyces* sp. CB03234, showed rapid and complete killing of cancer cells and could be used as a payload in antibody drug conjugates. The low yield of TNM A in the wild-type strain promoted us to use ribosome engineering and fermentation optimization for its yield improvement. The *Streptomyces* sp. CB03234-R-16 mutant strain with a L422P mutation in RpoB, the RNA polymerase  $\beta$ -subunit, was obtained from the rifamycin-resistant screening. After fermentation optimization, the titers of TNM A in *Streptomyces* sp. CB03234-R-16 reached to  $22.5 \pm 3.1$  mg L<sup>-1</sup> in shaking flasks, and  $13 \pm 1$  mg L<sup>-1</sup> in 15 L fermentors, which were at least 40-fold higher than that in the wild-type strain ( $\sim 0.3$  mg L<sup>-1</sup>). Quantitative real-time RT-PCR revealed markedly enhanced expression of key genes encoding TNM A biosynthetic enzymes and regulators in *Streptomyces* sp. CB03234-R-16. Our study should greatly facilitate the future efforts to develop TNM A into a clinical anticancer drug.

Compliance with ethical standards

This article does not contain any studies with human participants performed by any of the authors.

**Conflict of interest** All authors declare that he/she has no conflict of interest.

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## Keywords

Enediyne natural product; Tiansimycin A; Ribosome engineering; In situ resin adsorption; RpoB

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## Introduction

Enediyne natural products, characterized by the conjugation of a double bond with two acetylenic groups within a 9- or 10-membered macrocycle, are among the most cytotoxic natural products to date and several of them have been developed into clinical anticancer drugs (Fig. 1) [23, 34, 35, 37]. Among a dozen of enediynes discovered, the anti-tumor enediyne chromoprotein neocarzinostatin has been used for hepatoma treatment in Japan since 1994, while the chromoprotein C-1027 was reported to undergo phase II clinical trials in China [19, 32]. Most recently, gemtuzumab ozogamicin (GO), an anti-CD33 antibody–calicheamicin conjugate, was reapproved for acute myeloid leukemia (AML), and inotuzumab ozogamicin, an anti-CD22 antibody–calicheamicin conjugate, was approved for acute lymphoblastic leukemia [18, 31]. GO was actually the first antibody drug conjugates (ADC) on the markets, and the only AML drug to improve patient survival in the last three decades, despite its volunteering withdraw in 2010 [2, 8]. Therefore, highly potent payloads, such as enediynes, are promising drug leads to be used as warheads for ADC drugs [36]. Tiansimycin (TNM) A, a 10-membered enediyne natural product from *Streptomyces* sp. CB03234, was discovered through a novel genome mining approach (Fig. 1). It was the only new enediyne natural product discovered in the past decade and showed strong inhibitory activities against various tumor cell lines [7, 44]. Remarkably, TNM A exhibited rapid and complete killing of cancer cells, in contrast to the payloads of Adcetris and Kadcyła, two ADC drugs currently on the markets. Therefore, it has very high potential to be developed as effective anticancer drugs.

The *Streptomyces* sp. CB03234 wild-type strain produces TNM A in a low titer, with only 1.2 mg purified from 6-L fermentation [44]. The low titer of TNM A was similar to the initial titers of the known anthraquinone enediynes, such as dynemicin A and uncialamycin (Table S1). For example, dynemicin A was first isolated with an estimated yield of 0.1 mg L<sup>-1</sup>, and the production of dynemicin A in 10,000 L was reported to have a titer of 1.2–1.5 mg L<sup>-1</sup>, which was subsequently optimized to 24.7 mg L<sup>-1</sup> in shaking flasks [16, 17]. Uncialamycin was produced from *Streptomyces uncialis*, with ~0.3 mg isolated from 16 L fermentation on solid agar medium [7]. Since the direct access to these fascinating compounds through total synthesis remains impractical, the low titers significantly limit their clinical development [21, 22].

There are many effective strategies to improve the production of secondary metabolites in microorganisms [1, 3, 6, 24, 27, 30, 41]. Many factors would contribute to the titer or the production rate for the targeted secondary metabolites, including the metabolic potential of the producers, and fermentation factors, such as nutrient supply, dissolved oxygen (DO), temperature, and pH. Ribosome engineering is a cost-effective, rapid and a rational approach to obtain mutants with improved titers for secondary metabolite production [25, 26]. By screening for antibiotic resistance targeting bacterial ribosome or RNA polymerase, isolated

mutants could have hyperphosphorylated guanosine nucleotide (ppGpp) synthesis activities to induce the stringent response, resulting in extensively reprogramming of the cellular metabolic patterns and even the overexpression of certain pathway specific transcriptional regulators [4, 40].

In this report, we utilized a combination of ribosome engineering and fermentation optimization strategy to rapidly improve the TNM A titer from  $\sim 0.3 \text{ mg L}^{-1}$  in the *Streptomyces* sp. CB03234 wild-type strain to  $22.5 \pm 3.1 \text{ mg L}^{-1}$  (in shaking flasks) and  $13.0 \pm 1.0 \text{ mg L}^{-1}$  (in 15-L fermenters) in the *Streptomyces* sp. CB03234-R-16 mutant strain. Quantitative real-time PCR revealed markedly enhanced expression of key genes encoding enzymes and regulators for TNM A biosynthesis in the mutant, in comparison to the wild-type. The current titer of TNM A is comparable to those from industrial enediyne producing strains, such as dynemicin A ( $24.7 \text{ mg L}^{-1}$ ) [16] or esperamycin ( $8.1 \text{ mg L}^{-1}$ ) [15]. Our findings would greatly facilitate the future efforts to develop TNM A into a clinical anticancer drug.

## Materials and methods

### Bacterial strains, culture conditions and general methods

All chemical and biological reagents, such as antibiotics, inorganic salts, carbon and nitrogen sources used in this study were from common commercial sources, unless otherwise specified. The *Streptomyces* sp. CB03234 wild-type and mutant strains were grown at  $30 \text{ }^{\circ}\text{C}$  on ISP4 for sporulation. The *Streptomyces* sp. CB03234-R-16 mutant strain was deposited at China Center for Type Culture Collection under accession No. CCTCC M 2017556. The initial seed medium was tryptic soy broth (TSB), containing (per liter): tryptone 17 g, soya peptone 3 g, NaCl 5 g,  $\text{K}_2\text{HPO}_4$  2.5 g, dextrose 2.5 g, pH  $7.3 \pm 0.2$ . The initial production medium contained (per liter) soluble starch 10 g, pharmamedia 5 g,  $\text{CuSO}_4$  0.05 g, NaI 5 mg,  $\text{CaCO}_3$  2 g, pH adjusted to 7.0 before autoclaving [44]. The *Streptomyces* sp. CB03234 mutants identified from ribosome engineering were cultured in the TSB seed medium with  $1 \mu\text{g mL}^{-1}$  rifamycin (Rif). An aliquot of fresh spore suspension of *Streptomyces* sp. CB03234 or mutants (about  $1 \times 10^7$  spores) was inoculated into 50 mL TSB in 250 mL shaking flasks and grown for 36 h at  $30 \text{ }^{\circ}\text{C}$  and 250 rpm. Then 5 mL of seed culture was transferred to 50 mL production medium in a 250-mL flask and cultured for 6–9 days at  $30 \text{ }^{\circ}\text{C}$  and 230–250 rpm.

Tiancimycin A was analyzed on a waters ultra performance liquid chromatography (UPLC) system equipped with a PDA detector and an ACQUITY UPLC (Waters), C18 column ( $2.7 \mu\text{m}$ ,  $4.6 \text{ mm} \times 50 \text{ mm}$ , Waters). The mobile phase consisted of buffer A (ultrapure  $\text{H}_2\text{O}$  containing 0.1%  $\text{HCOOH}$  and 0.1%  $\text{CH}_3\text{CN}$ ) and buffer B (chromatographic grade  $\text{CH}_3\text{CN}$  containing 0.1%  $\text{HCOOH}$ ) was applied at a flow rate of  $0.4 \text{ mL min}^{-1}$ . A linear gradient program (60% buffer A and 40% buffer B to 50% buffer A and 50% buffer B for 2 mins, 50% buffer A and 50% buffer B to 0% buffer A and 100% buffer B for 5 mins, followed by 60% buffer A and 40% buffer B for 5 mins) was applied to detect TNM A at 540 nm.

### In vitro and in situ resin adsorption of TNM A

The resins HP20, HP2MGL, XAD-16, XAD-2, IRC-50, A30-B were washed prior to use based on the recommendation from the manufacturers. About 1% (w/v) of each type of resins (0.4 g) was added into *Streptomyces* sp. CB03234 fermentation supernatant (40 mL), respectively, mixing on a rotary shaker for 2 h at room temperature. After the resins were washed by deionized water (30 mL  $\times$  3), TNM A was eluted with methanol (MeOH) (30 mL  $\times$  3) from HP20, HP2MGL, XAD-16 and XAD-2, or with 20% (w/v) NaCl (10 mL  $\times$  3) from IRC-50 and A30-B, which was extracted by ethyl acetate (EtOAc) (30 mL  $\times$  3). The organic fractions were then dried in anhydrous Na<sub>2</sub>SO<sub>4</sub>, concentrated in vacuum and quantified via UPLC analysis based on the TNM A standard curve (Fig. S1). TNM A in the supernatant before and after resin treatment was extracted by EtOAc, dried in anhydrous Na<sub>2</sub>SO<sub>4</sub>, concentrated and similarly quantified. The absorption and deabsorption ratio, and the recovery ratio were then calculated.

The HP2MGL resin was chosen for the subsequent in situ resin addition experiments. Different amounts of HP2MGL resins (0.25, 0.5, 1, 2 and 3%, w/v) were added into 50 mL production medium in 250 mL shaking flasks before auto-claving. The *Streptomyces* sp. CB03234 strain was then fermented using the resin-containing production medium for 7 days, with production medium without resins as a control. The resins and the supernatant were separated, and TNM A was recovered as above and quantified using UPLC. TNM A in mycelium was obtained by treatment of the mycelium using MeOH (30 mL  $\times$  3). The extracts were then dried in anhydrous Na<sub>2</sub>SO<sub>4</sub>, concentrated in vacuum, and similarly quantified by UPLC.

### Generation of *Streptomyces* sp. CB03234 mutants by ribosome engineering

The *Streptomyces* sp. CB03234 wild-type strain was subjected to varying concentrations of Rif to isolate mutants by the ribosome engineering approach alone or in combination with random mutagenesis using diethyl sulfate (DES). The minimum inhibitory concentration (MIC) of Rif against *Streptomyces* sp. CB03234 was first determined using the agar dilution method [42]. Thus, about  $1 \times 10^9$  *Streptomyces* sp. CB03234 spores were treated with 0.46 M DES for 10–40 min at room temperature, resulting in a killing ratio of > 99.9%. Both the non-treated and DES-treated spores ( $1 \times 10^9$ ) were plated onto 12 GYM plates (per liter: glucose 4 g, yeast extract 4 g, malt extract 10 g, CaCO<sub>3</sub> 2 g, agar 20 g, pH 7.2), supplemented with 2, 5 or 10  $\mu\text{g mL}^{-1}$  Rif, respectively, and incubated at 30 °C. Rif-resistant colonies were observed after 6–7 days and confirmed by re-growing them on GYM plates containing 5  $\mu\text{g mL}^{-1}$  Rif. Chromosomal DNA was extracted and purified from the *Streptomyces* sp. CB03234 wild-type and Rif-resistant mutant strains following the standard protocol. To amplify the segment of *rpoB*, PCR experiments were performed by following the manufacturer's instructions and using Taq polymerase (Takara Ex Taq). An Eppendorf thermal cycler was used, and the PCR conditions were 5 min of incubation at 96 °C, followed by 30 cycles of 96 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min. The forward and reverse primers were 5'-CCC GAAGCGCTACGACCTCGC-3' and 5'-GAGGCGAGCGAGCCGATCAGAC-3'. The resulting PCR fragments were cloned into pMD18T (Takara) and subjected to DNA sequencing.

### Time course analysis of TNM A production in *Streptomyces* sp. CB03234 and CB03234-R-16, and the gene expression analysis by quantitative real-time RT-PCR

For the time course analysis of the *Streptomyces* sp. CB03234 wild-type and the CB03234-R-16 mutant over the 7-day fermentation, the respective cultures ( $3 \times 50$  mL) were individually harvested every day. TNM A in the resins was similarly quantified. The pH of the supernatant was measured on a pH meter. The mycelium of each culture was dried and weighed.

The mycelia from about 200  $\mu$ L culture from the above fermentation at 48, 120 and 168 h were used for total RNA extraction using the total RNA extraction kit (Roche), according to the manufacturer's instruction. Genomic DNA was removed by RNase-free DNase I (TaKaRa), and the concentration and quality of total RNA were determined using a Biophotometer Nanodrop (Analytik Jena). Reverse transcription was conducted using the cDNA Synthesis Kit (Roche). Quantitative real-time RT-PCR (qRT-PCR) was carried out on the Roche LightCycler 96 fluorescence quantitative PCR system using the Fast Start Essential DNA Green Master (Roche).

The PCR conditions consisted of one cycle of denaturation at 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 60 s at 55 °C. Each transcription assay was normalized to the corresponding transcriptional level of *hrdB*, and all qRT-PCR reactions were performed in triplicate. The primers used for qRT-PCR were listed in Table S3 in the Supporting Information.

### Fermentation medium development

Based on the original production medium, 13 types of carbon sources, including soluble starch (the original carbon source), corn flour, dextrin, D-fructose, D-galactose, D-maltose, D-xylose, glycerol, glucose,  $\alpha$ -lactose, mannitol, nonfat milk powder, sucrose, were individually tested at 10 g L<sup>-1</sup>. Then 14 nitrogen sources including pharmamedia (the original nitrogen source), beef extract, casamino acids, corn steep liquor, corn protein powder, fish peptone, fish meal, malt extract, oat meal, peptone, peanut protein powder, soya peptone, tryptone, and yeast extract, were individually tested at 5 g L<sup>-1</sup>, the *Streptomyces* sp. CB03234-R-16 mutant was cultured as before in the production medium without addition of HP2MGL resins. TNM A concentration in the above supernatants was estimated on day 2, 4 and 6 of the fermentation, by the agar diffusion assay against *Kocuria rhizophila* ATCC 9341.

Four different concentrations of soluble starch (15, 20, 30, 40 g L<sup>-1</sup>) and pharmamedia (10, 15, 20, 25 g L<sup>-1</sup>) were tested, respectively. The *Streptomyces* sp. 3234-R-16 strain was cultured in the production medium with addition of 1.5% (w/v) HP2MGL resins for 9 days, and TNM A was recovered from the resins and quantified by UPLC. Next the combination of soluble starch and pharmamedia, as well as the inorganic salts, including NaI (5, 15, 20, 25, 30, 35, 40 mg L<sup>-1</sup>), CuSO<sub>4</sub> (0.05, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4 g L<sup>-1</sup>) and CaCO<sub>3</sub> (2, 4, 6, 8, 16 g L<sup>-1</sup>) were investigated.

## Fermentation in 15-L fermentors

A 20  $\mu\text{L}$  *Streptomyces* sp. CB03234-R-16 spore suspension (about  $1 \times 10^9 \text{ mL}^{-1}$ ) was inoculated into 400 mL TSB seed medium with  $1 \mu\text{g mL}^{-1}$  Rif in two to four 2 L Erlenmeyer flasks and cultured at 30 °C for 36–48 h. The seed culture with varying amounts was transferred to 15 L fermentors with a working volume of 8.0 L using the following production medium: medium A (per liter: soluble starch 40 g, pharma-media 20 g,  $\text{CuSO}_4$  0.2 g, NaI 20 mg,  $\text{CaCO}_3$  8 g and 2 mL anti-foaming agent, pH 7.0); medium B (per liter: soluble starch 30 g, pharmamedia 15 g,  $\text{CuSO}_4$  0.15 g, NaI 15 mg,  $\text{CaCO}_3$  6 g, pH 7.0 and 2 mL anti-foaming agent) and medium C (per liter: soluble starch 30 g, pharmamedia 15 g,  $\text{CuSO}_4$  0.05 g, NaI 5 mg,  $\text{CaCO}_3$  2 g, pH 7.0 and 2 mL anti-foaming agent). The *Streptomyces* sp. CB03234-R-16 was fermented at 30 °C, air flow rate of 200 L/h and air pressure 0.05 MPa for 9 days. When the pH of the fermentation culture reached 7.0, 0.2 M HCl was added dropwise to control the pH of the culture around 7.0. For TNM A analysis from the fermentors, the resins from 50 mL fermentation culture were collected, and TNM A from the resins was quantified by UPLC. Medium C was used to obtain the optimal fermentation parameters. To determine the best volume of inoculum, different amounts of seed culture [(5, 10 and 15% (v/v))] was used. To determine the optimal dissolved oxygen (DO) level, 10% inoculum was used, and the agitation speed was correlated with the DO level.

The partial *rpoB* sequences from *Streptomyces* sp. CB03234-R-1 to *Streptomyces* sp. CB03234-R-18 reported in this manuscript were deposited with the GenBank Database under Accession NO MG241318–MG241335.

## Results

### In situ resin addition increased the titer of TNM A in *Streptomyces* sp. CB03234

The addition of solid-phase adsorbents during fermentation has increased the production of many valuable microbial natural products, including anticancer agents epothilones, leinamycin, and two enediynes dynemicin A and esperamicin A1, by avoiding certain feedback inhibition or the cytotoxicity of the products [28]. We first screened 4 macroporous resins ranging from different polymer chemistry, surface area, particle and pore sizes, including HP20, HP2MGL, XAD-16, and XAD-2, and two ion exchange resins, IRC-50 and A-30B. The choice of these resins was judicially based on their ability to adsorb TNM A from the fermentation broth of *Streptomyces* sp. CB03234 and the ease of TNM A to be eluted after the adsorption (Fig. 2a) (Table S2). Among them, the macroporous resin HP2MGL showed the highest TNM A recovery ratio and was thus chosen for the in situ resin addition.

The addition of 0.5–3% (w/v) of HP2MGL resins in *Streptomyces* sp. CB03234 fermentation medium had some positive effects on TNM A production in 250-mL shaking flasks (Fig. 2b). In comparison to the fermentation without addition of resins, no TNM A was detected in the supernatant and most TNM A was adsorbed to the resins and subsequently eluted. It seemed that the addition of larger amount of resins was instrumental to sequester TNM A from mycelium, since there was only very small amount of TNM A in

the mycelium when 1.0% (v/v) or more resins were added. Overall the addition of 1.5% (w/v) of HP2MGL resins led to the increase of TNM A titer to  $\sim 0.8 \text{ mg L}^{-1}$ , which was about threefold improvement over original titer of  $\sim 0.3 \text{ mg L}^{-1}$  in the absence of resins. The in situ resin adsorption of TNM A also streamlined the isolation procedure, since TNM A could be directly eluted from the resins after fermentation. Therefore, the HP2MGL resins were used in the following experiments.

### **Ribosome engineering of *Streptomyces* sp. CB03234 leading to the isolation of the *Streptomyces* sp. CB03234-R-16 mutant that overproduces TNM A**

Ribosome engineering alone, or in combination with random mutagenesis, is a rational method to increase the yield of many secondary metabolites, such as actinorhodin, erythromycin, milbemycin, sivefungin and fredericamycin A [12, 14, 39, 40, 43, 45]. Rif is a potent and selective antibiotic to inhibit DNA-dependent RNA polymerases in bacteria, and has often been used in ribosome engineering [10, 25]. We adopted a strategy to screen for Rif-resistant *Streptomyces* sp. CB03234 mutants, using spores freshly treated by chemical mutagen diethyl sulfate (DES) or spores untreated, to identify strains with higher TNM A production. First, the MIC of *Streptomyces* sp. CB03234 towards Rif was determined to be  $1 \mu\text{g mL}^{-1}$ . The spores were then spread onto GYM plates with varying concentrations of Rif, ranging from 2 to  $10 \mu\text{g mL}^{-1}$ . A total of 18 Rif-resistant colonies, named *Streptomyces* sp. CB03234-R-1 to CB03234-R-18, were isolated and their MICs towards Rif were subsequently determined (Table 1). The MICs for all the isolated mutants towards Rif were at least  $5 \mu\text{g mL}^{-1}$ , five times higher than that for the wild-type. Interestingly, there were several mutants with MICs above  $200 \mu\text{g mL}^{-1}$ . The Rif-resistance is often conferred by mutation in the *rpoB* gene, which encodes the RNA polymerase  $\beta$ -subunit [26]. We next cloned the *rpoB* genes of the *Streptomyces* sp. CB03234 wild-type and the mutants, and subjected them to DNA sequencing. Surprisingly, all mutants except one contain various type of mutation in *rpoB*, featuring a very high mutation ratio comparing to other reported Rif mutants. Besides the typical mutations on RpoB often identified from Rif-resistant *Streptomyces* species, such as L422P, D427V, H437Y, R440H and S442L (numbered based on the RpoB from *S. coelicolor*), there were two new mutations, H437N (*Streptomyces* sp. CB03234-R-10), and a deletion of a stretch of seven amino acids (FMDQNNP) from RpoB (*Streptomyces* sp. CB03234-R-5), identified from above mutants (Figs. S2, S3). Note that the H437Y mutation on RpoB was often the most effective mutation in enhancing the antibiotic production in various actinomycetes [25].

We next evaluated TNM A production in mutants bearing different type of mutations, in comparison to the wild-type strain, with the addition of HP2MGL resin in the production medium (Table 1 and Fig. S4). The mutants bearing L422P, D427V, H437N and H437Y mutations on RpoB, showed drastic increase of TNM A production, ranging from  $1.5 \pm 0.2$  to  $5.8 \pm 1.0 \text{ mg L}^{-1}$ , while the others showed no or attenuated TNM A production. Comparing to the wild-type strain with a titer of about  $0.8 \text{ mg L}^{-1}$ , the highest TNM A producing mutant *Streptomyces* sp. CB03234-R-16 with L422P mutation showed about 6–7 times improvement, which was selected for the following experiments.

### qRT-PCR analysis of *Streptomyces* sp. CB03234 wild-type and *Streptomyces* sp. CB03234-R-16 mutant strains revealing drastic increase of gene expression governing TNM A production

A time course analysis of TNM A production in both the *Streptomyces* sp. CB03234 wild-type and *Streptomyces* sp. CB03234-R-16 mutant strains was performed (Figs. 3, S5). The effects of RpoB (L422P) mutation on the transcription of *relA*, which encodes ppGpp synthetase, and selected genes governing TNM A biosynthesis, were assessed at mRNA level using qRT-PCR. The transcription of *hrdB*, which encodes the principal sigma factor of RNA polymer-ase, was used as the internal control [5].

The expression of *relA* gene was mainly elevated at the late stages of fermentation in the mutant in comparison to the wild-type strain, as exemplified by a 1.8-fold increase at 120 h and over 100-fold increase at 168 h, with the production of TNM A of  $6.6 \pm 1.0$  or  $5.8 \pm 1.0$  mg L<sup>-1</sup> in the mutant, in comparison to  $0.9 \pm 0.1$  or  $0.8 \pm 0.1$  mg L<sup>-1</sup> in the wild-type (Fig. 3b, c). This was consistent with the previous observation that certain ribosomal mutations would increase *relA* transcription and lead to antibiotic overproduction, although the intracellular ppGpp level was not measured in the present study [40]. The transcript amounts of *tnmL* (cytochrome P450 monooxygenase) and *tnmE10* (type II thioesterase) were very abundant, reflecting its direct involvement in TNM A biosynthesis (Fig. 3d, e). For example, the type II thioesterase TnmE10 might play a key role to remove aberrant or stalled biosynthetic intermediates in enediyne polyketide synthases, especially during the beginning of TNM A biosynthesis, since both the wild-type and mutant showed highest *tnmE10* expression at 48 h [9, 11, 13]. The constant overexpression of *tnmL* might reflect the requirement for oxidative modification in furnishing the final natural product, due to the presence of the hydroxylation in either the anthraquinone moiety or the enediyne core in TNM A (Fig. 1). For all the transcriptional factors examined, *tnmR4* and *tnmR7*, both encoding AraC family transcriptional regulator, and *tnmR2*, encoding a putative regulator, showed about threefold increase of the expression at 168 h in the mutant in comparison to the wild-type strain, while the other examined transcriptional factors showed slightly less variation (Fig. 3f, j). These findings are consistent with previous studies on enediyne biosynthesis and regulation, and the homologues of *TnmR4* and *TnmR7* have been shown as positive regulators to improve C-1027 production in *S. globisporus* [6].

### Medium optimization leading to overproduction of TNM A in *Streptomyces* sp. CB03234-R-16

During the 7-day fermentation in the original production medium, the *Streptomyces* sp. CB03234-R-16 mutant grew slower than the wild-type strain and produced less mycelia. In addition, some degradation of TNM A was also observed with the elevation of the pH in the culture, and the presence of the labile enediyne functional group might affect the stability of TNM A under basic conditions [23, 37] (Figs. 3b, S5). Therefore, we decided to develop an optimal medium for TNM A production in the *Streptomyces* sp. CB03234-R-16 mutant. Starting from the original medium used in the wild-type, we first evaluated 13 different carbon sources and 14 different nitrogen sources in shaking flasks without addition of the resins. The production of TNM A was monitored at day 2, 4, and 6, respectively, using agar diffusion test with *K. rhizophila* ATCC 9341 as the indication strain (Fig. S6). The original



carbon source and nitrogen source, soluble starch and pharmamedia, were still among the best to produce TNM A in *Streptomyces* sp. CB03234-R-16.

Then various concentrations of soluble starch, ranging from 10 to 40 g L<sup>-1</sup>, and pharmamedia, ranging from 10 to 25 g L<sup>-1</sup>, were individually investigated in the presence of 1.5% (w/v) HP2MGL resins. The increased concentration of soluble starch or pharmamedia in the production medium was beneficial to the growth of *Streptomyces* sp. CB03234-R-16, but delayed the production of TNM A. However, TNM A titers reached to 9.6 ± 0.2 or 11.1 ± 0.4 mg L<sup>-1</sup> when 30 g L<sup>-1</sup> of soluble starch or 15 g L<sup>-1</sup> pharmamedia was used, compared to 4.4 ± 0.9 mg L<sup>-1</sup> in the original medium after 9-day fermentation (Fig. 4a). Finally, the optimal fermentation medium to achieve the highest TNM A titer of 22.5 ± 3.1 mg L<sup>-1</sup> in *Streptomyces* sp. CB03234-R-16 in shaking flasks was the appropriate combination of soluble starch, pharmamedia, and certain inorganic salts, which consisted of 40 g L<sup>-1</sup> soluble starch, 20 g L<sup>-1</sup> pharmamedia, 8 g L<sup>-1</sup> CaCO<sub>3</sub>, 200 mg L<sup>-1</sup> CuSO<sub>4</sub>·5H<sub>2</sub>O, 20 mg L<sup>-1</sup> NaI and 15 g L<sup>-1</sup> HP2MGL resin (Fig. 4b).

### Production of TNM A from *Streptomyces* sp. CB03234-R-16 in 15 L fermentors

Production of TNM A from *Streptomyces* sp. CB03234-R-16 was first scaled up in 15 L fermentors using the optimal production medium based on the shaking flask experiments. But we quickly noticed that TNM A production could only reach to about 10 mg L<sup>-1</sup> in 15 L fermentors, mainly due to the high viscosity of the medium and the release of TNM A from some broken HP2MGL resins resulting from agitation (data not shown). Therefore, an alternative medium with less viscosity was selected, consisting of 30 g L<sup>-1</sup> starch soluble, 15 g L<sup>-1</sup> pharmamedia, 2 g L<sup>-1</sup> CaCO<sub>3</sub>, 50 mg L<sup>-1</sup> CuSO<sub>4</sub>·5H<sub>2</sub>O, 20 mg L<sup>-1</sup> NaI, and 2 mL L<sup>-1</sup> anti-foaming agent, along with 3% (w/v) HP2MGL resins to offset the broken resins. We evaluated the effects of inoculation volume of the seed culture (5, 10 and 15%), and DO levels (10–20, 20–60 and 60–80%) towards TNM A production (Fig. S7). We discovered that inoculation of 10% (v/v) of the seed culture and DO level at 20–60% would be optimal for TNM A production. Under the optimal fermentation conditions, the titers of TNM A in *Streptomyces* sp. CB03234-R-16 reached to 13.0 ± 1.0 mg L<sup>-1</sup> (Fig. 5).

## Discussion

Natural products are main sources of anticancer and anti-infective drugs, but one of the main hurdles to the development of natural products as clinical drugs is their limited sources [20, 33]. For microbial drug discovery, many micro-organisms can be isolated, preserved, and cultured, thereby offering potential of reliable supply by microbial fermentation. However, the initial titers of most microbial natural products are typically not high enough to warrant their future development. Probably due to the extremely toxicity and instability of the enediyne core, the titers of enediyne natural products are often extremely low, even for the industrial producers, in comparison to many other antibiotics of microbial origin (Fig. 1 and Table S1).

In this study, we have used a combinational approach to rapidly increase the titer of TNM A, a newly discovered enediyne natural product. Ribosome engineering of *Streptomyces* sp. CB03234 resulted the *Streptomyces* sp. CB03234-R-16 strain with L422P mutation on

RpoB, which afforded a TNM A titer of  $5.8 \pm 1.0 \text{ mg L}^{-1}$  aided by the in situ added macroporous resins (Table 1 and Fig. S5). Further fermentation optimization for *Streptomyces* sp. CB03234-R-16 mutant resulted in a TNM A titer of  $22.5 \pm 3.1 \text{ mg L}^{-1}$  in shaking flasks, and  $13.0 \pm 1.0 \text{ mg L}^{-1}$  in 15 L fermentors, an increase of ~ 60-fold or ~ 40-fold in comparison to the original titer in the wild-type, respectively (Figs. 4, 5). Optimal concentration of soluble starch, pharmamedia, and inorganic salts in the production medium was critical for higher TNM A production in *Streptomyces* sp. CB03234-R-16 in both the shaking flasks and 15 L fermentors. The original medium only contained  $10 \text{ g L}^{-1}$  soluble starch and  $5 \text{ g L}^{-1}$  pharmamedia, and showed highest TNM A production on day 5 (Fig. 3b). In contrast, the optimized nutrient rich medium contained  $40 \text{ g L}^{-1}$  soluble starch and  $20 \text{ g L}^{-1}$  pharmamedia in shaking flasks, and was thus advantageous for its growth, but delayed the production of TNM A (Fig. 4). However, the higher concentration of  $40 \text{ g L}^{-1}$  soluble starch and  $20 \text{ g L}^{-1}$  pharmamedia attenuated TNM A production in 15 L fermentors, probably due to the high viscosity in the fermentation medium and the insufficient oxygen supply. Further increase of the agitation speed was undesirable because it would break the macroporous resins in the medium. Therefore, an alternative production medium with less viscosity was finally selected. Our rapid improvement in TNM A titer was largely due to the use of the combinational approach, including ribosome engineering, in situ resin adsorption, medium optimization and fermentation engineering, which should guide the future strain improvement efforts for other enediyne production [44].

The success of ribosome engineering depended critically on the discovery of the so-called “antibiotic overproduction mutations” on RpoB, which have often been associated with increased secondary production [25]. In our study, a total of 17 out of 18 Rif-resistant *Streptomyces* sp. CB03234 mutants were obtained through ribosome engineering, and there were seven different types of mutations discovered (Table 1 and Fig. S3). The *Streptomyces* sp. CB03234 mutants bearing L422P and H437Y mutations on RpoB showed drastic increase in TNM A production, which have been found to increase the production of antibiotics from several other *Streptomyces* species, such as actinorhodin in both *S. coelicolor* and *S. lividans*, actinomycin in *S. parvulus* and *S. antibioticus*, formycin in *S. lavendulae*, piperidamycin in *S. mauvecolor* [25]. However, to our knowledge, H437N mutation in RpoB was reported to increase secondary metabolite production in *Streptomyces* for the first time. In addition to the understanding of the regulation of RNA polymerase by the pleiotropic second messenger ppGpp, a recent structure study has revealed the details of how RelA interacts with the stalled ribosome under amino acid starvation conditions, to initiate the synthesis of (p)ppGpp [4, 29, 38, 46]. Therefore, the observed overexpression of *relA* is consistent with the marked improved TNM A titer in *Streptomyces* sp. CB03234-R-16 mutant (Fig. 3). The mutated RNA polymerase in *Streptomyces* sp. CB03234-R-16 might also have an enhanced affinity towards the promoter regions of the TNM biosynthetic genes, which eventually leads to the TNM A overproduction, in collaborating with the ppGpp effects [12].

The strain *Streptomyces* sp. CB03234-R-5, with a seven amino acid deletion of FMDQNNP in RpoB, abolished TNM A production, while had a MIC of at least  $200 \mu\text{g mL}^{-1}$  towards Rif. Shorter deletion of RpoB has been discovered before, but how the mutant survived and how the removal of the seven amino acids in RpoB impacted the function the RNA

polymerase remained an interesting question. Along with the availability of the various *rpoB* mutants from Rif-resistant screening and many other mutations on ribosome discovered from ribosome engineering, rationally designed RpoB should facilitate the future metabolic engineering efforts for secondary metabolites production in microorganisms.

The yield of TNM A could be further improved by metabolic engineering of pathway specific regulators, such as the overexpression of putative transcriptional activators TnmR4 and TnmR7 in *Streptomyces* sp. CB03234-R-16 mutant, or screening for other antibiotic resistant mutants using the current Rif-resistant mutants. However, our current TNM A titer might already warrant the future pilot-scale production of TNM A, since gram quantity of TNM A would be sufficient to set the stage for preclinical testing of TNM A as the payload for the next generation of anticancer ADCs.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

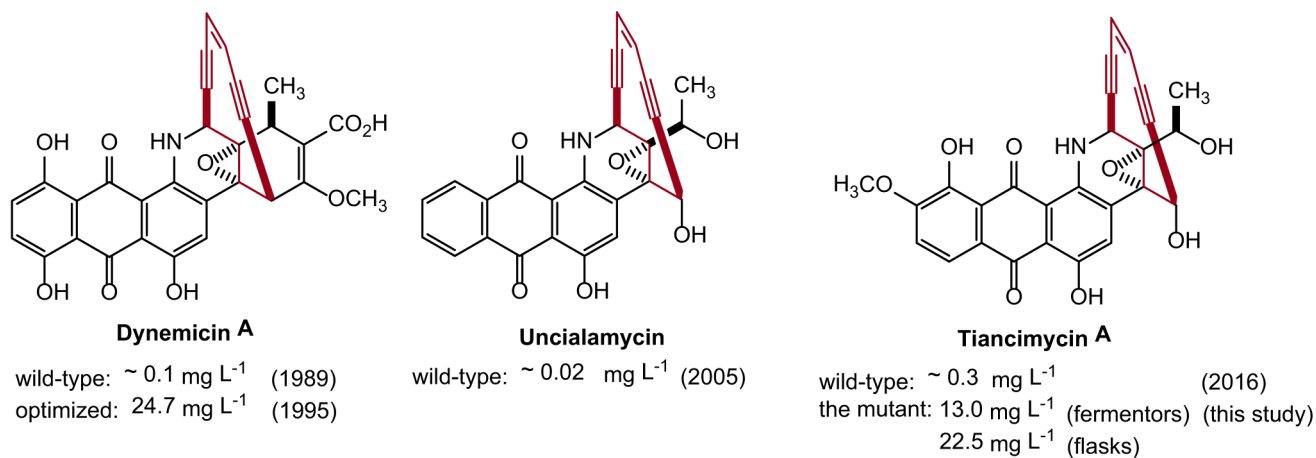
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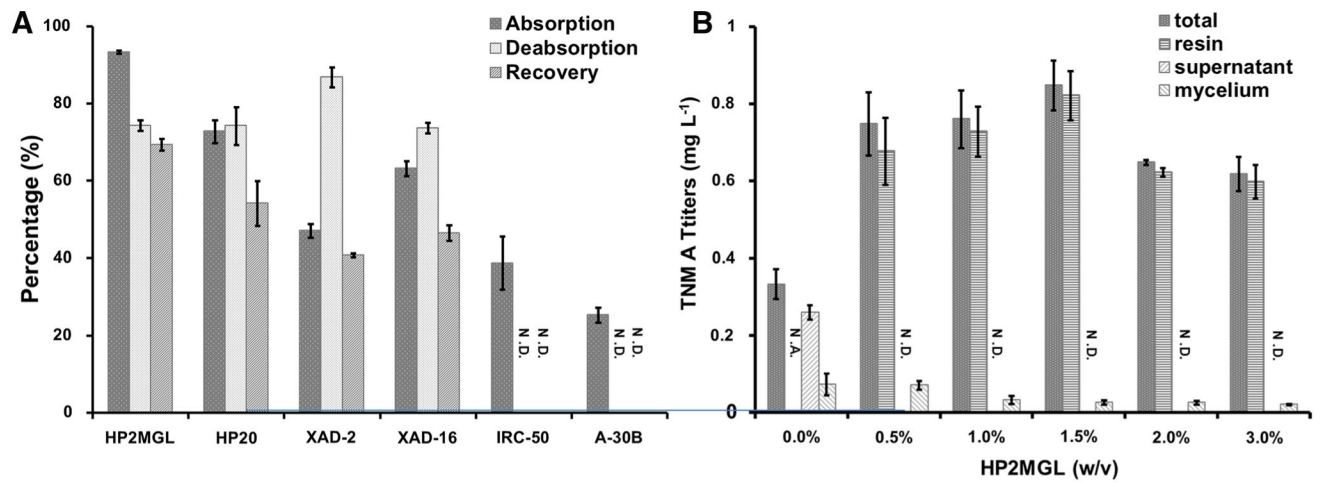
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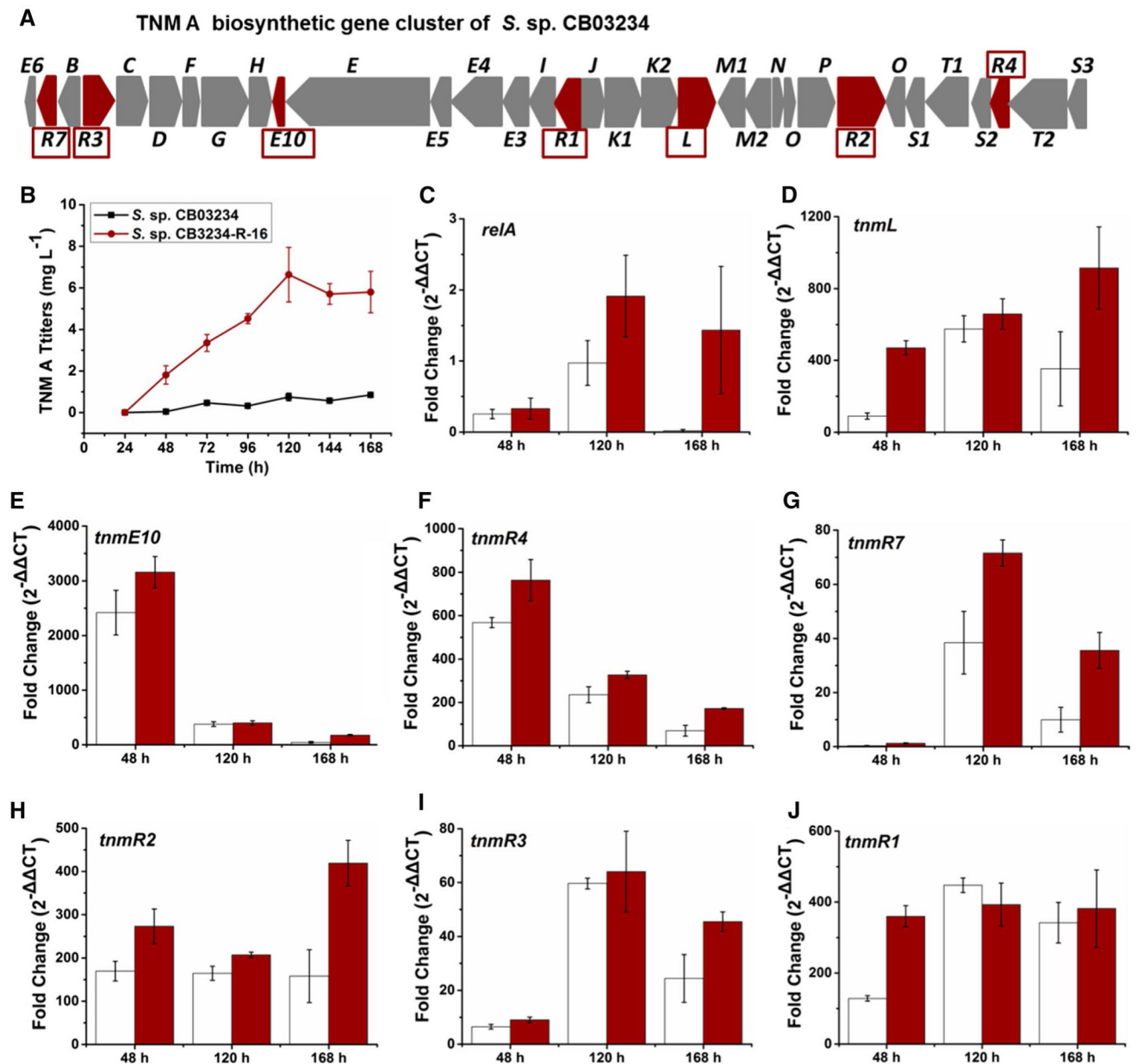


**Fig. 1.** The structures of the anthraquinone enediyne and their production yields, including dynemicin A, uncialamycin and tiansimycin A. The enediyne core was highlighted in red (color figure online)



**Fig. 2.**

The use of resin addition to improve TNM A production. **a** The adsorption, desorption and recovery ratio of TNM A from various types of resins. **b** The effect of different amounts of in situ added HP2MGL resin towards TNM A production in *Streptomyces* sp. CB03234 in shaking flasks. *N.D.* not detected, *N.A.* not applicable. There were three independent trials for the data bars

**Fig. 3.**

The time course analysis of TNM A production in *Streptomyces sp.* CB03234 wild-type and CB03234-R-16 mutant, and qRT-PCR analysis revealed the overexpression of key genes in *Streptomyces sp.* CB03234-R-16, comparing to the wild-type. **a** The biosynthetic gene cluster of TNM A in *Streptomyces sp.* CB03234 and the genes [5] evaluated in qRT-PCR were highlighted in red. **b** The production time course of TNM A in *Streptomyces sp.* CB03234 and *Streptomyces sp.* CB03234-R-16 in shaking flasks. **c–j** qRT-PCR analysis of *relA* and selected genes in TNM A biosynthetic gene cluster. The white bar stands for *Streptomyces sp.* CB03234 wild-type, and the red bar stands for CB03234-R-16 mutant. *hrdB* was used as an internal control. *tnmL*, cytochrome P450 monooxygenase; *tnmR7*, AraC family transcriptional regulator; *tnmR3*, unknown protein; *tnmE10*, type II



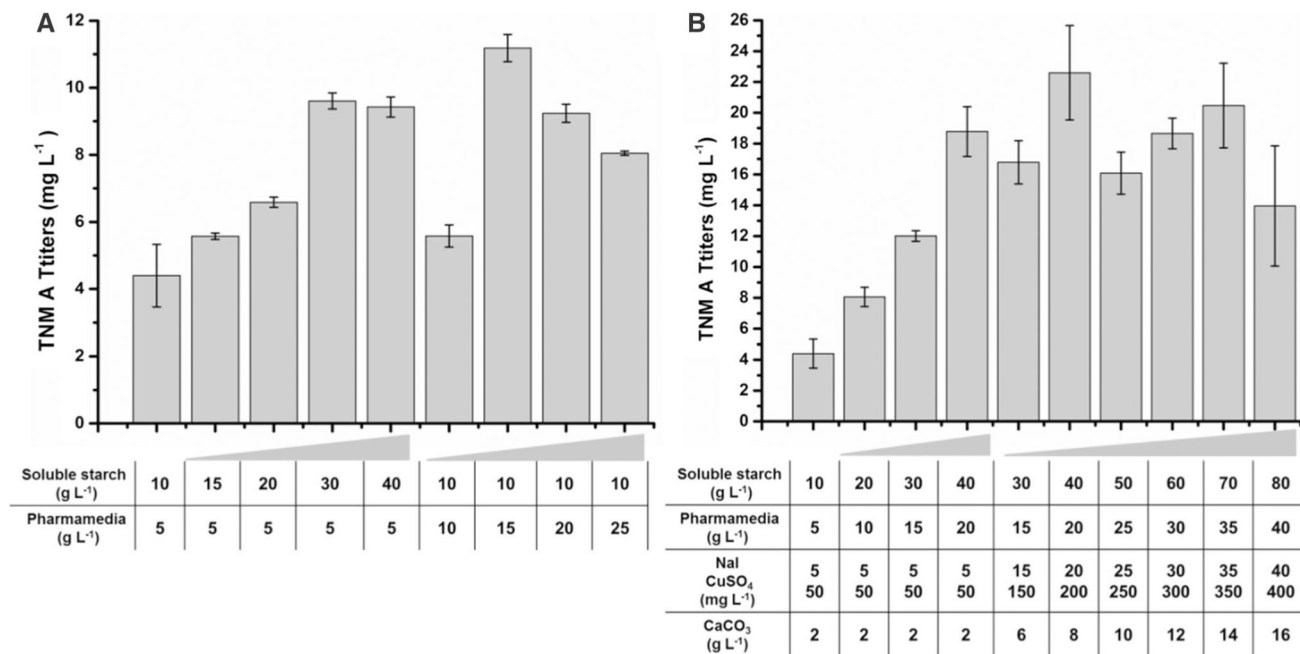
thioesterase; *tmmR1*, HxIR family transcriptional regulator; *tmmR2*, putative regulator; *tmmR4*, AraC family transcriptional regulator. Each data point represents the mean  $\pm$  SD of three biological replicates (color figure online)

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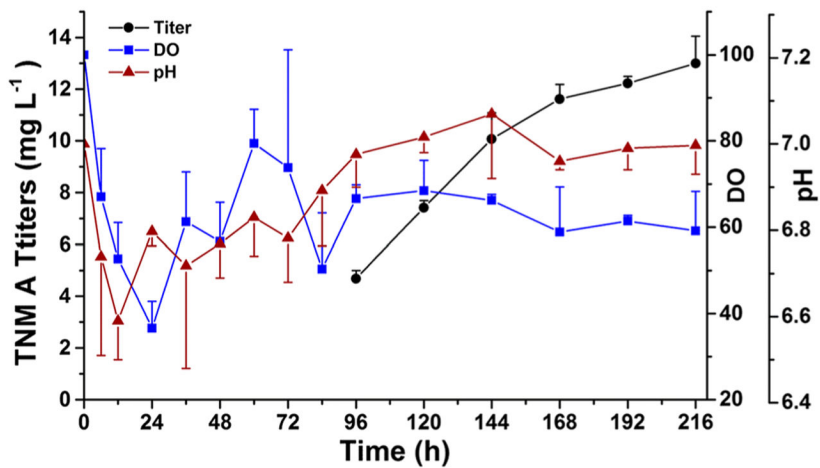
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**Fig. 4.** Medium optimization of *Streptomyces* sp. CB03234-R-16 for TNM A overproduction in shaking flasks. **a** Production of TNM A in different concentrations of soluble starch or pharmamedia. **b** Production of TNM A in different combinations of soluble starch, pharmamedia and inorganic salts, including NaI, CuSO<sub>4</sub> and CaCO<sub>3</sub>. There were at least three independent trials for the data bars



**Fig. 5.** The production of TNM A from *Streptomyces* sp. CB03234-R-16 in 15 L fermentors. There were at least two independent trials for the data bars. The medium consisted of 30 g L<sup>-1</sup> starch soluble, 15 g L<sup>-1</sup> pharmamedia, 2 g L<sup>-1</sup> CaCO<sub>3</sub>, 50 mg L<sup>-1</sup> CuSO<sub>4</sub>·5H<sub>2</sub>O, 20 mg L<sup>-1</sup> NaI, and 2 mL L<sup>-1</sup> anti-foaming agent, along with 2% (v/w) HP2MGL resins

Table 1

A ribosome engineering approach to identify *Streptomyces* sp. CB03234 mutants with higher TNM A production

<i>Streptomyces</i> sp.	Screening approach	Mutation in <i>rpoB</i>	Amino acids changes	Resistance to Rif ( $\mu\text{g mL}^{-1}$ )	TNM A ( $\text{mg mL}^{-1}$ )
CB03234	-	-	-	<1	0.8 $\pm$ 0.1
CB03234-R-1	DES + Rif	None	None	25	0.6 $\pm$ 0.1
CB03234-R-2	DES + Rif			25	NE
CB03234-R-11	Rif	1280A $\rightarrow$ T	Asp427 $\rightarrow$ Val	5	1.8 $\pm$ 0.1
CB03234-R-18	Rif			25	1.0 $\pm$ 0.2
CB03234-R-3	DES + Rif	1319G $\rightarrow$ A	Arg440 $\rightarrow$ His	25	0.3 $\pm$ 0.1
CB03234-R-4	DES + Rif			50	NE
CB03234-R-6	DES + Rif			25	NE
CB03234-R-7	DES + Rif	1325C $\rightarrow$ T	Ser442 $\rightarrow$ Leu	25	NE
CB03234-R-8	DES + Rif			25	0.1 $\pm$ 0.1
CB03234-R-9	DES + Rif			25	0.2 $\pm$ 0.1
CB03234-R-10	Rif	1309C $\rightarrow$ A	His437 $\rightarrow$ Asn	5	1.5 $\pm$ 0.2
CB03234-R-12	Rif			>200	NE
CB03234-R-17	Rif	1309C $\rightarrow$ T	His437 $\rightarrow$ Tyr	>200	3.9 $\pm$ 0.5
CB03234-R-13	Rif			25	NE
CB03234-R-14	Rif	1265T $\rightarrow$ C	Leu422 $\rightarrow$ Pro	5	NE
CB03234-R-15	Rif			5	5.7 $\pm$ 1.0
CB03234-R-16	Rif			5	5.8 $\pm$ 1.0
CB03234-R-5	DES + Rif	21-nt	FMDQNNP	>200	0

The titers of TNM A in selected strain represent the mean  $\pm$  SD of at least three biological replicates

DES diethyl sulfide, Rif rifamycin, nt nucleotides, - not applicable, NE not examined